SPANDIDOS PUBLICATIONS

MicroRNA-21 induces cell proliferation and invasion in esophageal squamous cell carcinoma

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Abstract. It has been suggested that microRNA-21 (miR-21) functions as an oncogene, as it is overexpressed in many types of tumors compared to adjacent normal tissues. However, the role of miR-21 has yet to be studied in esophageal squamous cell carcinoma (ESCC). miR-21 expression was quantified by real-time reverse transcription polymerase chain reaction in 38 ESCC specimens and their paired non-cancerous mucosa, and in 15 esophageal cancer cell lines (TE1-15). miR-21 expression levels in ESCC tissue were significantly higher than in the corresponding non-cancerous mucosa (6.873±12.664 vs. 1.000, p<0.0001). In patients with more advanced (T3 or T4) tumors, miR-21 expression levels were significantly higher than in those with less advanced (T1 or T2) tumors (P=0.0333). miR-21 expression levels in patients with more invasive infiltrative growth pattern (inf) ß tumors were significantly higher than in patients with less invasive infα tumors (P=0.0166). Among the cell lines studied, TE9 had the lowest and TE1 the highest expression of miR-21. Using the miRNA precursor or antisense miRNA inhibitor, we studied how the level of miR-21 influences the proliferation of ESCC cells. Cell proliferation of the anti-miR-21-transfected cell line was significantly lower, while that of the pre-miR-21-transfected cell line was significantly higher than in the control. In ESCC, miR-21 expression may be involved in tumor growth and invasion.

Introduction

In Japan, esophageal squamous cell carcinoma (ESCC) is the ninth most frequently occuring type of cancer. However, it is the sixth most frequent cause of death from malignant tumors, and the number of deaths it is responsible for has been steadily increasing. ESCC is often diagnosed at an advanced stage and its prognosis remains poor, prompting the search for new treatment strategies. Although pre-operative chemotherapy and chemoradiation therapy are currently used for patients with advanced-stage ESCC, their effectiveness is unsatisfactory. Even among patients with early-stage disease, we have noted that many develop locally recurrent tumors or distant metastases within a short period following curative surgery.

microRNAs (miRNAs) are a class of gene products that have been implicated in several types of cancer (1-3). Several hundred miRNAs have been described in humans (4). miRNAs function as potent regulators of gene expression, and altered miRNA levels result in the aberrant expression of gene products that may contribute to cancer biology (5). miR-21 has been found increased in several types of cancer tissue, and the aberrant expression of miR-21 has been found to contribute to cancer growth (5-8). In this study, we investigated miR-21 expression in 38 patients with ESCC, and evaluated its correlation with clinicopathological features and post-operative survival. We also investigated miR-21 expression in ESCC cell lines and analyzed the effects of anti-miR-21 or pre-miR-21 transfection on the proliferation of ESCC cell lines.

Materials and methods

Cell lines and cell culture. ESCC cell lines (TE1-15) were obtained from the Japanese Collection of Research Bioresources. Cultures were maintained in RPMI-1640 (Sigma) medium supplemented with 10% fetal bovine serum (Gibco) at 37˚C in a humidified 5% CO_2 incubator.

A human esophageal squamous epithelial cell line (Het-1A) was obtained from the American Type Culture Collection and served as the control. Het-1A was maintained in serum-free medium (LHC-9; BioSource, USA) at 37˚C in a humidified 5% CO_2 incubator.

Tissue samples. Samples were obtained from 38 patients with primary ESCC who had undergone radical esophagectomy at the Department of Surgery II, Nagoya City University Medical School, between 2001 and 2004. The study design was approved by the Institutional Review Board of our university hospital, and written consent was obtained from the patients.

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Tumors were classified according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus. Normal esophageal mucosa was taken from apparently non-cancerous mucosa at a distance ≥5 cm from the tumor. Samples were snap frozen in liquid nitrogen and stored at -80˚C until use. The characteristics of the 38 patients with ESCC are shown in Table I.

RNA extraction. Total RNA was extracted from ESCC tissue and its corresponding non-cancerous mucosa using the Absolutely RNA™ RT-PCR Miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Total RNA concentration was adjusted to 2 ng/μl using a spectrophotometer.

Quantitative reverse transcription-polymerase chain reaction. Taq Man miRNA assays (ABI PRISM, Forest City, CA) used the stem-loop method to detect the expression level of mature miR-21. For reverse transcription (RT) reactions, 10 ng total RNA was used in each reaction (5 μl) and mixed with the RT primer (3 μl). The RT reaction was carried out at 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min, and then maintained at 4˚C. Following the RT reaction, 1.33 μl cDNA was used for the polymerase chain reaction (PCR) along with Taq Man primers (2 μl). PCR was conducted at 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 60 sec in the ABI 7500 real-time PCR system. Real-time PCR results were analyzed and expressed as the relative miRNA expression of the threshold cycle (CT) value. RT and PCR primers for miR-21 were purchased from ABI PRISM. U6B was used for normalization.

Transfection. Nuclear transfection was performed using the Nucleofector system (Amaxa Biosystems, Koln, Germany). Cells ($1x10^5$) were suspended in 150 μl Nucleofector solution (Amaxa Biosystems) containing 100 nM miRNA precursor, antisense miRNA inhibitor or the respective controls at room temperature.

MTT assay. Transfected cells were seeded in 96-well plates at a density of $1x10^5$ cells/100 μl. Cell proliferation was measured using the MTT method. After 72 h, 20 μl of 5 mg/ml MTT solution was added to each well, and plates were incubated for 3 h at 37˚C. Absorbance at 490 nm was determined using a SPECTRAmax 340 (Molecular Devices Corporation). Six wells were assayed for each set of conditions and standard deviations (SDs) were determined.

Statistical analysis. Data are expressed as means ± SD. Statistical analyses were performed using the StatView software package (Abacus Concepts, Berkeley, CA). The Wilcoxon signed-rank, Mann-Whitney U and Kruskal-Wallis tests were used to evaluate the significance of differences in the expression levels of miR-21. In all analyses, $P<0.05$ was considered statistically significant.

Table I. Correlation of miR-21 expression with clinicopathological factors in esophageal cancer.

Characteristics	No. Patients (n=38)	miR-21 expression[a]	P-value
Normal	38	1.000	
Tumor	38	6.873±12.664	<0.0001
Age at surgery			
≤65 years	20	3.533±4.222	
>65 years	18	10.584±17.362	0.1438
Gender			
Male	31	7.221±13.698	
Female	7	5.335±6.887	0.9550
Tumor status			
T1	9	2.834±2.184	
T2	7	4.003±6.919	
T3	17	10.936±17.714	
T4	5	4.347±4.218	0.1464
T1, 2 vs. 3, 4			0.0333
Lymph node status			
N0	12	4.332±5.328	
N1	8	2.968±1.906	
N2	13	6.631±9.363	
N3	1	19.973	
N4	2	36.122±49.343	0.7202
Unknown	2	3.515±2.042	
Pathological stage			
0	3	2.423±1.240	
I	3	4.503±3.248	
II	14	3.692±5.013	
III	9	8.249±10.532	
IV	9	12.719±22.785	0.5494
Histological differentiation			
Well	12	11.034±20.719	
Moderate	19	5.262±6.709	
Poor	6	4.259±4.255	0.6828
Unknown	1		
Lymphatic invasion			
Negative	7	3.226±2.346	
Positive	24	9.303±15.462	0.5236
Unknown	7		
Blood vessel invasion			
Negative	9	6.289±9.946	
Positive	22	8.602±15.285	0.8277
Unknown	7		

[a]Expressed as the mean ± standard deviation (SD).

Results

miR-21 expression in esophageal cancer tissue. miR-21 expression was detectable in the ESCC and non-cancerous esophageal mucosal tissues. miR-21 expression was normalized to that of the control U6B small nuclear RNA gene (RNU6B). miR-21 expression levels were significantly higher in ESCC tissue than in the corresponding non-cancerous mucosa (6.873±12.664 vs. 1.000; $P<0.0001$, Wilcoxon signed-rank test) (Fig. 1 and Table I). The relationship between

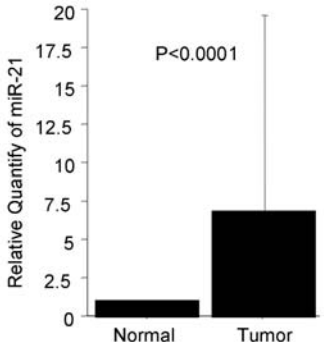

Figure 1. Comparison of miR-21 expression in ESCC tissue and non-cancerous mucosa. miR-21 expression levels were significantly higher in ESCC tissue than in the corresponding non-cancerous mucosa (n=38, p<0,0001).

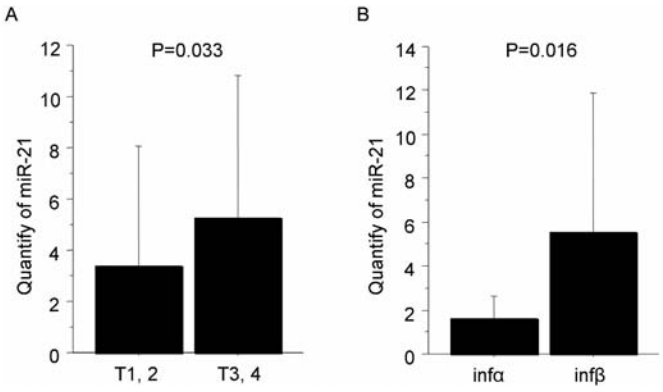

Figure 2. (A) Comparison of miR-21 expression in T1 and T2, as well as more advanced T3 and T4, ESCC. miR-21 expression levels were significantly higher in patients with more advanced T3 and T4 tumors than in those with T1 and T2 tumors (P=0.0333). (B) Comparison of miR-21 expression in infα and -ß ESCC. miR-21 expression levels were significantly higher in patients with infiltrative growth pattern (inf) ß tumors than in those with infα tumors (P=0.0166).

miR-21 expression and patient clinicopathological factors in 38 ESCC samples was examined (Table I). No significant differences were found in miR-21 expression with respect to age, gender, lymph node status, pathological stage, histological differentiation, lymphatic invasion and blood vessel invasion. miR-21 expression levels were significantly higher in patients with more advanced T3 and T4 tumors than in those with T1 and T2 tumors (P=0.0333, Mann-Whitney U test) (Fig. 2A). miR-21 expression levels were significantly higher in patients with infiltrative growth pattern (inf) ß tumors than in those with infα tumors (P=0.0166, Mann-Whitney U test) (Fig. 2B).

The correlation between miR-21 expression levels and survival in ESCC patients following surgery was investigated (median follow-up 27.4 months). No significant difference was observed in ESCC patient survival according to miR-21 expression in the tumor tissues (data not shown).

miR-21 expression in esophageal cancer cell lines and Het-1A. miR-21 expression was evaluated in 15 esophageal cancer cell lines (TE1-15) and one human esophageal squamous epithelial cell line (Het-1A) using quantitative RT-PCR. miR-21 and U6B expression was detectable in all cell lines. TE9 had the lowest and TE1 the highest expression of miR-21 compared with Het-1A in the cell lines studied (Fig. 3).

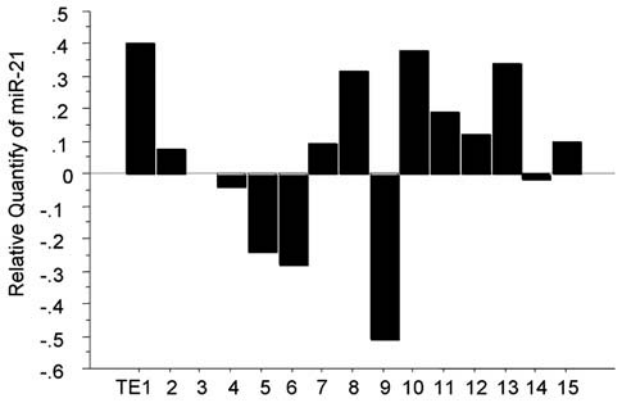

Figure 3. miR-21 expression in esophageal cancer cell lines. TE9 had the lowest and TE1 the highest expression of miR-21 among the cell lines studied as compared to Het-1A.

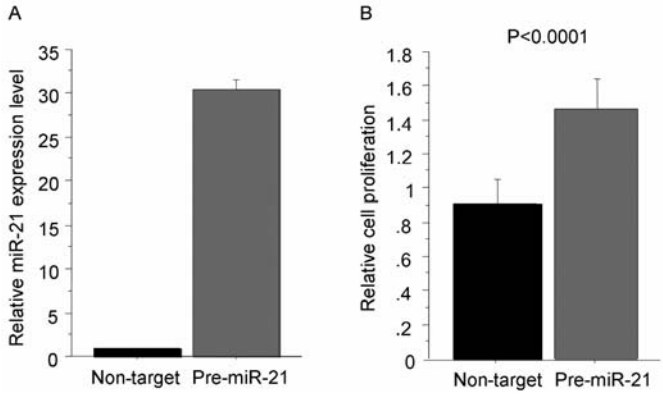

Figure 4. Effects of transfection with pre-miR-21 in TE9 cells. (A) TE9 cells were transiently transfected with 100 nM pre-miR-21 or control precursor. This effect was examined by quantitative RT-PCR on day 3. (B) Cell proliferation of pre-miR-21 or control precursor-transfected TE9 cells. TE9 cell proliferation rates were determined by MTT assay on day 3 and absorbance values were determined on a SPECTRAmax 340 at 490 nm. Proliferation of pre-miR-21-transfected TE9 cells was significantly increased as compared to control precursor-transfected cells.

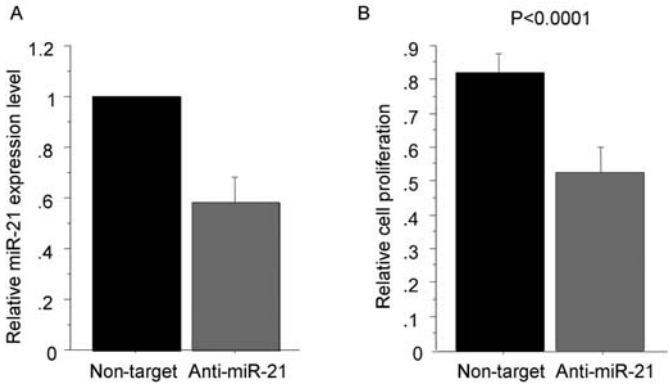

Figure 5. Effects of transfection with anti-miR-21 in TE1 cells. (A) TE1 cells were transiently transfected with 100 nM anti-miR-21 or control inhibitor. This effect was examined by quantitative RT-PCR on day 3. (B) Cell proliferation of anti-miR-21 or control inhibitor-transfected TE1 cells. TE1 cell proliferation rates were determined by MTT assay on day 3 and absorbance values were determined on a SPECTRAmax 340 at 490 nm. Proliferation of anti-miR-21-transfected TE1 cells was significantly decreased as compared to control inhibitor-transfected cells.

Effects of transfection with pre-miR-21 on TE9 cells. The effects of increased miR-21 expression on the proliferation of ESCC were investigated by transfecting the miRNA precursor or respective control into TE9 cells (the lowest expressor of miR-21 among the TE series). The effect of miR-21 expression was examined by quantitative RT-PCR 3 days after transfection. miR-21 expression levels were much higher in cells transfected with pre-miR-21 than in control precursor-transfected TE9 cells (Fig. 4A). MTT assay confirmed that the proliferation of pre-miR-21-transfected TE9 cells was significantly higher when compared with that of the control precursor-transfected cells on day 3 (Fig. 4B).

Effects of transfection with anti-miR-21 in TE1 cells. The effects of inhibiting miR-21 expression were investigated by transfecting the antisense miRNA inhibitor or respective control into TE1 cells (the highest expressor of miR-21 among the TE series). miR-21 expression levels in cells transfected with anti-miR-21 were down-regulated by 58% as compared to the control inhibitor-transfected cells (Fig. 5A). An MTT assay confirmed that the proliferation of anti-miR-21-transfected TE1 cells was significantly lower as compared to the control inhibitor-transfected cells on day 3 (Fig. 5B). Using TE10 cells (the second highest expressor of miR-21 among the TE series), similar effects on proliferation were obtained (data not shown).

Discussion

We have demonstrated that miR-21 expression plays a role in the proliferation of ESCC cells by serving as an accelerator of malignancy. miR-21 is one of the prominent miRNAs implicated in the oncogenesis and progression of human cancer. Not only has it been implicated in the promotion of tumor growth (7), proliferation (9), antiapoptosis (8) and response to gemcitabine-based chemotherapy (10), but it has also been shown to be overexpressed in a variety of tumor types, such as breast cancer (6,11), glioblastoma (8), cholangiocarcinoma (10), hepatocellular carcinoma (5), cervical cancer (12) and Barrett esophagus (13). We previously investigated the expression of a panel of 73 miRNAs in 30 human ESCC samples and their corresponding non-cancerous mucosa, and found miR-21 to be frequently overexpressed in ESCC.

There is a large body of information on putative targets of miR-21 predicted by different algorithm programs. For instance, the Sanger miRNA database target search reveals over 900 targets for miR-21, as miRNAs are believed to bind through a partial homologous sequence to a target gene at the 3'-UTR in animals (14). However, Zhu *et al* (7) suggested that oncogenic miRNAs have a relatively limited number of target genes. Reports have indicated that, in the case of miR-21, tropomyosin 1 (7), phosphatase and tensin homolog (5), as well as programmed cell death 4 (15,16), are the target genes.

miR-21 is located at 17q23.1. Amplification at 17q is associated with several malignancies, such as gastric cancer (17), pancreatic cancer (18) and neuroblastoma (19). In breast cancer, Kallioniemi *et al* (20) reported that the 17q23 amplification is observed in approximately 20% of cases by comparative genomic hybridization (CGH). Monni *et al* (21) showed that this amplification is associated with poor prognosis in breast cancer patients, suggesting that genes affected by it may play a crucial role in breast cancer progression. Amplification at 17q23 detected by CGH has also been reported in tumors of the brain (22-24), lung (25,26), bladder (27), testis (28) and liver (29), indicating that genes located at 17q23 may contribute to the development of various tumor types. Although there are no reports on amplification at 17q in ESCC by CGH, the abovementioned studies included only a small number of patients, which may have been insufficient for a statistically significant conclusion to be drawn.

In this study, we examined miR-21 expression in 38 ESCC tissues and their corresponding non-cancerous mucosa. miR-21 expression levels were significantly higher in the ESCC tissue than in the corresponding non-cancerous mucosa (Fig. 1 and Table I). Among the clinicopathological factors examined, we observed a significant correlation between miR-21 expression, depth of invasion (T factor) and infiltrative growth pattern (inf factor). These results indicate that miR-21 expression may be related to the invasiveness of ESCC. We were not able to demonstrate differences in patient survival according to miR-21 expression.

We have also demonstrated that cell proliferation in ESCC cell lines was inhibited by miR-21 inhibitor (Fig. 5) and enhanced by the overexpression of miR-21 (Fig. 4). Although the precise mechanism of enhanced cell growth by miR-21 is unclear, these results strongly suggest that miR-21 is related to esophageal cancer cell growth, making it a potential target for anticancer therapy.

The expression of many downstream mediators of cell proliferation are potentially modulated by targeting miR-21. To apply therapeutic strategies to decrease miR-21 expression in patients with ESCC, the miR-21 targets involved in this process must be identified. It is our expectation that this will occur in the near future.

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