Usefulness of microRNA-375 as a prognostic and therapeutic tool in esophageal squamous cell carcinoma

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Abstract. The aim of this study was to clarify the importance of microRNA-375 (miR-375) expression in patients with esophageal squamous cell carcinoma (ESCC) and to examine the *in vivo* antitumor effects of miR-375 in a model of ESCC using a non-viral delivery system. We estimated the miR-375 and LDHB and AEG-1/MTDH mRNA expression of the ESCC tumors from 85 patients. The correlation between the miR-375 expression and clinicopathological features, including the prognosis, were evaluated. The presence of high miR-375 expression was associated with lymphatic vessel invasion, while a low expression of miR-375 significantly correlated with a poor prognosis for the 85 ESCC patients. We also found that there was a significant inverse correlation between the expression of miR-375 and that of LDHB. Before the examination of miR-375 in the *in vivo* assay, we confirmed that atelocollagen prolonged the accumulation of miRNA by using fluorescently-labeled miRNA and an *in vivo* imaging system. We injected the miR-375/atelocollagen complex or a control-miRNA/atelocollagen complex into mice bearing TE2 and T.Tn xenografts via subcutaneous (s.c.) injections. The growth of both the TE2 and T.Tn tumors in the miR-375 groups was significantly suppressed compared with that in the control-miRNA groups. In addition, The LDHB mRNA expression of TE2 xenografts was significantly downregulated after miR-375 treatment. In conclusion, it might be possible for the level of miR-375 expression to be a utilized as a prognostic indicator for ESCC patients. The administration of miR-375 using a non-viral delivery might represent a powerful new treatment for ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in Japan. Despite recent advances in the use of combinations of chemo- and radiotherapy, ESCC remains one of the most lethal malignancies worldwide, and has a five-year survival rate of only 20-40% even after curative surgery (1,2). Nucleic acid-based therapy is a promising new approach, and the development of such a therapy might improve the morbidity and mortality of ESCC.

Epigenetic changes, such as DNA methylation, histone modifications and miRNA biogenesis, regulate gene expression (3). Histone deacetyltransferases, which deacetylate the lysine residues of histone proteins, facilitate the access of many transcriptional factors to DNA and consequently suppress the expression of the target genes (4,5). Histone deacetylase inhibitors (HDACIs) are anticancer agents with a potent HDAC-inhibiting activity (4,5). Numerous HDACIs have been identified and some of which have recently been used in clinical trials of cancer treatment (6). The microRNAs (miRs) are non-coding RNAs that are 21-25 nucleotides in length that silence gene expression, usually by targeting mRNAs for cleavage or translational repression (7). They are known to be involved in gene functions in a broad range of biological processes, including development, cell differentiation, proliferation, apoptosis, metabolism, carcinogenesis and growth control (8-11).

Studies have analyzed the function of microRNA-375 (miR-375) as a tumor suppressor in head and neck squamous cell carcinoma, maxillary sinus squamous cell carcinoma, hepatocellular cancer, pancreatic cancer and other cancers (12-15). Some of them reported that miR-375 plays a tumor-suppressive role in various cancers (12-15). On the other hand, miR-375 was upregulated in the tumor tissue from prostate cancer and papillary thyroid carcinoma, which revealed that the upregulation of miR-375 can serve as a novel marker of these cancers (16,17).

We have reported that the expression of miR-375 in HDACI-treated cells was upregulated >400-fold in ESCC cell lines (18). This result indicates the possibility that the transcription of miRs is regulated by histone modification, similar to that observed in other forms of gene transcription.

The miR-375 expression in ESCC was lower than that in the normal epithelium. miR-375 inhibits the proliferation, migration and invasion of ESCC cells. Moreover, we showed that *LDHB* and *AEG-1/MTDH* were miR-375-targeted genes. Clinical specimens of ESCC exhibited a high level of *LDHB* expression at both the mRNA and protein levels. Knockdown of *LDHB* by RNA interference (RNAi) showed that it had a tumor-suppressive function in ESCC cells. *LDHB*, which is regulated by the tumor-suppressive miR-375, may therefore act as an oncogene in ESCC (18).

However, the correlation between miR-375 expression and the clinicopathological features of ESCC have been unclear. In addition, the relationship between miR-375 expression and *LDHB* and *AEG-1/MTDH* mRNA expression in ESCC specimens has not yet been examined. Nor has the tumor-suppressive effect of miR-375 on ESCC been examined in *in vivo* assays.

The aim of this study was to clarify the correlations between the expression of miR-375 and the clinicopathological features of ESCC, the *LDHB* and *AEG-1/MTDH* mRNA expression in ESCC and to elucidate the tumor-suppressive effect of miR-375 *in vivo,* in assays using a non-viral delivery system.

Materials and methods

Clinical ESCC specimens. ESCC specimens and normal epithelial tissue specimens were collected from 85 patients who underwent surgical treatment for histologically proven ESCC in the Department of Frontier Surgery, Chiba University Graduate School of Medicine (Chiba, Japan), from 2001 to 2012. All patients gave their informed consent for tissue donation. Surgical treatments were performed without any preoperative radio- or chemotherapy. Normal esophageal epithelial tissue specimens were obtained far from the cancer. After excision, the tissue specimens were immediately frozen in liquid nitrogen until the subsequent analysis. The clinical stage of ESCC was assessed on the basis of the tumor-node-metastasis (TNM) classification system recommended by the International Union Against Cancer (7th edition, 2009).

RNA extraction. The tissue specimens were treated with the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, for total RNA extraction. The purity and concentration of all RNA samples were evaluated by their absorbance ratio at 260/280 nm, which was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE, USA).

Real-time quantitative RT-PCR for measuring the miR-375 expression. The expression of miR-375 was determined by quantitative RT-PCR using TaqMan® MicroRNA Assay kits (Applied Biosystems, Foster City, CA, USA). First-strand cDNA was synthesized from 10 ng of total RNA for each sample. The individual assays used a 15 μl reaction mixture containing 5 μl of RNA extract, 0.15 μl of 100 mM dNTPs, 1 μl of MultiScribe Reverse Transcriptase (50 U/μl), 1.5 μl of 10X reverse transcription buffer, 0.19 μl of RNase inhibitor (20 U/μl), 1 μl of gene-specific TaqMan primer and 4.16 μl of nuclease-free water according to the manufacturer's instructions. The reaction mixture was incubated at 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min and 4˚C until use. Subsequently, 1.33 μl of the DNA template was amplified using 10 μl of TaqMan Universal PCR Master Mix, 7.67 μl of nuclease-free water and 1 μl of gene-specific TaqMan primers/probe mix in a final volume of 20 μl. The amplification, detection and data analysis were performed with the iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). The reaction mixture was incubated at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The RT-qPCR experiments were performed in duplicate. The expression of RNU6B was used as internal control.

Real-time quantitative RT-PCR to assess the mRNA expression of LDHB and AEG-1/MTDH. The mRNA expression levels of *LDHB* and *AEG-1/MTDH* were examined by real-time quantitative PCR. The cDNA templates for real-time PCR were synthesized from 1 μg of total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems). The *Actin α1* (*ACTA1*) gene served as an internal control. The PCR reaction consisted of SsoFast EvaGreen Supermix (containing dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen dye and stabilizers; Bio-Rad), 1 μM each primer and cDNA. All reactions were run in duplicate on the MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). The PCR process was as follows: initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 55˚C for 10 sec. The following primer sequences were used: LDHB, 5'-TGG CGTGTGCTATCAGCATT-3' and 5'-GCTTATCTTCCA AAACATCCACAAG-3'; AEG-1/MTDH, 5'-TCCGAGAAG CCCAAACCAAAT-3' and 5'-CTTCACCCTCAGCCACTT CAA-3'; ACTA1, 5'-CCTTCATCGGTATGGAGTC-3' and 5'-GTTGGCATACAGGTCCTT-3'. The comparative cycle threshold (C_T) method was applied to quantify the expression levels of the mRNAs. The relative amount of *LDHB* and *AEG-1/MTDH* to *ACTA1* mRNA was calculated using the $2^{-\Delta CT}$ equation, where $\Delta C_T = (C_{T\ LDHB\ or\ AEG\text{-}1/MTDH} - C_{T\ ACTA1})$.

Statistical analysis. The correlation(s) between the expression of miR-375 and the clinicopathological factors were assessed by the χ^2 test. The overall survival was calculated as the time from the surgical treatment until death or the last follow-up date. The correlation between the overall survival and the expression of miR-375 was calculated using the log-rank test, and the results are presented as curves determined using the Kaplan-Meier method. A linear regression analysis was performed to determine whether there was a correlation between the expression of miR-375 and the mRNA expression of *LDHB* and *MTDH*.

Validation of the prolonged accumulation of miRNA in xenografts by atelocollagen. To examine the antitumor effects of miR-375 in an *in vivo* assay, we selected atelocollagen (Atelogene®; Koken Co., Ltd., Tokyo, Japan) as a non-viral delivery system. To explore the effects of atelocollagen on the accumulation of the miRNA, we prepared two types of tumor-bearing mice, those bearing a fluorescently-labeled miRNA (10 μM)/atelocollagen complex and those bearing a fluorescently-labeled miRNA (10 μM)/PBS complex. Alexa Fluor 555, premiR-Neg and Pre-miR miRNA Precursor (all from Life Technologies, Grand Island, NY,

USA) was used as the fluorescently-labeled miRNA. The fluorescently-labeled miRNA was adjusted to a concentration of 10 μM. Equal volumes of atelocollagen (0.1% in PBS at pH 7.4) and fluorescently-labeled miRNA solution were combined and mixed by rotating for 20 min at 4˚C. The labeled miR and PBS complex was prepared in an identical manner. We injected 200 μl of fluorescently-labeled miRNA (10 μM)/atelocollagen complex into one tumor-bearing mouse and the fluorescently-labeled miRNA (10 μM)/PBS complex into another mouse via subcutaneous (s.c.) injection. Fifteen minutes and 24 h after the injection, we observed the fluorescence signal using an *in vivo* imaging system (IVIS)®.

In vivo assay of the effects of the non-viral delivery of miR-375 on ESCC tumors. T.Tn and TE2 ($5.0x10^5$ cells each) human ESCC cells were injected into the backs of BALB/c nu/nu mice. When the estimated tumor volume reached ~300 mm^3, mice were randomly divided into four groups containing five animals each.

The miR-375 and negative control miRNA were obtained from Bonac Corp. (Fukuoka, Japan). Both the miR-375 and negative control miRNA levels were adjusted to a concentration of 10 μM. To prepare the miR-375 (10 μM)/atelocollagen and control-miR (10 μM)/atelocollagen complex, equal volumes of atelocollagen (0.1% in PBS at pH 7.4) and miR solution were combined and mixed by rotating for 20 min at 4˚C. Five weeks after tumor injection, we injected the mice with 200 μl of the miR-375/atelocollagen or control-miRNA/atelocollagen complex by s.c. injection. The treatment was performed twice a week for a total of six injections. The tumor size was measured in two dimensions using calipers, and the tumor volume (mm^3) was calculated as a^2 x b/2 mm^3 (a, minor axis; b, major axis). The tumor size was measured for 22 days.

The Animal Research Committee of the Chiba University, Japan, approved all studies. All animal procedures were performed according to the guidelines for the Animal Research Committee of the Chiba University, Japan. Either was used for mouse euthanasia and anesthesia.

Determination of the LDHB and AEG-1/MTDH mRNA expression in tumors treated with the miR-375/atelocollagen and control-miR/atelocollagen complex. We prepared TE2 and T.Tn tumor-bearing mice, and divided them into four groups containing three animals each. As stated above, we injected the miR-375/atelocollagen or control-miRNA/atelocollagen complex into the xenografts by s.c. injection. The treatment was conducted one time, and the tumors were excised 24 h after the treatment. We extracted total RNA from the tumors and determined the *LDHB* and *AEG-1/MTDH* mRNA expression level. The mRNA expression level of these genes was compared between the miR-375 and control-miRNA groups.

Results

The correlation between the expression of miR-375 in ESCC specimens and the clinicopathological factors. We performed RNA extraction and RT-PCR on ESCC specimens and normal epithelial tissue specimens from 85 patients (Table I). We examined the correlations between the miR375 expression and various clinicopathological factors, including the patient

Table I. Statistical features of ESCC patients.

Characteristics	No.
Total	85
Age	
≤65	37
>65	48
Gender	
Male	70
Female	15
Location	
Ph	1
Ce	3
Ut	8
Mt	32
Lt	36
Ae	4
Unknown	1
pT factor	
T1	27
T2	8
T3	38
T4	12
pN factor	
N0	35
N1	27
N2	14
N3	7
N4	2
Lymphatic vessel invasion	
0	39
1	26
2	12
3	8
Vessel invasion	
0	26
1	21
2	27
3	11
Pathology	
Wel	22
Mod	46
Por	13
Other	4
pUICC stage	
I	22
II	18
III	44
IV	1

The pT and pN factor, and pUICC stage are described according to the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) Classification (7th edition, 2009). ESCC, esophageal squamous cell carcinoma; Ph, pharynx; Ce, cervical esophagus; Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; Ae, abdominal esophagus; Wel, well differentiated; Mod, moderately differentiated; Por, poorly differentiated.

Table II. The correlation between the expression of miR-375 in ESCC specimens and clinicopathological factors.

Clinicopathological feature	Low	High	P-value
Age			
≤65	7	30	0.8108
>65	7	41	
Gender			
Male	11	59	0.982
Female	3	12	
pT factor			
T1	3	24	0.5327
T2, 3, 4	11	47	
pN factor			
N0	4	31	0.5327
N1, 2, 3	10	40	
Lymphatic vessel invasion			
0	3	36	0.0352
1, 2, 3	11	35	
Vessel invasion			
0	4	22	0.8901
1, 2, 3	10	49	
Pathology			
Wel	2	20	0.8803
Mod	9	37	
Por	3	10	
Other	0	4	
pUICC stage			
I	3	19	1
II, III, IV	11	52	

The significance was assessed by the χ^2 test. The pT and pN factor, and pUICC stage are described according to the UICC (International Union Against Cancer) tumor-node-metastasis (TNM) Classification (7th edition, 2009). miR-375, microRNA-375; ESCC, esophageal squamous cell carcinoma; Wel, well differentiated; Mod, moderately differentiated; Por, poorly differentiated.

age, gender, T and N factor, lymphatic vessel and vessel invasion, stage and differentiation. High expression of miR-375 was significantly correlated with lymphatic vessel invasion (P=0.0352). None of the other factors were found to be significantly related to the miR-375 expression (Table II).

Low expression of miR-375 was significantly correlated with a poor prognosis. We also performed a statistical analysis of the correlation between the expression of miR-375 and the prognosis of the 85 patients. The low expression of miR-375 was significantly correlated with a poor prognosis (P=0.007). In the group of patients with high miR-375 expression, the five-year survival rate was 58.2%, while that in the group with low miR-375 expression was 23.1% (Fig. 1).

A significant inverse correlation exists between the miR-375 expression and LDHB mRNA expression in ESCC specimens. We next examined the correlation between the expression of miR-375 and the mRNA expression levels of *LDHB* and *AEG-1/MTDH* using a linear regression analysis. We found that there was a significant inverse correlation between the expression of miR-375 and that of *LDHB* (P=0.039). No significant correlation was observed with the *AEG-1/MTDH* expression levels, although there was a tendency for an inverse correlation with the miR-375 expression (P=0.149) (Fig. 2).

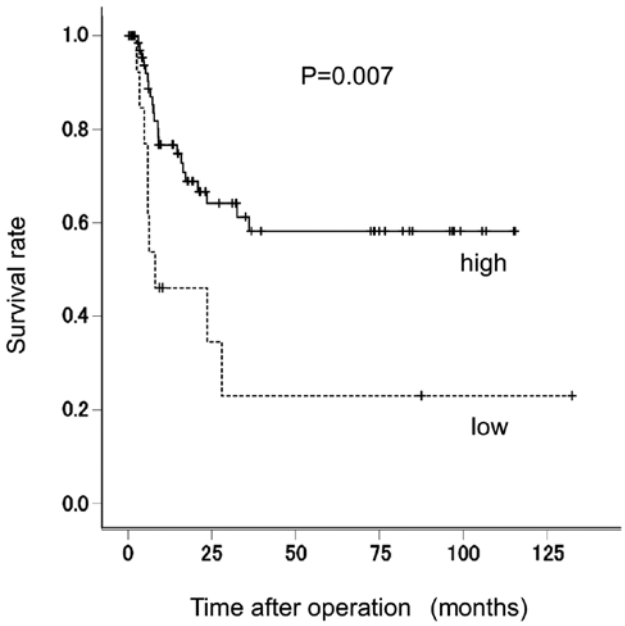

Figure 1. The correlation between the overall survival and the expression of microRNA-375 (miR-375) was determined by the log-rank test, and data are presented as curves determined using the Kaplan-Meier method. The low expression of miR-375 was significantly correlated with a poor prognosis (P=0.007). The five-year survival rate in the high miR-375 expression group was 58.2%, while it was 23.1% in the low miR-375 expression group.

Atelocollagen prolongs the accumulation of miRNA in xenograft tumors. We next examined whether atelocollagen could prolong the accumulation of miRNA in the tumor using a fluorescently-labeled miRNA and the IVIS imaging system. Fifteen minutes after injection of the atelocollagen or PBS-miRNA mixture, both xenografts exhibited the accumulation of fluorescence. After 24 h, we observed the accumulation of fluorescence in the tumors injected with the fluorescently-labeled miRNA (10 μM)/atelocollagen complex, but the fluorescence was not observed in another mouse that was injected with fluorescently-labeled miRNA (10 μM)/PBS (Fig. 3).

In vivo effects of miR-375 on ESCC tumors. As shown in Fig. 4, 22 days after the first treatment, the growth of both TE2 and T.Tn tumors in the miR-375/atelocollagen complex groups were significantly suppressed compared with the miR-control groups. The average volume of TE2 xenografts in the control-miR/atelocollagen complex group was 1,900.8 mm^3, while that of the TE2 xenografts in the miR-375/atelocollagen complex group was 668.0 mm^3. The miR-375/atelocollagen complex therefore suppressed the growth of the TE2 xenografts by 64.8%. For the T.Tn xenografts, the average volume

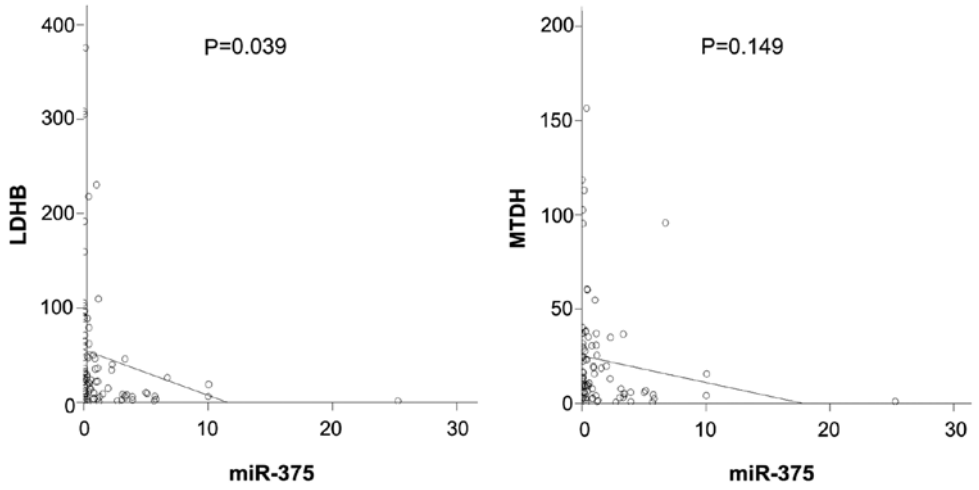

Figure 2. A linear regression analysis was performed to identify the correlation between the expression of microRNA-375 (miR-375) and the mRNA expression of *LDHB* and *AEG-1/MTDH*. The miR-375 expression was significantly inversely correlated with the *LDHB* mRNA expression (P=0.039). No significant correlation was observed with regard to the *AEG-1/MTDH* expression levels, although there was a tendency for an inverse correlation with the miR-375 expression (P=0.149).

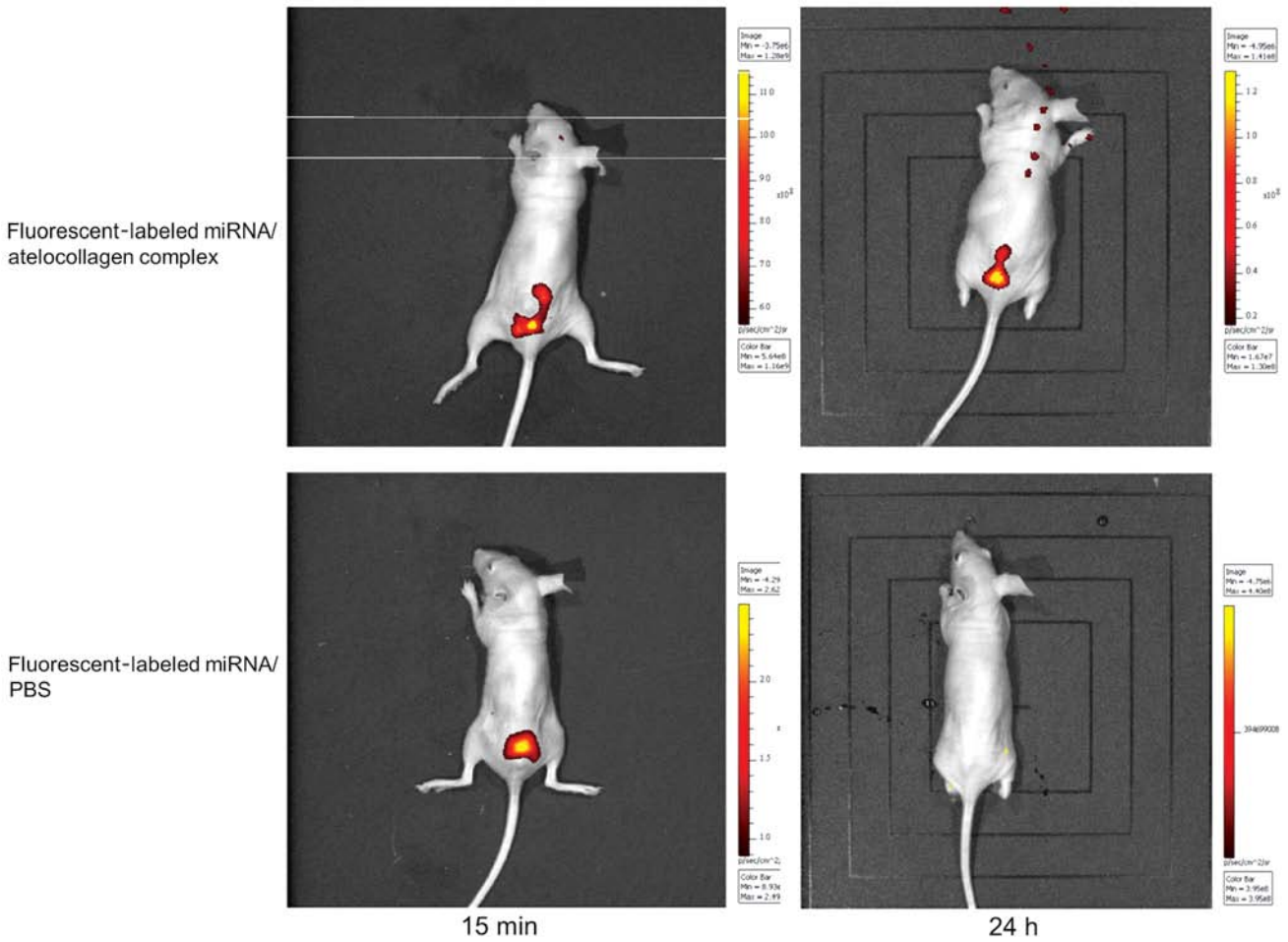

Figure 3. Using *an in vivo* imaging system, we confirmed that atelocollagen prolonged the accumulation of the fluorescently-labeled miRNA in the tumor. After 15 min, the tumor xenografts treated with both the fluorescently-labeled miRNA/atelocollagen and miRNA/PBS complex exhibited the accumulation of fluorescence. After 24 h, the accumulation of fluorescence was only observed in the tumor xenograft injected with the fluorescently-labeled miRNA/atelocollagen complex by IVIS.

in the control-miRNA/atelocollagen complex group was 1,938.5 mm^3, while that in the miR-375/atelocollagen complex group was 680.6 mm^3. The growth of the T.Tn xenografts was therefore reduced by 64.9% (Fig. 4A-C).

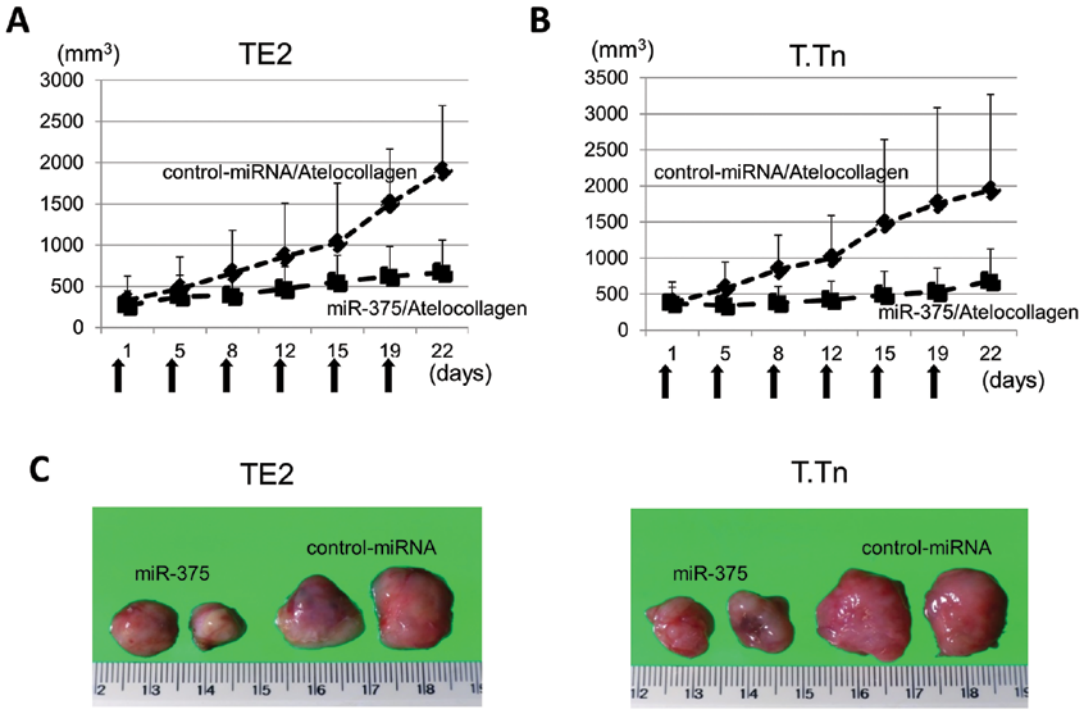

Figure 4. (A) The results of the *in vivo* assay using the non-viral delivery system to examine the antitumor effect of microRNA-375 (miR-375). The TE2 and T.Tn tumor-bearing mice were injected with 200 μl of miR-375/atelocollagen and control-miRNA/atelocollagen complex by subcutaneous (s.c.) injection. The treatment was performed twice a week for a total of six injections. The arrows indicate the days the mice were treated. The average volume of TE2 xenografts in the control-microRNA (miR)/atelocollagen complex group was 1,900.8 mm^3 on day 22, while that of the miR-375/atelocollagen complex group was 668.0 mm^3. The data points indicate the average of tumor sizes. (B) The average volume of T.Tn xenografts in the control-miRNA/atelocollagen complex group was 1,938.5 mm^3 on day 22, while that of the T.Tn xenografts in the miR-375/atelocollagen complex group was 680.6 mm^3. (C) The TE2 and T.Tn xenograft tumors removed on day 22 are shown. From the upper left panels, the xenografts were TE2 treated with: miR-375/atelocollagen, miR-375/atelocollagen, control-miRNA/atelocollagen and control-miRNA/atelocollagen. From the lower left panels, the xenografts were T.Tn treated with: miR-375/atelocollagen, miR-375/atelocollagen, control-miRNA/atelocollagen and control-miRNA/atelocollagen.

The mRNA expression levels of LDHB and AEG-1/MTDH were downregulated in xenografts treated with miR-375. The *LDHB* mRNA expression of the TE2 xenografts treated with miR-375 was significantly downregulated (P=0.0067). The level of the T.Tn xenografts treated with miR-375 group was not significantly suppressed. There were no significant differences in the *AEG-1/MTDH* mRNA expression levels between the miR-375 and control groups. However, there was a tendency for the *LDHB* expression of T.Tn xenografts and the *AEG-1/MTDH* expression of TE2 and T.Tn xenografts to be downregulated compared with the control groups (P=0.4240, P=0.1977, P=0.2549) (Fig. 5).

Discussion

Previously we reported that there was HDACI-induced miR-375 overexpression in ESCC cell lines (18). In the present study, we found that the miR-375 expression in ESCC was lower than that in the normal epithelium. miR-375 has a tumor-suppressive function in ESCC cells, and miR-375 downregulated *LDHB* and *MTDH* in ESCC cell lines. Kong *et al* previously revealed that the downregulation of miR-375 was frequently detected in primary ESCC, which was significantly correlated with advanced stage disease, distant metastasis, a poor overall survival and a shorter disease-free survival (19). In addition, a recent study estimated the expression of miR-375 in ESCC cell lines and tissues using a tissue microarray (TMA) that included 300 cases and confirmed the findings by miRNA *in situ* hybridization (MISH) (20). The MISH results also showed that miR-375 was significantly associated with an advanced clinical stage, tumor metastasis and a poor outcome of ESCC (19,20).

In this study, only lymphatic vessel invasion was found to significantly correlate with the high expression of miR-375. However, we found that a low expression of miR-375 was significantly correlated with a poor prognosis in our 85 patients. It might therefore be possible to use the level of miR-375 expression as a prognostic index for ESCC patients. Of interest, miR-375 might be regulated by epigenetic events, including histone acetylation. Supporting this possibility, in our previous study, miR-375 expression was restored by a HDACI in ESCC cells.

LDHB and *AEG-1/MTDH* were both identified as miR-375-targeted genes in our previous study. *AEG-1/MTDH* has been indicated to play a key role in the progression, invasion, metastasis and the resistance to chemotherapies of various types of tumors (21). In addition, the overexpression of *AEG-1/MTDH* is considered to be a valuable marker of ESCC progression (22).

In contrast to *AEG-1/MTDH*, there have been only a few reports on whether *LDHB* expression is related to the progression of cancers (23,24). Our previous report showed that ESCC clinical specimens exhibited a higher level of LDHB expression at both the mRNA and protein levels compared with the normal esophageal epithelium. Kaplan-Meier curves

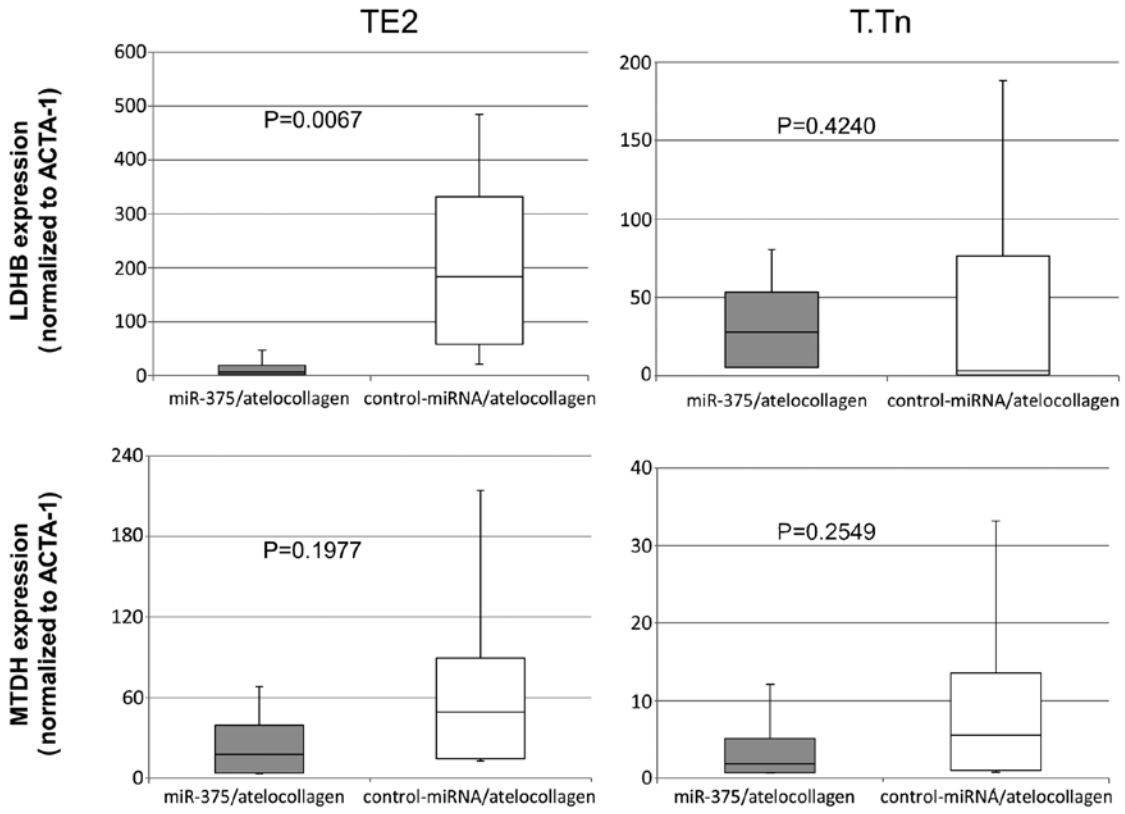

Figure 5. The LDHB mRNA expression of TE2 xenografts in the microRNA-375 (miR-375) group was significantly downregulated compared with the control-miRNA group (P=0.0067). The expression in the T.Tn xenografts in the miR-375 group was not significantly suppressed (P=0.4240). As shown in the lower diagrams, there were no significant differences between the AEG-1/MTDH mRNA expression in the miR-375 and control groups (P=0.1977, P=0.2549). However, there was a tendency for the LDHB expression of T.Tn xenograft and the AEG-1/MTDH expression of the TE2 and T.Tn xenografts to be downregulated compared with the control groups.

and log-rank tests revealed that positive immunoreactivity for the LDHB protein had a tendency to indicate a poor prognosis. Although it was verified that *LDHB* and *AEG-1/MTDH* were targeted by miR-375, and that these genes might act as oncogenes, the correlation between the miR-375 expression and *LDHB* and *AEG-1/MTDH* expression levels in ESCC clinical specimens was unclear. Therefore, in the present study, we evaluated the correlation between the miR-375 expression and either the *LDHB* or *AEG-1/MTDH* mRNA expression in ESCC specimens. We found that there was a significant inverse correlation between the expression of miR-375 and *LDHB*. Regarding *AEG-1/MTDH* mRNA expression, there was a tendency for an inverse correlation with the miR-375 expression. These results confirmed that miR-375 regulates *LDHB* and *AEG-1/MTDH* in clinical specimens and showed there is a possibility that *LDHB* and *AEG-1/MTDH* play a role as oncogenes in ESCC.

Although the use of miR-based therapy appears to be an effective strategy, there are unresolved issues, including the lack of tissue specificity, absence of an optimal delivery system, poor cellular uptake, and risk of systemic toxicity (3). Regarding miR-375, several studies have indicated that this miRNA has anticancer effects against various cancers. However, only one previous report exists so far on evaluation of the antitumor effect of miR-375 *in vivo* (14). It was revealed that therapeutic administration of cholesterol-conjugated 2'-O-methyl-modified miR-375 mimics (Chol-miR-375) could significantly suppress the growth of hepatoma xenografts in nude mice (14). Since no previous reports had examined the tumor-suppressive effect of miR-375 *in vivo* for ESCC xenografts, we determined whether miR-375 was effective for ESCC *in vivo* using an atelocollagen complex to deliver the miR-375.

Atelocollagen, which is prepared from the bovine dermis, increases the cellular uptake, nuclease resistance and the prolonged release of nucleic acids in various disease models *in vivo* (25-27). Previous studies have already shown the clear therapeutic efficacy of atelocollagen-mediated *in vivo* delivery of nucleic acids (25-27). In this study, we validated that the miR-375/atelocollagen complex could significantly suppress the growth of ESCC xenografts.

Although major barriers to the clinical application of miR-based therapeutics still exist, the administration of tumor-suppressive miRNA using a non-viral delivery system might provide a powerful new strategy for cancer therapy (3).

It was also found that the low expression of miR-375 was significantly correlated with a poor prognosis in ESCC patients. A significant inverse correlation between the expression of miR-375 and *LDHB* was observed, suggesting that *LDHB* may play a role as an oncogene in ESCC. Moreover, we validated that the miR-375/atelocollagen complex significantly suppressed the growth of ESCC xenografts. The administration of a tumor-suppressive miRNA using a non-viral delivery system might be a powerful treatment for cancer.

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