Biochemical and Biophysical Research Communications 421 (2012) 640–645

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-25 promotes cell migration and invasion in esophageal squamous cell carcinoma

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ARTICLE INFO

Article history:
Received 12 March 2012
Available online 17 March 2012

Keywords:
MicroRNA
Esophageal squamous cell carcinoma
Cell migration
Cell invasion
CDH1

ABSTRACT

MicroRNAs (miRNAs) as a species of small non coding single stranded RNA of about 21–25 nucleotides have important roles in the development of different cancers. In present study, we found that the expression of miR-25 was up-regulated in 60 esophageal squamous cell carcinoma (ESCC) tissues compared with matched adjacent non-cancer tissues. Moreover, we demonstrated that the up-regulation of miR-25 was significantly correlated with the status of lymph node metastasis and TNM (Tumor, Node and Metastasis) stage. Furthermore, over-expression of miR-25 markedly promoted migration and invasion of ESCC cells. On the contrary, down-regulation of miR-25 inhibited the migration and invasion of cells. E-cadherin(CDH1) is a very important tumor metastasis suppressor. We further identified that miR-25 directly targeted CDH1 3′-untranslated region (3′UTR) and repressed the expression of CDH1. These results, for the first time, demonstrate that miR-25 promotes ESCC cell migration and invasion by suppressing CDH1 expression.

1. Introduction

Esophageal cancer causes more than 200,000 deaths each year. It is the eighth most common cancer worldwide, and the sixth most common cause of death from cancer. China is one of the high-risk areas [1]. Esophageal cancer is developed from epithelia including two subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the most frequent subtype of esophageal cancer in China. Resection was previously considered the mainstay of treatment for ESCC. However, there is a very poor survival that is related to invasion and metastasis. ESCC has a high propensity for lymphatic spread via the rich network of submucosal lymphatic vessels [2]. Therefore, improved morbidity and mortality of ESCC will require effective treatments to metastatic pathway factors. Yet the molecular mechanism of metastasis is still not completely clear.

MiRNAs are an abundant class of small, well-conserved, non-coding RNA molecules that silence gene expression usually by interfering with mRNA stability or protein translation [3,4]. The function is by identifying 3′UTRs of target mRNAs with conserved complementarities to the seed (nucleotides 2–7) of the miRNA. Up to 30% of human protein-coding genes may be regulated by miRNAs [5]. They are known to be involved in gene functions in a broad range of biological processes including development, cell differentiation, proliferation, apoptosis, metabolism, carcinogenesis and growth control [6–8]. More than 50% of human miRNA genes are frequently located at fragile sites and genomic regions involved in cancers [9]. Accordingly, many functionally validated miRNA targets are oncogenes and tumor suppressors [10].

MiR-25 belongs to miR-106b-25 cluster. This cluster produces three mature miRNAs (miR-106b, miR-93, and miR-25). They accumulate in different types of cancers, including gastric, prostate, hepatocellular and pancreatic neuroendocrine tumors, neuroblastoma, and multiple myeloma [11–14]. MiR-106b and miR-93 have the same seed sequence and the similar 3′-half. In contrast, miR-25 without the same sequence as them is expected to have a separate function [15]. Petrocca et al. reported that miR-106b and miR-93 suppress the cell cycle inhibitor CDKN1A (p21) expression, miR-25 deregulates the proapoptotic gene BCL2L11 (BIM) expression [16].

Recently, several miRNAs were identified that played roles in various steps of metastasis [17]. We previously showed that miR-25 was up-regulated in ESCC tumor tissues [18]. We have identified by database analysis that the 3′UTR of CDH1 gene is a putative target of miR-25. CDH1 gene encodes the E-cadherin (CDH1) which has been shown to reduce the metastases and invasiveness of tumors [19]. Herein, in our study, we demonstrate that the relative expression of miR-25 between tumor and adjacent non-cancer tissues is correlated with lymph node metastasis and TNM stage in ESCC patients. In addition, miR-25 can promote cell migration and invasion by inhibiting the CDH1 expression in ESCC.

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http://dx.doi.org/10.1016/j.bbrc.2012.03.048

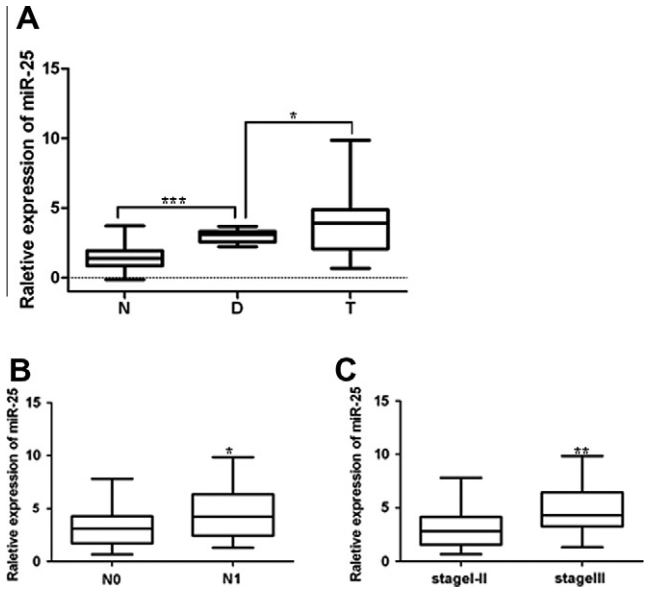

Fig. 1. Expression of miR-25 is up-regulated in ESCC tissues and correlated with lymph node metastasis and TNM stage in ESCC patients. A. The miR-25 expression was analyzed by qRT-PCR in normal epithelium (N, $n = 28$), dysplasia (D, $n = 24$), advanced tumor tissues (T, $n = 60$). RNU6B served as internal control. $-\triangle$Ct (CtRNU6B-CtmiR-25) was calculated. B. In the 60 patients with advanced tumor, the relative expression of miR-25 was compared between 30 patients with lymph node metastasis (N1) and 30 patients without lymph node metastasis (N0). C. In the 60 patients with advanced tumor, the relative expression of miR-25 was compared between 32 patients in stage I-II and 28 patients in stage III. Values are the average of triple determinations with the S.D. indicated by error bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2. Material and methods

2.1. Cell culture and transfection

Two human ESCC cell lines KYSE-150 and KYSE-410 were cultured in RPMI-1640 medium (Hyclone) with 10% FBS (Hyclone) supplemented with 0.2% penicillin–streptomycin (Invitrogen) in a humidified 5.0% CO_2 atmosphere at 37.0 °C. The precursor miR-25 molecule (pre-miR-25), miR-25 inhibitor (anti-miR-25), negative miR precursor and negative miR inhibitor (pre-miR-nc, anti-miR-nc), were purchased from Ambion, and transfected at a final concentration of 30 nM with Lipofectmine 2000 (Invitrogen).

2.2. Tissue samples

Normal esophageal epithelia tissues were obtained from normal persons, human ESCC tumor tissues (T) and matched adjacent non-cancer tissues were obtained from ESCC patients in Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS). All specimens were snap frozen in liquid nitrogen immediately after surgery and stored at −80 °C.

2.3. Quantitative real-time RT-PCR

Total miRNAs were isolated from frozen tissues using a mirVana miRNA isolation kit (Ambion), according to the manufacturer's instructions. The first strand cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Fermentas), which was then amplified using TaqMan gene expression master mix (Applied Biosystems) and Applied Biosystems 7300 Real Time PCR system following the manufacturer's instructions. The qRT-PCR specific primers were purchased from Ambion. RNU6B served as endogenous control. Data were presented as fold differences based on calculations of $2^{-\Delta\Delta Ct}$.

2.4. Antibodies

Rabbit anti-E-Cadherin (anti-CDH1) and mouse anti-β-Actin were obtained from Epitomics. The appropriate HRP- labeled secondary antibodies: HRP- goat anti-rabbit IgG and anti-mouse IgG were purchased from Abgent.

2.5. Western blot analysis

Cells were lysed in RIPA buffer (Santa Cruz Biotechnology, standard protocol). Equal amounts of protein were separated by 10% SDS–PAGE then transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were incubated at room temperature for 1 h with 10% non-fat milk, followed by incubation with rabbit anti-CDH1 (1:10000), or mouse anti-actin (1:10000) antibodies at 4.0 °C overnight. After washing, blots were treated with the appropriate HRP-conjugated secondary antibody (1:10000) at room temperature for 1 h, and then developed by enhanced chemiluminescence according to the manufacturer's protocol (Centro LB 960).

2.6. Migration and invasion assay

Twenty-four hours after transfection with miRNAs (pre-miR-25, pre-miR-nc, anti-miR-25, or anti-miR-nc), cells were starved with serum-free RPMI 1640 medium for 24 h. Then, 10^6 cells were suspended in 1 mL of serum-free medium, 100 μL (1×10^5) cells were plated to the upper compartment of transwell chambers (Corning, 24-well plate with 8.0 μm pores) uncoated or coated with Matrigel (BD Biosciences, 0.7 mg/mL). The lower compartment of the chamber was filled with 700 μL RPMI 1640 medium supplemented with 10% FBS. After incubating 20–24 h, cells on the lower surface of the filter were stained and fixed with 0.5% crystal violet dissolved in 100% methanol, and five fields across the membrane were counted at 100-fold magnifications. To restore the miR-25-resistant CDH1 expression, pCMV6-ENTRY-CDH1 containing CDH1 open reading frame (ORF) but lacking the 3′UTR including the putative miR-25 target site, was purchased from OriGene Technologies. Cells were transfected with pre-miR-25 for 24 h, then transfected with pCMV6-ENTRY-CDH1. Twenty-four hours later, cells were digested and added to the migration and invasion assay as discussed above.

2.7. Luciferase reporter assay

The truncated element of CDH1 mRNA 3′UTR containing predictive miR-25 responsive element (CDH1-3′-UTR-WT, 136 bp), and the mutant truncated element without the predictive binding site of miR-25 seed sequence (CDH1-3′-UTR- Mut, 129 bp), were inserted downstream of the firefly luciferase gene in pMIR (Ambion), and the resulting plasmids designated pMIR-CDH1-Wt/-Mut, respectively. Renilla luciferase expression construct (phRL-TK) was used as internal control (Promega). Plasmids pMIR-CDH1-Wt/-Mut (800 ng/well in 24-well plate) along with phRL-TK (16 ng per well), were transfected after 24 h transfection of miRNAs. Twenty-four hours later, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured by a dual-luciferase reporter system (Promega) using LB 960 Centro (Berthold). The luminescence intensity of Firefly luciferase was normalized to that of Renilla luciferase.

2.8. Statistical analysis

Student's t-test was used and $p < 0.05$ was considered significant. Results were displayed as mean ± SEM from at least triplicate experiments for each group.

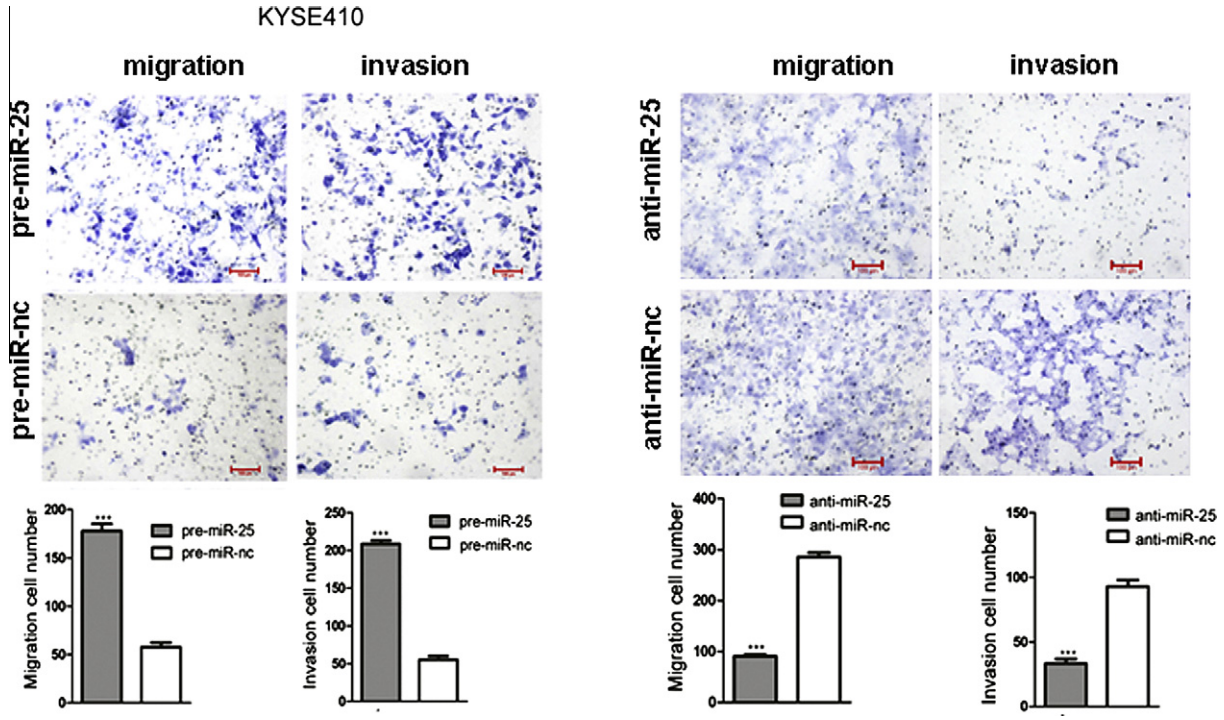

Fig. 2. MiR-25 regulates cell migration and invasion in ESCC cells. In KYSE410 cells, 48 h after expression of miRNAs, cells were seeded into transwell inserts at the density of 1×10^5 cells/well (invasion coated with Matrigel 0.7 mg/mL). After 20 h for migration and after 24 h for invasion, cells on the lower surface of the filter were stained and five different areas at 100-fold magnifications were counted. Values are the average of triple determinations with the S.D. indicated by error bars. $^{***}P < 0.001$.

3. Results

3.1. Expression of miR-25 is up-regulated in ESCC tissues and correlated with lymph node metastasis and TNM stage in ESCC patients

We detected 60 ESCC tissues to determine expression level of miR-25 by qRT-PCR. Then, the relationship between relative expression of miR-25 and clinicopathologic information of ESCC patients was analyzed. In order to investigate whether the expression of miR-25 is associated with the development of tumor, we assessed the level of miR-25 in 28 cases of normal esophageal epithelia tissues (N), 24 dysplasia tissues (D) using qRT-PCR at the same time. Notably, the expression of miR-25 was increased more than 1.5-fold in about 70% advanced ESCC tumor tissues compared with matched adjacent non-cancer tissues. In particular, the level of miR-25 was significantly increased in dysplasia tissues relative to normal esophageal epithelia tissues ($p < 0.001$) (Fig. 1A), it was increased in advanced ESCC tumor tissues relative to dysplasia tissues ($p < 0.05$) (Fig. 1A). The difference was significant. The relative expression of miR-25 increased in patients with lymph node metastasis compared with patients without lymph node metastasis ($p < 0.05$) (Fig. 1B, Supplementary Table.1). The level of miR-25 was higher in patients with stage I and II than patients with stage III ($p < 0.01$). No significant association was found between miR-25 expression and other characteristics such as age, gender, tobacco, alcohol, gross pathological type, tumor position, and T classification (Supplementary Table.1).

3.2. MiR-25 regulates migration and invasion in ESCC cell lines

To investigate the mechanism which miR-25 promoted lymph node metastasis, we examined the role of miR-25 in ESCC cell migration and invasion. To improve the expression of miR-25, KYSE150 and KYSE410 cells were transfected with pre-miR-25. To inhibit the expression of miR-25, the both cell lines were transfected with anti-miR-25. We tested cellular migration and invasion levels using transwell chambers coated with Matrigel or without Matrigel. It was found that over-expression of miR-25 markedly enhanced migration and invasion of cells compared with negative controls ($p < 0.001$) (Fig. 2, Supplementary Fig. 1). Conversely, the inhibition of miR-25 expression led to considerable decrease of the migration and invasion of cells compared with negative controls ($p < 0.001$) (Fig. 2, Supplementary Fig. 1). These results indicate that miR-25 promotes ESCC cell migration and invasion.

3.3. MiR-25 regulates CDH1 expression by directly targeting its 3′ UTR in ESCC cell lines

By computer searches (TargetScan, miRanda and MicroCosm), we found that the 3′ UTR of CDH1 mRNA contains a highly conserved binding site from position 499 to 524 for miR-25. It was showed that CDH1 might be a target for miR-25. In cell lines with high miR-25 expression, a low amount of CDH1 protein was observed, whereas with low miR-25 expression, a high amount of CDH1 protein was observed (Fig. 3A). Then KYSE150 cells were transfected with increasing concentration of pre-miR-25. The expression of CDH1 was decreased along with the increased miR-25 level. On the other hand, when the KYSE150 cells were transfected with increasing concentration of anti-miR-25, the expression of CDH1 was increased along with the decreased miR-25 level. There was a inverse correlation between miR-25 and CDH1 protein levels (Fig. 3A). These results provide evidence that abnormal expression miR-25 regulates CDH1 expression in ESCC cells.

To evaluate whether miR-25 directly targets the CDH1 mRNA, we performed dual luciferase reporter assays. In KYSE410 and KYSE150 cells, the reporter plasmids that were fused to the CDH1-3′UTR-Wt (pMIR-CDH1-Wt)/CDH1-3′UTR-Mut (pMIR-CDH1-Mut) were transfected. A significant decrease luciferase activity was observed in cells transfected with pre-miR-25 versus

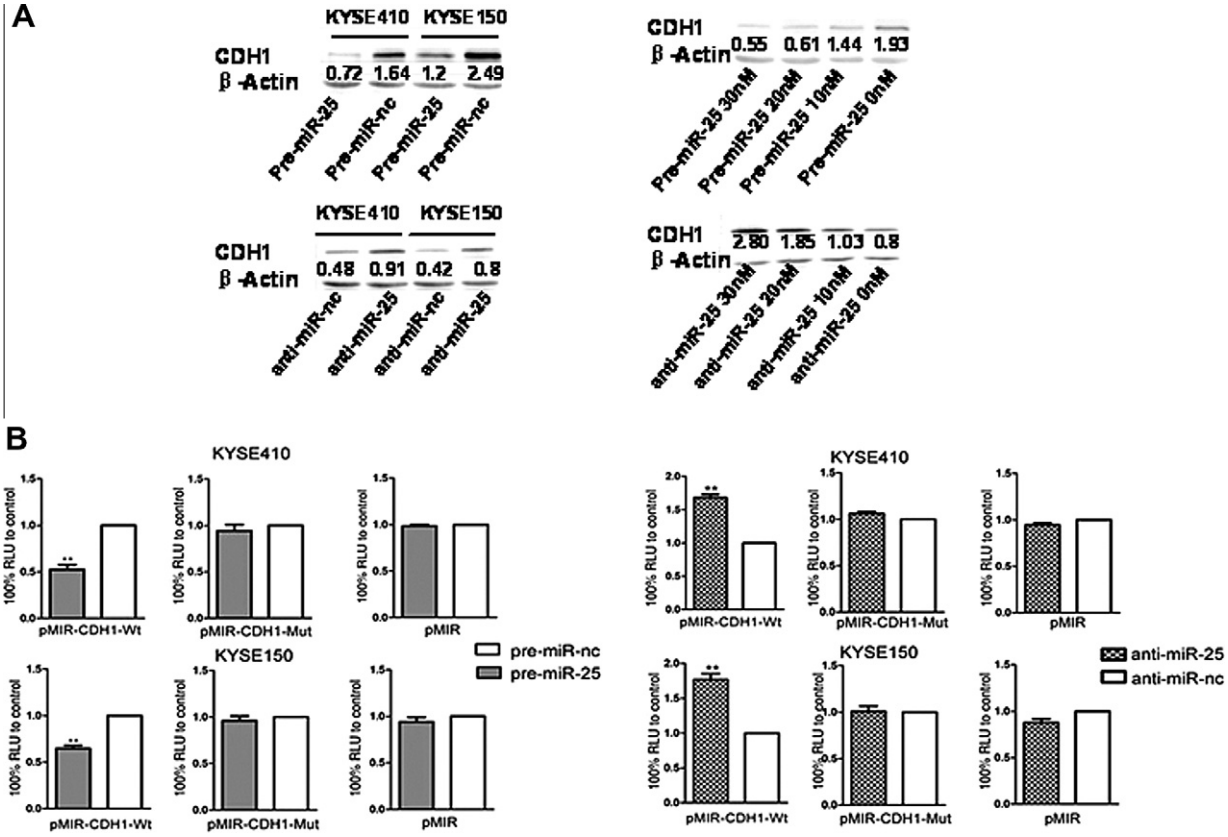

Fig. 3. MiR-25 regulates CDH1 expression by targeting its 3′UTR in ESCC cell lines. A. KYSE150 and KYSE410 cells were transfected with 30 nmol/L pre-miR-25/anti-miR-25. After 48 h, CDH1 expression was analyzed by western blotting. KYSE150 cells were transfected with the increasing doses of pre-miR-25/anti- miR-25 (0, 10, 20 and 30 nmol/L). After 48 h, the expression of CDH1 was measured. The density of each protein band was quantified by LANE 1D Analyzer V4.0 software (Beijing Sage Creation) and β-Actin served as loading control. B. Cells were transfected with miRNAs for 24 h, and then were transfected with 800 ng Wt/Mut reporter plasmids along with phRL-TK (16 ng/well). Luciferase activity was measured by a dual-luciferase reporter assay. The result was expressed as relative luciferase activity (firefly LUC/renilla LUC). Values are the average of triple determinations with the S.D. indicated by error bars. $^{**}P < 0.01$.

negative controls (Fig. 3B). In contrast, the luciferase activity was significantly increased in cells transfected with anti-miR-25 versus negative controls (Fig. 3B). There were no differences between pre-/anti-miR-25 and negative controls transfected with pMIR-CDH1-Mut and pMIR. It is indicated that 3′ UTR of CDH1 mRNA is a direct target of miR-25.

3.4. Cell migration and invasion induced by miR-25 is reversed by CDH1

To further investigate the contribution of CDH1 to the biological function of miR-25, 24 h after transfection with pre-miR-25, the KYSE410 and KYSE150 cells were transfected with pCMV6-ENTRY-CDH1. Twenty-four hours later, cells were digested and added to the migration and invasion assay. We examined whether reconstitution of CDH1 had an effect on miR-25-induced ESCC cell migration and invasion. It was showed that the ability of migration and invasion of ESCC cells can be reversed by CDH1 (Fig. 4, Supplementary Fig. 2). It demonstrates that inhibition of CDH1 expression is at least partially responsible for the miR-25 ability to promote cell invasion and migration.

4. Discussion

Metastasis is the major cause of death in cancer patients. Epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion and metastatic processes [20]. One of the hallmarks of EMT and the concomitant induction of cell migration and invasion is the loss of CDH1 [21]. CDH1 as a major cell-to-cell adhesion molecule plays a critical role in orchestrating cellular signals mediating cell survival and differentiation [22,23]. The reduced expression of CDH1 in ESCC tissues is correlated with an increased number of lymph node metastases [24,25]. Our study indicates that miR-25 represses the expression of CDH1 in ESCC.

Recently, some miRNAs were found to be associated with metastasis. The metastasis-suppressing miRNAs included miR-335, miR-206, miR-146a/b and miR-31 [26–28]. The metastasis-promoting miRNAs included miR-10b, miR-373, miR-520c, miR-21, miR-143 and miR-182 [29–34]. However, the effect of miR-25 on tumor metastasis has not been reported. Using miRNA microarray, our previous study demonstrated that three miRNAs (hsa-miR-25, hsa-miR-424, and has-miR-151) showed up-regulation in ESCC tissues. In particular, miR-25 was correlated with gross pathologic and differentiation classifications [18].

In this study, we assessed miR-25 level in 28 normal esophageal epithelia tissues, 24 dysplasia and 60 advanced ESCC tumor tissues. It was found that the expression of miR-25 was significantly up-regulated in dysplasia tissues compared with normal epithelium, and it was up-regulated in advanced ESCC tissues compared with dysplasia tissues. We also showed that the relative expression of miR-25 was significantly correlated with lymph node metastasis, TNM stage, of ESCC patients, but not the T stage and the size of tumors. These results suggest that the over-expression of miR-25 is along with the development of the ESCC tumorigenesis by promoting the metastatic ability of tumor.

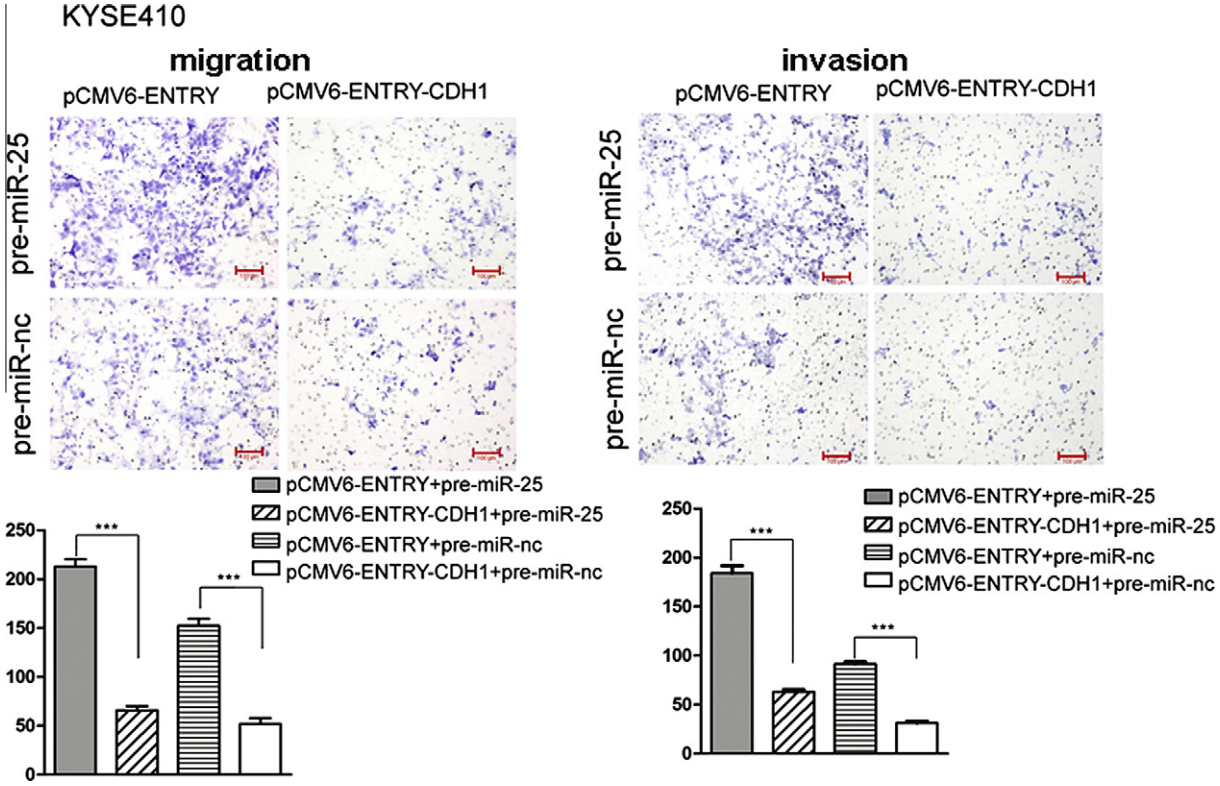

Fig. 4. Cell migration and invasion induced by miR-25 is reversed by CDH1. KYSE410 cells were transfected with pre-miR-25/pre-miR-nc for 24 h, then transfected with pCMV6-ENTRY-CDH1/pCMV6-ENTRY. After 24 h, cells were suspended with serum-free medium and added to the migration and invasion assay. Values are the average of triple determinations with the S.D. indicated by error bars. $^{***}P < 0.001$.

Our study indicates that miR-25 inhibits the translation of CDH1 mRNA by targeting its 3′ UTR, which adds a new explanation of deregulation of CDH1 expression in ESCC. Moreover, overexpression of miR-25 leads to promoting cell migration and invasion of KYSE150 and KYSE410 cells. We also showed that loss of miR-25 expression reduces migration and invasion in the two ESCC cell lines *in vitro*. It is consistent with our correlation study in ESCC tissues which the level of miR-25 is associated with lymph note metastasis. In addition, we uncovered an inverse correlation between miR-25 and CDH1 in ESCC cell lines. Furthermore, CDH1 reversing cell migration and invasion induced by miR-25 overexpression indicated that CDH1 functioned as a mediator of miR-25 in cell migration and invasion.

The study by the Croce CM laboratory addressed that overexpression of miR-25 interferes with TGFβ-induced apoptosis by inhibiting the proapoptotic gene BCL2L11 (BIM) expression [16]. In hepatocellular carcinoma, high expression of miR-25 may be one means of down-regulating expression of the BIM [12]. Overexpression of miR-25 plays a role in gastric cancer tumorigenesis by targeting the cell-cycle inhibitor p57 [15]. In human prostate cancer, miR-25 was inversely correlated with tumor suppressor PTEN abundance [13]. MiR-25 plays a role in the development successive stages of neoplasia from normal esophageal to Barrett's esophagus and finally to esophageal adenocarcinoma by targeting BIM [35]. In order to explore the phenotypes, we assayed the cell proliferation, apoptosis and cell cycle in ESCC cell lines by pre-miR-25/anti-miR-25 transfection. However, we did not observe altered cell proliferation, apoptosis and cell cycle in ESCC cells. These results indicate that deregulated miR-25 expression plays different roles in different types of cancers.

In conclusion, we have provided strong evidence that miR-25 is frequently up-regulated in ESCC tissues. MiR-25 promotes cell migration and invasion in ESCC by suppressing CDH1 expression.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.048.

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