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

YUSAKU OSAKO1, NAOHIKO SEKI2, YOSHIKI KITA1, KEIICHI YONEMORI1, KEIICHI KOSHIZUKA2, AKIRA KUROZUMI2, ITARU OMOTO1, KEN SASAKI1, YASUTO UCHIKADO1, HIROSHI KURAHARA1, KOSEI MAEMURA1 and SHOJI NATSUGOE1

1Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Sakuragaoka, Kagoshima 890-8520; 2Department of Functional Genomics, Chiba University Graduate School of Medicine, Chuo-ku, Chiba 260-8670, Japan

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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.

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-dependent 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...
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 BioResource 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) and RNU48 (assay ID: 001006) were 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.

| 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  | IIIB  | 0  | 0  | -          |
| 4   | 62          | Male   | Well           | 2  | 2  | 0  | IIIA  | 3  | 1  | +          |
| 5   | 66          | Male   | Moderate       | 3  | 0  | 0  | IA    | 1  | 1  | -          |
| 6   | 74          | Male   | Moderate       | 2  | 2  | 0  | IIIA  | 1  | 1  | -          |
| 7   | 56          | Male   | Moderate       | 2  | 0  | 0  | IB    | 0  | 1  | -          |
| 8   | 79          | Male   | Moderate       | 2  | 1  | 0  | IIIB  | 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  | IIIA  | 1  | 1  | +          |
| 14  | 66          | Male   | Moderate       | 3  | 0  | 0  | IIIA  | 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           | 2  | 1  | 0  | IIIB  | 1  | 1  | -          |
| 18  | 78          | Male   | Well           | 3  | 0  | 0  | IIIA  | 1  | 2  | -          |
| 19  | 71          | Male   | Well           | 3  | 0  | 0  | IIIA  | 1  | 2  | -          |
| 20  | 75          | Male   | Moderate       | 3  | 2  | 0  | IIIB  | 1  | 1  | +          |
| 21  | 60          | Male   | Moderate       | 2  | 1  | 0  | IIIB  | 1  | 2  | -          |
| 22  | 62          | Male   | Well           | 1a | 1  | 0  | IIIB  | 0  | 0  | -          |
| 23  | 71          | Male   | Moderate       | 1b | 1  | 0  | IIIB  | 0  | 0  | -          |
| 24  | 69          | Male   | Moderate       | 1b | 0  | 0  | IA    | 1  | 0  | -          |
| 25  | 84          | Male   | Well           | 2  | 1  | 0  | IIIB  | 1  | 1  | -          |
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 XhoI and PmeI 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
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. IC). 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).

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.
In gene expression analyses, 2,897 and 1,007 genes were downregulated (log₂ 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
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 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. *\(P<0.0001\), **\(P<0.05\).

**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. *\(P<0.0001\).
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).

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<0.0001, 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 (log2) | GEO data (GSE20347) FC (log2) |
|----------------|-------------|-------------|-----------------------------------------------|-------------------------------|
| 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        | Fascilitation and elongation protein zeta 1 (zygin I) | -2.27 -2.97 | 1.42 |
| 9918           | NCPAD2      | 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 PWBP domain 1 | -3.19 -3.44 | 1.25 |
| 81624          | DIAP3       | 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          | NCPAG       | 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           | DHRF        | 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 |
In the present study, we confirmed that studies have shown that the expression of enhanced cancer cell aggressiveness in ESCC. Many previous expression downregulated in cancer tissues and that ectopic expression functions as an antitumor miRNA (14,23). 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 pediatreric 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 tenasin 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 SPC23. 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 CENPF 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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