Tumor suppressive *microRNA-375* regulates *lactate dehydrogenase B* in maxillary sinus squamous cell carcinoma

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Abstract. The expression of *microRNA-375 (miR-375)* is significantly reduced in cancer tissues of maxillary sinus squamous cell carcinoma (MSSCC). The aim of this study was to investigate the functional significance of *miR-375* and a possible regulatory role in the MSSCC networks. Restoration of *miR-375* significantly inhibited cancer cell proliferation and invasion in IMC-3 cells, suggesting that *miR-375* functions as a tumor suppressor in MSSCC. Genome-wide gene expression data and luciferase reporter assays indicated that *lactate dehydrogenase B (LDHB)* was directly regulated by *miR-375*. Cancer cell proliferation and invasion were significantly inhibited by transfection of si-*LDHB* into IMC-3 cells, suggesting that *LDHB* may play a role in MSSCC oncogenic function. In clinical MSSCC specimens, *LDHB* mRNA levels were up-regulated in cancer tissues, which were inversely correlated with the expression of *miR-375*. In addition, Kaplan-Meier curves and log-rank tests revealed that the high mRNA expression levels of *LDHB* had a significant adverse effect on survival rate. The identification of a cancer network regulated by the *miR-375* tumor suppressor could provide new insights into the molecular mechanisms of MSSCC oncogenesis.

Introduction

Maxillary sinus squamous cell carcinoma (MSSCC) is a malignant epithelial tumor originating in the respiratory mucosa of the maxillary sinus. MSSCC comprises 2-3% of all head and neck tumors, with an annual incidence of 0.5-1.0 per 100,000 people (1,2). Since the clinical symptoms of patients with MSSCC are very insidious, tumors are often diagnosed at advanced stages. Despite advances in multimodality therapy including surgery, radiotherapy and chemotherapy, the 5-year survival rate for MSSCC has remained ~50%. Although regional lymph node metastasis and distant metastasis are uncommon (20%), the high rate of locoregional recurrence (60%) contributes to poor survival (3). The results of epidemiological studies have suggested that occupational exposures to leather, wood dust, nickel, arsenic and formaldehyde are risk factors for MSSCC (4-6); a point of divergence from head and neck squamous cell carcinomas (HNSCC) of the tongue, oral cavity and pharynx. Few reports have performed genome analysis of MSSCC. Understanding the molecular oncogenic pathways underlying MSSCC could significantly improve diagnosis, therapy, and disease prevention.

Growing evidence suggests that carcinogenesis is induced by overactivation of pro-oncogenic pathways and inactivation of tumor-suppressive pathways (7). In cancer pathways, normal regulatory mechanisms are disrupted by aberrant expression of microRNA (miRNA) (8). miRNAs are a class of small non-coding RNA molecules consisting of 19-22 nucleotides that are involved in a variety of biological processes including development, differentiation, apoptosis, and cell proliferation. They regulate gene expression through translational repression and mRNA cleavage (9). Bioinformatic predictions indicate that miRNAs regulate >30% of protein coding genes (10). So far, 1424 human miRNAs have been registered at miRBase release 17.0 (http://microrna.sanger.ac.uk/). miRNAs contribute to the initiation and development of various types of cancers (11). miRNAs are aberrantly expressed in many human cancers, and can function either as tumor suppressors or oncogenes (12).

Down-regulated miRNAs in cancer cells might normally function as tumor suppressive miRNAs that negatively regulate oncogenes. Based on this hypothesis, we have profiled the miRNA expression signatures of human cancers, including HNSCC (13-17). In human squamous cell carcinoma (SCC), tumor suppressive miRNAs such as *miR-1, miR-133a* and

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miR-489 were identified through direct repression of several oncogenes (15,16,18-20). The miRNA expression signatures of hypopharyngeal SCC, esophageal SCC and MSSCC revealed that *miR-375* is significantly reduced in cancer cells (15,16). *miR-375* was originally cloned and identified as an evolutionarily conserved pancreatic islet-specific miRNA (21). *miR-375*$^{-/-}$ mice were hyperglycemic and exhibited an increase in the number of pancreatic α cells (22). Although several studies have indicated that *miR-375* is down-regulated in human cancers, the function of *miR-375* and its target genes in MSSCC remains unclear (23-26).

The aim of this study was to clarify the functional significance of *miR-375* in MSSCC and to identify molecular pathways regulated by *miR-375*. We analyzed the effect of *miR-375* on cell proliferation and cell invasion in the MSSCC cell line IMC-3. Genome-wide expression analysis identified *lactate dehydrogenase B (LDHB)* as a candidate gene target of *miR-375*. The identification of a novel *miR-375*-regulated tumor suppressive pathway could further reveal the molecular mechanism of MSSCC oncogenesis.

Materials and methods

MSSCC cell culture. IMC-3, a human MSSCC cell line, was derived from a primary lesion of MSSCC (27). MSSCC cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37˚C.

Clinical specimens of MSSCC. Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Chiba University. Twenty pairs of primary tumor tissues and corresponding normal epithelial tissues were obtained from patients with MSSCC in Chiba University Hospital (Chiba, Japan) from 2007 to 2010. The normal tissue was confirmed to be free of cancer cells by pathologic examination. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at -20˚C until RNA was extracted. Clinical information of the twenty patients is shown in Table I.

RNA isolation. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration was determined spectrophotometrically, and molecular integrity was checked by gel electrophoresis. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Mature miRNA transfection and small interfering RNA treatment. The following RNA species were used in this study: mature miRNAs, Pre-miRTM miRNA Precursors (*hsa-miR-375*; Pre-miR ID: PM10327), negative control miRNA (P/N: AM17111) (Applied Biosystems, Foster City, CA, USA), small interfering RNA (Stealth Select RNAi™ siRNA; si-*LDHB* Cat. no.; HSS106003 and HSS106004) (Invitrogen) and negative control siRNA (Stealth™ RNAi Negative Control Medium GC Duplex; 12935-300) (Invitrogen). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine™ RNAiMax Reagent (Invitrogen) as described previously (13). Transfection efficiency of Pre-miRTM in the IMC-3 cell line was confirmed based on down-regulation of *TWF1 (PTK9)* mRNA following transfection with *miR-1* as previously reported (14,15).

Cancer cell proliferation and invasion assays. Cells were transfected with 10 nM miRNA and siRNA by reverse transfection and plated in 96-well plates at $3x10^3$ cells per well. After 72 or 96 h, cell proliferation was determined with the XTT assay, using the Cell Proliferation kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported (15,16). Triplicate wells were measured for cell viability in each treatment group.

A cell invasion assay was carried out using modified Boyden chambers containing transwell-precoated Matrigel membrane filter inserts with 8-μm pores in 24-well tissue culture plates at $1x10^5$ cells per well (BD Biosciences, Bedford, MA, USA) (14). Triplicate wells were measured for cell invasion in each treatment group.

Microarray expression analysis. A genome-wide screen was performed to identify gene targets of *miR-375* in the IMC-3 cell line. Oligo-microarray Human 44K (Agilent Technologies) was used for expression profiling of *miR-375* transfectants in comparison with miRNA-negative-control transfectants. Microarray hybridization and data collection were performed as previously described (28). TargetScan (release 5.1, http://www.targetscan.org/) was used to identify predicted target genes and their miRNA binding site seed regions. Sequences of the predicted mature miRNAs were confirmed using miRBase (release 17.0, http://microrna.sanger.ac.uk/).

Quantitative reverse-transcription-PCR (qRT-PCR). First-strand cDNA was synthesized from 1.0 μg of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene-specific PCR products were assayed continuously using a 7900-HT Real-Time PCR System according to the manufacturer's protocol. TaqMan® probes and primers for *LDHB* (P/N: Hs00929956_m1), and *GUSB* (P/N: Hs99999908_m1) internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of *miR-375* (Assay ID: 000564) was analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to *RNU48* (Assay ID: 001006). All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

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

Table I. Clinical information of 20 patients with MSSCC.

No.	Age	Gender	Differentiation	T	N	M	Stage
1	68	Male	Well	4b	0	0	IVB
2	77	Male	Poor	3	0	0	III
3	76	Male	Moderate	3	0	0	III
4	61	Male	Well	3	0	0	III
5	54	Male	Poor	3	0	0	III
6	65	Female	Poor	4b	0	0	IVB
7	65	Male	Moderate	4a	0	0	IVA
8	64	Male	Poor	4b	0	0	IVB
9	74	Male	Well	4a	0	0	IVA
10	71	Male	Moderate	3	1	0	III
11	64	Male	Moderate	4a	0	0	IVA
12	80	Male	Moderate	4a	0	0	IVA
13	66	Female	Poor	4a	2c	0	IVA
14	67	Male	Moderate	4a	0	0	IVA
15	60	Male	Poor	4a	0	0	IVA
16	66	Female	Moderate	4a	0	0	IVA
17	85	Male	Poor	4a	0	0	IVA
18	69	Male	Well	4a	0	0	IVA
19	57	Male	Poor	4a	0	0	IVA
20	69	Male	Poor	4a	2b	0	IVA

Plasmid construction and dual-luciferase reporter assay. The wild-type sequence of the *LDHB* 3'-UTR (WT-3'UTR) and a sequence with deleted *miR-375* target sites (DEL-3'UTR) were inserted between the *Xho*I-*Pme*I restriction sites in the 3'UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI). IMC-3 cells were transfected with 15 ng of vector, 10 nM of *miR-375* (Applied Biosystems), and 1 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of Opti-MEM (Invitrogen). The activities of Firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the quotient of *Renilla*/Firefly luciferase activities.

Statistical analysis. The relationships between two groups and the numerical values obtained by qRT-PCR were analyzed using the nonparametric Mann-Whitney U test or the paired t-test. The relationship among more than three variables and numerical values was analyzed using the Bonferroni adjusted Mann-Whitney U test. Spearman's rank test was used to evaluate the relationships among the relative expression levels of *miR375* and *LDHB* mRNA. The Kaplan-Meier method was performed using a median split method in which patients were divided into 2 groups on the basis of whether mRNA expression levels were higher or lower than the median. The log-rank test was used to compare curves. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA).

Results

Effect of miR-375 transfection on proliferation and invasion in IMC-3 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 72 h (% of cell proliferation, 60.9±1.0, 100.0±3.3 and 103.0±2.6, respectively, P<0.0001) and after 96 h (% of cell proliferation, 51.0±2.6, 100.0±2.6 and 117.3±8.3, respectively, P<0.0001) (Fig. 1A).

The Matrigel invasion assay demonstrated that invading cell numbers were significantly decreased in *miR-375* transfectants compared to mock and miRNA-control transfectants (% of cell invasion, 46.4±2.4, 100.0±14.2 and 98.7±3.9, respectively, P=0.0005) (Fig. 1B).

Screening of miR-375 target genes using gene expression analysis. To gain further insight into which protein-coding genes are regulated by *miR-375* in IMC-3 cells, we performed comprehensive gene expression analysis with *miR-375* transfectants. miRNA-control transfectants that produced raw signal values of <3,000 were excluded before comparisons were made. Fifty-two genes were down-regulated less than -1.0 (Log_2 ratio) in the transfectants (Table II). The 3'UTR of these down-regulated genes were examined for the existence of *miR-375* target sites using the TargetScan database. TargetScan revealed that 28 of the 52 genes had putative *miR-375* target sites in their 3'UTR (Table II).

miR-375 regulation of lactate dehydrogenase B (LDHB). The *LDHB* gene was chosen for further study because it ranked at the top of the list of candidate genes in the genome-wide gene expression analysis and had one putative miR-375 target site. qRT-PCR revealed that the expression level of *LDHB* mRNA

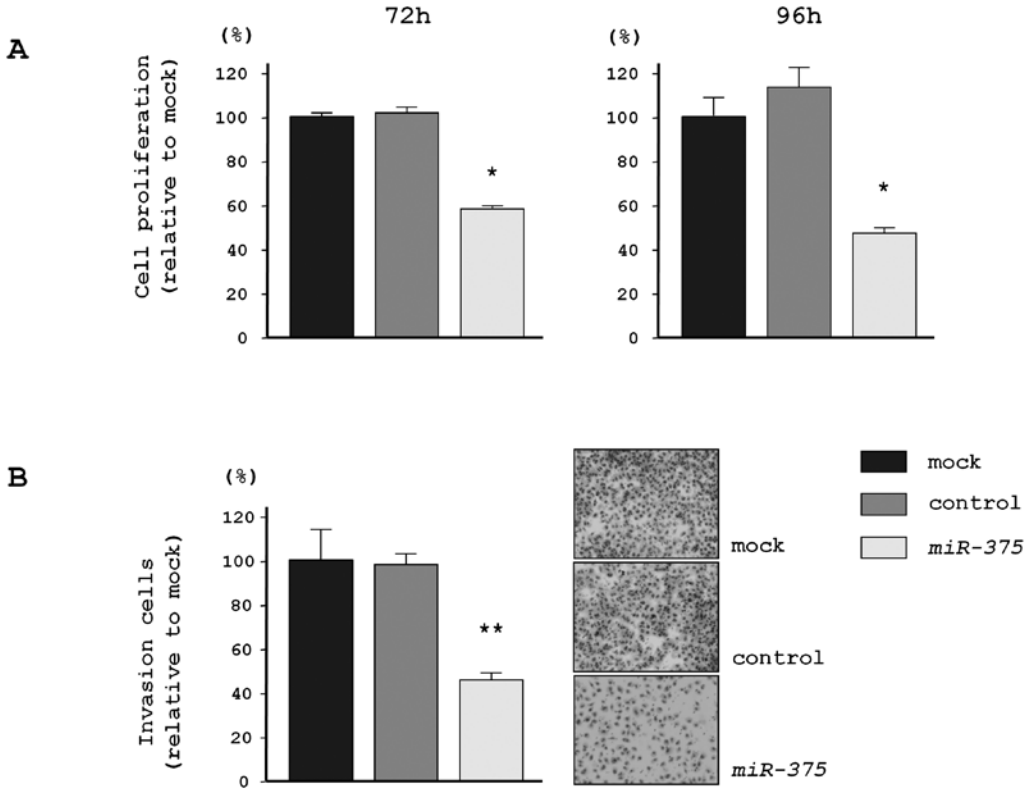

Figure 1. Gain-of-function studies using *miR-375* transfected IMC-3 cells. (A) Cell proliferation as revealed by the XTT assay 72 and 96 h after transfection. *P<0.0001. (B) Cell invasion activity (Matrigel invasion assay) in IMC-3 cells 48 h after transfection with *miR-375*. **P=0.0005.

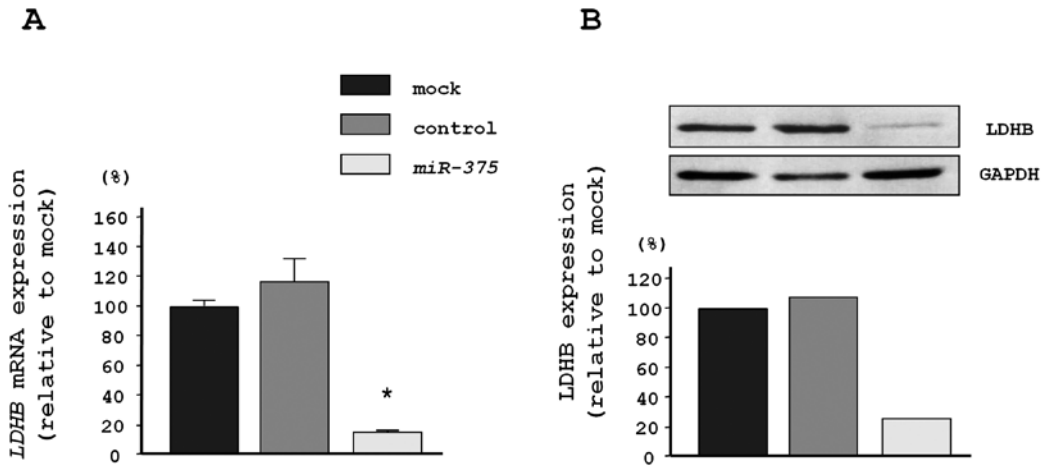

Figure 2. LDHB mRNA and protein expression in IMC-3 cells. (A) qRT-PCR revealed that *LDHB* mRNA was significantly repressed in *miR-375* transfectants compared with mock and control. *GUSB* was used as an internal control. *P<0.0001. (B) Western blotting revealed that LDHB protein expression was also decreased in the *miR-375* transfectant. GAPDH was used as a loading control.

was significantly repressed in *miR-375* transfected IMC-3 cells compared to mock controls (Fig. 2A). Protein expression was also markedly reduced in *miR-375* transfectants (Fig. 2B).

A luciferase reporter assay was then used to confirm that the predicted binding site was an actual target of *miR-375*. We used a vector encoding either the total sequence of the *LDHB* 3'UTR, including the predicted *miR-375* target site (positions 171-177), or a vector lacking the *miR-375* target site. Luminescence intensity was significantly reduced by transfection of the entire *LDHB* 3'UTR, while deletion of positions 171-177 blocked the decrease in luminescence (Fig. 3).

Effect of LDHB silencing on cell proliferation and invasion. The functional role of *LDHB* was examined with loss-of-function studies using IMC-3 cells transfected with two different si-*LDHB*s. The mRNA and protein levels were markedly reduced in si-*LDHB* transfectants (Fig. 4A and B).

The XTT assay revealed significant inhibition of cell proliferation in si-*LDHB* transfectants in comparison with mock

Table II. Down-regulated genes in *miR-375* transfectant of IMC-3.

No.	Entrez gene ID	Gene symbol	Gene name	Log_2 ratio	*miR-375* target site
1	3945	*LDHB*	Lactate dehydrogenase B	-1.82	1
2	7534	*YWHAZ/ 14-3-3ζ*	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide/14-3-3ζ	-1.69	1
3	3516	*RBPJ*	Recombination signal binding protein for immunoglobulin κ J region	-1.65	1
4	57179	*KIAA1191*	KIAA1191	-1.60	1
5	114569	*MAL2*	Mal, T-cell differentiation protein 2	-1.60	1
6	5420	*PODXL*	Podocalyxin-like	-1.57	2
7	84650	*EBPL*	Emopamil binding protein-like	-1.52	1
8	10857	*PGRMC1*	Progesterone receptor membrane component 1	-1.52	0
9	4023	*LPL*	Lipoprotein lipase	-1.51	1
10	92140	*AEG-1/MTDH*	Astrocyte elevated gene-1/metadherin	-1.47	1
11	5052	*PRDX1*	Peroxiredoxin 1	-1.47	1
12	6418	*SET*	SET nuclear oncogene	-1.40	1
13	4609	*MYC*	V-Myc myelocytomatosis viral oncogene homolog (avian)	-1.40	0
14	708	*C1QBP*	Complement component 1, q subcomponent binding protein	-1.39	1
15	55177	*FAM82A2*	Family with sequence similarity 82, member A2	-1.33	1
16	5504	*PPP1R2*	Protein phosphatase 1, regulatory (inhibitor) subunit 2	-1.27	0
17	10314	*LANCL1*	LanC lantibiotic synthetase component C-like 1 (bacterial)	-1.21	2
18	114971	*PTPMT1*	Protein tyrosine phosphatase, mitochondrial 1	-1.20	1
19	9601	*PDIA4*	Protein disulfide isomerase family A, member 4	-1.20	0
20	9221	*NOLC1*	Nucleolar and coiled-body phosphoprotein 1	-1.19	2
21	442249	*LOC442249*	Similar to keratin 18	-1.18	0
22	50999	*TMED5*	Transmembrane emp24 protein transport domain containing 5	-1.17	1
23	5037	*PEBP1*	Phosphatidylethanolamine binding protein 1	-1.16	0
24	159	*ADSS*	Adenylosuccinate synthase	-1.16	0
25	9156	*EXO1*	Eexonuclease 1	-1.16	0
26	284085	*FLJ40504*	Keratin 18 pseudogene	-1.16	0
27	22856	*CHSY1*	Chondroitin sulfate synthase 1	-1.14	1
28	90133	*KRT8P12*	Keratin 8 pseudogene 12	-1.13	0
29	9802	*DAZAP2*	DAZ associated protein 2	-1.13	1
30	5557	*PRIM1*	Primase, DNA, polypeptide 1 (49 kDa)	-1.12	0
31	55536	*CDCA7L*	Cell division cycle associated 7-like	-1.12	2
32	9343	*EFTUD2*	Elongation factor Tu GTP binding domain containing 2	-1.12	0
33	116228	*FAM36A*	Family with sequence similarity 36, member A	-1.11	0
34	9709	*HERPUD1*	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-1.11	1
35	10360	*NPM3*	Nucleophosmin/nucleoplasmin 3	-1.10	0
36	3856	*KRT8*	Keratin 8	-1.09	0
37	528	*ATP6V1C1*	ATPase, H^+ transporting, lysosomal 42 kDa, V1 subunit C1	-1.08	1
38	5955	*RCN2*	Reticulocalbin 2, EF-hand calcium binding domain	-1.08	0
39	9493	*KIF23*	Kinesin family member 23	-1.08	0
40	26521	*TIMM8B*	Translocase of inner mitochondrial membrane 8 homolog B (yeast)	-1.08	0
41	332	*BIRC5*	Baculoviral IAP repeat-containing 5	-1.07	0
42	1063	*CENPF*	Centromere protein F, 350/400 ka (mitosin)	-1.06	0
43	10960	*LMAN2*	Lectin, mannose-binding 2	-1.06	0
44	1478	*CSTF2*	Cleavage stimulation factor, 3' pre-RNA, subunit 2, 64 kDa	-1.05	1
45	112950	*MED8*	Mediator complex subunit 8	-1.05	1
46	3099	*HK2*	Hexokinase 2	-1.05	0
47	11335	*CBX3*	Chromobox homolog 3 (HP1 γ homolog, *Drosophila*)	-1.04	1
48	10440	*TIMM17A*	Translocase of inner mitochondrial membrane 17 homolog A (yeast)	-1.03	1
49	11113	*CIT*	Citron (rho-interacting, serine/threonine kinase 21)	-1.03	1
50	8727	*CTNNAL1*	Catenin (cadherin-associated protein), α-like 1	-1.01	0
51	3875	*KRT18*	Keratin 18	-1.01	0
52	9804	*TOMM20*	Translocase of outer mitochondrial membrane 20 homolog (yeast)	-1.00	2

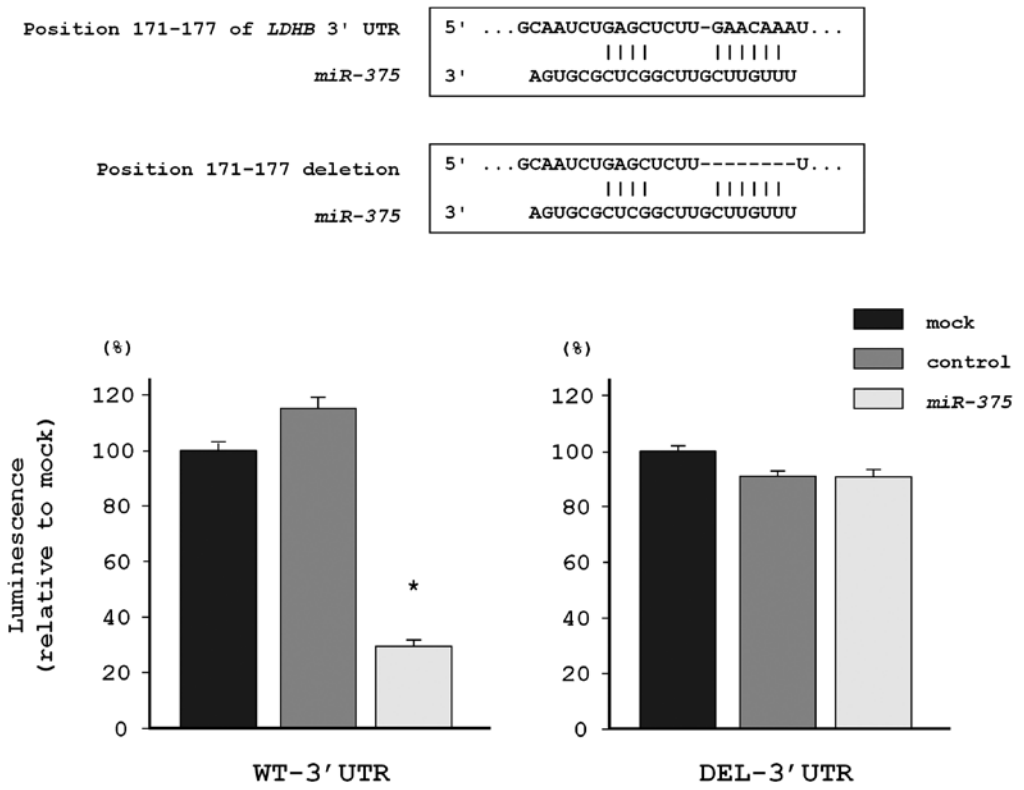

Figure 3. Interaction of *miR-375* with the *LDHB* 3'UTR. At 24 h after transfection (with 10 nM *miR-375* or miRNA-control) or mock transfection, a reporter plasmid containing *LDHB* WT-3'UTR or DEL-3'UTR and a plasmid expressing *Renilla* luciferase (hRluc) were co-transfected into IMC-3 cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity. *P<0.0001.

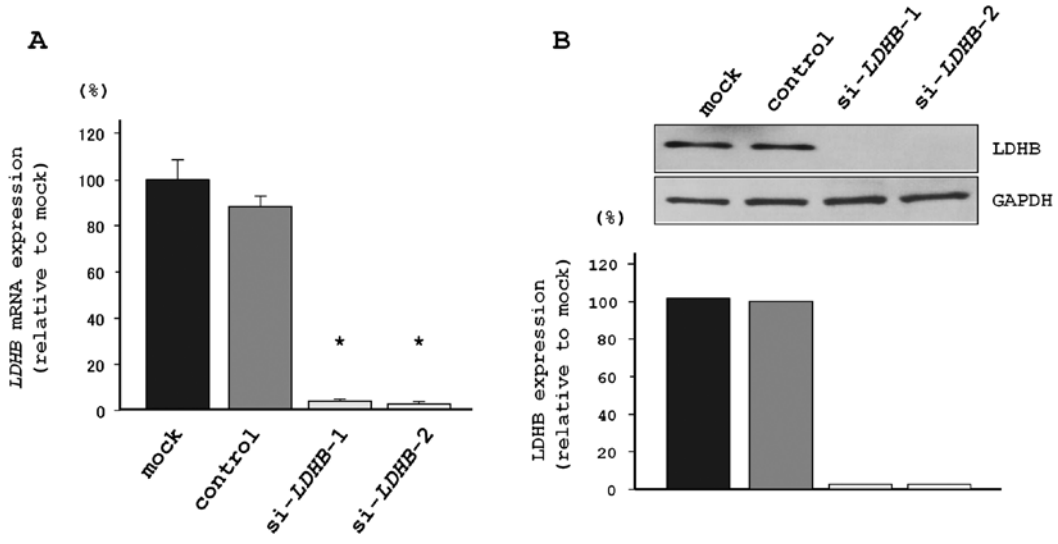

Figure 4. siRNA knockdown of *LDHB* in IMC-3 cells. (A) qRT-PCR revealed that *LDHB* mRNA was significantly repressed in si-*LDHB* transfectants compared with mock and si-controls. *GUSB* was used as an internal control *P<0.0001. (B) Western blot analysis revealed that LDHB protein expression was also decreased in si-*LDHB* transfectants. GAPDH was used as a loading control.

and si-control transfectants after 72 h (% of cell proliferation: 83.6±2.1, 83.4±3.0, 100.0±5.0 and 99.3±3.0, respectively, P<0.0005; Fig. 5A) and 96 h (% of cell proliferation: 64.0±1.1, 67.8±2.6, 100.0±1.4 and 96.8±0.8, respectively, P<0.0001; Fig. 5A).

The Matrigel invasion assay demonstrated that the number of invading cells was significantly lower in the si-*LDHB* transfectants compared with mock and si-control transfectants (% of cell invasion, 15.3±2.8, 12.2±3.2, 100.0±14.7 and 77.8±4.8 respectively, P<0.0001) (Fig. 5B).

Expression levels of miR-375 and LDHB in MSSCC clinical specimens. The expression level of *miR-375* was significantly down-regulated in clinical MSSCC specimens compared with

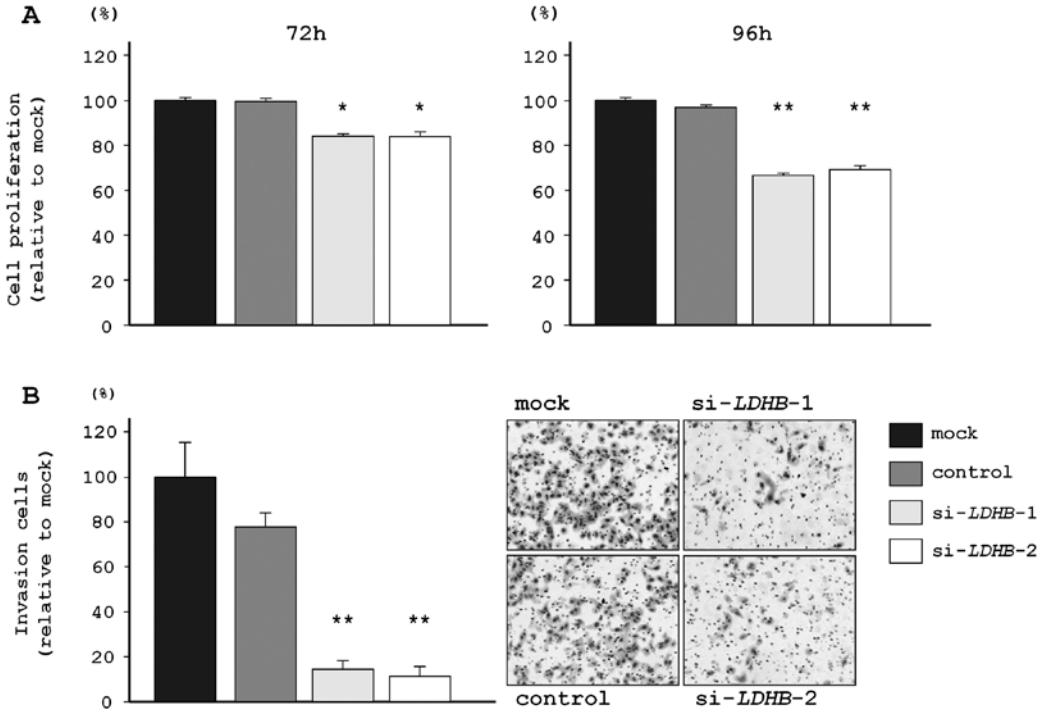

Figure 5. Loss of function studies using si-*LDHB* transfected IMC-3 cells. (A) Cell proliferation as revealed by the XTT assay. *P<0.0005, **P<0.0001. (B) Cell invasion activity (Matrigel invasion assay) of cells transfected with si-*LDHB*. **P<0.0001.

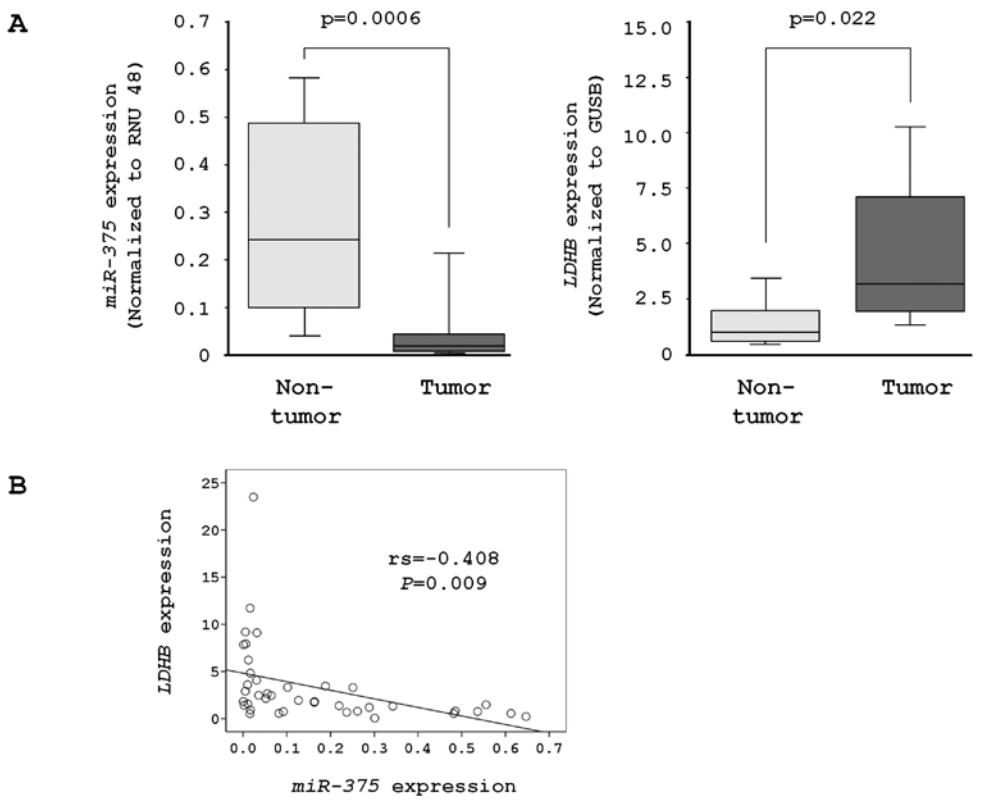

Figure 6. *miR-375* and *LDHB* mRNA expression in 20 clinical MSSCC specimens. (A) *miR-375* expression in normal adjacent issues and tumor tissues. (B) *LDHB* mRNA expression in tumor tissues and normal adjacent tissues. (C) Correlation between *miR-375* expression and *LDHB* mRNA expression.

normal tissues (P=0.0006, Fig. 6A). Conversely, *LDHB* mRNA was significantly up-regulated in tumor tissues (P=0.022, Fig. 6A). The expression of *miR-375* was inversely correlated with that of *LDHB* (Fig. 6B).

To determine if the levels of *LDHB* of tumor tissues correlate with the survival of the MSSCC patients, we categorized two groups according to *LDHB* mRNA expression. As the results of statistical analysis, patients with high *LDHB* expression group

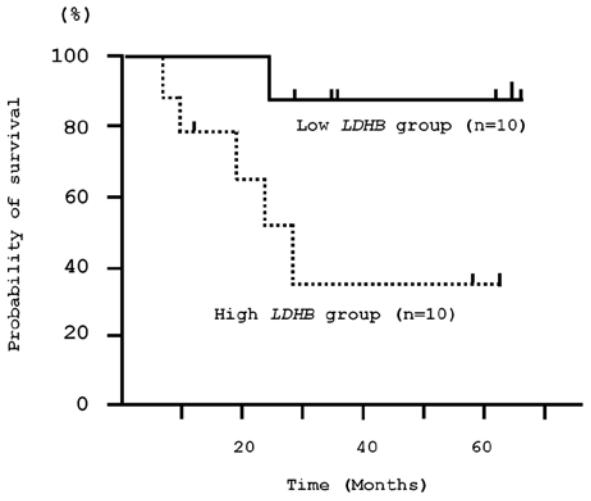

Figure 7. Kaplan-Meier analysis of overall survival in patients with MSSCC. Twenty MSSCC patients were divided into 2 groups on the basis of whether *LDHB* mRNA expression levels were higher or lower than the median. Kaplan-Meier analysis indicates the correlation between the overall survial of MSSCC patients and mRNA expression levels of *LDHB*. The log-rank test showed statistically significant difference between the 2 groups. (P=0.035).

showed worse survival rate than low *LDHB* expression group (five-year overall survival rate: 35 and 88%, respectively). The log-rank test showed statistically significant difference between the 2 groups (P=0.035; Fig. 7).

Discussion

Aberrant expression of miRNAs and uncontrolled gene expression of tumor suppressors and oncogenes contribute to the initiation and development of human cancers (11). We have identified tumor suppressive miRNAs and their associated cancer networks based on miRNA expression signatures (13-16). A comparison of these expression signatures (hypopharyngeal and esophageal SCC) with reports from other institutes found that *miR-375* is significantly down-regulated in several types of cancers (23,25,26,29,30). An expression signature of MSSCC in our recent study revealed that *miR-375* is also reduced (third down-regulated miRNA in this profile) in cancer tissues (data not shown).

Therefore, we investigated the functional significance of *miR-375* in MSSCC in this study. The data revealed that restoration of *miR-375* suppressed cancer cell proliferation and invasion in IMC-3 cells, confirming results from an analysis of other region of HNSCC (31). The tumor suppressive function of *miR-375* has also been reported with other cancers, such as gastric cancer and liver cancer (23,25,26,32). These data strongly indicate that *miR-375* acts as a tumor suppressor in human cancers.

The unique point of miRNA biogenesis is that one miRNA regulates many protein-coding genes. That is why the elucidation of a miRNA-regulated cancer network is important in understanding human cancer. According to the past studies, *miR-375* directly regulates the *YWHAZ/14-3-3ζ, PDK1, YAP* and *JAK2* genes in several cancers (23,25,26). More recently, we also reported that *miR-375* directly regulates an oncogene *AEG-1/MTDH* in HNSCC (31). Microarray analyses of MSSCC (this study) found that the expression levels of *YWHAZ/14-3-3ζ* was conspicuously reduced in *miR-375* transfectants. There are many studies that *YWHAZ/14-3-3ζ* functions as an oncogene in several human cancers, including HNSCC (33,34). In addition, *YWHAZ/14-3-3ζ* is located on human chromosome 8q22.3, a region that is frequently the site of copy number gain in HNSCC (35). Overexpression of *AEG-1/MTDH*, an oncogene under direct regulation of *miR-375*, was observed in several types of cancers (36-38). Many studies have indicated that *AEG-1/MTDH* contributes to several steps in human oncogenesis, including cancer cell progression, invasion, metastasis, and chemoresistance (39). It is reported that overexpression of *AEG-1/MTDH* promotes activation of the pro-survival pathway of PI3K/AKT via up-regulation of PIP3 (40). These facts suggest that *miR-375* is a direct regulator of the *YWHAZ/14-3-3ζ* and *AEG-1/MTDH* oncogenes, and that this phenomenon is important to HNSCC and MSSCC oncogenesis.

The novel finding of this study is that *LDHB* is directly regulated by *miR-375* in MSSCC. *LDHB* and *LDHA* are the glycolytic enzymes that catalyze the formation of lactic acid from pyruvate (41). It has been reported that *LDHA* is elevated and activated in many cancers (42,43). However, a definitive relationship between *LDHB* and cancer has not been established. In non-small cell lung cancer (NSCLC), the levels of LDHB were significantly elevated in NSCLC sera. Furthermore, the serum levels of LDHB correlated with the clinical stage of NSCLC (41), and concomitant protein expression of LDHB and CCNB1 was a predictor of very poor prognosis in medulloblastoma patients (44). Our data also indicated that mRNA levels of *LDHB* were elevated in cancer tissues. More interestingly, Kaplan-Meier curves and log-rank tests revealed the high mRNA expression levels of *LDHB* had a significant adverse effect on survival rate. Our results show the possibility that *LDHB* becomes the prognostic marker of MSSCC. However, our cohort was too small to evaluate this relationship.

A recent study on the role of *LDHB* in cancer signaling pathways reported that *LDHB* is a downstream target of *mTOR*, which is critical for oncogenic *mTOR*-mediated tumorigenesis (45). Our data revealed that cell proliferation was significantly reduced in si-*LDHB* transfectants, suggesting that down-regulation of *miR-375* and up-regulation of *LDHB* is an indispensable event for MSSCC initiation and development. A detailed study of *miR-375*-regulated molecular networks is needed for full understanding of human oncogenesis.

In conclusion, the reduction of *miR-375* and the increased expression of *LDHB* are frequent events in MSSCC cancer cells. *miR-375* functions as a tumor suppressor and direct regulator of *LDHB* in MSSCC. The *miR-375* regulation of novel cancer pathways could provide new insights into the molecular mechanisms involved in the development of MSSCC and contribute to the development of new therapeutic strategies for the disease.

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