miR-1291 targets mucin 1 inhibiting cell proliferation and invasion to promote cell apoptosis in esophageal squamous cell carcinoma

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Abstract. MicroRNAs (miRNAs) are well known as important regulators in various cancer development. In the present study, we focused on the expression and biological function of miR-1291 in esophageal squamous cell carcinoma (ESCC). Compared with adjacent non-tumorous tissue samples, qRT-PCR data showed significant downregulation of miR-1291 in 54 ESCC tissue samples (P<0.05), which was also significantly associated with lymph node metastases and clinical stage (P<0.05). Cell Counting Kit-8 (CCK-8), colony formation, Transwell and flow cytometric apoptosis assays were performed to detect the effect of miR-1291 upregulation, and the results showed inhibition of the proliferation, invasion and promotion of apoptosis in EC9706 and EC-1 cells. Using bioinformatic analyses, we found that mucin 1 (MUC1) was a potential target for miR-1291. Luciferase assays were performed to reveal that miR-1291 inhibited MUC1 expression by targeting the seed region of MUC1 3'-untranslated region (3'UTR). We also found that the expression of MUC1 lacking in 3'UTR abrogated the anti-invasion and pro-apoptosis function of miR-1291. Our results demonstrated the importance of miR-1291 in targeting MUC1 for the regulation of esophagus cancer growth, invasion and apoptosis, and may be helpful for developing new targets for early diagnosis or new therapeutic targets for ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a common malignancy worldwide (1). As a result of lymph node metastases, deep tumor invasion and difficulty in early diagnosis, the majority of esophageal cancer patients have a relatively low survival rate (2,3). Despite the many advances in surgery, chemotherapy and nutritional aid therapies at present, the long-term ESCC survival rate has only slightly improved in recent years (4). Therefore, it is a formidable task to study and explore novel biomarkers or therapeutic targets for ESCC patients. Recently some research findings have indicated that the initiation and development of ESCC involved the mutation of numerous oncogenes and anti-oncogenes, and thus were characterized by the synergetic effect of multiple genes, factors and steps (5,6), yet more effort is still needed to study the genetic and molecular changes underlying the development of ESCC.

MicroRNAs (miRNAs) are small non-coding RNAs with 19-23 nucleotides (7,8). Numerous studies have been performed on the role and related mechanism of miRNAs in numerous different kinds of diseases (9-12). miRNAs bind primarily to the 3'-untranslated region (3'UTR) of their target messenger RNAs (mRNAs) to reduce their stability and decrease the expression of target mRNAs at the post-transcriptional level (13), which play important roles in various biological processes including cell growth, proliferation, differentiation and death (14-19). Concerning chemotherapy in various types of cancers such as breast cancer, lung adenocarcinoma, glioblastoma, and ovarian cancer, recent studies have shown that miRNAs also act in important roles (20-24). In ESCC, altered expression of miR-1290, miR-655, miR-375 and others, has been observed (25-27), suggesting that miRNA deregulation plays an important role in ESCC development.

Recently, miR-1291 was found to be significantly downregulated in pancreatic and renal cell carcinomas, and restoration of miR-1291 function repressed tumorigenesis. To the best of our knowledge, there is no previous study concerning miR-1291 in ESCC biology. To gain insight into the potential mechanisms of miR-1291 in ESCC, we performed the relevant bioinformatic analyses using TargetScan and miRanda (28), and found that mucin 1 (MUC1) is a potential target of miR-1291. A previous study showed that the alteration of MUC1 is correlated with regional lymph node metastasis, and...
high-expression of MUC1 is associated with poor prognosis for esophageal cancer patients (29). Based on the above information, miR-1291 and MUC1 may play roles in the development of ESCC. The present study investigated miR-1291 and MUC1 expression levels in ESCC tissues from 54 patients, and evaluated the effect of miR-1291 upregulation on proliferation, invasion and apoptosis of ESCC cells, which provides new insight into the potential mechanisms of ESCC and identify a new possible target for early diagnosis or therapy.

Materials and methods

Clinical sample collection. Paired tumorous and adjacent non-tumorous tissues were obtained from 54 patients with ESCC who underwent radical resection in the First Affiliated Hospital of Zhengzhou University and the First Affiliated Hospital of Luohu Medical College between 2012 and 2014 (Table 1). Tissues were snap-frozen in liquid nitrogen and identified by pathological examinations after resection. None had received chemotherapy or radiotherapy before surgery. All patients consented to the use of their tissue samples in the present study. Human Research Ethics Committee of Zhengzhou University and Luohu Medical College approved the present study.

Cell lines and cell culture. Human ESCC EC9706 and EC-1 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were maintained in a RPMI-1640 medium containing 100 U/ml penicillin, 100 µl/ml streptomycin and 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA) and incubated at 37°C and 5% CO₂.

RNA oligo-ribonucleotides and cell transfection. miR-1291 mimic and a negative control (NC) were chemically synthesized by Shanghai GenePharma Co. Ltd. The amount of miR-1291 mimic and NC is 7.92 µg/4x10⁶ cells, respectively. Transfection was performed with a BTX ECM 2001 square wave electroporation (Genetronics Inc., San Diego, CA, USA) with electroporation settings adjusted according to the BTX ECM 2001 protocol. After transfection, EC9706 and EC-1 cells were seeded in 6-well plates (2x10⁵ cells/well). Cells from each cell line were subdivided into three groups: the non-transfected blank (blank), a mimic NC-transfected (NC) and the miR-1291 mimic-transfection groups (miR-1291). Cells were harvested for further experiments after 24-48 h post-transfection.

RNA extraction and quantitative real-time PCR. Relative levels of miR-1291 and MUC1 mRNA in ESCC tissue samples and adjacent non-tumorous tissue samples were determined by quantitative real-time PCR (qRT-PCR) assays. Total RNA was extracted with an RNA extraction kit (Qiagen, Venlo, The Netherlands), and RNA quality was confirmed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed using MMLV RTase cDNA synthesis kit (Takara, Dalian, China). A cDNA library of miRNAs was constructed by QuantiMir cDNA kit (Takara). All protocols were performed according to the manufacturers' instructions, and qPCR was performed using the ABI Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The relative expression of miR-1291 was calculated using the comparative cycle threshold (CT, 2-ΔΔCT) method with U6 snRNA as an endogenous control to normalize the data. β-actin was used as normalization for the relative levels of MUC1 mRNA. The calculation method was the same as presented.

Western blotting. Proteins were precipitated and determined by western blot analysis following the previously described procedures (26). Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were incubated overnight at 4°C with the diluted (1:500) primary antibody (polyclonal rabbit anti-MUC1; Santa Cruz). Following extensive washing, the membranes were incubated with the diluted (1:3,000) horse-radish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Signals were detected using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). An antibody against β-actin (Santa Cruz) served as an endogenous reference. Relative protein levels were calculated using β-actin as a loading control.

Cell Counting Kit-8 (CCK-8) assay. We used the CCK-8 assay (Dojindo Laboratories, Japan) according to the manufacturer's instructions to determine cell viability. Briefly, cells were seeded at a density of 2x10⁴ cells/well in 96-well plates (in three replicate wells) and treated daily for four consecutive days with 10 µl/well of CCK-8 solution. Optical density was measured at 450 nm to estimate the number of viable cells.

Colony formation assay. Twenty-four hours after RNA transfection, cells were suspended in 0.3% agarose with RPMI-1640 containing 10% FBS and plated into four 6-cm cell culture dishes on top of an existing layer of 0.6% agarose. Twenty-four hours after RNA transfection, cells were suspended in 0.3% agarose with RPMI-1640 containing 10% FBS and plated into four 6-cm cell culture dishes on top of an existing layer of 0.6% agarose. The plates were incubated at 37°C in a 5% CO₂ incubator for 12 days. Colonies were stained with crystal violet, and those with >50 cells were scored as surviving colonies. The cloning efficiency was calculated by dividing the average number of colonies/dish by the number of plated cells.

Cell apoptosis detection. Flow cytometric assays were conducted using the Annexin V-FITC apoptosis detection kit I (BestBio, Shanghai, China), according to the manufacturer's instructions. Cells from the two groups (miR-1291 and NC) were harvested by trypsinization and resuspended at a density of 1x10⁶ cells/ml in 1X binding buffer. After double staining with propidium iodide (PI) and Annexin V-FITC, the samples were analyzed by flow cytometry and the data were analyzed using CellQuest software.

Transwell invasion assay. We assayed the invasion ability of cells using 6.5 mm diameter Transwell chambers with 8-µm membranes (Corning, USA). Twenty-four hours after post-transfection, EC9706 or EC-1 cells were added to the upper chambers, and the bottom wells were coated with 1 mg/ml Matrigel for the invasion assays, while a medium containing 10% FBS was added to the lower chamber. After 24 h at 37°C in a 5% CO₂ humidified atmosphere, cells in the upper chamber were carefully scraped off using a cotton swab,
and cells that had migrated to the basal side of the membrane were fixed in 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 (magnification, x200). Each test was performed in triplicate.

Luciferase reporter assays. The wild-type MUC1 3’UTR fragments containing putative seed region for miR-1291 were amplified from the human genomic DNA by PCR. The mutagenesis of the seed region was achieved by overlap PCR. The wild-type (wt) MUC1 3’UTR and mutant (mut) MUC1 3’UTR fragments were inserted into the pmirGLO promoter vector (Promega) downstream of the luciferase gene to generate the recombinant luciferase reporter vectors pmirGLO-MUC1-wt and pmirGLO- MUC1-mut, respectively. For the luciferase reporter assay, the recombinant plasmids were co-transfected with miR-1291 mimic or NC into EC9706 cells. After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer’s instructions.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software. Data are expressed as mean ± standard deviation (SD). The Student’s t-test was performed to compare sample means. One-way analysis of variance (ANOVA) was used to analyze the significance between different samples. P<0.05 was considered to indicate a statistically significant result. Pearson’s correlation analysis was used to analyze the correlation between different variable elements.

Results

Expression of miR-1291 and MUC1 mRNA in ESCC samples. We investigated the expression of miR-1291 and MUC1 in ESCC samples by qRT-PCR array. Compared with adjacent non-tumorous tissues, miR-1291 expression was significantly lower in ESCC tissues (P<0.05; Fig. 1B), while MUC1 mRNA showed the opposite trend (P<0.05; Fig. 1A). These observations demonstrate that the expression of miR-1291 and MUC1 was negatively correlated in ESCC tissues. In addition, we further analyzed our data and found that miR-1291 expression level in ESCC tissues was associated with lymph node metastases and TNM stage (P<0.05; Table I; Fig. 1C and D). No significant differences were observed between miR-1291 expression and gender, age, differentiation or tumor location (P>0.05; Table I). Distinctly, the MUC1 mRNA expression level in ESCC tissues was associated with lymph node metastases, differentiation and tumor location (P<0.05; Table I). There were no significant differences between MUC1 mRNA expression and TNM stage, gender or age (P>0.05; Table I).

Upregulation of miR-1291 inhibits cell proliferation in EC9706 and EC-1 cells. The CCK-8 and colony formation
assays in EC9706 and EC-1 cell lines were performed to evaluate the effect of miR-1291 on cell growth. According to the CCK-8 assay results, the absorbance of EC9706 or EC-1 cells transfected with miR-1291 mimic was markedly decreased compared to the control groups (NC and blank control) from the second day onwards (P<0.05; Fig. 2A and B). In the colony formation assay, the colony-forming activity of the miR-1291 mimic group was lower than that of the NC group in both EC9706 (Fig. 3A) and EC-1 (Fig. 3B) cells (P<0.05). Based on these results, we concluded that upregulation of miR-1291 inhibited the proliferation of EC9706 and EC-1 cells.

Upregulation of miR-1291 promotes apoptosis in EC9706 and EC-1 cells. In order to determine whether apoptosis resulted in proliferation inhibition, we performed a flow cytometric apoptosis assay. Our data showed that in contrast to NC groups the number of apoptotic EC9706 or EC-1 cells transfected with miR-1291 mimic increased significantly after 48 h (P<0.05; Fig. 4A and B). Based on these results, we concluded that upregulation of miR-1291 promotes apoptosis of EC9706 and EC-1 cells.

Upregulation of miR-1291 restricts cell invasion ability of EC9706 and EC-1 cells. A Transwell assay was performed
to evaluate the role of miR-1291 in regulating the invasion activity of EC9706 and EC-1 cells. Compared to the NC group, the number of EC9706 or EC-1 cells transfected with miR-1291 mimic that penetrated the Transwell membrane was significantly lower (P<0.05; Fig. 5A and B). These results indicated that upregulation of miR-1291 restricted the invasion capacity of EC9706 or EC-1 cells.

miR-1291 downregulates MUC1 expression by binding to its 3’UTR. The TargetScan and miRanda prediction algorithms showed that the MUC1 3’UTR may be directly targeted by miR-1291 (Fig. 6A). In order to verify this targeting relationship, the wild-type and mutant human MUC1 3’UTR fragments were cloned downstream of the firefly luciferase reporter gene in pmirGLO vector (pmirGLO-MUC1-wt and pmirGLO-MUC1-mut) and then co-transfected with a miR-1291 mimic (or NC) into EC9706 cells. The relative luciferase activity of the reporter gene in EC9706 cells co-transfected with pmirGLO-MUC1-wt and miR-1291 mimic was significantly lower (P<0.05) compared with the control group (co-transfected pmirGLO-MUC1-wt and NC) (Fig. 6C).

Figure 3. Overexpression of miR-1291 inhibits colony formation in ESCC cells. EC9706 and EC-1 cells were transfected with miR-1291 mimic or NC. Number of (A) EC9706 and (B) EC-1 colonies in the miR-1291 group was significantly reduced when compared to the NC groups, as measured by the colony-forming assay (P<0.05). Significance (*P<0.05). ESCC, esophageal squamous cell carcinoma; NC, negative control.

Figure 4. Overexpression of miR-1291 promotes apoptosis in ESCC cells. Flow cytometric apoptosis assays were used to assess the apoptosis effect of ESCC cells after transfection. (A) Statistically significant increases in the number of apoptotic cells in miR-1291 mimic-transfected EC9706 cells compared with that in the NC group (P<0.05). (B) Statistically significant increases of apoptotic cells in miR-1291 mimic-transfected EC-1 cells compared with that in the NC group (P<0.05). Statistical significance (*P<0.05). ESCC, esophageal squamous cell carcinoma; NC, negative control.
no difference was found between the relative luciferase activity of cells co-transfected with miR-1291 mimic and cells co-transfected with NC. This result was confirmed by western blot analysis showing that MUC1 expression was downregulated in the EC9706 cells following transfection with miR-1291 mimic (Fig. 6B). These results indicated that miR-1291 negatively regulated MUC1 expression by directly binding to the 3'UTR seed region in ESCC.
Expression of MUC1 abrogates the anti-invasion and pro-apoptosis function of miR-1291. To further clarify MUC1 as a direct target of miR-1291, the function of MUC1 in miR-1291-mediated invasion and apoptosis was investigated. We constructed a recombinant expressing vector pcDNA3.1-MUC1, which contains MUC1 lacking the 3'UTR. The EC9706 cells were transfected with miR-1291 mimic or pcDNA3.1-MUC1 or co-transfected with both. Western blotting showed that co-transfection with pcDNA3.1-MUC1 (lacking the 3'UTR) and miR-1291 mimic increased MUC1 expression. β-actin was used as an endogenous reference. (B) Apoptosis assay showed that transfection with pcDNA3.1-MUC1 (lacking the 3'UTR) and miR-1291 mimic decreased the percentages of apoptotic cells (P<0.05). (C) Transfection with pcDNA3.1-MUC1 (lacking the 3'UTR) and the miR-1291 mimic increased the average number of invading cells, as measured by the Transwell assay (P<0.05). MUC1, mucin 1; 3'UTR, 3'-untranslated region.

Discussion

Increasing studies have shown that miRNAs play important roles in carcinogenesis and progression of esophagus cancer (EC). For instance, miR-1290 was significantly upregulated in ESCC tissue samples, and ectopic miR-1290 expression potently promoted ESCC cell growth, migration and invasion in vitro (25). miR-140 expression was decreased in the EC tissues and regulated the cell invasion of EC via controlling Slug expression (30). It has also been shown that miR-625 expression is lower in EC tissues than in the corresponding adjacent tissues and may regulate the proliferation and invasion of EC cells by targeting Sox2 (31). miR-103/107 expression has been associated with poor survival rates for ESCC patients (32).

In the present study, miR-1291 expression was explored in ESCC. We analyzed the relationship between the miR-1291 expression levels and clinicopathological characteristics of ESCC. The results showed that miR-1291 was significantly downregulated in ESCC tissues and was correlated with lymph node metastases and TNM stage. These results indicated that miR-1291 may be a new diagnosis molecule or therapeutic target for ESCC. Moreover, cell biology experiments in EC9706 and EC-1 cells showed overexpression of miR-1291 inhibited proliferation, restricted cell invasion and resulted in an increased rate of cell apoptosis. These observations suggested that miR-1291 functioned as a tumor-suppressor in ESCC.

Bioinformatics analysis using TargetScan and miRanda revealed that MUC1 is one of the targets of miR-1291. MUC1 is a transmembrane protein that has been identified by its marked overexpression in human carcinomas (33,34). Studies have shown that epithelial Muc1/MUC1 facilitates mucosal wound healing by enhancing cell migration and proliferation, protecting against apoptosis and mediating expression of mucosal modulators (35). In most epithelial-derived cancer cells, MUC1 is overexpressed and loses its apical polarity (36,37). In various adenocarcinoma including ESCC, MUC1 overexpression is correlated with tumor growth, lymph node metastasis and resistance to apoptosis (38), involved in multiple cancer-associated pathways. MUC1 suppresses activation of the ARF-MDM2-p53 pathway (39). In MUC1-expressing cells, a MUC1 co-operating NF-κB signaling pathway plays a critical role in cancer cell invasion (40). MUC1 deficiency impairs NFkB p65, Akt and MAPK pathways, which indicated that MUC1 appears to be a good therapeutic target to slow down esophageal tumor progression (41). In the present study, we detected MUC1 mRNA of tumorous and adjacent non-tumorous human esophagus tissues from 54 patients with ESCC. Our data showed that MUC1 mRNA levels were significantly increased in ESCC tissues and related to lymph node metastasis. In addition, we found that altered MUC1 expression levels were associated with ESCC differentiation status and tumor location. Our results clarified the
relationship between the MUC1 expression levels and clinicopathological characteristics of ESCC.

To confirm that MUC1 was one of the direct functional targets of miR-1291, the 3'UTR region of MUC1 was amplified from human genomic DNA and inserted into the pmirGLO vector to construct a luciferase reporter plasmid, and qRT-PCR, western blotting, luciferase reporter and knockdown assays were performed. Further, restoration assays on invasion and apoptosis were performed and the results showed expression of MUC1 abrogates the anti-invasion and pro-apoptosis function of miR-1291, which confirmed that miR-1291 bound to the 3'UTR of MUC1 mRNA reduced stability and/or inhibited translation involved in proliferation, invasion and apoptosis of ESCC cells.

Collectively, our investigation identified significantly lower expression of miR-1291 in ESCC tissues and that overexpression of miR-1291 suppressed cell growth, invasion and promoted apoptosis in ESCC cells. Our results indicated that miR-1291 acts as a tumor suppressor by targeting MUC1 in ESCC. These new findings suggest that miR-1291 plays an important role in regulating carcinogenesis and development of ESCC and is of value to the diagnosis and therapy of ESCC.

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References


