Identification of novel molecular targets regulated by tumor suppressive miR-375 induced by histone acetylation in esophageal squamous cell carcinoma

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Abstract. The aim of this study was to determine whether histone acetylation regulates tumor suppressive microRNAs (miRNAs) in esophageal squamous cell carcinoma (ESCC) and to identify genes which are regulated by these miRNAs. We identified a miRNA that was highly upregulated in an ESCC cell line by cyclic hydroxamic acid-containing peptide 31 (CHAP31), one of the histone deacetylase inhibitors (HDACIs), using a miRNA array analysis. *miR-375* was strongly upregulated by CHAP31 treatment in an ESCC cell line. The expression levels of the most upregulated miRNA, *miR-375* were analyzed by quantitative real-time PCR in human ESCC specimens. The tumor suppressive function of *miR-375* was revealed by restoration of *miR-375* in ESCC cell lines. We performed a microarray analysis to identify target genes of *miR-375*. The mRNA and protein expression levels of these genes were verified in ESCC clinical specimens. *LDHB* and *AEG-1/MTDH* were detected as *miR-375*-targeted genes. The restoration of miR-375 suppressed the expression of *LDHB* and *AEG-1/MTDH*. The ESCC clinical specimens exhibited a high level of LDHB expression at both the mRNA and protein levels. A loss-of-function assay using a siRNA analysis was performed to examine the oncogenic function of the gene. Knockdown of *LDHB* by RNAi showed a tumor suppressive function in the ESCC cells. The correlation between gene expression and clinicopathological features was investigated by immunohistochemistry for 94 cases of ESCC. The positive staining of LDHB correlated significantly with lymph node metastasis and tumor stage. It also had a tendency to be associated with a poor prognosis. Our results indicate that HDACIs upregulate miRNAs, at least some of which act as tumor suppressors. *LDHB*, which is regulated by the tumor suppressive miR-375, may therefore act as an oncogene in ESCC.

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Introduction

Esophageal cancer occurs in humans worldwide with a variable geographic distribution, and it ranks eighth among cancers in the order of occurrence (1). Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in Japan. Despite recent advances in cancer therapy, esophageal cancer remains one of the least responsive malignancies (2). The overall 5-year survival rate for esophageal cancer is approximately 20-25% for all stages (3), therefore, the development of a molecular oncogenic therapy that can provide a higher response rate than the current combinations of chemotherapy and radiotherapy is urgently required.

Epigenetics is a rapidly expanding field that focuses on stable changes in gene expression that are not accompanied by any changes in the DNA sequence, and that are mediated primarily by DNA methylation, histone modifications and small non-coding RNA molecules (4). Histone deacetylation is known to correlate with transcriptional silencing and with the downregulation of the expression of proapoptotic genes, especially in cancer cells. The histone deacetylase inhibitors (HDACIs) were mainly thought to act by modulating the gene expression patterns, including those of genes associated with cell cycle arrest and apoptosis, by inhibiting the activity of histone deacetylases (HDACs)(5). Previous we reported that depsipeptide (FK228) and cyclic hydroxamic acid-containing peptide 31 (CHAP31) have potent antitumor effects against ESCC *in vitro* and *in vivo* (6-8).

Table I. The clinicopathological features of patients with ESCC.

No.	Gender	Age	Location[a]	UICC[b] T	UICC[b] N	UICC[b] Stage
1	F	48	Mt	4	4	4a
2	M	69	Mt	1b	0	1
3	M	75	Mt	3	3	3
4	M	73	Mt	3	2	3
5	M	67	Lt	4	1	4a
6	F	70	Mt	1b	0	1
7	M	65	Mt	1b	0	1
8	M	53	Ae	1b	3	3
9	M	65	Mt	3	1	3
10	M	67	Lt	2	2	3
11	M	71	Mt	3	2	3
12	M	77	Mt	3	0	2
13	M	70	Lt	3	1	3
14	M	66	Lt	1b	0	1
15	M	47	Mt	3	0	2
16	M	68	Lt	3	3	3
17	M	53	Ae	4	1	4a
18	M	60	Lt	4	0	3
19	M	71	Lt	3	1	3

RNU48 was used as an internal control. [a]Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; Ae, abdominal esophagus. [b]UICC, the N and stage are described according to UICC (International Union Against Cancer) TNM Classification (Sixth Edition, 2002).

Numerous studies have demonstrated that microRNAs (miRNAs), non-coding RNAs 21-25 nucleotides in length, control gene expression by targeting mRNAs for cleavage or translational repression (9). These miRNAs are associated with important biological processes, including development, differentiation, apoptosis, and proliferation (9,10). A growing body of evidence indicates that the miRNA expression profiles associated with particular types of cancer could serve as useful biomarkers for both disease prognosis and diagnosis (11,12).

The purpose of this study was to determine whether histone acetylation is associated with the regulation of the expression of tumor-suppressive miRNAs in ESCC, and to identify the target genes that are regulated by these miRNAs.

Materials and methods

Clinical ESCC specimens. RNA extraction was performed for 19 pairs of primary ESCC and corresponding normal esophageal epithelium. All specimens were obtained from patients who underwent surgical treatment at the Department of Frontier Surgery, Graduate School of Medicine, Chiba University, Japan from 2004 to 2005. The clinicopathological characteristics of the patients and samples are listed in (Table I). The staging of the tumors was carried out according to the TNM classification. The tissues were frozen in liquid nitrogen immediately, and stored at -80˚C.

Immunohistochemical staining was performed for 94 patients wh underwent surgical resection from 1997 to 2005. Normal esophageal epithelial tissue specimens were obtained far from the cancer in the specimens. All patients gave their informed consent for tissue donation. Surgical treatments were performed without any preoperative radiotherapy or chemotherapy.

ESCC cell culture and reagents. The human ESCC cell lines were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS in a humidified incubator containing 5% CO_2 at 37˚C. The T.Tn cells were provided by the Japanese Cancer Research Resources Bank. TE2 cells were provided by Tohoku University. CHAP31 was provided by Dr M. Yoshida (RIKEN Advanced Science Institute, Wako, Saitama, Japan), and was dissolved in dimethyl sulfoxide.

Total RNA preparation and miRNA analysis. The cells were seeded into 225 cm^2 flasks and incubated for 48 h, then treated with or without an IC_{50} concentration of CHAP31 and harvested after 12 h of treatment. The cells were washed with PBS and processed for RNA extraction with TRIzol. The miRNA expression patterns were evaluated using the TaqMan Low Density Array Human MicroRNA Panel v1.0 (Applied Biosystems, Foster City, CA). The assay was conducted in 2 steps: generation of cDNA by reverse transcription, and a TaqMan real-time PCR assay. Briefly, the miRNAs in the samples were converted into cDNA using 365 specific stem-loop reverse transcription primers. The quantity of mature miRNAs was evaluated using specific TaqMan real-time PCR primers and probes. Real-time PCR was performed in duplicate using the GeneAmp Fast PCR Master Mix (Applied

Table II. Upregulated and downregulated miRNAs in ESCC cell lines treated with an IC_{50} concentration of CHAP31.

No.	microRNA	Accession no.	Fold change (CHAP31/control)		Average
			T.Tn	TE2	
1	miR-375	MIMAT0000728	1724.872	473.217	1099.045
2	miR-449a	MIMAT0001541	184.607	9.238	96.922
3	miR-449b	MIMAT0003327	27.045	19.514	23.280
4	miR-192	MIMAT0000222	17.483	15.143	16.313
5	miR-497	MIMAT0002820	28.623	2.569	15.596
6	miR-132	MIMAT0000426	13.077	10.885	11.981
7	miR-194	MIMAT0000460	12.253	8.502	10.378
8	miR-146b-5p	MIMAT0002809	2.813	3.394	3.103
9	miR-183	MIMAT0000261	3.222	2.697	2.959

Biosystems) and the ABI 7900HT Real-Time PCR System. The comparative CT method was used to determine the expression levels. The relative miRNA expression data were analyzed using the GeneSpring GX version 7.3.1 software package (Agilent Technologies), as previously described (13). Normalization to an endogenous gene (RNA48) was used to normalize the expression data.

RNA isolation. The tissue specimens and cells were treated with the TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol, for total RNA extraction. The RNA concentrations were determined spectrophotometrically, and the molecular integrity was checked by gel electrophoresis. The RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Mature miRNA transfection and small interfering RNA treatment. The RNA sequences used in this study included mature miR-375, Pre-miR™ miRNA precursors (hsa-miR-375; Pre-miR ID: PM10327), miRNA-control (P/N: AM17111; Applied Biosystems), small interfering RNA [Stealth Select RNAi™ siRNA; si-LDHB Cat#; HSS106003 and HSS106005 (Invitrogen)], and siRNA-control (Stealth™ RNAi negative control medium GC Duplex; 12935-300). The RNA sequences were incubated with Opti-MEM (Invitrogen) and Lipofectamine™ RNAiMax reagent (Invitrogen) as previously described (14).

Cell proliferation assay. The cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 96-well plates at $3x10^3$ cells per well. Cell proliferation was evaluated by the XTT assay after 72 h, using the Cell proliferation kit II (Roche Molecular Biochemicals, Mannheim, Germany). Triplicate wells were measured for cell viability in each treatment group.

Cell migration assay. Cell migration was evaluated using modified Boyden Chambers (Transwells; Corning/Costar #3422, NY, USA) containing uncoated transwell-polycarbonate membrane filters with 8 μm pores in 24-well tissue culture plates. Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 10 cm dishes at $8x10^5$ cells. The cells were cultured for 48 h, and then $5x10^4$ cells were added to the upper chamber of each well and allowed to migrate for 48 h. The non-migratory cells were gently removed from the filter surface of the upper chamber, and the cells that migrated to the lower side were fixed and stained with Diff-Quick (Sysmex Corporation, Tokyo, Japan). The number of cells migrating to the lower surface was determined microscopically by counting 4 constant areas per well. Triplicate wells were measured for cell migration in each treatment group.

Cell invasion assays. The cell invasion assays were carried out using modified Boyden Chambers containing transwell-precoated Matrigel membrane filter inserts with 8 μm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA) as previously described (13). All experiments were performed in triplicate.

Screening for miRNA-375 target genes by a microarray analysis. The expression profiles of TE2 and T.Tn cells transfected with miRNA-375 were displayed and compared against miRNA-negative control transfectants using the Oligo-microarray human 44K platform (Agilent Technologies) as previously described (15). The hybridization and washing steps were performed as previously described (16). The arrays were scanned using a Packard GSI Lumonics ScanArray 4000 (Perkin Elmer, Boston, MA, USA) and the data were analyzed. Data from each microarray study were subjected to global normalization (16).

The predicted target genes and their target miRNA site seed regions were explored using the TargetScan software program (release 5.1, http://www.targetscan.org/). The sequences of predicted mature miRNAs were confirmed using the miRBase software program, release 13.0 (http://microrna.sanger.ac.uk/).

Real-time quantitative RT-PCR. First-strand cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems). The gene-specific PCR products were assayed continuously using a 7900-HT Real-Time PCR system according to the manufacturer's protocol. The first PCR step was a 10 min denaturation at 95˚C, followed by 40 cycles of a 15 sec denaturation at 95˚C

and a 1 min annealing/extension at 63˚C. The TaqMan probes and primers used to amplify LDHB (Hs00929956_m1), MTDH (Hs00757841_m1), PRDX1 (Hs03044567_g1), CXCL1 (Hs00236937_m1), MAL2 (Hs00294541_m1), CHSY1 (Hs00208704_m1) and GAPDH (Hs02758991_g1) were Assay-On-Demand Gene Expression Products (Applied Biosystems). All reactions were performed in triplicate and included a negative control lacking cDNA. The expression levels of miRNA-375 (Assay ID: 000564) were analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to RNU48 (Assay ID, 001006).

Western blot analysis. The cells were harvested and lysed 72 h after transfection. Each cell lysate (50 μg of protein) was separated by electrophoresis using Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with a monoclonal antibody against LDHB (1:10000; 2090-1; Epitomics, Burlingame, CA, USA) and a polyclonal antibody against MTDH (1:200; HPA015104; Sigma-Aldrich). A GAPDH antibody (1:1000; ab8245; AbCam, Cambridge, UK) was used as an internal loading control. The membranes were washed and incubated with a goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad). The complexes were visualized with the Immun-Star WesternC chemiluminescence kit (Bio-Rad), and the expression levels of these proteins were evaluated using the ImageJ software package (version 1.44; http://rsbweb.nih.gov/ij/index.html).

Expression of LDHB and MTDH determined by immunohistochemistry in clinical esophageal squamous cell carcinoma specimens. Immunohistochemical staining was performed to detect the expression of LDHB and MTDH in the cancerous and normal epithelial regions in 19 ESCC clinical specimens. The LDHB expression was also evaluated for 94 cases to assess the immunohistochemical features of LDHB during the progression of ESCC. Paraffin blocks were cut into 3 μm-thick sections, and mounted after staining with hematoxylin and eosin.

Immunohistochemical staining was performed with a monoclonal LDHB antibody (1:250; 2090-1; Epitomics, Burlingame, CA, USA) and a polyclonal AEG-1/MTDH antibody (1:350; HPA015104; Sigma-Aldrich). Secondary antibodies (biotinylated rabbit anti-rabbit immunoglobulins, Dako K4003) were applied to all slides for 60 min at 37˚. The color was developed by 2 min of incubation with DAB chromogen on the slides. The slides were all counterstained with hematoxylin.

The proportion of the specimen showing positive staining for LDHB in five representative fields at magnification x100 was evaluated independently by two observers who were blinded to the clinicopathological characteristics and prognosis of the patients. The LDHB expression was graded according to the percentage of LDHB positive cells using the scale: 0-10% (-), 10-50% (1+), 50-100% (2+).

Comparisons between the groups were performed using the chi-square test. The overall survival was calculated from the time of the surgical treatment until mortality or the last follow-up date. The correlation between the overall survival and LDHB expression was computed by the log-rank test and presented as curves determined using the Kaplan-Meier method.

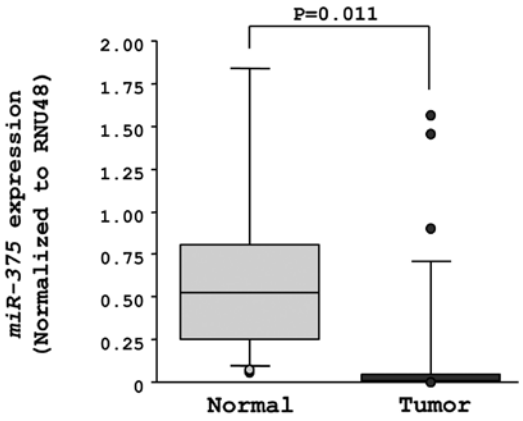

Figure 1. The expression levels of miR-375 were significantly downregulated in tumor tissues in comparison to normal tissues. RNU48 was used as an internal control.

Results

Identification of upregulated miRNAs in ESCC cell lines treated with CHAP31. The raw data were normalized using an internal reference, RNU44, and 11 upregulated and 9 downregulated miRNAs we identified using a cutoff p-value of <0.05 and a fold-change of the with/without CHAP31 treatment value <0.5 (Log2 ratio; Table II). The expression of miR-375 in CHAP31-treated cells was upregulated more than 400-fold in both cell lines, and was then identified to be a potential tumor suppressor.

Expression of miR-375 in ESCC clinical specimens. The expression levels of miR-375 were significantly downregulated in clinical ESCC specimens in comparison to neighboring normal tissue sections (Fig. 1).

Effect of miR-375 transfection on the proliferation, migration and invasion in ESCC cells. The functional significance of miR-375 was evaluated with a gain-of-function assay using miR-375 transfectants. The XTT assay showed significant inhibition of cell proliferation in miR-375 transfectants in comparison with mock and miRNA-control transfectants after a 72-h treatment (% cell proliferation, T.Tn; 43.7±1.4, 100.0±1.0 and 120.0±3.2, respectively, P<0.0001, TE2; 75.5±2.3, 100.0±1.5 and 101.5±1.7, respectively, P<0.0001; Fig. 2A).

The migration assay demonstrated that the number of cells that migrated was significantly decreased in miR-375 transfectants in comparison to mock and miRNA-control transfectants (% cell migration, T.Tn; 36.9±4.3, 100.0±11.1 and 125.2±14.8, respectively, P<0.0001, TE2; 44.1±2.4, 100.0±2.7 and 93.7±5.3, P<0.0001; Fig. 2B).

Similarly, the Matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in miR-375 transfectants in comparison to mock and miRNA-control transfectants (% cell invasion, T.Tn; 24.8±3.4, 100.0±13.8 and 112.2±5.9, respectively, P<0.0001, TE2; 70.4±6.1, 100.0±10.3 and 96.1±6.0, respectively, P=0.0132; Fig. 2C).

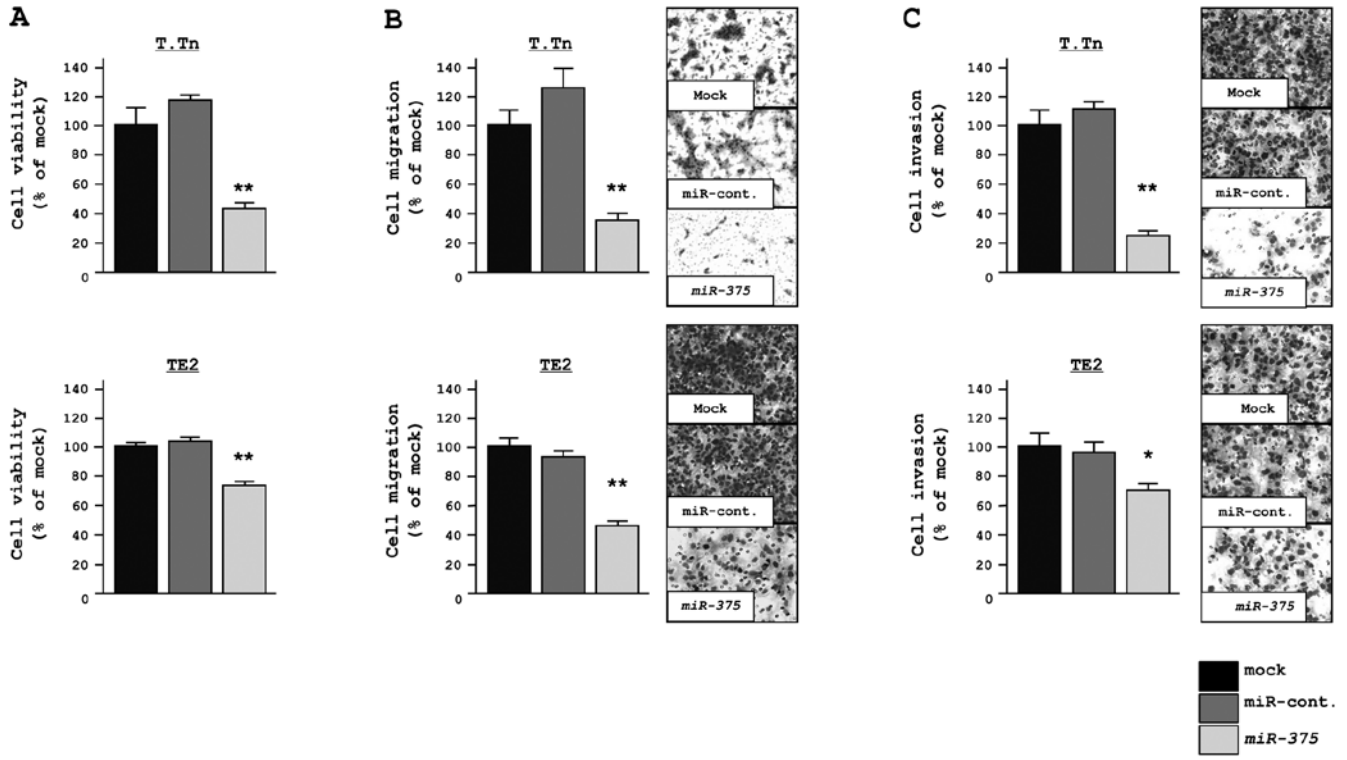

Figure 2. Gain-of-function studies were performed using miR-375-transfected TE2 and T.Tn cell lines. (A) Inhibition of cell proliferation by miR-375 transfection into TE2 and T.Tn cells. The cells were transfected with 10 nM of miR-375 or miR-control. Cell proliferation was determined using the XTT assay after 72 h of treatment. (B) Inhibition of cell migration by miR-375 transfection into TE2 and T.Tn cells. The data for cell migration at 48 h after transfection were expressed as the relative values compared to the expression in the mock-transfected cultures. (C) Inhibition of cell invasion by miR-375 transfection into TE2 and T.Tn cells. Cell invasion was determined using the Matrigel invasion assay after 72 h of treatment. *P<0.0132, **P<0.0001.

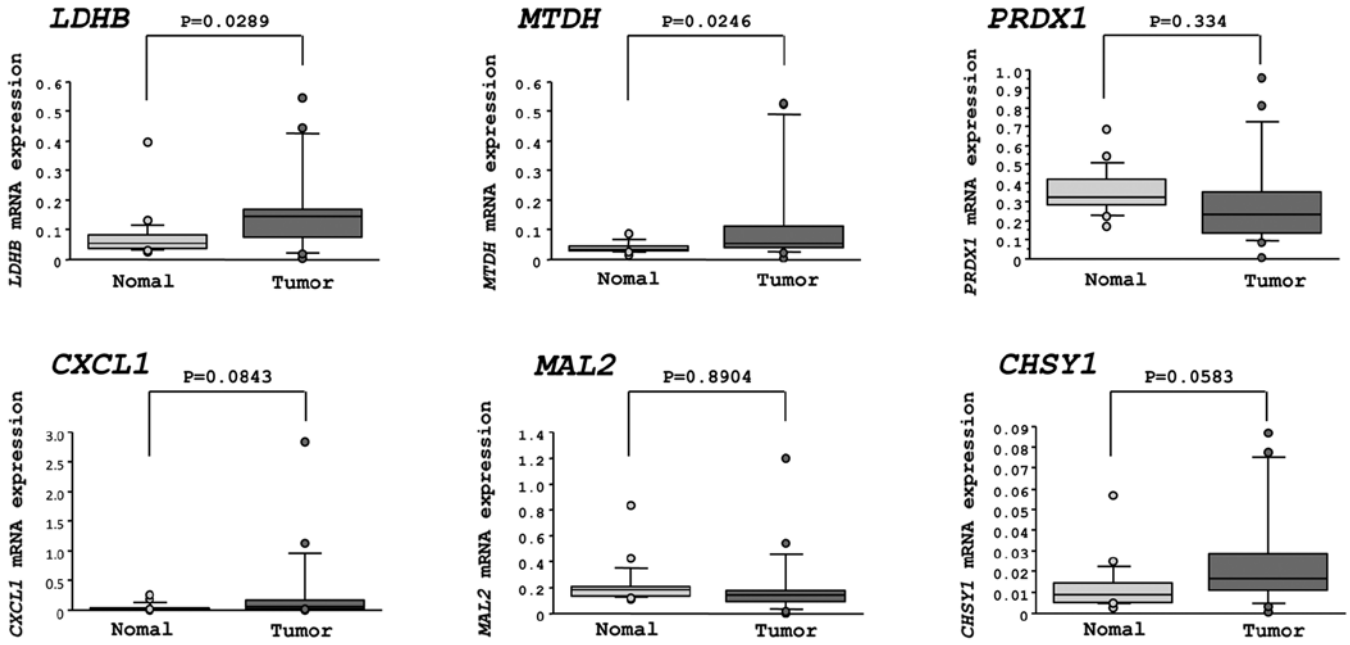

Figure 3. Measurement of the mRNA expression level of candidate miR-375 target genes in ESCC clinical specimens. The expression levels of LDHB and AEG-1/MTDH mRNA were significantly upregulated in cancer tissues in comparison to normal tissues. PRDX1, CXCL1, MAL2 and CHSY1 were not significantly upregulated. GAPDH was used as an internal control.

Screening of miR-375 target genes by a genome-wide gene expression analysis. The effect of miR-375 on protein-coding genes was examined to identify candidate molecular targets of miR-375 in ESCC cells. A comprehensive gene expression analysis was performed with miR-375 transfectants in both the T.Tn and TE2 cell lines. MiR-control transfectants that

Table III. Genes downregulated by miR-375 treatment in ESCC cell lines.

No.	Entrez gene ID	Gene name	Gene symbol	Log2 ratio			miR-375
				T.Tn	TE2	Average	Target
1	8000	Prostate stem cell antigen	PSCA	-1.50	-1.95	-1.72	-
2	642587	NPC-A-5	LOC642587	-1.58	-1.60	-1.59	-
3	3945	Lactate dehydrogenase B	LDHB	-1.48	-1.60	-1.54	1
4	8581	Lymphocyte antigen 6 complex, locus D	LY6D	-1.59	-1.33	-1.46	-
5	92140	Metadherin	MTDH	-1.24	-1.63	-1.43	1
6	5052	Peroxiredoxin 1	PRDX1	-1.43	-1.35	-1.39	1
7	218	Aldehyde dehydrogenase 3 family, memberA1	ALDH3A1	-1.36	-1.32	-1.34	-
8	2919	Chemokine (C-X-C motif) ligand 1 (melanoma Growth stimulating activity, α)	CXCL1	-1.24	-1.37	-1.30	1
9	1789	DNA (cytosine-5-)-methyltransferase 3 β	DNMT3B	-1.28	-1.31	-1.29	-
10	1475	Cystatin A (stefin A)	CSTA	-1.02	-1.57	-1.29	-
11	114569	Mal, T-cell differentiation protein 2	MAL2	-1.23	-1.34	-1.29	1
12	445	Argininosuccinate synthetase 1	ASS1	-1.26	-1.18	-1.22	-
13	216	Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	-1.11	-1.31	-1.21	-
14	84958	Synaptotagmin-like 1	SYTL1	-1.20	-1.21	-1.20	-
15	10857	Progesterone receptor membrane component 1	PGRMC1	-1.32	-1.02	-1.17	-
16	22856	Chondroitin sulfate synthase 1	CHSY1	-1.09	-1.01	-1.05	1

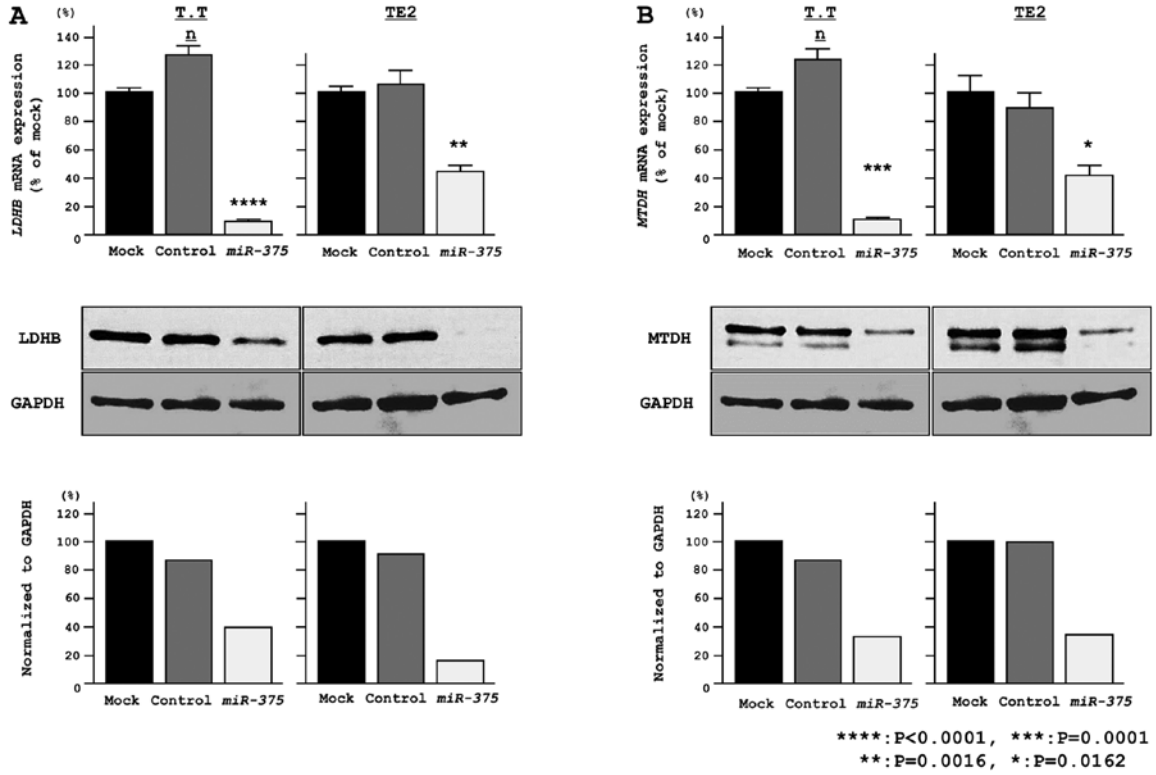

Figure 4. Restoration of miR-375 in TE2 and T.Tn cells inhibited LDHB expression at both the mRNA and protein levels. (A) RT-PCR analyses revealed that the *LDHB* mRNA expression was markedly repressed in both the TE2 and T.Tn miR-375 transfected cells. (B) Western blot analyses showed that the LDHB protein expression was decreased in miR-375 transfectants.

produced raw signal values of <3,000 were excluded before comparisons were made. Sixteen genes were downregulated by <-1.0 (Log2 ratio) in the miR-375 transfectants in both the T.Tn and TE2 cell lines (Table III). The 3'UTR of these genes were screened for miR-375 target sites using the TargetScan database. Six of these 16 genes had miR-375 target sites in their 3'UTR, and were identified as putative target genes of miR-375.

Expression levels of candidate miR-375 target genes in ESCC clinical specimens. The mRNA expression levels of the six candidate genes were measured in clinical specimens of ESCC by quantitative real-time reverse-transcription-PCR. Two genes, lactate dehydrogenase B (LDHB) and astrocyte elevated gene-1/metadherin (AEG-1/MTDH), were significantly upregulated in cancer tissues (P=0.0289 and P=0.0246, respectively). The other four genes (PRDX1, CXCL1, MAL2 and CHSY1) were not significantly upregulated in the specimens of ESCC (Fig. 3).

LDHB and MTDH mRNA and protein levels are repressed by miR-375. Gain-of-function studies were conducted using miR-375-transfected T.Tn and TE2 cells, and the mRNA and protein expression levels of LDHB (Fig. 4A) and MTDH (Fig. 4B) were found to be markedly downregulated in the transfectants in comparison to the mock controls.

The expression levels of LDHB and MTDH by IHC in ESCC clinical specimens. The expression of LDHB and MTDH was observed in all the specimens examined, but the expression in tumors was much higher in comparison to that in the corresponding normal epithelium (Fig. 5).

The correlation between LDHB expression and the clinicopathological characteristics. Positive staining for LDHB was found in 68% of the cases. The correlation between LDHB expression and the clinicopathological features, including patient age, gender, tumor depth, lymph node metastasis, distant metastasis, tumor stage and tumor differentiation was investigated (Table IV).

The level of LDHB staining correlated significantly with lymph node metastasis (P<0.05) and the tumor stage (P<0.005). There was no significant correlation between LDHB staining and other factors.

Relationship between LDHB expression and patient prognosis. No significant differences in survival were observed according to the LDHB expression levels, although there was a tendency for the patients with high immunoreactivity for LDHB to have a poorer prognosis (Fig. 6A and B).

Effect of LDHB loss-of-function in ESCC cell lines. A loss-of function assay using a siRNA analysis was performed to examine the oncogenic function of LDHB. The effects of si-LDHB on the mRNA and protein expression levels were evaluated 72 h after transfection into both T.Tn and TE2 cells. The LDHB mRNA and protein levels were both reduced after transfection. The XTT assay revealed significant inhibition of cell proliferation in si-*LDHB* transfectants in comparison to mock and si-control transfectants after 72 h. The Matrigel

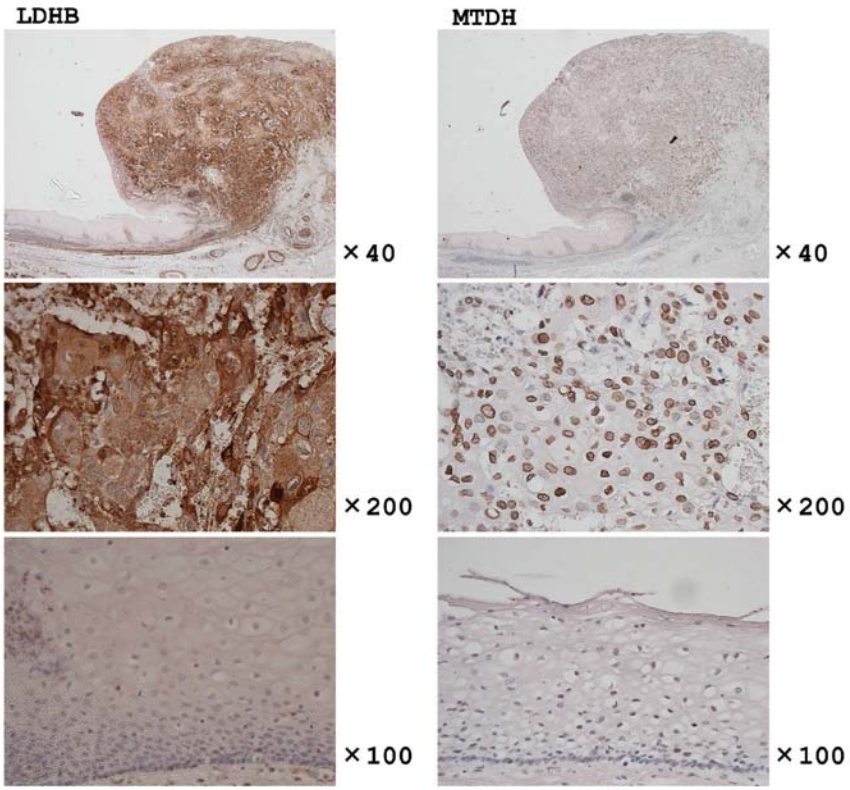

Figure 5. LDHB and MTDH protein expression in clinical ESCC specimens. The immunohistochemical staining for LDHB is shown in the left column, and that for MTDH is shown in the right column. Upper panel, the immunohistochemical staining for LDHB and MTDH in a low power field; middle panel, high power field images of the tumor lesion; lower panel, high power field images of neighboring normal epithelium.

invasion assay demonstrated that the number of invading cells was significantly lower in the si-*LDHB* transfectants compared to mock and si-control transfectants (Fig. 7).

Discussion

This study showed that HDACIs induced miR-375 overexpression in ESCC cell lines, and that miR-375 downregulated *LDHB* and *AEG-1/MTDH* in ESCC cell lines. HDACs are associated with numerous types of cancer and regulate cancer development (5). Histone deacetylation correlates with transcriptional silencing and with the downregulation of the expression of proapoptotic genes, especially in cancer cells (5-8). HDACIs cause changes in the acetylation status of chromatin, resulting in changes in gene expression, induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis and metastasis (17,18).

Dysregulation of miRNAs is associated with dysregulated gene expression of tumor suppressors and oncogenes in several types of cancer (9). miRNAs are differentially expressed in several cancers, including ESCC, as indicated by their expression signatures (13-15,19-21). A previous study analyzed the function of miR-375 as a tumor suppressor in head and neck squamous cell carcinoma and maxillary sinus squamous cell carcinoma, and investigated the target genes and their function (15,22). Another study showed significantly lower expression of miR-375 in ESCC (19).

The correlation(s) between DNA demethylation or histone acetylation and miRNAs has not been fully elucidated, and few reports exist on this correlation regarding miR-375 (23-25).

Table IV. Correlation between LDHB expression and clinicopathological characteristics.

Clinicopathological features	$LDHB^-$	$LDHB^+$	P-value
Age			
≤65 years	18	39	0.9309
>65 years	12	25	
Gender			
Male	27	54	0.6775
Female	3	10	
Tumor depth			
Tis/T1	15	20	0.0796
T2/T3/T4	15	44	
Lymph node metastasis			
N0	19	23	<0.05
N1	11	41	
Distant metastasis			
M0	25	56	0.8219
M1	5	8	
Stage			
0/I	13	10	<0.005
II/III/IV	16	55	
Tumor differentation			
Well	10	16	0.3558
Moderate	11	37	
Poor	9	10	
Other	0	1	

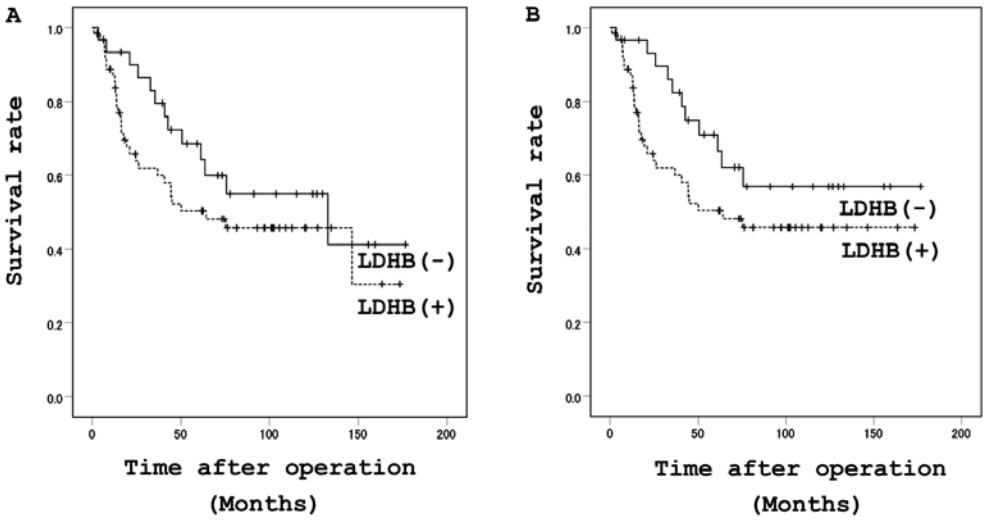

Figure 6. Cumulative survival curves of the patients are shown. (A) The survival curves did not shown any significant differences between LDHB positive and negative patients. (P=0.194). (B) The cause-specific survival of LDHB positive patients tended to be associated with a poor prognosis compared with LDHB negative patients (P=0.119).

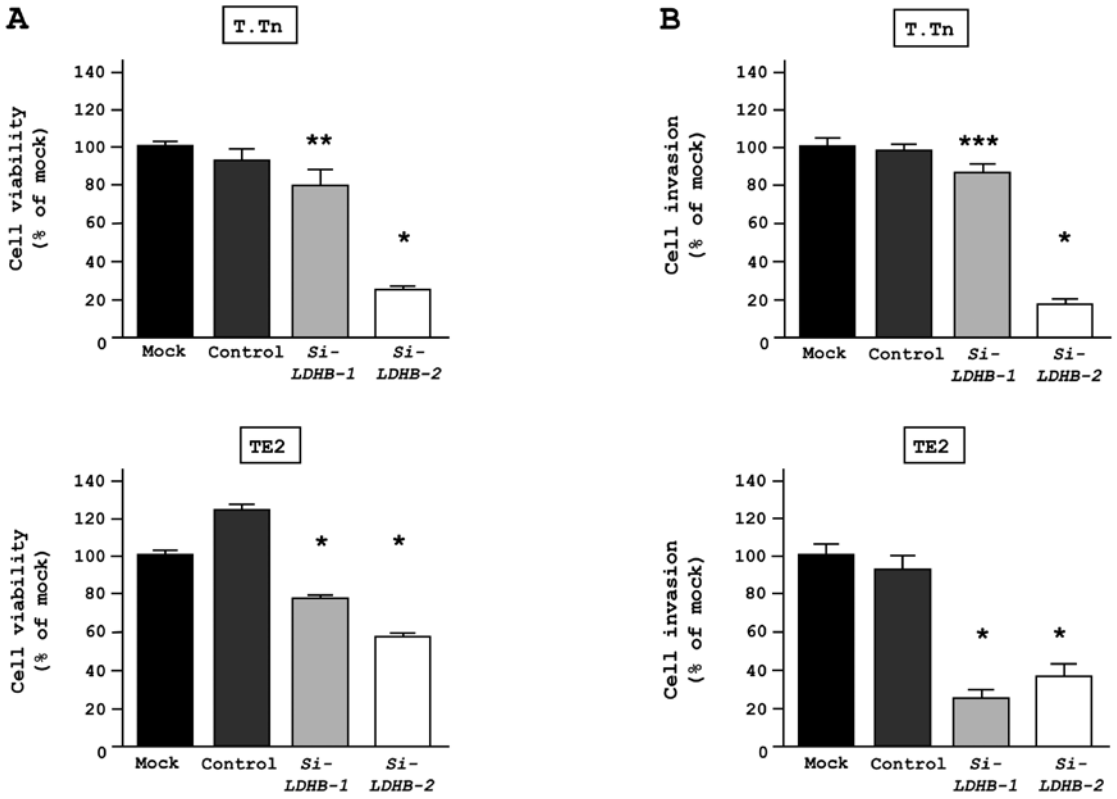

Figure 7. Loss of function studies using si-*LDHB*-transfected TE2 and T.Tn cells. (A) Cell proliferation was determined by the XTT assay. *P<0.0001, **P<0.046, ***P<0.0369. (B) Cell invasion (Matrigel invasion assay) of cells transfected with si-*LDHB*.

The downregulation of miR-375 is caused by promoter hypermethylation.

A computational analysis revealed that miR-375 is located in a CpG island on chromosome 2q35 (National Center for Biotechnology Information) (24). The acetylation of lysine residues in the N-terminal histone tail of the unmethylated CpG island induces an open structure of the chromatin and increased the transcription of that region (5,26). Although histone acetylation might directly upregulate miR-375, further experiments are required to confirm this.

We showed that the reinstatement of miR-375 could inhibit cancer cell proliferation and invasion in ESCC cell lines. In a recent study, it was shown that miR-375 inhibits tumor growth and metastasis in ESCC *in vivo* and *in vitro*. That study also revealed that the downregulation of miR-375 significantly correlated with a poor prognosis in ESCC (25).

In the present study, the genome-wide gene expression analysis revealed six candidate genes that were regulated by miR-375(*LDHB*, *MTDH*, *PRDX1*, *CXCL1*, *MAL2* and *CHSY1*). In this analysis, the criterion used for selection was upregulation in cancer tissues. Two genes, *LDHB* and *MTDH*, were of particular interest as they had also been identified in a search for miR-375 targets in HNSCC, an indication that these genes may have a role in the oncogenesis of human squamous cell carcinoma (15,22).

LDHB is known to convert lactase to pyruvate, which is then further oxidized (27). A correlation between LDHB expression and cancer has been reported (27). It was also revealed that the serum levels of LDHB are specifically elevated in non-small cell lung carcinoma patients, and are progressively increased with clinical stage (28). Kinoshita *et al* reported that the mRNA expression of LDHB might serve as a predictor of a poor prognosis in maxillary sinus squamous cell carcinoma (22). In our study, the knockdown of *LDHB* by RNAi showed a tumor suppressive effect in ESCC cells. In addition, ESCC clinical specimens exhibited a high level of LDHB expression at both the mRNA and protein levels compared with the normal esophageal epithelium. Kaplan-Meier curves and log-rank tests revealed that positive immunoreactivity for the LDHB protein had a tendency to indicate a poor prognosis. The current results indicate that LDHB plays an important role in cancer signaling pathways in ESCC.

Recent studies have shown that Metadherin (*MTDH*)/Astrocyte Elevated Gene 1 (*AEG-1*) plays a key role in tumor progression, invasion, metastasis, and resistance to chemotherapies (29). There is overexpression of AEG-1/MTDH in ESCC, and a multivariate analysis indicated that AEG-1/MTDH expression is a valuable marker of ESCC progression (30).

The current study suggested the possibility that histone deacetylase inhibition, the downregulation of miR-375, and the upregulation of LDHB and AEG-1/LDHB are involved in the initiation and development of ESCC. Further studies are required to elucidate the additional roles of miR-375-regulated molecular networks and to characterize the epigenetic cross-talk between histone acetylation and miRNAs, and also to determine the mechanism underlying the involvement of LDHB and MTDH in human oncogenesis.

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