GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Characterization and effects of miR-21 expression in esophageal cancer

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Genet. Mol. Res. 14 (3): 8810-8818 (2015)
Received November 12, 2014
Accepted April 7, 2015
Published August 3, 2015
DOI http://dx.doi.org/10.4238/2015.August.3.4

ABSTRACT. The aim of this study was to investigate the expression of miR-21 in esophageal cancer and the impact of miR-21 on apoptosis, invasion, and the expression of target genes in esophageal cancer cells. Fluorescence quantitative polymerase chain reaction analysis was used to detect the expression of miR-21 in human esophageal tissues, adjacent tissues, and an esophageal cancer cell line (TE-13). The antisense miR-21 oligonucleotide was generated commercially using the solid-phase chemical synthesis method. Transient transfection was used to transfect esophageal cancer cells (TE-13 antisense and TE-13 control cells). Flow cytometry and Transwell cell assays were used to detect the apoptosis and invasion of esophageal cancer cells, respectively. The western blot method was used to detect the expression of PTEN, PDCD4, and K-ras proteins. These analyses determined that mir-21 expression significantly increased in esophageal cancer tissues and in TE-13 cells, and that this phenomenon was not associated with staging or lymph node metastasis. The apoptosis rate of TE-13 control cells was lower than that of antisense TE-13 cells indicating an enhanced

invasive ability. In tissues adjacent to esophageal cancer and in TE-13 antisense cells, the expression of PTEN and PDCD4 was found to be higher than that in the control group, whereas the expression of K-ras showed the opposite pattern. Together, these results suggest that miR-21 might be involved in the development and metastasis of esophageal cancer, through interaction with its *PDCD4* and *K-ras* target genes.

Key words: Esophageal cancer; MiR-21; Esophageal cancer cell lines; Transfection

INTRODUCTION

Esophageal cancer is a common malignancy. At present, no sensitive indicators of the characteristics of esophageal cancer that can be used for its early detection are available, and most clinical treatments available for patients with advanced esophageal cancer provide a 5-year survival rate of less than 20%. Now many studies have shown that the serum and plasma levels of miR-21 in patients with esophageal cancer are significantly different from those in normal individuals, and it has been suggested that this could be used as a valuable new esophageal cancer biomarker. MicroRNAs (miRNAs) are a class of non-coding small molecule RNA that can regulate gene expression functions at the post-transcriptional level. Abnormal expression of miRNAs in a variety of tumor cells is involved in cell differentiation, proliferation, and apoptosis and is closely associated with tumor development, diagnosis, treatment, and prognosis; thus, these miRNAs have clinical significance (Gramantieri et al., 2008; Selaru et al., 2009). Recent studies have found that miR-21 can enhance the radiosensitivity of esophageal cancer down TE-1 cells, and a number of studies have shown that miRNA expression is significantly associated with esophageal cancer prognosis (Hummel et al., 2011; Yang et al., 2013). In this study, we examined miR-21 expression in esophageal tissue and cultured cells and investigated the effects of miR-21 on apoptosis and invasion of esophageal cancer cells using cell transfection techniques. In addition, the relationship between specific oncogenes and tumor suppressor genes was also studied.

MATERIAL AND METHODS

Materials

Samples were obtained from 76 patients who had esophageal squamous cell carcinoma and had undergone surgical treatment from September 2009 to October 2010 at the 4th Hospital of Hebei Medical University. The pathological cancer types were confirmed by preoperative endoscopy and biopsy. The samples originated from 45 men and 31 women aged from 50 to 75 years with a mean age of 59.2 ± 5.5 years. Tumor grade and tumor, lymph nodes, and metastasis (TNM) stage were designated according to the seventh edition of the Esophageal Cancer Staging Criteria of 2009 by the Union for International Cancer Control. Medium and high differentiations were observed in 48 patients, and 22 had low differentiation; 28 were stage T1/T2, and 48 were T3/T4. Lymph node metastasis was present in 41/76 patients. The clinical grading of 10 patients was stage I, 43 were in stage II, and 23 were in stage III. During

the surgery, tissues collected included the central section, for removal of necrotic tissue, as well as peripheral esophagus tissues, which were at least 5 cm from the edge of the tumor. The stub-end of each esophagus was confirmed to have normal esophageal epithelium by pathologic examination. Tissues were immediately frozen in liquid nitrogen and stored at -80°C in a freezer after removal.

Cell culture and oligonucleotide transfection

The human esophageal cancer cell line TE13 was provided by the Research Center of the Fourth Hospital of Hebei Medical University. RPMl l640 medium containing 10% fetal bovine serum was used for conventional culture at 37°C and 5% CO_2 in a humidified incubator; cells in logarithmic growth phase were taken as the normal group. The experimental group underwent transfection of the antisense miR-2l (AS-miR-21) as follows: 5 μg AS-miR-21 oligonucleotide (5'-UCA ACA UCA GUC UAU AAG CUA-3', synthesized by Guangzhou Funeng Gene Co., Ltd., Guangzhou, China) and l mL Oligofectamine were mixed in a 1:1 ratio. The mixture was added into the cell culture medium; cells in logarithmic growth phase were taken as the antisense group. The same method was used to transfect the negative control plasmid (5'-CAG UAC UUU UGU GUA GUA CAA-3') as the control group.

Detection of miR-21 expression

Cells in the TE-13 antisense and control groups were adjusted to 1 x 10^5/mL, and divided and cultured in 25 mL flasks until confluent. The RNA extraction reagent TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. Total RNA from 100 g esophageal carcinoma and adjacent tissues that had been crushed in liquid nitrogen was extracted using the same method. cDNA was synthesized using AMV reverse transcriptase. The proper amount of cDNA was taken as the template for polymerase chain reaction (PCR) amplification using Taq DNA polymerase. The miR-21 primers used were according to Tang et al. (2006): upstream primer: 5'-ACA CTC CAG CTT AGT GCT TAT CAG ACT GA-3'; downstream primer: 5'-GTG TCG TGG AGT CGG CAA TTC-3'.

The PCR amplification conditions were as follows: denaturation at 95°C for 5 min, and 22 cycles of 95°C for 30 s, 60°C for 30 s, and 68°C for 30 s. The size of the PCR product was 67 bp. All reactions were run in triplicate. The number of cycles or the Q value when the fluorescence signal reached the set threshold value in each reaction tube were recorded; *GAPDH* was used as an internal reference. The Ct values of specimens in each reaction tube were recorded, and the results were analyzed using the relative quantitation method of quantitative PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate miR-21 expression in the cells and the tissues.

Transwell cell assay for cell invasion

The bottom of a Transwell chamber was pre-coated with 40 μL artificial Matrigel, evenly spread in each hole. Cells in each group were used and subjected to 24-h starvation after withdrawal of serum. After completion of digestion, centrifugation was performed to remove the culture medium, and the pelleted cells were washed twice with phosphate-buffered

saline (PBS), followed by adjustment of the cell density to 10^5/mL. A 100-µL cell suspension aliquot was added to the Transwell upper chamber, and 500 µL medium containing 10% serum was added to the lower chamber. After routine culture for 24 h, the Matrigel was carefully wiped from the upper chamber, and placed in 75% ethanol for 15 min fixation, crystal violet staining for 15 min, and repeated rinses with PBS to wash away the excess crystal violet. Samples were observed under a microscope at 200X; cells were counted in five random fields, with the means taken for calculation.

Cell cycle and apoptosis detection with FCM

TE-13 antisense and control cells were collected. The number of cells in each sample was adjusted to 1 x 10^6, and 1 mL phosphoinositol (PI) staining liquid was added (50 µg/mL PI, 10 µg/mL RNA enzyme and 1% Triton-X 100). Chicken red blood cells were taken as an internal reference; they were stained simultaneously with the sample, and normal lung cells were taken as the diploid type. For staining, 1 mL staining liquid was added to each sample at 4°C, and the mixture was incubated in the dark for 30 min. The cells were then filtered through a 500-mesh sieve cloth to reduce the sample to a qualified single cell suspension before apoptosis detection.

Cell-associated protein detection

Cells in each group were collected to extract total protein; the Coomassie brilliant blue method was used for protein quantification. A total of 100 µg protein was collected for SDS-polyacrylamide gel electrophoresis and membrane transfer. For protein detection, the membrane was blocked with 50 g/L skim milk powder and kept in a sealed 37°C shaker for 1 h, followed by addition of programmed cell death gene (PDCD4), PTEN, or K-ras rabbit anti-human monoclonal antibodies (1:200, Zhongshan Company, Zhongshan, China) with incubation overnight at 4°C. The membranes were then washed with PBS for 3 x 15 min, and horseradish peroxidase labeled goat anti-rabbit IgG secondary antibody (1:l000, Zhongshan Company) was added and incubated at 37°C for 1 h. The membranes were subsequently washed in Tris-buffered saline plus 0.05% Tween-20 for 3 x 15 min, incubated in ECL detection reagent, and set on preservation film (chemiluminescence substrate) for 3-5 min. The membrane was exposed to x-ray film in a cassette for 1 min, and the film was developed and scanned. The Lab Works 4.5 software provided by UVP (Upland, CA, USA) was used for quantitative analysis of western blot bands. The relative absorbance value of the hybridization band was determined, and the ratio of the absorbance value of PTEN, PDCD4, and K-ras versus the absorbance value of β-actin was used to represent the relative expression level of each protein. Each indicator in each sample was repeated three times, and the mean was taken for calculation.

Statistical analysis

The SPSS 17.0 statistical software (SPSS, Chicago, IL, USA) was used for analysis, and continuous data are reported as means ± SD and compared using the *t*-test; $P < 0.05$ was considered to be statistically significant.

RESULTS

miR-21 expression

Real time reverse transcription (RT)-PCR was used to detect miR-21 expression in cells in the TE-13 antisense and TE-13 control groups in lung cancer tissues and adjacent tissues. The real time RT-PCR product melting curve showed a single peak (Figure 1A), suggesting that stem-loop RT-PCR could specifically amplify miR-21 fragments. The amplification curves in Figure 1B showed that the Ct values of miR-21 in cancer tissues were significantly lower than those in corresponding adjacent tissues, indicated that the expression of miR-21 in cancer tissues was higher than in adjacent tissues. The Ct value of miR-21 in T1/T2 samples was 7.25 ± 2.3, which was significantly higher than the value in T3/T4 samples (29.54 ± 13.13) (t = 8.57, P = 0.00). The Ct value of miR-21 in patients with lymph node metastasis was 29.12 ± 15.52, which was significantly lower than the value (10.74 ± 8.64) in patients without lymph node metastasis (t = 8.57, P = 0.00). The Ct value of miR-21 in the TE-13 control cells was 34.65 ± 11.59, which was significantly lower than that of the TE-13 antisense cells (8.65 ± 2.76); this indicated that the expression miR-21 in the TE-13 control cells was higher than that in the TE-13 antisense cells.

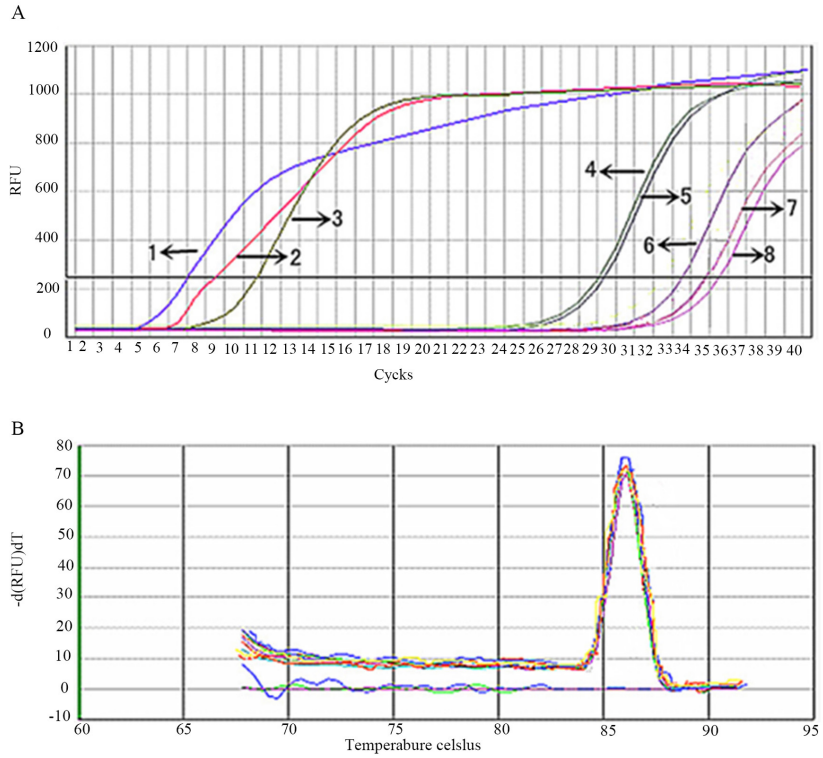

Figure 1. Mir-21 expression. **A.** Mir-21 dissolution curves; **B.** mir-21 amplification curves. Curve 1: mir-21 Ct values at T1/T2; curve 2: TE-13 antisense cells; curve 3: without lymph node metastasis; curve 4: with lymph node metastasis; curve 5: at T3/T4; curve 6: lung cancer; curve 7: TE-13 control cells; curve 8: adjacent tissues.

Cell invasion assessment via the Transwell cell assay

The numbers of cells moving through the polycarbonate film in three random fields in the central part of each Transwell chamber were counted. The cell numbers of the TE-13 control and antisense groups in each field were (50.47 ± 12.89) and (31.86 ± 10.23); the difference was statistically significant (t = 4.23, P = 0.00).

Cell apoptosis in the TE-13 control and antisense groups

The cell apoptosis rates of the two groups were measured using flow cytometry. This analysis showed G2/M in DNA square figure. The apoptotic peak represents the number of cells undergoing apoptosis, and appears before the cell cycle peaks. Quantitative analysis of the apoptotic peak was performed to calculate the apoptosis rate. The apoptosis rate was 10.4 ± 0.6% in the TE-13 control group, which was significantly lower than that in the TE-13 antisense group (50.5 ± 1.2%; P < 0.05) (Figure 2A and B).

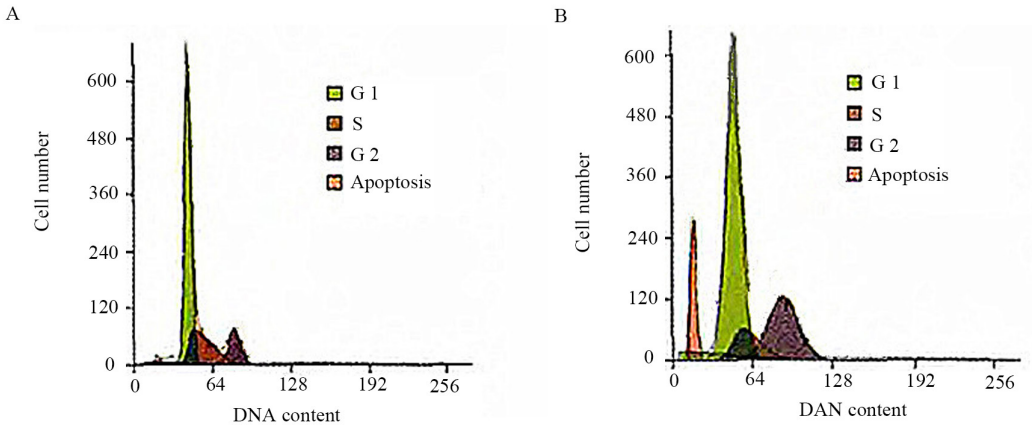

Figure 2. Cell apoptosis in the TE-13 control group (**A**) and antisense TE-13 group (**B**) as measured by flow cytometry.

Protein expression

Western blot analysis was performed to detect the levels of PTEN, PDCD4, and K-ras protein expression in the esophageal cancer tissues and adjacent tissues, and in the TE-13 control and antisense cells, as shown in Figure 3. The expression is shown in Table 1.

DISCUSSION

miRNAs are non-coding single-stranded small molecule RNAs with a size of about 21 to 25 bp; they can regulate gene expression in a variety of eukaryotes. Lee et al. (1993) first discovered the miRNA coding gene *lin-4* in *Caenorhabditis elegans*; since then, a large

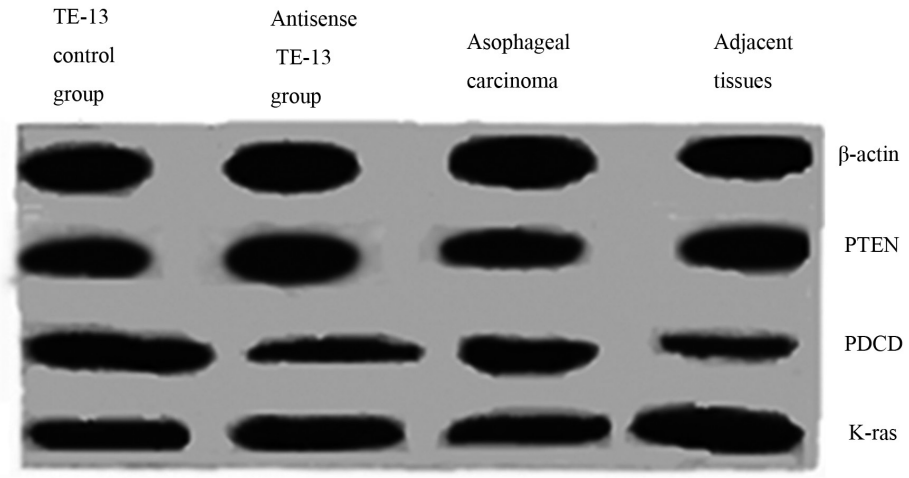

Figure 3. Western blot analysis of PTEN, PDCD4, and K-ras protein expression.

Table 1. PTEN, PDCD4, and K-ras protein expression.

Group	PTEN	PDCD4	K-ras
TE-13 control group	0.35 ± 0.12*	0.21 ± 0.07**	0.48 ± 0.19**
Antisense TE-13 group	0.64 ± 0.11*	0.48 ± 0.43**	0.21 ± 0.04**
Esophageal carcinoma	0.41 ± 0.13*	0.27 ± 0.05**	0.55 ± 0.15**
Adjacent tissues	0.75 ± 0.15*	0.54 ± 0.55**	0.26 ± 0.07**

*P < 0.05; **P < 0.05; TE-13 = esophageal cancer cell line.

number of miRNA genes have been identified in tobacco, fruit flies, nematodes, zebra fish, mammals, and humans. Bioinformatic prediction has shown that there are approximately 1000 miRNA genes in the human genome; these might regulate a third of the gene expression within the genome (Bartel, 2009).

Esophageal squamous cell carcinoma is a common malignancy, with a particularly high incidence and mortality in China. Most patients are already in an advanced phase upon diagnosis, and available treatments are ineffective. Increasing amounts of evidence have shown that miRNAs are associated with esophageal cancer, and its development and invasion. Though the molecular genetics of esophageal cancer has been extensively studied, the molecular mechanisms are still not clear. In recent years, the role of miR-21 in tumors has received ever increasing amounts of attention. As for other miRNAs, miR-21 is transcribed and generated by RNA polymerases II and III in the nucleus. It is then processed by the nuclease Drosha into hairpin precursor pre-miR-21s and is finally transported to the cytoplasm and cleaved into mature miR-21 by the Dicer enzyme. Increased expression of miR-21 has been seen in many tumor tissues (Hiyoshi et al., 2009). For example, studies have shown that elevated miR-21 in breast cancer tissues was associated with breast cancer staging, lymph node metastasis, and poor prognosis in patients (Yan et al., 2008).

This study detected miR-21 expression in esophageal cancer and adjacent tissues, as well as in TE-13 antisense and control cells. The results showed that miR-21 expression in

esophageal cancer tissues was significantly higher than that in the adjacent tissues, and that miR-21 expression in TE-13 cells was significantly higher than that in miR-21 antisense TE-13 cells. In addition, miR-21expression was found to be associated with tumor invasion depth and lymph node metastasis. miR-21 expression in phase T3/T4 samples was significantly higher than that in phase T1/T2 samples, suggesting that higher miR-21 expression occurred upon deeper tumor invasion. Furthermore, miR-21 expression in the esophageal cancer tissues of patients with lymph node metastasis was higher than that in patients without lymph node metastasis. We also used a Transwell cell invasion assay to further detect the impact of miR-21 on esophageal invasion ability. The results of this analysis showed that TE-13 cells had stronger invasion ability when compared to miR-21antisense TE-13 cells.

Flow cytometry was used to measure the apoptosis in esophageal cancer tissues, adjacent tissues, TE-13 cells, and antisense TE-13 cells. The results showed that the apoptosis rate in adjacent tissues was significantly higher than that in cancer tissues. The apoptosis rate in phase T1/T2 cancer tissues was significantly higher than that in phase T3/T4 cancer tissues. Further analysis of the linear relationship between miR-21 expression and apoptosis showed that the two had negative correlation. The results suggested that high miR-21 expression was associated with abnormal esophageal cancer proliferation and was protective against apoptosis.

In summary, the results of this study indicated that miR-21 is likely involved in the incidence, development, invasion, and metastasis of esophageal cancer, and plays the role of an oncogene. However, the mechanisms underlying this function of miR-21 are still unknown. In colon cancer cell lines, miR-21 expression levels were shown to be negatively correlated with PDCD4 (Asangani et al., 2008). miR-21 knockout induced the upregulation of PDCD4 protein and decreased cell invasion ability.

In glioma cells, miR-21 was shown to negatively regulate matrix metalloproteinase inhibitors; activation of matrix metalloproteinases would thereby promote tumor cell invasion (Gabriely et al., 2008). We further detected PTEN, PDCD4, and Bcl-2 protein expression in esophageal cancer tissues, adjacent tissues, TE-13 cells, and TE-13 antisense cells. These analyses showed that the expression of PTEN in TE-13 cells, TE-13 antisense cells, and cancer tissues was significantly lower than that in the normal adjacent tissues, and that PDCD4 and Bcl-2 expression significantly increased in cancer tissues and in TE-13 cells. *PTEN* can act as a tumor suppressor gene, and some studies have shown that deletion of chromosome 10q results in *PTEN* gene mutations in some tumors, thereby eliminating gene expression (Ohgaki and Kleihues, 2007; Suvasini et al., 2011). One study determined that *PTEN* gene expression was decreased in esophageal cancer tissues, and that the level of PTEN protein expression was negatively correlated with tumor size, lymph node metastasis, and the staging of esophageal cancer (Hou et al., 2011). The study found that the carcinogenicity of mir-21 was independent of low *PTEN* gene expression. *PDCD4* is an apoptosis-related gene (Shibahara et al., 1995). Our findings regarding PDCD4 elevated expression is consistent with the findings by Liu et al. (2011), that downregulated miR-21 expression can cause upregulation of PDCD4. Among ras oncogenes, K-ras had the greatest impact on human cancers. Highly expressed miR-21 in lung cancer tissues has been associated with K-ras mutations and been suggested as a tumor marker of lung cancer in clinical applications (Capodanno et al., 2013). Our study showed that miR-21 antisense expression in TE-13 cells can cause decreased expression of K-ras protein, suggesting that downregulation of miR-21 expression can inhibit K-ras gene expression.

Conflicts of interest

The authors declare no conflict of interest.

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