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Significance and Function of MicroRNA-7 in Oesophageal Squamous Cell Carcinoma

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Abstract. *Background/Aim: We detected microRNA-7 (miR-7) as being specific for oesophageal squamous cell carcinoma (ESCC) by using database analysis. However, the significance of miR-7 in clinical ESCC remains unexplored. This study aimed to clarify the clinicopathological significance of miR-7 in ESCC, and investigate miR-7 function. Materials and Methods: Quantitative TaqMan reverse transcription polymerase chain reaction was used to evaluate miR-7 expression in 85 ESCC samples to determine the clinicopathological significance of miR-7 expression. The regulation of proliferation by miR-7 was examined with miR-7 precursor-transfected cells. Results: The expression of miR-7 in ESCC was higher than that in normal tissues. Low expression levels of miR-7 were associated with poor prognosis. Multivariate analysis indicated that low miR-7 expression was an independent prognostic factor for poor survival. In vitro assays showed miR-7 precursor treatment suppressed the proliferation of ESCC cells. Conclusion: miR-7 might be a promising prognostic marker and therapeutic target in ESCC.*

Survival of patients with advanced oesophageal squamous cell carcinoma (ESCC) remains poor regardless of improvements in perioperative management and definitive and adjuvant therapy (1-3). Locally advanced ESCC directly invades adjacent organs; this increases local recurrence and makes radical surgical resection difficult. Moreover, such ESCC cases exhibit early lymphatic and frequent haematogenous dissemination compared with other types of gastrointestinal cancer (4, 5). Therefore, markers that can predict ESCC progression and prognosis correctly and reliably are needed for improving survival of patients with ESCC.

MicroRNAs (miRs) have attracted attention as important regulators of gene expression. These small non-coding RNAs are 18-25 nucleotides in length and partially bind to the 3'-untranslated region of target mRNAs. This binding of miR and mRNA leads to mRNA degradation or translational repression (6). Depending on their targets, many miRs play important roles in proliferation, apoptosis, differentiation, and cancer progression. In order to find cancer-specific miRs in ESCC, we re-analyzed data with the series accession number GSE6188 in the Gene Expression Omnibus public microarray database (7). As a result, we detected *miR-196a*, *miR-7*, and *miR-503* as being up-regulated by 6.64-, 4.31- and 3.65-fold, respectively, in ESCC compared to normal oesophageal mucosa. The function and expression significance of *miR-196a* and *miR-503* in ESCC have been reported (8-10). In addition, Fu *et al.* reported that the expression level of *miR-7* in primary ESCC tissue was higher than that in non-cancerous tissues (11), and Dong *et al.* described serum *miR-7* expression in patients with ESCC as being higher than that in healthy volunteers (12). However, few studies are available on the significance and function of *miR-7* expression in ESCC.

The purpose of this study was to clarify the clinicopathological significance of *miR-7* expression in ESCC, and to investigate the function of *miR-7* in ESCC cells *in vitro*. Therefore, we examined the expression of *miR-7* in clinical ESCC samples and the *miR-7*-mediated regulation of proliferation in ESCC cells *in vitro*.

Materials and Methods

Cancer-specific miR detection by re-analysis of GSE6188. Re-analysis for GSE6188 was performed by Subio Platform (Subio Inc. Tokyo, Japan). Probes significantly different between cancerous tissues (153 samples) and normal tissues (104 samples) were extracted with a fold-change greater than 3.5 and a *t*-test (equal variance) with $p<0.05$. The probes corresponding to over half of 48 donors with two cancerous tissues and two adjacent normal tissues

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and whose clinical characteristics were significantly different were selected as the three up-regulated miRs.

Clinical samples and RNA isolation. Samples of primary ESCC (n=85) and corresponding normal oesophageal epithelium (n=85) were obtained from patients with ESCC who had undergone potentially curative surgery at the Department of General Surgical Science, Gunma University, Japan, between 1999 and 2006. These samples were used after obtaining written informed consent in accordance with institutional guidelines and the Declaration of Helsinki (approval number 1457). The pathological features of the samples were classified according to the sixth edition of the TNM Classification of the International Union against Cancer (13). The surgical procedures were classified as curative; there was no evidence of residual tumour tissue, and the resected margins were microscopically free of tumour (R0). Normal tissue samples were obtained away from the centre of the tumour in surgical specimens. All samples were immediately frozen in liquid nitrogen and stored at −80˚C until RNA extraction. Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Evaluation of miR-7 expression in clinical ESCC samples. For quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) of *miR-7*, cDNA was synthesized from 10 ng total RNA using the TaqMan MicroRNA Reverse Transcription kit and specific stem-loop reverse transcription primers (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol. PCR was performed in a LightCycler™ 480 system (Roche Diagnostics GmbH, Mannheim, Germany). The 20 μl PCR mixture included in the LightCycler 480 Probes Master kit (Roche Diagnostics GmbH, Mannheim, Germany) was incubated in a 96-well optical plate at 95˚C for 10 min, and then subjected to 45 cycles of 95˚C for 10 seconds and 60˚C for 30 sec. Expression levels of *miR-7* were normalized to that of the small nuclear RNA *RNU6B* and analysed using the $2^{-\Delta\Delta Ct}$ method.

Cell line. The human ESCC cell line TE-8 was obtained from the RIKEN BioResource Center (RIKEN BRC, Tukuba, Ibaraki, Japan). TE-8 cells were cultured in RPMI_1640 medium (Wako, Osaka, Japan) supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA).

Transfection of the miR-7 precursor. Pre-miR™ miRNA Precursor Molecule mimicking *miR-7* (premiR-7; Applied Biosystems) or non-specific control miR (Pre-miR™ miRNA Negative Control #1; premiR-nc; Applied Biosystems) was transfected at 30 pmol/l into TE-8 cells by using an electroporator (CUY 21 EDIT II; BEX, Tokyo, Japan) according to protocol. Before conducting assays, we confirmed that *miR-7* expression in premiR-7 precursor-treated cells was significantly higher than that in the parent TE-8 cells and control miR-treated cells using RT-PCR.

Proliferation assay. Cell proliferation analysis was performed using cells that had been transfected with negative control or premiR-7. The cells were plated in 96-well plates in 100 μl of medium at approximately 3,000 cells per well. For the quantification of cell viability in the WST-8 assay (Dojindo Lab., Tokyo, Japan), 10 μl of the cell counting solution was added to each well after 0, 24, 48, and 72 h and plates were incubated at 37˚C for another 2 hours. The cell proliferation rate was then determined by measuring the absorbance of the well at 450 nm with the reference wavelength set at 650 nm. Absorbance was read using a microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA).

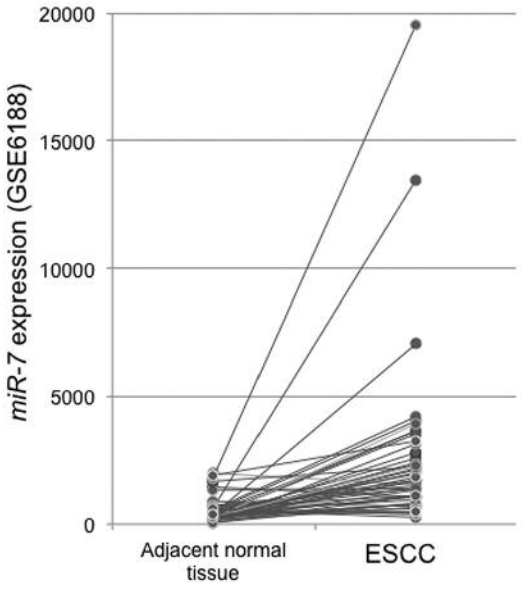

Figure 1. *Clinical significance of miR-7 expression in oesophageal squamous cell carcinoma (ESCC) samples from the re-analysed GSE6188 data. miR-7 levels were shown to be higher in ESCC than those of the adjacent normal tissues.*

Statistical analysis. Differences between groups were estimated using Student's *t*-test, the chi-square test, and the repeated measures ANOVA. Kaplan–Meier curves were generated for overall survival, and statistical significance was determined using the log-rank test. A probability value of less than 0.05 was considered significant. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA).

Results

Clinical significance of miR-7 expression in patients with ESCC. Re-analysis of GSE6188 revealed *miR-196a*, *miR-7* and *miR-503* to be up-regulated in ESCC Among them, we focused on *miR-7* (Figure 1) in order to clarify the clinical significance of *miR-7* expression in clinical ESCC samples. We assessed *miR-7* expression in 85 ESCC samples and 85 paired non-cancerous samples. The expression of *miR-7* was significantly greater in tumour than in normal tissue ($p<0.001$) (Figure 2A).

We divided the 85 patients with ESCC into two groups according to the tumour/normal tissue ratio (T/N) for *miR-7* expression, and the cut-off point was defined as T/N=8.5 (high-expression group, T/N≥8.5, n=31; low-expression group, T/N<8.5, n=54). Clinicopathological factors did not differ between the *miR-7* low-expression and high-expression groups (Table I).

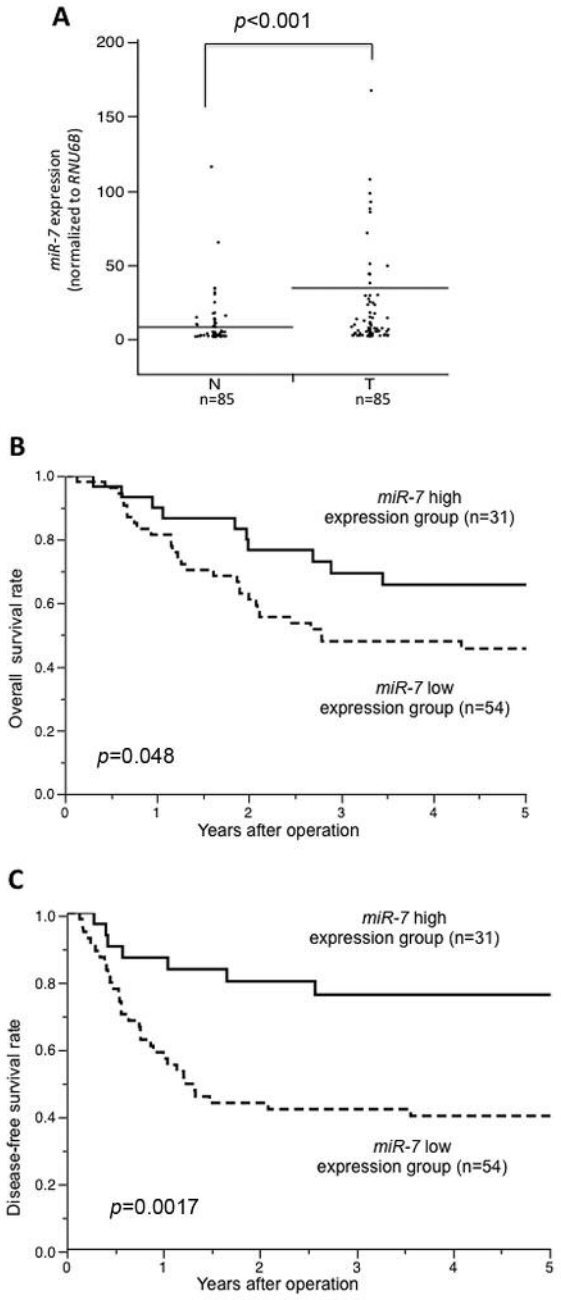

Figure 2. *miR-7 expression in 85 oesophageal squamous cell carcinoma (ESCC) samples of our cohort and its relationship with survival. A: The expression levels of miR-7 in tumour (T) and normal (N) tissue. Overall (B) and disease-free (C) survival curves of patients with ESCC according to miR-7 expression using a cut-off of 8.5.*

Prognostic significance of miR-7 expression in patients with ESCC. The overall survival curve revealed that patients in the low-*miR-7* expression group had a significantly poorer prognosis than those in the high-expression group (p=0.048) (Figure 2B). In univariate and multivariate analyses, low expression of *miR-7* in ESCC was found to be a significant independent prognostic factor (Table II). In multivariate analysis, other investigated clinicopathological factors were not revealed as significant independent predictors of poor prognosis in this ESCC cohort, whereas low expression of *miR-7* in primary ESCC remained prognostically significant with regard to overall survival.

Table I. *Relationship between miR-7 expression and clinicopathological features.*

Factors	*miR-7/RNU6B* Low expression n=54	*miR-7/RNU6B* High expression n=31	*p*-Value
Age			
<65 Years	22	14	0.72
≥65 Years	26	14	
Gender			
Male	47	29	0.34
Female	7	2	
T Factor			
T1	12	7	0.96
T2, T3, T4	42	24	
N Factor			
Absent	17	10	0.94
Present	37	21	
Lymphatic invasion			
Absent	5	1	0.29
Present	49	30	
Venous invasion			
Absent	8	8	0.21
Present	46	23	
M Factor			
Absent	44	26	0.78
Present	10	5	
Stage			0.43
1	8	3	
2	15	14	
3	20	9	
4	11	5	

Regulation of proliferation by miR-7 in ESCC cells. We confirmed that the expression of *miR-7* in *miR-7* precursor-transfected cells was higher than that in control cells (Figure 3A). Proliferation of *miR-7* precursor-transfected cells was significantly reduced from 48 h compared with control cells ($p<0.05$) (Figure 3B).

Discussion

In this study, we clarified that a low expression level of *miR-7* in ESCC was an independent prognostic factor in spite of cancer-specific high expression and weak association with

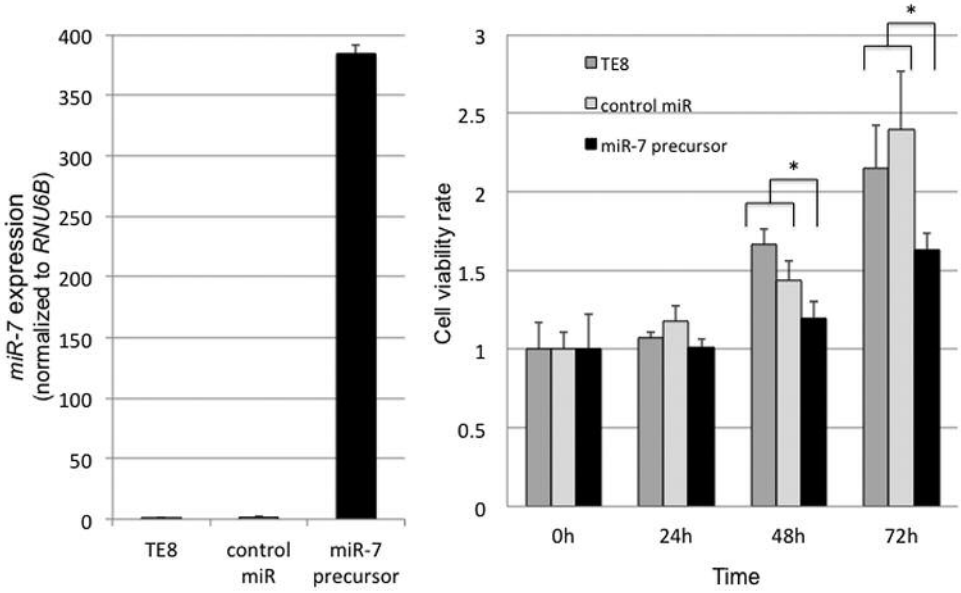

Figure 3. *Proliferation potency of miR-7 precursor-transfected cells. A: Expression of miR-7 was shown to be significantly increased in miR-7 precursor-treated cells. B: The proliferation rate was reduced in miR-7 precursor-treated cells in comparison with that of control miR-treated cells. The data represent mean±SD. *Significantly different at p<0.05.*

Table II. *Results of univariate and multivariate analyses of clinicopathological Factors affecting the overall survival rate following surgery.*

Clinicopathologic variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
T Factor (T1/T2, T3, T4)	0.4	0.15-0.89	0.022*	0.57	0.2-1.33	0.204
N Factor (negative/positive)	0.4	0.19-0.87	0.019*	0.6	0.25-1.3	0.205
M Factor (negative/positive)	0.6	0.29-1.25	0.157	0.88	0.42-2.0	0.76
Lymphatic invasion (negative/positive)	0.21	0.01-0.99	0.0489*	0.4	0.01-3.4	0.428
Venous invasion (negative/positive)	0.46	0.15-1.08	0.0789	0.98	0.28-2.6	0.969
miR-7 expression (high/low)	0.5	0.23-0.97	0.0426*	0.48	0.18-0.86	0.0389*

RR: Relative risk, CI: confidence interval, *$p<0.05$.

increasing severity of clinicopathological factors. On *in vitro* analysis, we found that the proliferation of *miR-7* precursor-treated ESCC cells decreased compared with the control cells.

We focused on *miR-7* as an ESCC-specific miR, and initially predicted that *miR-7* might function as a tumour promoter in ESCC. The expression of *miR-7* was found to be increased in ESCC compared with normal tissue; however, patients with ESCC with low *miR-7* had a poorer prognosis than those with high expression of *miR-7*. Similar to our findings in ESCC, previous study revealed that miR-7 expression in colorectal cancer cells was higher than that in normal cells but that the prognosis was worse in the patients with low expression than in those with high expression (14). On the other hand, down-regulation of *miR-7* expression has been described in thyroid cancer and mesothelioma (15, 16). The detailed mechanism of *miR-7* regulation in ESCC is unclear; however, other researchers have reported that E2F transcription factors regulate *miR-7* expression (17) and that USP18 negatively regulate *miR-7* expression (18). Moreover, Chuo *et al*. reported that the activation of epidermal growth factor receptor (EGFR) in lung cancer cells stimulated *miR-7* expression *via* extracellular signal-regulated kinase (19). E2F and EGFR have been reported to be expressed in ESCC (20, 21); this suggests that *miR-7* might be at least partially regulated by the activation of cancer-specific signals such as E2F and EGFR.

Down-regulation of *miR-7* in ESCC samples was not associated with progression of existing clinicopathological factors; however, patients with *miR-7* down-regulation had

poorer prognosis than those with high miR-7 expression. *miR-7* was reported to be suppressed in chemotherapy-resistant cancer cells (22-24). Moreover, Lee *et al*. described that *miR-7* induction suppressed EGFR and protein kinase B expression, and induced increased sensitivity to radiation treatment in several cancer cell lines (25). Multiple targets have been identified for *miR-7*, including EGFR, human epidermal growth factor receptor 2, Raf-1 proto-oncogene, multidrug resistance-associated protein 1, focal adhesion kinase, insulin-like growth factor 1 receptor, B-cell lymphoma 2, and SATB homeobox1, which are associated with therapeutic resistance and malignant potential in several cancer types (14, 23, 26-30). In this study, we found that *miR-7* precursor treatment suppressed cell growth by ESCC cells. From these observations, we suggest that *miR-7* induction in patients with ESCC might be useful as a therapeutic strategy to overcome resistance to chemotherapy or radiation therapy, which are very important therapeutic tools for patients with ESCC.

In this study, we showed that *miR-7* treatment induced an anticancer effect on ESCC cells *in vitro*. This finding indicates that administration of *miR-7* to patients with ESCC might be a useful means of attacking the ESCC cells. In fact, previous reports have demonstrated that small RNAs administered systemically to humans were able to inhibit specific genes *via* an RNA interference mechanism, and these therapeutic strategies have attracted attention (31-35). Currently, the role of *miR-7* in the human circulatory system is not well understood; however, it has been reported that high levels of serum *miR-7* were associated with high sensitivity to chemoradiation in patients with ESCC (12). Administration of *miR-7* to the circulation might function as a sensitizer to chemotherapy or radiation therapy. In future, determination of an effective means to deliver miRs to cancerous tissue is an important task.

In conclusion, our data indicate that down-regulation of *miR-7* is associated with poor prognosis and reduced proliferative ability in ESCC. The level of *miR-7* in ESCC might be a powerful prognostic factor independent of existing clinicopathological factors. The regulation of miR-7 could provide a promising novel possibility for targeted therapeutic strategies in ESCC.

Conflicts of Interest

None of the Authors has any conflict of interest to declare in regard to this study.

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