Regulation of *MMP13* by antitumor *microRNA-375* markedly inhibits cancer cell migration and invasion in esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies. Recently developed molecular targeted therapies are not available for patients with ESCC. After curative surgical resection, patients frequently suffer distant metastasis and recurrence. Exploration of novel ESCC metastatic pathways may lead to the development of new treatment protocols for this disease. Accordingly, we have sequentially identified microRNA (miRNA)-mediated metastatic pathways in several cancers. Our past studies of miRNA expression signatures have shown that *microRNA-375* (*miR-375*) is frequently reduced in several types of cancers, including ESCC. In the present study, we aimed to investigate novel *miR-375*-mediated metastatic pathways in ESCC cells. The expression of *miR-375* was downregulated in ESCC tissues, and ectopic expression of this miRNA markedly inhibited cancer cell migration and invasion, suggesting that *miR-375* acted as an antimetastatic miRNA in ESCC cells. Our strategies for miRNA target searching demonstrated that matrix metalloproteinase 13 (*MMP13*) was directly regulated by *miR-375* in ESCC cells. Overexpression of *MMP13* was observed in ESCC clinical tissues, and the expression of *MMP13* promoted cancer cell aggressiveness. Moreover, oncogenic genes, including *CENPF*, *KIF14* and *TOP2A*, were shown to be regulated downstream of MMP13. Taken together, these findings demonstrated that the antitumor *miR-375*/oncogenic MMP13 axis had a pivotal role in ESCC aggressiveness. These results provide novel insights into the potential mechanisms of ESCC pathogenesis.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers and the major histological type of esophageal cancer in Japan and East Asia (1-3). ESCC cells frequently metastasize to the lymph nodes, liver, lungs and bone (2-4). Despite the use of multimodality therapies, the prognosis of patients with ESCC is still poor, with an overall 5-year survival rate of approximately 20-30% (2,4). Recently developed molecularly targeted therapeutics have not been shown to have beneficial effects in patients with ESCC (2). Additionally, the molecular pathogenesis of the aggressive phenotype in ESCC remains unclear. Thus, in order to improve disease outcomes in patients with ESCC, it is necessary to elucidate the molecular mechanisms of ESCC cell aggressiveness using advanced genomic approaches.

The discovery of microRNAs (miRNAs) has resulted in major advancements in cancer research (5,6). miRNAs are small non-coding RNAs that function to fine tune the expression of protein coding/non-coding RNAs by repressing translation or cleaving RNA transcripts in a sequence-depending manner (7). The unique characteristic function of miRNAs is to regulate RNA transcripts in human cells. Therefore, dysregulated expression of miRNAs can disrupt tightly regulated RNA networks in cancer cells. Currently, numerous studies have shown that miRNAs are aberrantly expressed in several cancers, including ESCC (6,8). Using miRNA expression signature analyses, we have sequentially identified tumor-suppressive miRNAs and shown that these miRNAs mediate novel cancer networks (9-13).

Our miRNA expression signatures revealed that *microRNA-375* (*miR-375*) is frequently downregulated in several types of squamous cell carcinoma (10,13,14). Moreover, our previous studies demonstrated that ectopic expression of *miR-375* suppressed cancer cell aggressiveness in several types of cancer cells (15). In ESCC cells, several studies have

Table I. Clinical features of patients with ESCC.

No.	Age (years)	Gender	Differentiation	T	N	M	Stage	ly	v	Recurrence
1	68	Male	Poor	1b	2	0	IIIA	1	3	+
2	72	Male	Moderate	1b	0	0	IA	0	1	-
3	69	Male	Moderate	1b	0	0	IIIA	0	0	-
4	62	Male	Well	3	2	0	IIIB	1	1	+
5	66	Male	Moderate	3	0	0	IIA	1	1	-
6	74	Male	Moderate	2	2	0	IIIA	3	1	+
7	56	Male	Moderate	2	0	0	IB	0	1	-
8	79	Male	Moderate	2	1	0	IIB	1	1	-
9	68	Male	Moderate	1b	2	0	IIIA	1	1	-
10	52	Male	Poor	1b	0	0	IA	1	1	+
11	67	Male	Well	3	2	0	IIIB	2	2	+
12	57	Male	Poor	3	3	0	IIIC	1	1	+
13	70	Male	Moderate	3	0	0	IIA	1	1	+
14	66	Male	Moderate	3	0	0	IIA	1	1	-
15	63	Male	Well	3	3	0	IIIC	2	1	+
16	55	Male	Moderate	3	2	0	IIIB	1	1	+
17	60	Male	Well	1b	1	0	IIB	1	1	-
18	78	Male	Well	3	0	0	IIA	1	2	-
19	71	Male	Well	3	0	0	IIA	1	2	-
20	75	Male	Moderate	3	2	0	IIIB	1	1	+
21	60	Male	Moderate	2	1	0	IIB	1	2	-
22	62	Male	Well	1a	1	0	IIB	0	0	-
23	71	Male	Moderate	1b	1	0	IIB	0	0	-
24	69	Male	Moderate	1b	0	0	IA	1	0	-
25	84	Male	Well	2	1	0	IIB	1	1	-

indicated that *miR-375* has antitumor roles through targeting oncogenic genes (16,17). Moreover, *miR-375*-mediated cancer pathways are essential for cancer cell initiation, development and aggressiveness.

Accordingly, in the present study, we aimed to investigate the novel cancer networks regulated by *miR-375* in ESCC cells. Our present data showed that matrix metalloproteinase 13 (*MMP13*) was directly regulated by *miR-375* in ESCC cells. Overexpression of *MMP13* was observed in ESCC clinical tissues, and knockdown of *MMP13* expression markedly inhibited ESCC cell migration and invasion, indicating that *MMP13* acted as a cancer-promoting gene in ESCC cells. Moreover, the oncogenic genes *CENPF*, *KIF14* and *TOP2* were found to function downstream of *MMP13*. Taken together, these results showed that the antitumor *miR-375*/oncogenic *MMP13* axis had a pivotal role in ESCC aggressiveness.

Materials and methods

Clinical ESCC specimens and ESCC cell lines. Clinical specimens were collected from 25 patients with ESCC. All patients underwent primary surgical treatment and were pathologically proven to have ESCC at the Kagoshima University Hospital from 2010 to 2014. The present study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were obtained from all patients. The clinicopathological characteristics of the patients are shown in Table I.

We used two ESCC cell lines: TE-8, which was moderately differentiated; and TE-9, which was poorly differentiated. Both of these cells lines were provided by Riken BioResourse Center (Tsukuba, Japan).

Extraction of total RNA from clinical specimens and cell lines was performed using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The quality of RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The procedure for PCR quantification was previously described (13,18-20). The expression levels of *miR-375* (assay ID: 000564; Applied Biosystems, Foster City, CA, USA) were analyzed by TaqMan qRT-PCR assays (TaqMan MicroRNA assays; Applied Biosystems) and *RNU48* (assay ID: 001006) was used for normalization. TaqMan probes and primers for *MMP-13* (assay ID: Hs00233992_m1; Applied Biosystems), *CENPF* (assay ID: Hs01118845_m1), *KIF14* (assay ID: Hs00978236_m1) and *GUSB* (the internal control; assay ID: Hs00939627_ml; Applied Biosystems) were used for gene expression analysis.

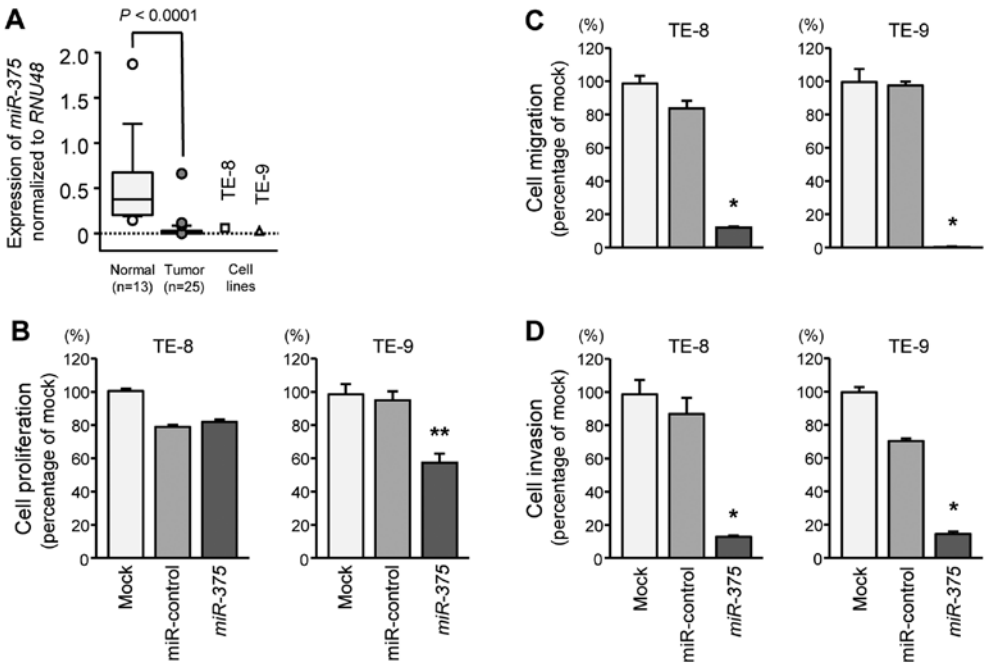

Figure 1. Expression levels of *miR-375* and functional assays of *miR-375* transfection in ESCC cell lines. (A) Expression levels of *miR-375* in ESCC or normal esophageal tissues and ESCC cell lines. (B) Cell proliferation was determined by XTT assays. *P<0.0001, **P<0.05. (C) Cell migration activity was determined by migration assays. (D) Cell invasion activity was determined by Matrigel invasion assays.

Transfection with mature miRNAs and small interfering RNAs (siRNAs). The following mature miRNA was used: Ambion Pre-miR miRNA precursor for *hsa-miR-375* (product ID: PM10327; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi siRNA, *si-MMP13* (cat nos. HSS106637 and HSS106638; Invitrogen, Carlsbad, CA, USA), and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax transfection reagent (Invitrogen), as previously described (13,18-20).

Cell proliferation, migration and invasion assays. TE-8 and TE-9 cells were transfected with 10 nM miRNAs or siRNAs by reverse transfection. Cell proliferation, migration and invasion assays were performed as previously described (13,18-20).

Screening of miR-375 target genes using in silico analysis and gene expression data. To identify *miR-375* target genes, a combination of genome-wide gene expression and *in silico* analyses was conducted as previously described (13,18-20). The microarray data were deposited into the GEO repository under accession number GSE77790. Next, we selected putative miRNA target genes using microRNA.org (August, 2010 release, http://www.microrna.org) databases. Our strategy for identification of *miR-375* target genes is shown in Fig. 2.

Western blot analysis. Anti-human MMP-13 rabbit polyclonal IgG (1:1,000; sc30073; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody. Anti-human GAPDH mouse monoclonal IgG (1:5,000; 010-25521; Wako Pure Chemical Industries, Osaka, Japan) was used as an internal loading control. The membrane was washed and incubated with a horseradish peroxidase-conjugated secondary antibody. Bands were visualized using Amersham ECL Prime Western Blotting detection reagent (GE Healthcare Life Sciences, Uppsala, Sweden).

Immunohistochemistry. Tumor samples were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), embedded in paraffin and sectioned into 4-μm-thick slices. The sections were incubated with rabbit polyclonal anti-MMP-13 IgG (1:200; ab84594; Abcam, Cambridge, UK) at 4˚C overnight. The procedure for immunohistochemistry was previously described (21).

Plasmid construction and Dual-luciferase reporter assays. Partial wild-type sequences of the 3' untranslated region (UTR) of *MMP13* containing the *miR-375* target site (positions 100-113 of the *MMP13* 3' UTR) or sequences with a deleted *miR-375* target site were inserted between the *Xho*I and *Pme*I restriction sites in the 3' UTR of the *hRluc* gene in the psiCHECK-2 vector (product ID: C8021; Promega, Madison, WI, USA). TE-8 and TE-9 cells were transfected with 50 ng of the vector and 10 nM *miR-375* using Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific). The activities of firefly and *Renilla* luciferases were determined in lysates of transfected cells using a Dual-luciferase reporter assay system according to the manufacturer's recommendations (product ID: E1960; Promega). Data were normalized to firefly luciferase activity (ratio of *Renilla*/firefly luciferase activities).

Identification of downstream genes mediated by MMP13 in ESCC cells. Gene expression analyses of *si-MMP13*-transfected TE-8 and TE-9 cells revealed molecular targets mediated by *MMP13* in ESCC cells. This method is described in more detail in previous studies (13,18-20). Microarray

Table II. Highly expressed genes putatively regulated by *miR-375* in ESCC.

Entrez Gene ID	Gene symbol	Description	*miR-375* target sites	Expression in *miR-375* transfectants FC (Log_2)		GEO data (GSE20347) FC (Log_2)
				TE-8	TE-9	
4322	*MMP13*	Matrix metalloproteinase 13	1	-2.24	-1.76	5.12
6004	*RGS16*	Regulator of G-protein signaling 16	3	-1.50	-0.92	2.45
4920	*ROR2*	Receptor tyrosine kinase-like orphan receptor 2	1	-0.80	-0.59	2.14
10202	*DHRS2*	Dehydrogenase/reductase (SDR family) member 2	3	-3.07	-0.83	2.02
1956	*EGFR*	Epidermal growth factor receptor	1	-0.93	-0.78	1.58
655	*BMP7*	Bone morphogenetic protein 7	1	-0.85	-0.74	1.54
23363	*OBSL1*	Obscurin-like 1	1	-0.80	-0.71	1.52
23035	*PHLPP2*	PH domain and leucine rich repeat protein phosphatase 2	1	-0.69	-0.64	1.15
1896	*EDA*	Ectodysplasin A	1	-0.72	-0.63	1.09

results were deposited in the GEO database (accession number GSE82108).

Statistical analysis. Relationships between two or three variables and numerical values were analyzed using the Mann-Whitney U test or the Bonferroni-adjusted Mann-Whitney test. Spearman's rank test was used to evaluate the correlations between the expression levels of *miR-375* and *MMP13*. Expert StatView version 5.0 (SAS Institute, Inc., Cary, NC, USA) was used in these analyses.

Results

Expression levels of miR-375 in ESCC clinical specimens and cell lines. We evaluated the expression levels of *miR-375* in ESCC tissues (n=25), normal esophageal specimens (n=13), and ESCC cell lines (TE-8 and TE-9). The patient backgrounds and clinicopathological characteristics are shown in Table I. The expression levels of *miR-375* were significantly downregulated in cancer tissues and ESCC cell lines compared with those in normal tissues (P<0.0001; Fig. 1A). Additionally, there were no significant relationships between the expression level of *miR-375* and any of the clinicopathological parameters examined in this study (recurrence, T stage, N stage, vascular invasion, or survival rate).

Effects of miR-375 restoration on cell proliferation, migration and invasion in ESCC cell lines. To investigate the antitumor functions of *miR-375*, we performed gain-of-function studies using mature miRNA transfection of TE-8 and TE-9 cells.

Cell proliferation was significantly suppressed by *miR-375* transfection in TE-9 cells in comparison with that of mock or miR-control transfectants (Fig. 1B). However, no changes were detected in TE-8 cells (Fig. 1B).

Migration assays showed that cell migration activity was significantly inhibited by *miR-375* transfection in TE-8 and TE-9 cells in comparison with that in mock or miR-control transfectants (Fig. 1C). Additionally, Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited by *miR-375* transfection in TE-8 and TE-9 cells in comparison with that in mock or miR-control transfectants (Fig. 1D).

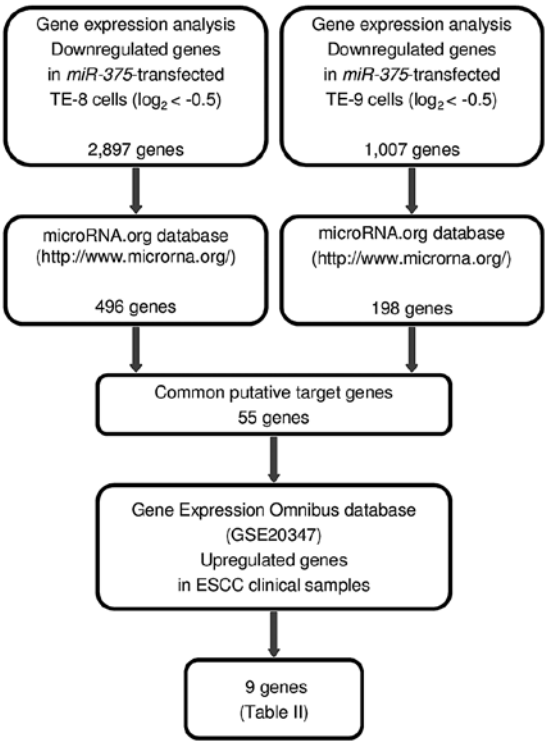

Figure 2. The strategy for analysis of *miR-375* target genes.

Identification of putative target genes regulated by miR-375 in ESCC cells. To gain additional insights into the molecular pathways regulated by antitumor *miR-375* in ESCC cells, we used a combination of *in silico* and gene expression analyses. The strategy for identification of the *miR-375*-regulated genes in ESCC cells is shown in Fig. 2.

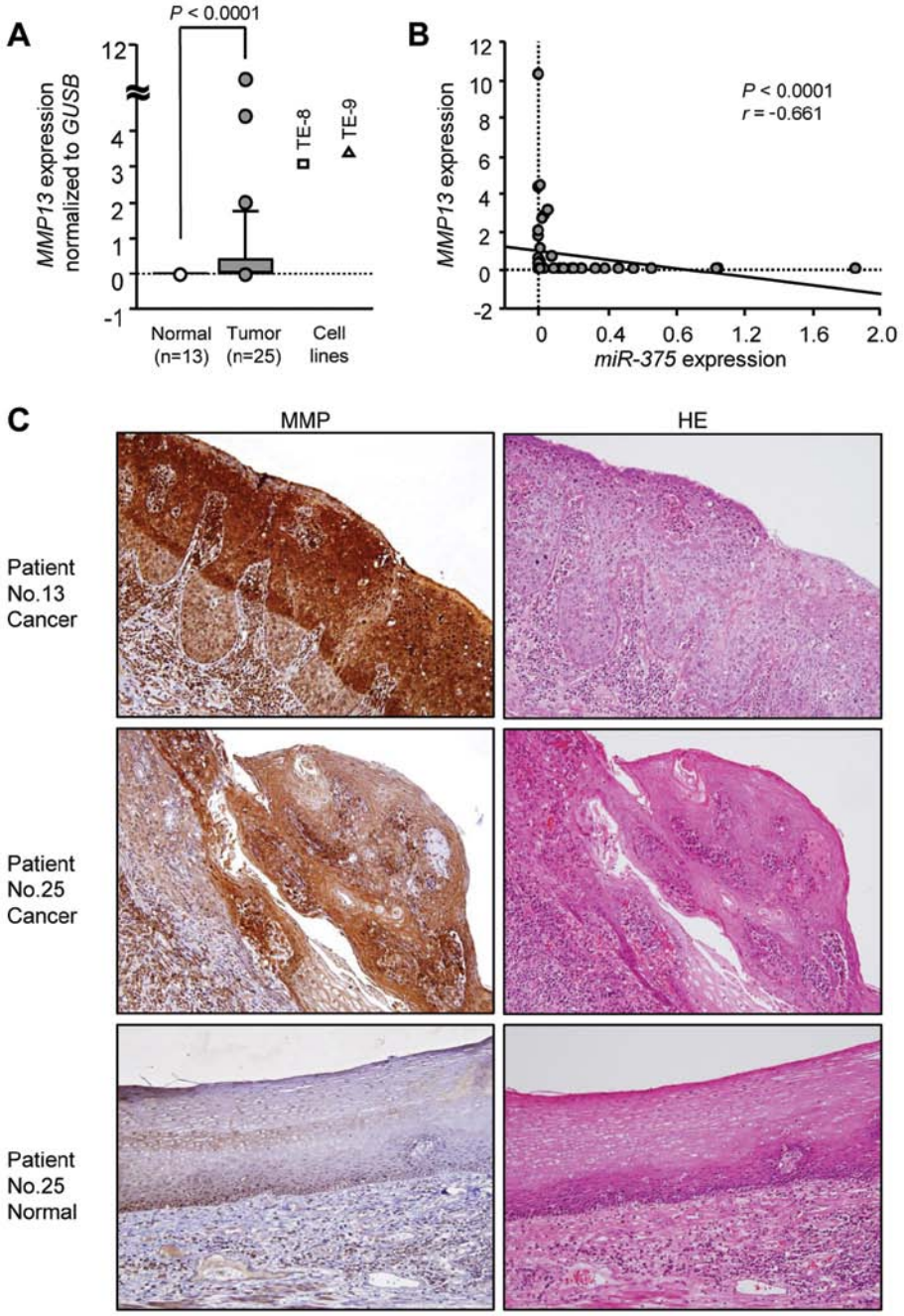

Figure 3. Expression levels of *MMP13* mRNA and immunohistochemical staining of MMP13 protein in ESCC specimens. (A) Expression levels of *MMP13* mRNA in ESCC or normal esophageal tissues and ESCC cell lines. (B) The expression of *miR-375* and *MMP13* mRNA was negatively correlated (r=-0.661 and P<0.0001). (C) Immunohistochemical staining of *MMP13* in ESCC specimens. All ESCC tissues were stained positively, whereas normal esophageal tissues were stained negatively or weakly (left panel, MMP13 staining; right panel, hematoxylin-eosin staining; original magnification, x200).

In gene expression analyses, 2,897 and 1,007 genes were downregulated ($\log_2$ ratio <-0.5) in TE-8 and TE-9 *miR-375* transfectants, respectively, in comparison with that in control transfectants. Our present expression data were deposited in the Gene Expression Omnibus (GEO accession number GSE77790). Among these downregulated genes, we searched for genes having putative *miR-375* binding sites in their 3' UTRs using the microRNA.org database. A total of 55 genes were identified as putative target genes of *miR-375*, and nine genes were upregulated in ESCC clinical specimens, as determined using ESCC expression data (GEO accession number: GSE20347; Table II).

In this study, we focused on *MMP13* because its expression was most upregulated in ESCC clinical specimens and most downregulated in *miR-375* transfectants. Moreover, previous studies have shown that the activation of MMPs is associated with cancer cell aggressiveness (22).

Expression of MMP13 in ESCC clinical specimens. Next, we validated the upregulation of *MMP13* in the ESCC clinical specimens at both the mRNA and the protein levels. The expression of *MMP13* was significantly upregulated in 25 ESCC specimens and ESCC cell lines compared with that in 13 normal specimens (P<0.0001; Fig. 3A). The

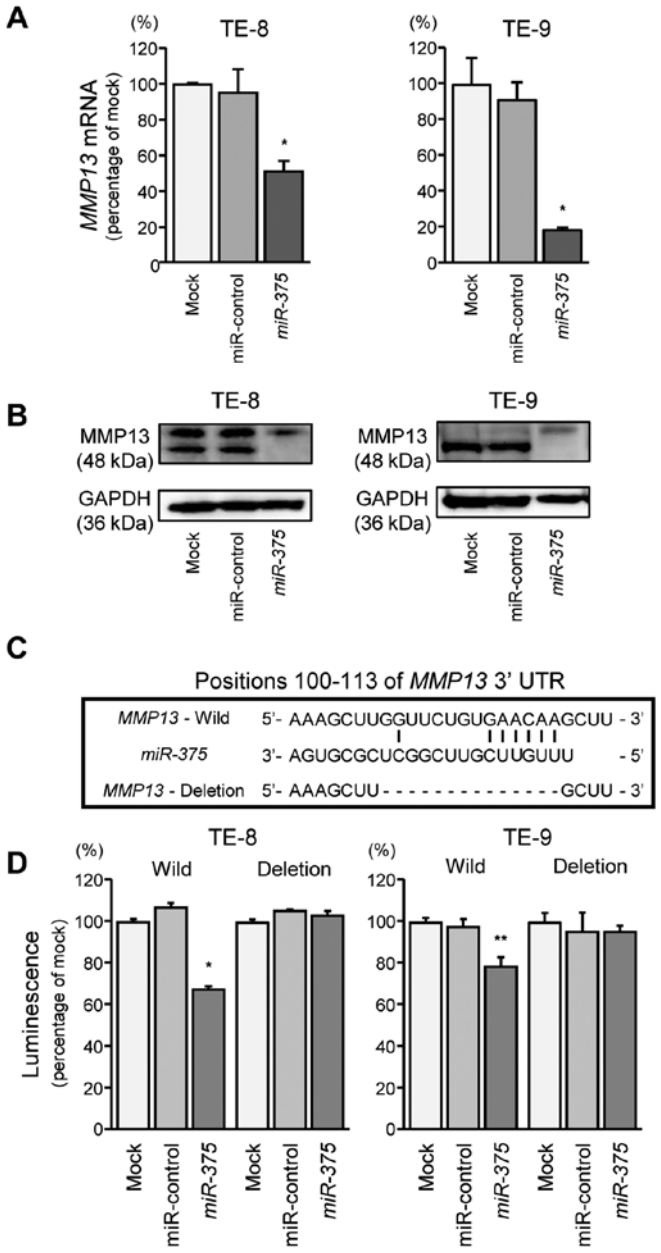

Figure 4. Direct regulation of *MMP13* by *miR-375* in ESCC cell lines. (A) Expression levels of *MMP13* mRNA 72 h after transfection with *miR-375*. (B) *MMP13* protein expression 72 h after transfection with *miR-375*. (C) Putative *miR-375* binding sites in the 3' UTR of *MMP13* mRNA. (D Luciferase reporter assay using vectors encoding putative *miR-375* target sites at positions 100-113 for both wild-type and deletion-type constructs. *Renilla* luciferase values were normalized to firefly luciferase values. *P<0.0001, **P<0.05.

Spearman's rank tests showed negative correlations between the expression of *miR-375* and that of *MMP13* (r=-0.661, P<0.0001; Fig. 3B).

Immunohistochemistry showed that MMP13 tended to be strongly expressed in ESCC lesions, whereas low expression was observed in normal esophageal epithelium (Fig. 3C).

Direct regulation of MMP13 by miR-375 in ESCC cells. We performed qRT-PCR to validate *miR-375*-mediated repression of *MMP13* expression in ESCC cell lines. Our results showed that *MMP13* mRNA was significantly reduced in *miR-375*

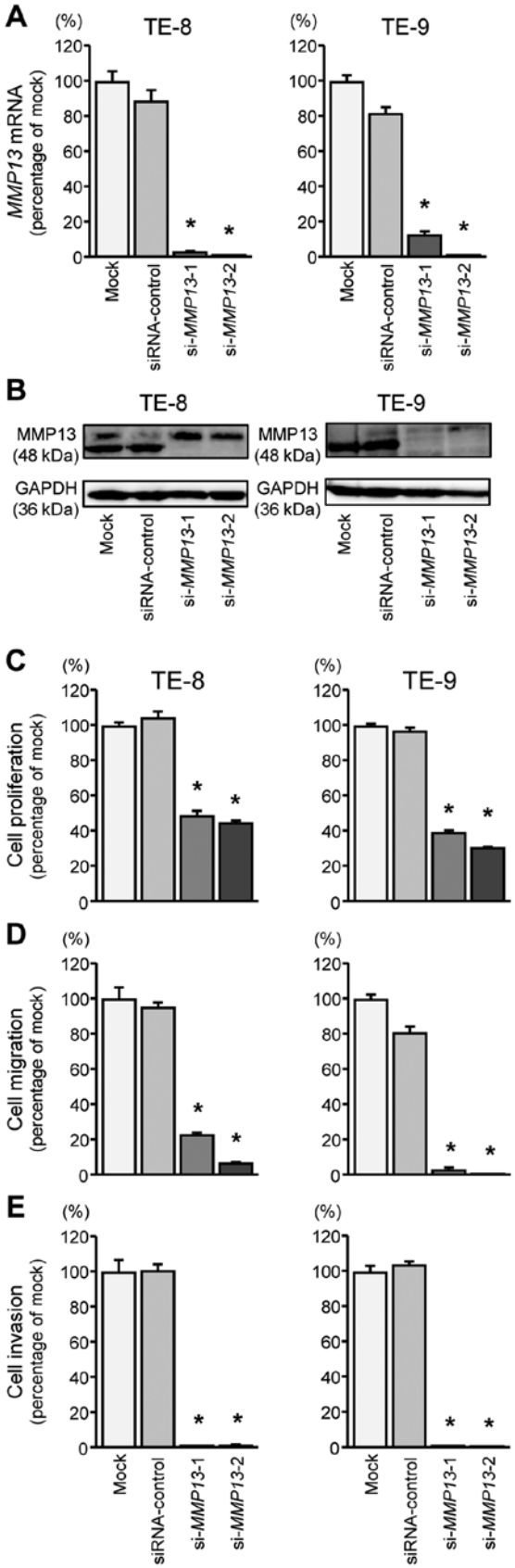

Figure 5. Loss of function studies using siRNAs. (A) Expression levels of *MMP13* mRNA after transfection with *si-MMP13* in ESCC cell lines. (B) *MMP13* protein expression 72 h after transfection with *si-MMP13*. (C) Cell proliferation was determined by XTT assays. Inhibition of cell proliferation was observed in *si-MMP13*-transfected cell lines. (D) Cell migration activity was determined by migration assays. (E) Cell invasion was determined by Matrigel invasion assays. Inhibition of migration and invasion was observed in *si-MMP13*-transfected cell lines. *P<0.0001.

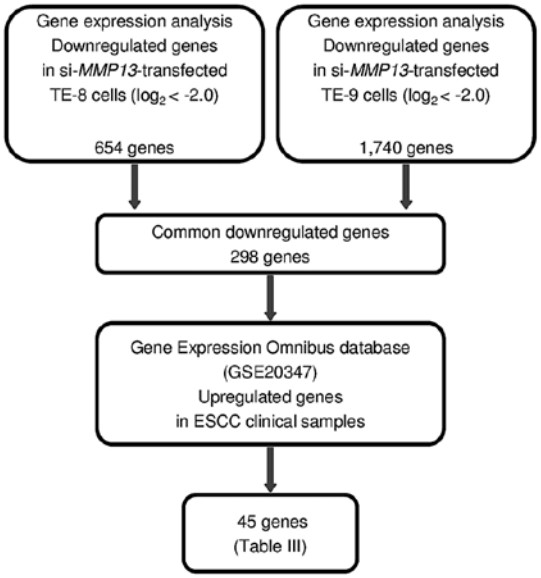

Figure 6. The strategy for analysis of *MMP13* downstream genes.

transfectants in comparison with that in mock or miR-control transfectants (P<0.0001; Fig. 4A). MMP13 protein expression was also repressed in *miR-375* transfectants (Fig. 4B).

Next, we performed luciferase reporter assays using TE-8 and TE-9 cells to determine whether *MMP13* had an actual target site for *miR-375* binding. The microRNA.org database predicted that there was one putative target site in the 3' UTR of *MMP13* (Fig. 4C). Compared with the miR-control, luminescence intensity was significantly reduced by transfection with *miR-375* at the *miR-375* target site, positions 100-113, in the 3' UTR of *MMP13* (Fig. 4D).

Effects of silencing MMP13 on proliferation, migration and invasion in ESCC cells. To investigate the functional roles of MMP13 in ESCC cell lines, we performed loss-of-function assays by transfection of *si-MMP13* into TE-8 and TE-9 cells.

First, we evaluated the knockdown efficiency of *si-MMP13* transfection in ESCC cell lines. In the present study, we used two siRNAs targeting *MMP13* (*si-MMP13-1* and *si-MMP13-2*). According to qRT-PCR and western blot analyses, both siRNAs effectively downregulated *MMP13* expression in both cell lines (Fig. 5A and B).

Cell proliferation, migration and invasion assays demonstrated that cell proliferation, migration, and invasion were inhibited in *si-MMP13*-transfected cells compared with those in mock- or siRNA-control-transfected cells (Fig. 5C-E).

Identification of downstream genes regulated by MMP13 in ESCC cells. To determine which downstream genes were regulated by MMP13, genome-wide gene expression and *in silico* analyses were performed in TE-8 and TE-9 cells transfected with *si-MMP13*.

Our expression analysis showed that a total of 298 genes were commonly downregulated (log_2 ratio <-2.0) in TE-8 and TE-9 cells following *si-MMP13* transfection. Among these genes, 52 were upregulated in ESCC clinical specimens, as determined using ESCC expression data (GEO accession number: GSE20347; Fig. 6 and Table III).

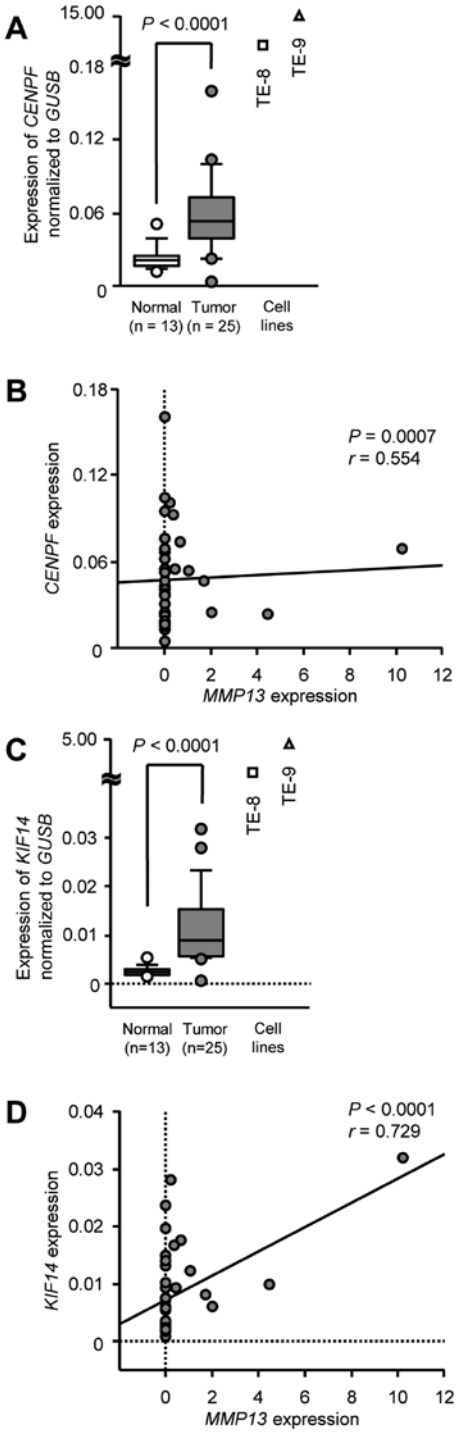

Figure 7. mRNA expression levels of *MMP13* downstream genes (*CENPF* and *KIF14*) in ESCC specimens. (A and C) Expression levels of *CENPF* and *KIF14* mRNA in ESCC or normal esophageal tissues and ESCC cell lines. (B and D) The expression levels of *MMP13*/*CENPF* and *MMP13*/*KIF14* mRNAs were positively correlated (P<0.0001).

We then validated the upregulation of *CENPF* and *KIF14* mRNAs in ESCC clinical specimens. The expression of *CENPF* and *KIF14* mRNAs was significantly upregulated in 25 ESCC specimens and ESCC cell lines compared with that in 13 normal specimens (P<0.0001; Fig. 7A and C). The Spearman's rank tests showed correlations between the expression of *MMP13* and that of *CENPF* or *KIF14* (CENPF: r=0.554, P=0.0007, Fig. 7B; KIF14: r=0.729, P<0.0001, Fig. 7D).

Table III. Downregulated genes in *si-MMP13*-transfected ESCC cell lines.

Entrez gene ID	Gene symbol	Description	Expression in *si-MMP13* transfectants FC (log_2)		GEO data (GSE20347) FC (log_2)
			TE8	TE9	
4322	*MMP13*	Matrix metallopeptidase 13 (collagenase 3)	-4.42	-4.47	5.12
1063	*CENPF*	Centromere protein F, 350/400 kDa	-2.96	-5.18	2.31
9928	*KIF14*	Kinesin family member 14	-2.28	-4.66	2.14
2842	*GPR19*	G protein-coupled receptor 19	-2.67	-3.74	2.12
983	*CDK1*	Cyclin-dependent kinase 1	-2.07	-3.78	1.95
55165	*CEP55*	Centrosomal protein 55 kDa	-3.33	-4.79	1.94
1033	*CDKN3*	Cyclin-dependent kinase inhibitor 3	-2.08	-3.73	1.94
7153	*TOP2A*	Topoisomerase (DNA) II alpha 170 kDa	-3.36	-5.01	1.91
10403	*NDC80*	NDC80 kinetochore complex component	-2.19	-3.69	1.76
9787	*DLGAP5*	Discs, large (*Drosophila*) homolog-associated protein 5	-2.27	-3.32	1.72
55215	*FANCI*	Fanconi anemia, complementation group I	-2.27	-3.97	1.70
23306	*TMEM194A*	Transmembrane protein 194A	-2.31	-2.79	1.68
4751	*NEK2*	NIMA-related kinase 2	-2.70	-3.84	1.66
2735	*GLI1*	GLI family zinc finger 1	-2.70	-3.31	1.63
3161	*HMMR*	Hyaluronan-mediated motility receptor (RHAMM)	-4.06	-5.29	1.60
259266	*ASPM*	Asp (abnormal spindle) homolog, microcephaly associated (*Drosophila*)	-2.17	-3.81	1.56
4998	*ORC1*	Origin recognition complex, subunit 1	-2.23	-3.08	1.53
57405	*SPC25*	SPC25, NDC80 kinetochore complex component	-2.16	-4.12	1.48
28951	*TRIB2*	Tribbles pseudokinase 2	-2.28	-2.35	1.44
9603	*NFE2L3*	Nuclear factor, erythroid 2-like 3	-2.00	-2.51	1.42
9638	*FEZ1*	Fasciculation and elongation protein zeta 1 (zygin I)	-2.27	-2.97	1.42
9918	*NCAPD2*	Non-SMC condensin I complex, subunit D2	-2.12	-2.79	1.38
7468	*WHSC1*	Wolf-Hirschhorn syndrome candidate 1	-2.43	-3.36	1.33
100288413	*ERVMER34-1*	Endogenous retrovirus group MER34, member 1	-2.76	-3.78	1.32
1062	*CENPE*	Centromere protein E, 312 kDa	-2.60	-3.91	1.29
55063	*ZCWPW1*	Zinc finger, CW type with PWWP domain 1	-3.19	-3.44	1.25
81624	*DIAPH3*	Diaphanous-related formin 3	-2.22	-3.54	1.25
6119	*RPA3*	Replication protein A3, 14 kDa	-2.34	-3.42	1.24
8318	*CDC45*	Cell division cycle 45	-2.13	-4.07	1.23
64151	*NCAPG*	Non-SMC condensin I complex, subunit G	-3.25	-3.92	1.22
7083	*TK1*	Thymidine kinase 1, soluble	-2.11	-3.86	1.22
55732	*C1orf112*	Chromosome 1 open reading frame 112	-2.06	-2.62	1.22
1058	*CENPA*	Centromere protein A	-2.02	-3.86	1.18
55635	*DEPDC1*	DEP domain containing 1	-2.33	-3.44	1.18
3925	*STMN1*	Stathmin 1	-2.66	-4.51	1.17
3092	*HIP1*	Huntingtin interacting protein 1	-2.71	-3.51	1.17
5427	*POLE2*	Polymerase (DNA directed), epsilon 2, accessory subunit	-2.18	-4.37	1.15
1719	*DHFR*	Dihydrofolate reductase	-2.46	-3.63	1.14
54830	*NUP62CL*	Nucleoporin 62 kDa C-terminal like	-2.17	-2.22	1.10
5062	*PAK2*	p21 protein (Cdc42/Rac)-activated kinase 2	-2.37	-2.60	1.09
100129361	*LOC100129361*	Chromosome X open reading frame 69-like	-2.57	-2.46	1.09
5933	*RBL1*	Retinoblastoma-like 1	-3.24	-4.43	1.08
4288	*MKI67*	Marker of proliferation Ki-67	-2.14	-4.87	1.03
81691	*LOC81691*	Exonuclease NEF-sp	-2.62	-3.61	1.03
675	*BRCA2*	Breast cancer 2, early onset	-2.90	-4.04	1.00

Discussion

Numerous studies of miRNA expression signatures in ESCC have shown that *miR-375* is frequently downregulated in cancer tissues and functions as an antitumor miRNA (14,23). In the present study, we confirmed that *miR-375* was markedly downregulated in cancer tissues and that ectopic expression of *miR-375* significantly suppressed cancer cell migration and invasion. Thus, we found that loss of *miR-375* expression enhanced cancer cell aggressiveness in ESCC. Many previous studies have shown that the expression of *miR-375* is markedly decreased in several types of cancers and that *miR-375* functions as an antitumor miRNA (15,24). In contrast to these antitumor activities, *miR-375* is upregulated in pediatric acute myeloid leukemia (AML) and prostate cancer, suggesting that *miR-375* acts as an oncogenic miRNA in these diseases (25,26). The dual function of *miR-375* is very unique; thus, it is important to identify *miR-375*-regulated pathways in various cancer types.

It is also important to elucidate novel RNA networks regulated by antitumor *miR-375* in ESCC cells. Previous studies have shown that insulin-like growth factor 1 receptor (*IGF1R*), lactate dehydrogenase B (*LDHB*), and astrocyte elevated gene-1/metadherin (*AEG-1/MTDH*) are directly regulated by *miR-375* in ESCC cells (16,17). These target genes are upregulated in ESCC clinical specimens and functioned as oncogenes in this disease. Another unique characteristic of miRNAs is that a single miRNA can regulate a large number of RNA transcripts in human cells (27,28). Thus, the continuous identification of *miR-375*-regulated oncogenes in ESCC cells is important for elucidation of the molecular pathogenesis of ESCC.

In this study, we identified *MMP13* as a direct target of antitumor *miR-375* in ESCC cells. *MMP13* (also known as collagenase 3) is a member of the collagenase subfamily of MMPs and functions to degrade a wide range of extracellular matrix components, including tenascin C, fibronectin and type I-IV collagen (29). Thus, MMP13 has a wide range of proteolytic functions, suggesting that MMP13 is involved in several physiological and pathological processes (30). High expression of MMP13 has been reported in rheumatoid arthritis, osteoarthritis and several types of cancers (22). Previous studies have also shown that high expression of MMP13 is associated with vascular invasion and lymph node metastasis in ESCC (31). Our present data demonstrated that knockdown of *MMP13* markedly reduced cancer cell migration and invasion in ESCC cells.

The *MMP13* gene has also been reported to be epigenetically regulated by several other miRNAs, including *miR-125b* and *miR-143*, in cancer cells (32-34). Notably, our miRNA signatures have shown that *miR-125b* and *miR-143* are downregulated in ESCC and in oral and hypopharyngeal squamous cell carcinoma (12-14). Moreover, functional assays have indicated that these miRNAs act as tumor suppressors in several cancers, including ESCC cells (32-35). Loss of the expression of several antitumor miRNAs and activation of *MMP13* may enhance cancer cell aggressiveness and metastasis. Thus, identification of *miR-375/MMP13*-mediated cancer pathways may facilitate the discovery of candidate therapeutic targets in ESCC.

Based on the above, we further investigated the downstream genes mediated by MMP13 in ESCC cells using genome-wide gene expression analysis. Our data showed that several centromere-associated proteins were regulated by MMP13-mediated genes, such as *CENPF*, *CENPE*, *CENPA*, *CEP55*, *NDC80* and *SPC25*. Moreover, cell cycle-promoting genes, e.g., *KIF14*, *CDK1*, *TOP2A*, *CDC45* and *PAK2*, were also downregulated by *si-MMP13* in this study. Recent studies have reported that several genes encoding mitotic apparatus components are upregulated in cancer cells and contribute to cancer cell phenotypes (36,37). Therefore, overexpression of genes encoding mitotic apparatus components may represent a potential target for cancer drug development (38). Several compounds that inhibit centromere proteins and mitotic kinesins are being tested as potential cancer therapies in clinical trials (39).

Among these genes, we validated the overexpression of *CENPF* and *KIF14* in ESCC clinical specimens. Previous studies have shown that *CENPF* is a master regulator of prostate cancer malignancy and that high expression of *CEPNF* is a prognostic indicator of poor survival and metastasis in patients with ESCC (40). *KIF14* is a member of the kinesin superfamily of proteins and functions as a microtubule motor protein involved in cytokinesis and chromosome segregation (41). Overexpression of *KIF14* has been reported in several cancers, and its expression is associated with cancer cell phenotypes (42,43). An in-depth functional analysis of these genes in ESCC cells is necessary to further characterize these pathways. Identification of the downstream genes regulated by the *miR-375/MMP13* axis may lead to a better understanding of ESCC aggressiveness.

In conclusion, downregulation of *miR-375* was frequently observed in ESCC clinical specimens, and *miR-375* was shown to function as an antitumor miRNA in ESCC cells. To the best of our knowledge, this is the first report demonstrating that *MMP13* is directly regulated by antitumor *miR-375* and acts to regulate several cell cycle promoting genes. The identification of novel molecular pathways and targets regulated by the *miR-375/MMP13* axis may lead to a better understanding of ESCC molecular pathogenesis.

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