MicroRNA-21 Regulates the Proliferation and Invasion in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: MicroRNAs are ~22 nucleotide noncoding RNA molecules that posttranscriptionally regulate gene expression. The aim of this study was (*a*) to determine a role of microRNA-21 in esophageal squamous cell carcinoma and (*b*) to elucidate the regulation of the programmed cell death 4 (*PDCD4*) gene by microRNA-21.

Experimental Design: MicroRNA-21 expression was investigated in 20 matched normal esophageal epitheliums and esophageal squamous cell carcinomas and seven esophageal squamous cell carcinoma cell lines (TE6, TE8, TE10, TE11, TE12, TE14, KYSE30) by TaqMan quantitative real-time PCR and *in situ* hybridization. To evaluate the role of microRNA-21, cell proliferation and invasion were analyzed with anti–microRNA-21–transfected cells. In addition, the regulation of PDCD4 by microRNA-21 was elucidated to identify the mechanisms of this regulation.

Results: Of 20 paired samples, 18 cancer tissues overexpressed microRNA-21 in comparison with matched normal epitheliums. Specifically, patients with lymph node metastasis or venous invasion showed significantly high expression of microRNA-21. *In situ* hybridization for microRNA-21 showed strong positive staining in paraffin-embedded esophageal squamous cell carcinoma tissues. All seven esophageal squamous cell carcinoma cell lines also overexpressed microRNA-21, and anti–microRNA-21–transfected cells showed significant reduction in cellular proliferation and invasion. The PDCD4 protein levels in esophageal squamous cell carcinoma cells have an inverse correlation with microRNA-21 expression. Anti–microRNA-21–transfected cells increased PDCD4 protein expression without changing the PDCD4 mRNA level and increased a luciferase-reporter activity containing the PDCD4-3′ untranslated region construct.

Conclusions: MicroRNA-21 targets PDCD4 at the posttranscriptional level and regulates cell proliferation and invasion in esophageal squamous cell carcinoma. It may serve as a novel therapeutic target in esophageal squamous cell carcinoma.

Esophageal cancer occurs worldwide with a variable geographic distribution and ranks eighth in order of occurrence and the sixth leading cause of cancer mortality, affecting men more than women (1). It has two main forms, each with distinct etiologic and pathologic characteristics, esophageal squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma is the most frequent subtype of esophageal cancer, although the incidence of adenocarcinoma in the Western world is increasing faster than other esophageal malignancies. A large number of investigations on esophageal cancer have identified a variety of molecules, such as p53, cyclin D1, BRCA2, FAS, vascular endothelial growth factor, Wnt-1, and DEC1, which are associated with the development of esophageal squamous cell carcinoma (2–8). However, precisely how specific molecules regulate the initiation and progression of esophageal squamous cell carcinoma and are utilized for a clinical target therapy are aspects that still remain to be elucidated.

MicroRNAs are a class of naturally occurring small noncoding RNAs that control gene expression by targeting mRNAs for translational repression or cleavage (9–11). Primary transcripts are cleaved by Drosha, thus leading to pre-microRNAs of ~70 nucleotides and processed to 17- to 24-nucleotide microRNAs via Dicer (12). MicroRNAs have important regulatory functions in processes such as differentiation, proliferation, and inhibiting apoptosis (13, 14). Furthermore, it has recently been shown that some microRNAs may act as either oncogenes or tumor suppressor genes (oncomirs; ref. 15). Tumor suppressive microRNAs are usually underexpressed in tumors. For instance, let-7, miR-15, and miR-16 are down-regulated or deleted in lung cancer and leukemia (16–18). In contrast, oncogenic microRNAs are frequently overexpressed in tumors. Specifically, microRNA-21 stands out as the microRNA most often overexpressed in very diverse types of malignancy, and

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Translational Relevance

In the present study, we showed that microRNA-21 was overexpressed in esophageal squamous cell carcinoma tissues and anti-microRNA-21 inhibited both cellular proliferation and invasion *in vitro*. Some previous studies showed a possibility that anti-microRNA-21 may have potential therapeutic value. For example, it was shown that high microRNA-21 expression was associated with poor survival for those who received adjuvant chemotherapy in patients with stage II or III colon cancer. In addition, it was shown that inhibition of microRNA-21 increased sensitivity to anticancer drugs in cancer cell lines. These findings may show that chemotherapy combined with microRNA-21 suppression is more effective than chemotherapy alone. Therefore, we suggest that microRNA-21 may serve as potential target for cancer therapy in patients with not only esophageal squamous cell carcinoma but also various types of cancer.

microRNA-21 expression and cancer-related processes such as proliferation, apoptosis, invasion, and metastasis have been shown (19–31). In recent studies, several target genes of microRNA-21 associated with cancer progression have been identified: the phosphatase and tensin homologue (23, 24), tropomysin 1 (32), maspin (33), and the programmed cell death 4 (PDCD4; refs. 33–36). In human esophageal squamous cell carcinoma, although there are a few reports about microRNA expression profiling (25, 37), neither a functional analysis nor the target genes of microRNAs have yet been documented.

In a bioinformatic analysis, PDCD4 has been known to contain the microRNA-21 binding site, which acts as a tumor suppressor gene regulating various aspects associated with cancer progression (cell proliferation, invasion, metastasis, and neoplastic transformation; refs. 33–36). In this study, we present data indicating the correlation of overexpression of microRNA-21 with lymph node metastasis or venous invasion of esophageal squamous cell carcinoma *in vivo* and the promotion of cell proliferation and invasion of esophageal squamous cell carcinoma *in vitro*. Based on these various aspects of the microRNA-21 function in our study, we hypothesized that microRNA-21 played an important role in the progression of esophageal squamous cell carcinoma and regulated PDCD4 as one of the several targets of microRNA-21. The present study was done (*a*) to determine the role of microRNA-21 in human esophageal squamous cell carcinoma and (*b*) to elucidate the regulation of PDCD4 by microRNA-21 and identify target motifs and mechanisms of this regulation. This is the first report to show that microRNA-21 negatively regulates PDCD4 while also promoting proliferation and invasion in esophageal squamous cell carcinoma.

Materials and Methods

Tissue samples, cell lines, and reagents. Twenty pairs of primary esophageal squamous cell carcinoma tissues and matched normal esophageal epitheliums were obtained from patients in Kumamoto University Hospital from 2007 to 2008, with informed consent and agreement. All tissue samples were from untreated patients undergoing surgery and were snap frozen in liquid nitrogen and stored at -80°C until the extraction of RNA. For all the samples, clinicopathologic information (age, gender, pathology, differentiation, tumor-node-metastasis classification) was available. The study was approved by the medical ethics committee of Kumamoto University. Human esophageal squamous cell carcinoma cell lines TE6, TE8, TE10, TE11, TE12, and TE14 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. KYSE30 was purchased from American Type Cell Collection. All cells were grown in RPMI 1640 (Cambrex) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mmol/L glutamine, 100 units of penicillin/mL, and 100 μg of streptomycin/mL (Cambrex), and incubated at 37°C in a humidified chamber supplemented with 5% CO_2. Probes of human microRNA-21 and scramble for *in situ* hybridization were purchased from Exiqon. Anti–microRNA-21 oligonucleotide and negative control oligonucleotide were purchased from Ambion. Anti-PDCD4 antibody was purchased from Rockland. Anti–β-actin antibody was from Cell Signaling Technology, Inc. All PCR primers were purchased from Genenet.

Real-time PCR–based detection of microRNA-21 and PDCD4 mRNA. Total RNA was obtained from tissue samples using the MELT Total Nucleic Acid Isolation System (Ambion) and from cell lines using the mirVana microRNA isolation kit (Ambion), according to the manufacturer's instructions. The expression of mature microRNAs was determined by TaqMan quantitative real-time PCR using the TaqMan microRNA assay (Applied Biosystems) and normalized using the $2^{-\Delta\Delta}$CT method (38) relative to U6–small nuclear RNA. PDCD4 mRNA was quantified by SYBR-Green–quantitative real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. PCR primers used were as follows: PDCD4, 5′-agtgacgcccttagaagtgg-3′ (forward) and 5′-tcatatccacctcctccaca-3′ (reverse), and glyceraldehyde-3-phosphate dehydrogenase, 5′-tgaccacagtccatgccatc-3′ (forward) and 5′-ccaccctgttgc-tgtagcc-3′ (reverse). All PCRs were done in triplicates.

In situ *hybridization of microRNA.* *In situ* hybridization was done according to the manufacturer's protocol for formalin-fixed, paraffin-embedded tissue written by Kloosterman et al. (39) on human esophageal tissue. The probes were 5-labeled with digoxigenin with the DIG tailing kit (Roche). Tailed LNA oligonucleotides were purified and used for overnight hybridization at 52°C.

The staining was carried out as previously described. After deparaffinization, the specimens were subjected to proteinase K (20 μg/mL) digestion for 20 mins. The postfixed tissues were subsequently incubated overnight with the locked nucleic acid–modified probes. For the immunodetection, tissues were incubated overnight at 4°C in anti–DIG-AP FAB fragment (Roche; 1/2,000). The final visualization was carried out with NBT/BCIP (Pierce).

Cell proliferation assay. For cell proliferation assay, transfections were carried out in triplicate. To transfect, 5 pmol inhibitor was diluted into 10 mL Opti-MEM into each well. Next, 0.3 mL NeoFx (Ambion) was diluted into 10 mL Opti-MEM for each sample, incubated for 10 mins at room temperature and 10 mL of diluted transfection mixture was added to wells (96-well plate) that already contain the inhibitors and incubate for another 10 mins at room temperature. Then, 100 mL of diluted cell suspension mixture containing 5,000 cells was added on top of the complex, the medium was changed after 24 h, and total RNA was isolated after 48 h from transfections. The cell proliferation assay (96-well plate) was carried out using the WST-8 assay with a Cell Counting Kit-8 (Dojin Laboratories) after 72 h from transfections, as previously described (40). Absorbance was measured at 450 nm.

Invasion assay. Cell invasion was assessed using the Matrigel Invasion Chamber (BD Biosciences) in triplicate. Cells (1.0×10^5) transfected with either control anti-microRNA or anti–microRNA-21 plated on transwell chambers precoated with Matrigel. Medium

Table 1. The clinicopathologic characteristics in 20 patients with esophageal squamous cell carcinoma

Factors	
Age (median)	59-85 (69.0)
Sex (male/female)	15/5
Tumor size (mm, median)	18-90 (45.0)
Differentiation (well/moderate/poor)	11/17/2
Tumor depth (sm/mp/ad)	6/4/10
Lymph node metastasis (negative/positive)	6/14
Lymphatic invasion (negative/positive)	10/10
Venous invasion (negative/positive)	5/15
P stage (I/II/III/IV)	1/10/6/3

Abbreviations: sm, submucosa; mp, muscularis propria; ad, adventitia.

containing 10% fetal bovine serum in the lower chamber served as the chemoattractant. After the cells were incubated for 22 h at 37°C in a humidified incubator with 5% CO_2, the noninvading cells were removed with cotton swabs. The invasive cells attached to the lower surface of the membrane insert were fixed in 100% methanol at room temperature for 2 mins and stained with toluidine blue. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

Western blotting. For isolating the proteins, cells harvested in 6-well plates were washed once in PBS and lysed in the lysis buffer [Tris-HCl (pH 7.4), 25 mmol/L; NaCl, 100 mmol/L; EDTA, 2 mmol/L; Triton X, 1%; with 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonylfluoride]. SDS-PAGE and Western blotting were done according to standard procedures. Western blotting of β-actin on the same membrane was used as a loading control. The signals were detected by secondary antibodies labeled with ECL Detection System (GE Healthcare), and signal intensity was determined by NIH-ImageJ.

Luciferase reporter assay. The full-length 3′ untranslated region of PDCD4 mRNA containing the microRNA-21 binding site were amplified by PCR [primers, 5′-ggggagctcatataagaactcttgcagtct-3′ (forward) and 5′-gggaagcttggtgtacattcttctagaac-3′ (reverse)] and cloned into the *Sac*I-*Hin*dIII site of the pMIR-REPORT Kit (Applied Biosystems) and named Luc-PDCD4-Wt. To make microRNA-21 binding site deletion mutants, the seed sequences were deleted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), with Luc-PDCD4-Wt as a template and named Luc-PDCD4-d. For the reporter assays, the cells were transiently transfected into 24-well plates with the luciferase vector control vector, and either anti–microRNA-21 oligonucleotide or negative control using Lipofectamine 2000 (Invitrogen). Reporter assays were done at 24 h posttransfection using the luciferase assay kit (Promega). β-Galactosidase activity was used for normalizing the transfection efficiency.

Statistical analysis. A statistical analysis was done using the StatView5.0 software program (SAS Institute). Values are expressed as the mean ± SEM. Differences/correlations between groups were calculated with Student's *t* test, the Mann-Whitney *U* test, and Pearson's correlation test. $P < 0.05$ was defined as being significant.

Results

MicroRNA-21 is overexpressed in esophageal squamous cell carcinomas in comparison with matched normal epitheliums. The clinicopathologic findings of 20 patients are shown in Table 1. Total RNA was isolated from matched normal esophageal epitheliums and esophageal squamous cell carcinoma tissues, and the microRNA-21 levels were determined by TaqMan real-time PCR (Fig. 1). Of 20 matched normal and cancer tissues, 18 cancer tissues overexpressed microRNA-21 in comparison with the matched normal tissues (Fig. 1A). The microRNA-21 expression was significantly higher in esophageal squamous cell carcinoma tissues than in normal tissues ($P < 0.001$; Fig. 1B). Next, the correlation of T/N ratios for microRNA-21 expression with the clinicopathologic factors listed in Table 1 was examined. The T/N ratios in patients with venous invasion or lymph node metastasis were significantly higher than those without them ($P < 0.05$; Fig. 1C and D).

In situ ***hybridization showed microRNA-21 expression in esophageal squamous cell carcinomas and matched normal epitheliums.*** To visualize microRNA-21 expression in tumor and adjacent normal epithelium, we performed *in situ* hybridization in formalin-fixed, paraffin-embedded tissue (Fig. 2). As shown in the H&E staining protocol, this sample was resected from a patient with T_3 stage esophageal squamous

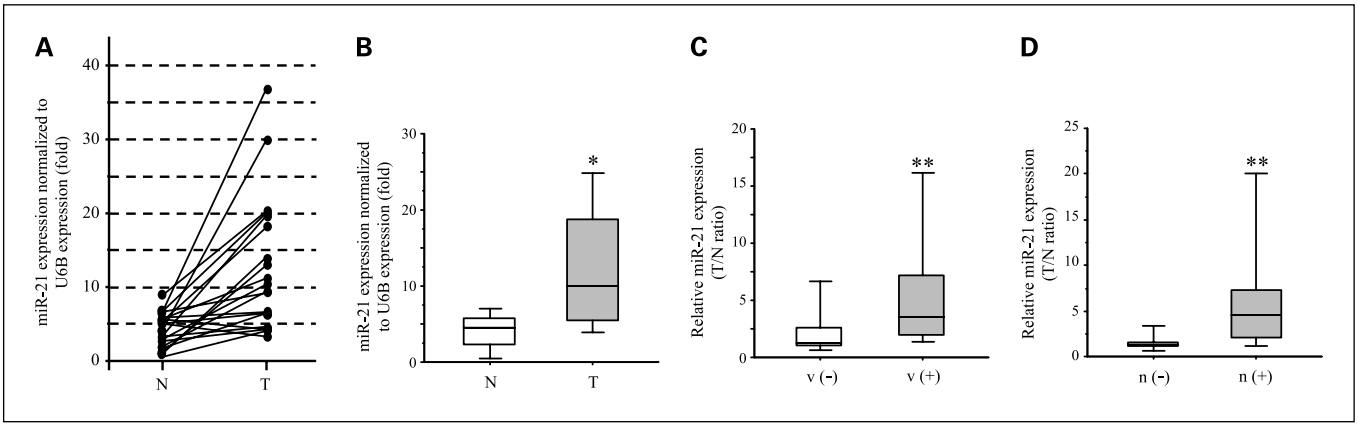

Fig. 1. Overexpression of mature microRNA-21 in esophageal squamous cell carcinoma tissue specimens. Total RNA was isolated from normal and esophageal squamous cell carcinoma tissues using the MELT Total Nucleic Acid Isolation System. Mature microRNA-21 expression was analyzed by TaqMan – quantitative real-time PCR and normalized to U6B expression. *A,* a comparison of microRNA-21 expression between matched normal epitheliums and esophageal squamous cell carcinoma tissues in 20 patients. N, normal tissue; T, tumor tissue. *B,* the data of microRNA-21 expression were analyzed statistically using the Mann-Whitney *U* test. *, $P < 0.001$. *C,* the ratios of tumor to normal tissue for microRNA-21 expression were presented as relative T/N ratio of microRNA-21 expression. The T/N ratios were analyzed statistically in patients with venous invasion or without. v, venous invasion. **, $P < 0.05$. n, lymph node metastasis (*D*).

cell carcinoma (Fig. 2A). An overexpression of microRNA-21 was observed in the same part of the tumor (Fig. 2B). In normal squamous epithelium, there was weakly positive staining for microRNA-21 around a basal layer (shown by an arrow; Fig. 2C). On the other hand, esophageal squamous cell carcinoma showed strongly positive staining. In addition, the staining was stronger in esophageal squamous cell carcinoma cells than in cancer-related stroma (Fig. 2D). The scramble control probe showed no significant staining in either the tumor or nontumor tissue (Fig. 2E-G).

Anti–microRNA-21 inhibitor transfection leads to reduced cell proliferation and invasion in esophageal squamous cell carcinoma cell lines. To test whether microRNA-21 may function as an oncogene, the effect of suppression of microRNA-21 on esophageal squamous cell carcinoma cell proliferation and invasion was examined *in vitro* (Fig. 3). TE6, TE8, and TE10 cells were used for the proliferation assay and TE11 cells for the invasion assay. TE11 cells were suitable for the invasion assay because they showed well invasion to the Matrigel membranes. TaqMan real-time PCR revealed anti–microRNA-21 to significantly reduce the microRNA-21 level after 48 hours (Fig. 3A and C). The anti–microRNA-21–transfected cells showed a significant reduction in the cellular proliferation and invasion (Fig. 3B and D).

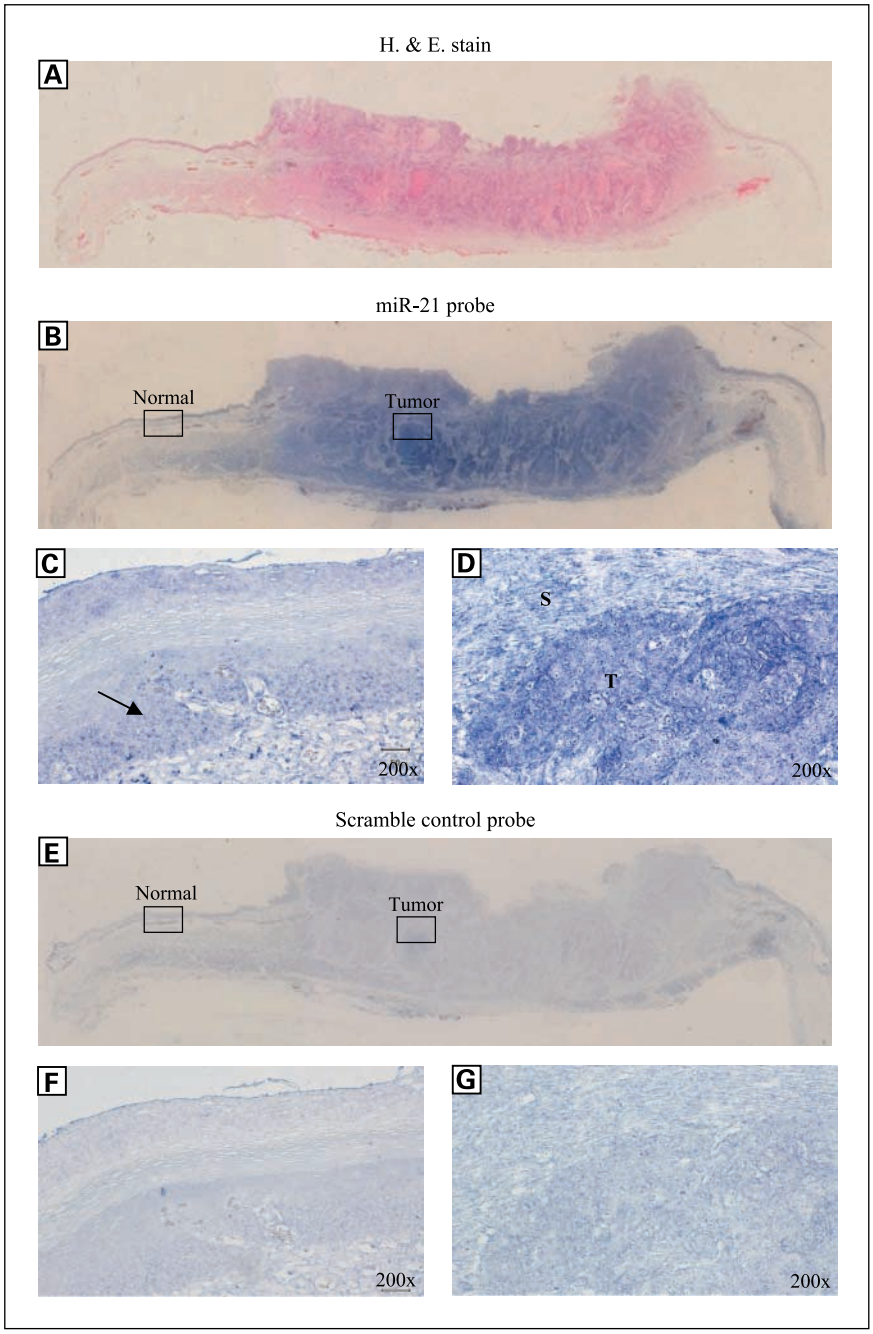

Fig. 2. Detection of microRNA-21 in esophageal squamous cell carcinoma tissues by *in situ* hybridization. *A,* by H&E staining, normal epitheliums and tumor (T_3 stage) were observed in esophageal tissue. *In situ* hybridization with DIG-labeled microRNA-21 LNA probe (*B-D*) and scramble probe (*E-G*) in the serial section. A high-magnification field of normal epithelium (*C* and *F*) and cancer tissue (*D* and *G*). Original magnification, ×200. *B,* microRNA-21 was detected as a strongly positive staining in tumor. Arrowhead, weak positive staining of the normal epithelium (*C*). T, tumor; S, related stromal tissue (*D*). *E, F, G,* the scramble control probe showed no significant staining in the tumor or nontumor tissue.

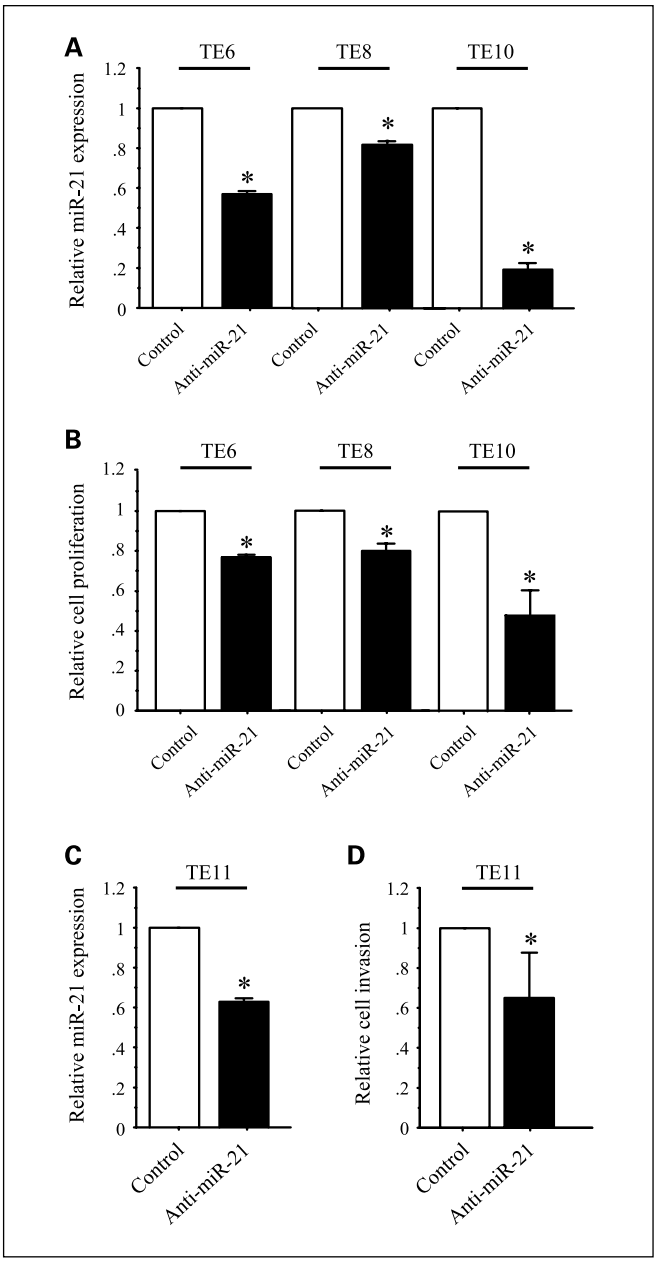

Fig. 3. Inhibition of cell proliferation and invasion by anti – microRNA-21 oligonucleotide. Cancer cells were treated with anti – microRNA-21 oligonucleotide containing Opti-MEM and NeoFX for transfection for 24 h. Cells were further incubated with RPMI 1640 containing 10% fetal bovine serum for the indicated time after rinsing. *A* and *C,* cellular total RNA was isolated after transfection with anti – microRNA-21 oligonucleotide and then the microRNA-21 expression was analyzed by TaqMan – quantitative real-time PCR in TE6, TE8, TE10, and TE11 cells. The results are normalized to U6B expression and are presented as the relative microRNA-21 expression. *, $P < 0.05$. *B,* cell proliferation was analyzed by a WST-8 assay after transfection with anti – microRNA-21 oligonucleotide in TE6, TE8, and TE10 cells. The data are standardized against control each cell lines and are presented as relative cell proliferation. *D,* cell invasion was evaluated in TE11 cells using the 24-well Matrigel Invasion Chamber as described in Materials and Methods. The data are presented as relative cell invasion. The data are representative of three experiments.

PDCD4 protein correlates inversely with microRNA-21 expression in esophageal squamous cell carcinoma cell lines. The expression levels of microRNA-21 and PDCD4 protein were determined in seven different esophageal squamous cell carcinoma cell lines (Fig. 4). All seven esophageal squamous cell carcinoma cell lines showed markedly elevated microRNA-21 expression in comparison with U6B expression (Fig. 4A) by TaqMan real-time PCR. In cell lines with higher endogenous

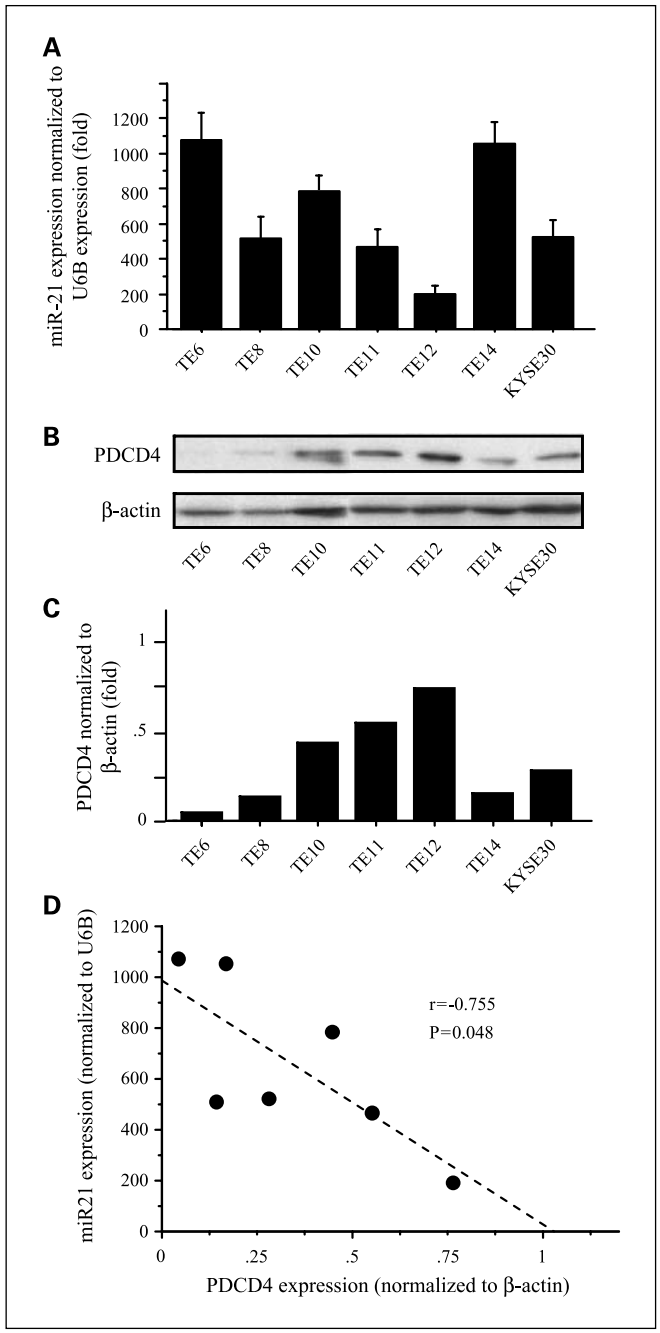

Fig. 4. Correlation between microRNA-21 expression and PDCD4 protein in esophageal squamous cell carcinoma cell lines. Cancer cells were cultured to 70% to 80% confluence in RPMI 1640 containing 10% fetal bovine serum. Cellular total RNA and protein extracts were collected simultaneously and stored at -80°C until analysis. *A,* microRNA-21 expression was analyzed by TaqMan – quantitative real-time PCR. The results are normalized to U6B expression and are presented as relative microRNA-21 expression. *B,* cells lysates were prepared and analyzed by Western blotting. The membranes were blotted by anti-PDCD4 IgG and anti – β-actin IgG. *C,* the autoradiographic densities of each protein band were quantified using NIH-ImageJ. The results were standardized against the levels of β-actin and are presented as the relative density. *D,* correlation between microRNA-21 expression and PDCD4 protein levels (Pearson correlation = -0.755; $P = 0.048$).

microRNA-21 expression (for example, TE6, TE14; Fig. 4A, *lines 1 and 7*), a low amount of PDCD4 protein was observed (Fig. 4B and C, *lines 1 and 7*), whereas cell lines with low microRNA-21 expression (for example, TE12; Fig. 4A, *line 5*) showed high amounts of PDCD4 protein (Fig. 4B and C, *line 5*). Across all seven cell lines tested, there was a significant inverse correlation between microRNA-21 and PDCD4 protein levels (Pearson correlation, $r = -0.755$; $P < 0.05$; Fig. 4D).

MicroRNA-21 regulates PDCD4 expression at the posttranscriptional level by targeting the PDCD4-3′ untranslated region. TE10 cells were used to determine whether the suppression of microRNA-21 affected PDCD4 expression. The down-regulation of endogenous microRNA-21 with anti–microRNA-21 (Fig. 5A) led to a significant increase in PDCD4 protein (Fig. 5C and D) without any change in PDCD4 mRNA (Fig. 5B). Next, to determine whether the 3′ untranslated region of PDCD4 mRNA is a functional target of microRNA-21, a reporter plasmid driven by the SV40 basal promoter, harboring the full-length 3′ untranslated region of PDCD4 mRNA at the 3′ position of the luciferase reporter gene, was cloned (Fig. 5E). The transient transfection of TE10 cells with the reporter plasmid and anti–microRNA-21 inhibitor led to a significant increase of reporter activity in comparison with the negative control (Fig. 5F). However, the activity of the reporter construct deleted at the seed sequences of microRNA-21 target site was unaffected by a simultaneous transfection with anti–microRNA-21 (Fig. 5F).

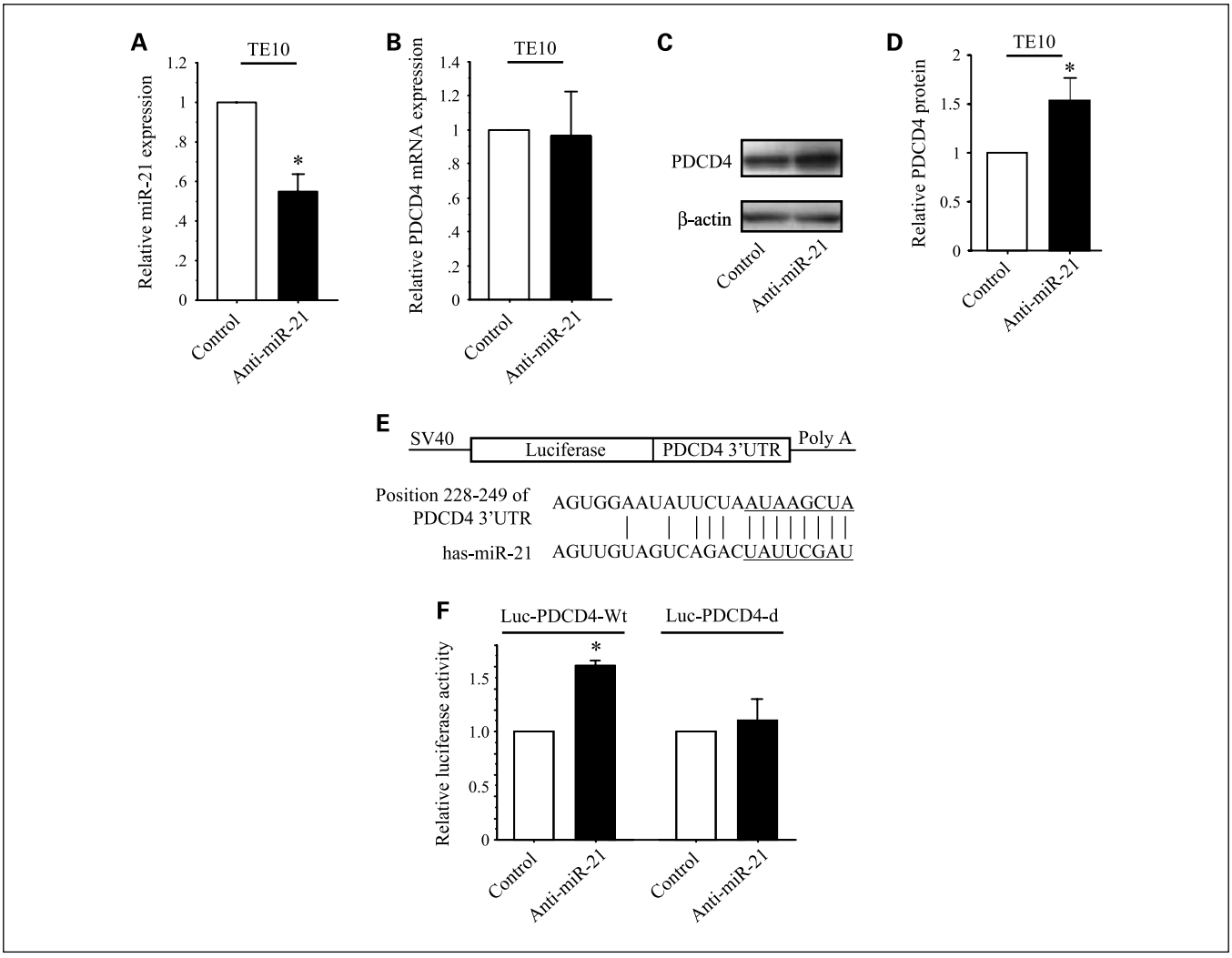

Fig. 5. MicroRNA-21 regulates the PDCD4 expression at the posttranscriptional level by targeting the 3′ untranslated region of PDCD4 mRNA. TE10 cells were transfected with 50 nmol/L anti–microRNA-21 oligonucleotide. After incubation for 48 h, cellular total RNA was isolated and analyzed for microRNA-21 and PDCD4 mRNA. Proteins from the same experiment were isolated 72 h after transfection and used to detect PDCD4 protein by Western blotting. *A,* microRNA-21 expression was analyzed by TaqMan–quantitative real-time PCR. The results are normalized to U6B expression and are presented as relative microRNA-21 expression. *, $P < 0.05$. *B,* PDCD4 mRNA expression was analyzed by quantitative real-time PCR. The results are normalized to β-actin expression and are presented as relative PDCD4 expression. *C,* the cells lysates were analyzed by Western blotting. The membranes were blotted by anti-PDCD4 IgG and anti–β-actin IgG. *D,* autoradiographic densities of each protein band were quantified using NIH-ImageJ. The results were standardized against the levels of β-actin and are presented as the relative density. *E,* the construction of full-length 3′ untranslated region of PDCD4 cDNA containing microRNA-21 binding site for the reporter plasmid. The seed sequences are shown with underline. *F,* the cells were transfected with 200 ng reporter plasmid and 50 nmol/L anti–microRNA-21 oligonucleotide or negative control. After transfection for 24 h, the luciferase activity was measured. The results were normalized to the transfection efficiency using the β-galactosidase activity and were presented as the relative luciferase activity.

Discussion

This is the first study to show that microRNA-21 regulates cellular proliferation and invasion in esophageal squamous cell carcinoma cells. It is also the first study to show that tumor suppressor PDCD4 is negatively regulated by microRNA-21 at the posttranscriptional level via binding to 3′ untranslated region of PDCD4 mRNA in esophageal squamous cell carcinoma cells.

MicroRNAs regulate a variety of cellular pathways through the regulation of the expression of multiple target genes (12). In this regard, microRNA-21 has been suggested to function as an oncogene because it is overexpressed in many types of malignancy such as breast cancer (19, 21), brain tumor (20), lung cancer (19, 30), prostate cancer (19), ovarian cancer (29), pancreatic cancer (19, 22), colon cancer (19, 31), gastric cancer (19, 41), cholangiocarcinoma (23), hepatocellular cancer (24), head and neck cancer (28), and esophageal cancer (25). Furthermore, an association between microRNA-21 expression and prognosis has been shown in some types of cancer. It has been shown that high expression of microRNA-21 was associated with poor survival in patients with colon cancer (42), pancreatic cancer (43), and non–small cell lung cancer (30). However, microRNA-21 expression has been shown to not be associated with the prognosis in gastric cancer patients (41). In the present study, microRNA-21 expression of esophageal squamous cell carcinoma samples was significantly higher than that of matched normal epitheliums as shown in previous studies. When determining the relationship between the microRNA-21 expression and the clinicopathologic factors or prognosis in cancer patients, it remains controversial which is better, n amely, only the cancer expression is used or the cancer/normal expression is used. Some previous reports have analyzed the microRNA-21 expression of cancer tissue in comparison with that of normal tissue (30, 31, 41), whereas others have analyzed only the cancer expression (42, 43). In the present study, we used the T/N ratio of the microRNA-21 expression to determine any associations with clinicopathologic findings because the differences (Fig. 1C and D) were more significant than when the cancer expression was analyzed. The T/N ratio of microRNA-21 expression in patients with lymph node metastasis or venous invasion was significantly higher than in those without them. Although the observation period was too short to evaluate the prognosis of 20 patients enrolled in the present study, these findings show that a high expression of microRNA-21 may therefore be associated with a poor survival in esophageal squamous cell carcinoma patients.

In situ hybridization using LNA probe is useful method to visualize the microRNA-21 expression in formalin-fixed, paraffin-embedded tissues (39). Dillhoff et al. (43) did *in situ* hybridization with tissue microarrays in 80 pancreatic cancer specimens and showed strong expression of microRNA-21 predicted limited survival in patients with node-negative disease. Although TaqMan real-time PCR method easily enables the analysis of the microRNA expression in cancer tissues, it is impossible to distinguish the microRNA expression that originates from the cancer cells or noncancer cells: tumor-related stroma, surrounding normal tissue, blood cells, etc. Therefore, it is important to visualize the microRNA-21 expression, and *in situ* hybridization was used in the current study. As shown in Fig. 2, an overexpression of microRNA-21 was observed in the cancer cells in comparison with either the matched normal epithelium or cancer-related stroma. On the other hand, there was slightly positive staining for microRNA-21 around a basal layer in normal squamous epithelium (Fig. 2F). This finding may show that microRNA-21 plays a role in the differentiation and growth of the normal epithelium.

The tumor suppressor gene *PDCD4* was originally characterized as an inhibitor of cellular transformation in a mouse cell culture model (44). PDCD4 expression is down-regulated or lost in several tumor types (45, 46), and ectopic expression of Pdcd4 reduces tumor formation in a mouse skin cancer model (47). At the molecular level, PDCD4 binds and inhibits the translation initiation factor eukaryotic initiation factor 4a, thereby impacting protein translation (48, 49). In addition, PDCD4 has been found to inhibit activator protein–mediated transactivation (44) and to induce the expression of the cyclin-dependent kinase inhibitor p21 (46). As a result, the loss of PDCD4 confers growth advantages to the cells by several means, thereby facilitating the development of cancer. In the recent studies, PDCD4 was reported as a functional target of microRNA-21 in various aspects of tumor progression: cell proliferation, invasion, metastasis, and neoplastic transformation in breast cancer (33, 34, 36), and invasion, intravasation, and metastasis in colon cancer (35). In the present study, a high expression of microRNA-21 was associated with venous invasion and lymph node metastasis (Fig. 1C and D). Furthermore, suppression of microRNA-21 *in vitro* led to reduction of cellular proliferation and invasion. Therefore, we hypothesized that PDCD4 was also an important target of microRNA-21 in esophageal squamous cell carcinoma. As shown in Fig. 4, microRNA-21 expression correlated inversely with PDCD4 protein levels significantly. In addition, as shown in Fig. 5, anti–microRNA-21–transfected cells led to a significant increase in PDCD4 protein without any change in PDCD4 mRNA. Transient transfection of cells with reporter plasmid containing 3′ untranslated region of PDCD4 mRNA and anti–microRNA-21 inhibitor led to a significant increase of reporter activity. These findings suggest that the PDCD4 is negatively regulated by microRNA-21 at the posttranslational level via binding the 3′ untranslated region of PDCD4 mRNA.

In summary, microRNA-21 is overexpressed in esophageal squamous cell carcinoma tissues, and anti–microRNA-21 inhibits cellular proliferation and invasion *in vitro*. These effects are possibly due to down-regulation of the tumor suppressor PDCD4 by microRNA-21. These findings raise the possibility that anti–microRNA-21 may have potential therapeutic value in esophageal squamous cell carcinoma patients. It has been shown that anti-microRNA oligonucleotides could stay a relatively long period of time in animals (50). Therefore, microRNAs, in particular microRNA-21, may serve as potentially useful targets for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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