Silencing of Long Noncoding RNA MALAT1 by miR-101 and miR-217 Inhibits Proliferation, Migration, and Invasion of Esophageal Squamous Cell Carcinoma Cells*

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Malat1, a highly conserved long noncoding RNA, is deregulated in several types of cancers. However, its role in esophageal squamous cell carcinoma (ESCC) and its posttranscriptional regulation remain poorly understood. In this study we provide first evidences that a posttranscriptional regulation mechanism of MALAT1 by miR-101 and miR-217 exists in ESCC cells. This posttranscriptional silencing of MALAT1 could significantly suppress the proliferation of ESCC cells through the arrest of G2/M cell cycle, which may be due to MALAT1-mediated up-regulation of p21 and p27 expression and the inhibition of B-MYB expression. Moreover, we also found the abilities of migration and invasion of ESCC cells were inhibited after overexpression of miR-101, miR-217, or MALAT1 siRNA. This might be attributed to the deregulation of downstream genes of MALAT1, such as MIA2, HNF4G, ROBO1, CCT4, and CTHRC1. A significant negative correlation exists between miR-101 or miR-217 and MALAT1 in 42 pairs of ESCC tissue samples and adjacent normal tissues. Mice xenograft data also support the tumor suppressor role of both miRNAs in ESCCs.

Malat1, a highly conserved long non-coding RNA (lncRNA), acts as oncogene in multiple human cancers.

Results: miR-101 and miR-217 can silence MALAT1 expression and then inhibit esophageal cancer proliferation, migration and invasion.

Conclusion: Tumor suppressor miR-101 and miR-217 can negatively regulate MALAT1 expression.

Significance: These data provide a new mechanism for MALAT1 regulation.

Esophageal squamous cell carcinoma (ESCC)2 is one of the most common and lethal malignancies all around the world. In eastern Asia ESCC shows a relatively high morbidity and mortality compared with Western countries (1). Epidemiological evidences indicate that heavy alcohol drinking, tobacco smoking, micronutrient deficiency, and dietary carcinogen exposure are major environmental risk factors of this fatal disease (2, 3). However, only a part of exposed individuals eventually develop ESCC, demonstrating that host genetic components may also contribute to ESCC etiology (2–7).

Multiple cancer causal protein-coding genes, either as oncogenes or tumor suppressors, have been identified in the majority of cancer-associated human genomic loci (8). Accumulated evidences indicate that noncoding RNA (ncRNA) genes also play a crucial role in malignant transformation and/or cancer progression (9–11). Among different kinds of ncRNAs, the discovery of endogenous ~22 nucleotides ncRNAs, named microRNAs (miRNAs), not only disclosed a new layer of gene expression regulation but also revealed the direct involvement of ncRNAs in tumorgenesis (12, 13). Recently, many long ncRNAs (lncRNAs) ranging in size from several hundred base pairs (bp) to tens of thousands bp have been identified as a new class of cancer-associated ncRNAs in human (9–11). Among >3000 human lncRNAs, <1% have been functionally characterized (14). Metastasis associated in lung adenocarcinoma transcript 1 (MALAT1; also known as NEAT2) is a highly conserved mRNA-like IncRNA that was originally identified with high expression in metastatic non-small-cell lung cancer (15). It has also been found that MALAT1 is overexpressed in many other human malignancies, including breast, pancreas, colon, prostate, and liver (16). Functional studies showed that its deregulation influences proliferation, invasion, and/or metastasis of multiple cancer cells (17–23). Therefore, fine-regulation of MALAT1 is critical for cancer development.

Interestingly, lncRNAs may potentially interact with miRNAs and modulate each other’s expression. On the one
hand, IncRNAs may function as a competing endogenous RNAs to miRNAs. On the other hand, miRNAs could inhibit expression of IncRNAs through Argonaute 2 (Ago2)-mediated pathway (24–28). However, how IncRNA MALAT1 is regulated by miRNAs at the transcriptional level and its involvement in ESCC remains largely unknown. In the current study we found that miR-101 and miR-217 could silence MALAT1 in ESCCs, and this posttranscriptional regulation may lead to inhibition of growth, invasion, and metastasis of ESCC cells.

**MATERIALS AND METHODS**

**Quantitative Real-time PCR**—Total RNA was isolated from either culture cells or tissue samples using TRIzol reagent (Invitrogen). RNA samples were reverse-transcribed (RT) into cDNA with different RT primers using Revert Ace kit (Invitrogen). RNA samples were reverse-transcribed into cDNA using Revert Ace transcriptase by specific stem-loop RT primers according to the manufacturer’s instruction. The stem-loop PCR was performed as previously described (26). Transcript levels were measured against an endogenous control by quantitative PCR using the SYBR® Green I fluorogenic dye using the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany).

**Plasmid Construction**—Total RNA was extracted from KYSE30 cell line and reverse-transcribed into cDNA. The sequence corresponding to the wild-type MALAT1 3′ end was amplified by PCR and inserted in the XbaI restriction site of the reporter plasmid pGL3-control (Promega). The primers used were as follows: 5′-AACCTCTAGACCTGGCTTTCTTCTGTTC-3′/5′-AACCTCTAGACCTCAACACTCGCCTTATTAC-3′. The plasmid was named as pGL3-MAL. For construction of MALAT1 reporter gene plasmids with a mutant miR-101 binding site or a mutant miR-217 binding site, the QuikChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s construction. These mutant plasmids were named as pGL3-mut101 or pGL3-mut217. Wild-type and mutant inserts were confirmed by DNA sequencing.

**Dual Luciferase Reporter Gene Assays**—A firefly luciferase reporter plasmid (pGL3-MAL, pGL3-mut101, or pGL3-mut217) and a renilla luciferase vector (pRL-SV40, Promega) plus small RNAs (miR-101 mimics, miR-217 mimics, or negative control RNAs) were co-transfected into KYSE30, KYSE150, or KYSE450 cells with Lipofectamine® 2000 (Invitrogen). Three independent transfection experiments were performed, and each was done in triplicate. Firefly luciferase activities derived from pGL3-control-derived plasmids were normalized to renilla luciferase activity from pRL-SV40 using a luciferase assay system (Promega) as reported previously (29, 30).

**Cell Proliferation Assays**—Human ESCC cell lines (KYSE30, KYSE150, and KYSE450) were cultured in RPMI 1640 medium (Invitrogen) supplemented with penicillin-streptomycin and 10% fetal bovine serum (Hyclone) at 37 °C with 5% CO₂. KYSE30 and KYSE150 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well. KYSE450 cells were seeded in a 12-well plate at a density of 2 × 10⁵ cells per well. Cells were transfected with 10 nm miR-101 mimics, miR-217 mimics, negative control RNA (NC), or MALAT1 siRNAs (siM) (GenePharma, Shanghai, China) combined with Lipofectamine® RNAi Max (Invitrogen). Cells were then harvested by trypsin digestion, washed in cold PBS twice, dyed with trypan blue, and counted under microscopy at 24 and 48 h after transfection.

**Colony Formation Assays**—A total of 8000 KYSE150 cells were transfected with 10 nm NC RNA, miR-101 mimics, miR-217 mimics, or siM into a 6-well cell culture plate. After 14 days, cells were washed with cold PBS twice and fixed with 3.7% formaldehyde. After cancer cells were dyed with crystal violet, colony number in each well was counted.

**Wound-healing Assays and Transwell Assays**—When reaching ~90% confluence, the cell layer was scratched. Cells were then continued cultured at 37 °C. The average extent of wound closure was quantified. During transwell assays, the transwell chambers were coated with 100 μl of BD Biosciences Matrigel™ overnight in cell incubator. Cells (1 × 10⁵ cells in 200 μl of medium with 0.2% BSA) transfected with 10 nm miR-101 mimics, miR-217 mimics, siM, or NC were added to upper transwell chambers (pore 8 μm, Corning). A medium containing 10% FBS (650 μl) was added to the lower wells. After 48 h of incubation, cells were fixed and stained, and the nonmigratory cells were scraped from the upper part of the filter. Cells migrated to the lower wells through pores were stained with 0.2% crystal violet solution and counted.

**Flow Cytometry**—Cells were transfected with 10 nm miR-101 mimics, miR-217 mimics, or NC and harvested at 48 h after transfection. After washing with cold PBS twice, cells were fixed with ethanol at −20 °C overnight and washed with cold PBS twice again. After treatment with RNase A at 37 °C for 0.5 h and dyed with propidium iodide, the samples were detected with the FACSCalibur flow cytometer (BD Biosciences). During apoptosis assays, apoptosis was determined using the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis kit (Invitrogen) with the FACSComp flow cytometer.

**Tumor Tissue Specimens**—Twenty-four pairs of ESCC tissues and esophagus normal tissues adjacent to the tumors were obtained from surgically removed specimens of ESCC patients in Shandong Cancer Hospital, Shandong Academy of Medical Sciences. Eighteen pairs of ESCC and normal tissues were obtained from Huainan No. 2 Hospital. The tumor tissues and adjacent normal tissues were frozen in liquid nitrogen after resection. No patients in the current study received chemotherapy or radiation therapy before the surgery. This study was approved by the institutional Review Boards. A part of the tissue samples has been reported previously (31, 32).

**ESCC Xenograft**—Five-week-old female nude BALB/c mice were purchased from Vital River Laboratory (Beijing, China). To evaluate the tumor suppressor role of miR-101 and miR-217 in vivo, 1 × 10⁵ KYSE150 cells transfected with 30 nm miR-101, miR-217, or siM were inoculated subcutaneously into fossa axillaris of 12 nude mice (n = 3 per group). Tumor volumes were measured three times a week. All procedures involving mice were approved by the institutional Review Board of Huainan No. 2 Hospital.
miR-101 and miR-217 Silence MALAT1 in ESCC

FIGURE 1. Identification of candidate miRNAs targeting lncRNA MALAT1. A, bioinformatics prediction of candidate miRNAs targeting MALAT1. Left panel, the flow chart on selection of candidate miRNAs. Right panel, photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) data indicated that Ago2 bound to MALAT1 3’ end (6615–6650 nucleotides (nt)). miRcode prediction indicated that this region might be targeted by miR-217, miR-101, miR-383, and miR-503. Homologous analysis of the potential miRNA binding sequence in the 100 vertebrates by PhastCons is shown. B, knockdown of Ago2 increases MALAT1 RNA expression. 10 nM siAgo2 duplexes or negative control RNA (NC) was transfected into KYSE30, KYSE150, and KYSE450 cells. MALAT1 (left) and Ago2 (right) expression was detected at 48 h after transfection. C, knockdown of Dicer results in elevated MALAT1 RNA expression. 10 nM siDicer duplexes or NC RNA was transfected into cells. MALAT1 (left) and Dicer (right) expression was detected at 48 h after transfection. D, miR-101 and miR-217 inhibit MALAT1 RNA expression. 10 nM miR-101, miR-217, miR-383, miR-503, MALAT1 siRNA (siM), or NC RNA was transfected into cells. MALAT1 RNA expression was detected at 48 h after transfection (left). The transfection efficiency of different miRNAs was confirmed by quantitative PCR (right). Data were normalized to log10 scale. E, inhibition of miR-101 or miR-217 up-regulates MALAT1 RNA expression. 20 nM antagamiR-101 (anti101), antagomiR-217 (anti217), and NC RNA were transfected into cells. MALAT1 and miRNA expression was detected at 48 h after transfection. All data of MALAT1, Ago2, or Dicer expression were normalized to β-actin mRNA expression levels. All miRNA expression data were normalized to U6 small RNA expression. All results of the mean of triplicate assays with S.D. are presented. *, p < 0.05; **, p < 0.01.
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Statistical Analyses—All data are presented as the means ± S.D. unless stressed. Student’s t test was used to examine the differences in luciferase reporter gene expression, and Spearman’s correlation was used to test the significance of association between miR-101 or miR-217 expression and MALAT1 RNA expression. p < 0.05 was considered statistically significant. All analyses were performed with SPSS software package (Version 16.0, SPSS Inc.).

RESULTS

Identification of Candidate miRNAs Targeting MALAT1—Photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) is a biochemical method used for identifying the binding sites of cellular RNA-binding proteins and microRNA-containing ribonucleoprotein complexes. Using this method, Hafner et al. (33) drew a precise map binding sites of Ago proteins across the transcriptome of HEK293 cells. Based on these data, Jalali et al. evaluated systematic transcriptome wide lncRNA-miRNA interactions (24), which provide a good resource for exploring the potential miRNA-dependent lncRNA regulation in human cells. Interestingly, we found that Ago2 protein probably binds to the 3’ end region (from 6615 to 6650 nt) of MALAT1 lncRNA in human cells (Fig. 1A). This MALAT1 6615–6650-nt region was highly conserved in different mammals (Fig. 1A), suggesting its potential function significance as a posttranscriptional regulation region. MiRcode was then utilized to predict potential miRNA candidates, which may target this region. As a result, miR-101, miR-217, miR-383, and miR-503 were identified as candidates for further evaluations (Fig. 1A).
To verified whether MALAT1 might be regulated by miRNAs, we first knocked down Ago2 (the catalytic subunit of RNA-induced silencing complex, RISC) as well as Dicer (an essential enzyme during miRNA maturing) and examined expression changes of MALAT1 in ESCC cells (Fig. 1, B and C). As expected, we found 1.2–1.5-fold elevated MALAT1 RNA expression after depressed Ago2 expression in KYSE30, KYSE150, and KYSE450 cells. Also, there was a 1.2–1.4-fold increased MALAT1 RNA expression after inhibition of Dicer expression in these cell lines. These results gave some clues that miRNAs might participate in MALAT1 regulation.

We then tested the possible regulation of MALAT1 RNA expression by miR-101, miR-217, miR-383, and miR-503 in ESCC cells (Fig. 1D). Although overexpression of miR-101 and
miR-217 could significantly inhibit MALAT1 RNA expression, miR-383 and miR-503 showed little impact. To validate this observation, antagomirs of miR-101 and miR-217 were employed to inhibit endogenous miR-101 and miR-217 expression. We found a 1.2–1.6-fold up-regulation of MALAT1 RNA expression in ESCC cells after transfection with antagomirs of both miRNAs (Fig. 1E). Given the relative low endogenous expression of miR-101 and miR-217 in ESCC cell lines, it was possible that further inhibition of miR-101 and miR-217 by antagomirs only yielded a marginal effect on MALAT1 RNA expression, whereas their low expression in ECSS cells also provided a clue that miR-101 and miR-217 might be tumor suppressors.

Interaction of miR-101 or miR-217 and the MALAT1 RNA—
To examine the potential miRNA-lncRNA interaction experimentally, a 343-bp human MALAT1 3’ end region (from 6423 to 6765 nucleotides) was subcloned downstream of the firefly luciferase gene (named as pGL3-MAL) and co-transfected into KYSE30, KYSE150, and KYSE450 cells. MiR-101 produced a 42.8, 58.4, and 41.5% decrease in relative luciferase activity compared with

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**FIGURE 4.** MiR-101 and miR-217 reduce migration and invasion ability of ESCC cells through MALAT1 depression. A, miR-101, miR-217, and MALAT1 siRNA (siM) inhibited wound-healing in KYSE30, KYSE150, and KYSE450 cells. Wound fields were observed directly after removal of inserts (0 h), and cell migration was followed for 24 and 48 h. Wound-healing area in KYSE30, KYSE150, and KYSE450 cells is presented as a histogram. B, miR-101, miR-217, and siM inhibit invasion ability of KYSE30, KYSE150, and KYSE450 cells. Cells on the lower surface of the chamber were stained by crystal violet at 48 h after transfection. Cell counts data are presented as a histogram. All results of the mean of triplicate assays with S.D. are presented. *, p < 0.05; **, p < 0.01.
NC RNA-transfected KYSE30, KYSE150, and KYSE450 cells (all \( p < 0.05 \)) (Fig. 2B, left panel). Similarly, there was 30.3, 86.7, and 35.1\% decreased relative luciferase activity in these ESCC cells with miR-217 overexpression compared with the NC RNA group (all \( p < 0.05 \)) (Fig. 2B, left panel). We also measured firefly luciferase mRNA expression with RT-quantitative PCR. As expected, pGL3-MAL firefly luciferase mRNA level was significantly inhibited by miR-101 or miR-217 (Fig. 2B, right panel). An analogous reporter with point substitutions disrupting the target sites of miR-101 or miR-217 (Fig. 2A) was also co-transfected with miR-101 mimics, miR-217 mimics, or NC RNA. There was no significant decrease in relative luciferase activity for cells transfected with miR-101 or miR-217 mimics compared with NC RNA-transfected cells (Fig. 2B, left panel).

**MiR-101 and miR-217 Inhibit Proliferation of ESCC Cells by G2/M Cell Cycle Arrest**—It has been reported that lncRNA MALAT1 can modulate cellular proliferation through regulating cell cycle progression. Therefore, miR-101 and miR-217 may also be involved in control of ESCC cell growth through silencing MALAT1 RNA expression. We found that miR-101, miR-217, or MALAT1 siRNA could significantly inhibit proliferation of KYSE150 and KYSE30 cells in both a dose-dependent and time-dependent way (all \( p < 0.01 \)) (Fig. 3A). Colony formation assays also support the tumor suppressor role of both miRNAs (Fig. 3B).

To gain insight into the functional relevance of miR-101 and miR-217 through MALAT1 depletion, we examined the impacts of these miRNAs on KYSE30 cell cycle progression and apoptosis (Fig. 3C). Compared with NC RNA-transfected KYSE30 cells, both miR-101 and miR-217 could result in a significantly reduced \( G_1 \) population (NC, 59.4\%; miR-101, 31.7\%; miR-217, 32.1\%), marginally increased replication (S-phase) (NC, 30.9\%; miR-101, 34.2\%; miR-217, 42.5\%), and significantly elevated \( G_2/M \) population (NC, 9.6\%; miR-101, 34.1\%; miR-217, 25.4\%). Similar results have been found in KYSE150 cells (Fig. 3C and D). However, no significant miRNA-induced apoptosis was observed in both cell lines (data not shown). Moreover, we did not observe a growth depression in KYSE450 cells (data not shown).
miR-101 and miR-217 Silence MALAT1 in ESCC
MiR-101 and miR-217 Inhibit Migration and Invasion of ESCC Cells—Because impacts of IncRNA MALAT1 on ESCC invasion and metastasis were still largely unclear, we investigated how siM, miR-101, and miR-217 regulate migration and invasion of ESCC cells. The wound-healing assays demonstrated that siM, miR-101, and miR-217 impaired the motility of the KYSE30, KYSE150, and KYSE450 cells compared with control cells transfected with NC RNA (Fig. 4A). Next, the impact of miR-101 and miR-217 on invasiveness of KYSE30, KYSE150, and KYSE450 cells was determined using the Matrigel invasion assay system. Reduced invasion ability of ESCC cells was observed after elevated expression of miR-101 and miR-217 (Fig. 4B). In line with this, MALAT1 siRNA can also inhibit the invasion of these ESCC cells (Fig. 4B). We also confirmed this observation using antagonisms of miR-101 and miR-217 and found enhanced invasion ability of ESCC cells transfected with these antagonisms (Fig. 4B).

miR-101 and miR-217 Modulate Expression of MALAT1 Downstream Genes—Considering the regulation of miR-101 and miR-217 on cell cycle progression as well as metastasis of ESCC cells, we examined expression of multiple MALAT1 downstream genes involved in cell cycle control (p21, p27, and B-MYB) and metastasis regulation (MIA2, ROBO1, CTHRC1, and CCT4) (Fig. 5). Neither miR-101 nor miR-217 has been proven or has potential binding sites in these genes predicted by Targetscan. We found that overexpression of miR-101 and miR-217 or siM up-regulated expression of p21 and p27 and inhibited B-MYB expression in both KYSE30 and KYSE150 cell lines but not in KYSE450, which was consistent with our results on how these miRNAs influence ESCC cell proliferation and cell cycle. Moreover, miR-101, miR-217, or MALAT1 siRNA could not only increase expression of MIA2, HNF4G, and ROBO1, which are negative regulators of migration and invasion, but also depress expression of CCT4 and CTHRC1, which are positive regulators of metastasis, in all three ESCC cell lines.

miR-101 and miR-217 Are Negatively Correlated with MALAT1 RNA Expression in ESCC Tissues—We further examined expression of miR-101, miR-217, and IncRNA MALAT1 in 42 pairs of ESCC tissue samples, and adjacent normal tissues from two different medical centers. For Shandong cohort, significant up-regulation of MALAT1 in ESCC tissues was observed compared with normal tissues (p < 0.01) (see Fig. 6A). There was significant down-regulation of miR-101 or miR-217 in ESCC specimens than normal tissues (Fig. 6A). Interestingly, we found significant negative correlation between miR-101 or miR-217 expression and MALAT1 RNA expression in ESCC or normal specimens using Spearman’s correlation tests (all p < 0.05) (Fig. 6A). Similar results were found in Huaian cohort (Fig. 6B).

miR-101 and miR-217 Inhibit ESCC Growth in Vivo—We found that the growth of tumors from miR-101-up-regulated or miR-217-up-regulated xenografts was inhibited significantly compared with that of tumors from control xenografts after 2 weeks (both p < 0.01) (Fig. 7, A and B). The growth of tumors from MALAT1-down-regulated xenografts was also inhibited significantly when compared with that of tumors from control xenografts (p < 0.01) (Fig. 7, A and B). However, there were no significant differences of mice weight between controls or miRNA- or siM-treated grouped (Fig. 7C).

**DISCUSSION**

Although IncRNA MALAT1 has been investigated in multiple human cancers (15, 17–23), little is known about its involvement and regulation in ESCC development. Our results demonstrate that an Ago2-dependent posttranscriptional regulation of MALAT1 by miR-101 and miR-217 exists in ESCC cells for the first time. Data from human malignant or normal esophageal tissues strongly support this observation as there was significantly negative correlation between these two miRNA expression levels and the MALAT1 RNA expression level. This posttranscriptional regulation of MALAT1 could lead to significantly depressed proliferation, migration, and invasion abilities of ESCC cells assuming the tumor suppressor role of miR-101 and miR-217.

As a highly conserved IncRNA across mammalian species, MALAT1 shows extreme abundance in many human cancers, underlining its functional importance during carcinogenesis. It has been shown that MALAT1 may play its part through several different mechanisms. The 3’ end of MALAT1 could be cleaved by RNase P and RNase Z, which produces the cytoplasmic MALAT1-associated small cytoplasmic RNA (a new tRNA-like ncRNA) (34). After localizing to nuclear speckles (35), MALAT1 might modulate alternative splicing of a subset of pre-miRNAs by regulating serine/arginine splicing factors activity (36). Moreover, MALAT1 can bind to CBX4 (Chromobox homolog 4), also referred to as Pc2 (Polycomb 2), a component of the polycomb repressive complex 1 (PRC1). This interaction controls the re-localization of PRC1 on interchromatin granules and silences or activates gene expression (37). In this way MALAT1 influences proliferation, invasion, and migration of cancer cells through regulation of multiple known downstream genes, including several cell cycle control genes (p21, p27, and B-MYB) and metastasis-related genes (MIA2, ROBO1, CTHRC1, and CCT4), which we examined in this study.

Our data revealed that IncRNAs can be regulated by miRNAs at the posttranscriptional level. Dicer is a key enzyme during miRNA maturation. Therefore, knockdown of Dicer expression would result in decreased miRNA levels in cells. Consistent with this, we found increased MALAT1 RNA expression after Dicer depression in ESCC cells. Additionally, because Ago2 is essential for incorporation of mature miRNAs into RISC, decreased Ago2 expression might lead to attenuated function of miRNAs. In accordance with this, we observed increased MALAT1 RNA expression after Ago2 silencing.

**FIGURE 6.** MiR-101 and miR-217 are negatively correlated with MALAT1 RNA expression in ESCC tissues. A, MiR-101, miR-217, and MALAT1 were quantified using SYBR real-time PCR in Shandong cohort. U6 was used as an endogenous reference for miR-101 and miR-217 normalization. B-Actin was used as endogenous reference for MALAT1 RNA expression. Correlations between miR-101 and MALAT1 or miR-217 and MALAT1 are presented. B, similar results were observed in the Huaian cohort. All results are the mean of triplicate assays with S.D. presented. *, p < 0.05; **, p < 0.01.
which might be due to inhibition of miR-101 and miR-217 function. Interestingly, Leucci et al. (27) and Han et al. (28) reported that miR-9 and miR-125b can target MALAT1 for degradation, which also supports the hypothesis that miRNAs are involved in regulation of lncRNA MALAT1.

As an important tumor-suppressive miRNA, miR-101, takes part in development of multiple human cancers through targeting several genes including EZH2, Cox-2, Mcl-1, and Fos (38–40). Similarly, it has been reported that miR-217 could function as a tumor suppressor in pancreatic ductal adenocarcinoma and clear cell renal cell carcinoma (41, 42). However, the role of miR-101 or miR-217 in ESCC is still largely unclear. Our results indicate that miR-101 and miR-217 act as vital tumor suppressors at least partially through silencing MALAT1 in ESCC, which are consistent with their functions in other malignancies.

In the current study KYSE450 cells did not behave like other cells, which may be due to different ESCC cell lines are from different cancer patients with different genetic backgrounds. It is quite common to observe cellular behavior discrepancies among different cell lines after the same treatment even they belong to the same type of malignancy.

Taken together we identified lncRNA MALAT1 as a novel target of miR-101 and miR-217. As a result, this posttranscriptional regulation shows a significant impact on proliferation, invasion, and metastasis of ESCC cells. Our findings highlight the interaction between miRNAs and lncRNA MALAT1 during tumorigenesis and progression of esophageal cells.

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REFERENCES
1. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M. J. (2009) Cancer statistics, 2009. CA Cancer J. Clin. 59, 225–249
2. Gao, Y. T., McLaughlin, J. K., Blot, W. J., Ji, B. T., Benichou, J., Dai, Q., and Fraumeni, J. F., Jr. (1994) Risk factors for esophageal cancer in Shanghai, China. I. Role of cigarette smoking and alcohol drinking. Int. J. Cancer 58, 192–196
3. Hu, J., Nyрен, O., Wolk, A., Bergström, R., Yuen, J., Adami, H. O., Guo, L., Li, H., Huang, G., and Xu, X. (1994) Risk factors for oesophageal cancer in northeast China. Int. J. Cancer 57, 38–46
4. Wu, C., Hu, Z., He, Z., Jia, W., Wang, F., Zhou, Y., Liu, Z., Zhan, Q., Liu, Y., Yu, D., Zhai, K., Chang, J., Qiao, Y., Jin, G., Liu, Z., Shen, Y., Guo, C., Fu, J., Miao, X., Tan, W., Shen, H., Ke, Y., Zeng, Y., Wu, T., and Lin, D. (2011) Genome-wide association study identifies three new susceptibility loci for esophageal squamous-cell carcinoma in Chinese populations. Nat. Genet. 43, 679–684
5. Wu, C., Kraft, P., Zhai, K., Chang, J., Wang, Z., Li, Y., Hu, Z., He, Z., Jia, W., Abnet, C. C., Liang, L., Hu, N., Miao, X., Zhou, Y., Liu, Z., Zhan, Q., Liu, Y., Qiao, Y., Zhou, Y., Jin, G., Guo, C., Lu, C., Yang, H., Fu, J., Yu, D., Freedman, N. D., Ding, T., Tan, W., Goldstein, A. M., Wu, T., Shen, H., Ke, Y., Zeng, Y., Chanock, S. J., Taylor, P. R., and Lin, D. (2012) Genome-wide association analyses of esophageal squamous cell carcinoma in Chinese identify multiple susceptibility loci and gene-environment interactions. Nat. Genet. 44, 1090–1097
6. Wang, L. D., Zhou, F. Y., Li, X. M., Sun, L. D., Song, X., Jin, Y., Li, J. M., Kong, G. Q., Qi, H., Cui, J., Zhang, L. Q., Yang, J. Z., Li, J. L., Li, X. C., Ren, J. L., et al. (2010) Genome-wide association study of esophageal squamous cell carcinoma in Chinese subjects identifies susceptibility loci at PLCE1 and C20orf54. Nat. Genet. 42, 759–763
7. Abnet, C. C., Freedman, N. D., Hu, N., Wang, Z., Yu, K., Shu, X. O., Yuan, J. M., Zheng, W., Dawsey, S. M., Dong, L. M., Lee, M. P., Ding, T., Qiao, Y. L., Gao, Y. T., Koh, W. P., Xiang, Y. B., Tang, Z. Z., Fan, J. H., Wang, C., Wheeler, W., Gail, M. H., Yeager, M., Yuen, J., Hutchinson, A., Jacobs, K. B., Giffen, C. A., Burdett, L., Fraumeni, J. F., Jr., Tucker, M. A., Chow, W. H., Goldstein, A. M., Chanock, S. J., and Taylor, P. R. (2010) A shared susceptibility locus in PLCE1 at 10q23 for gastric adenocarcinoma and
esophageal squamous cell carcinoma. Nat. Genet. 42, 764–767
8. Eichler, E. E., Flint, J., Gibson, G., Kong, A., Leal, S. M., Moore, J. H., and Nadeau, J. H. (2010) Missing heritability and strategies for finding the underlying causes of complex disease. Nat. Rev. Genet. 11, 446–450
9. Esteller, M. (2011) Non-coding RNAs in human disease. Nat. Rev. Genet. 12, 861–874
10. Tsai, M. C., Spittle, R. C., and Chang, H. Y. (2011) Long intergenic non-coding RNAs: new links in cancer progression. Cancer Res. 71, 3–7
11. Rinn, J. L., and Chang, H. Y. (2012) Genome regulation by long noncoding RNAs. Annu. Rev. Biochem. 81, 145–166
12. Ambros, V. (2001) microRNAs: tiny regulators with great potential. Cell 107, 823–826
13. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233
14. Ponting, C. P., Oliver, P. L., and Reik, W. (2009) Evolution and functions of long noncoding RNAs. Cell 136, 629–641
15. Ji, P., Diederichs, S., Wang, W., Böing, S., Metzger, R., Schneider, P. M., Tidev, N., Brandt, B., Buerger, H., Buek, T., Thomas, M., Berdal, W. E., Serve, H., and Muller-Tidow, C. (2003) MALAT-1, a novel noncoding RNA, and thyminos m4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22, 8031–8041
16. Lin, R., Maeda, S., Liu, C., Karin, M., and Edgington, T. S. (2007) A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. Oncogene 26, 851–858
17. Tripathi, V., Shen, Z., Chakraborty, A., Giri, S., Freier, S. M., Wu, X., Zhang, Y., Gorospe, M., Prasanth, S. G., Lal, A., and Prasanth, K. V. (2013) Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. PLoS Genet. 9, e1003368
18. Gutschner, T., Hammerle, M., Eissmann, M., Hsu, J., Kim, Y., Hung, G., Wiebe, K., Berdel, W. E., Wiwrodt, R., and Muller-Tidow, C. (2011) The unknown function of MALAT1. Mol. Cell 39, 358–369
19. Zhu, L., Zhou, C., Li, M., Tang, X., Li, H., Yuan, Q., and Yang, M. (2013) Association of a genetic variation in a miR-191 binding site in MDM4 with risk of esophageal squamous cell carcinoma. PLoS ONE 8, e64331
20. Liu, L., Zhou, Z., Zhou, L., Peng, L., Li, D., Zhang, X., Zhou, M., Kuang, P., Yuan, Q., Song, X., and Yang, M. (2012) Functional FEN1 genetic variants contribute to risk of hepatocellular carcinoma, esophageal cancer, gastric cancer, and colorectal cancer. Carcinogenesis 33, 119–123
21. Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hauser, J., Berninger, P., Rotheballer, A., Ascana, M., Is., Jungkamp, A. C., Munschauer, M., Ulrich, A., Wardle, G. S., Dewell, S., Zavolan, M., and Tuschl, T. (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 141, 129–141
22. Wulcz, J. E., Freier, S. M., and Spector, D. L. (2008) 3’ end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA. Cell 135, 919–932
23. Hutchinson, J. N., Ensminng, A. W., Clemson, C. M., Lynch, C. R., Lawren, J. B., and Chess, A. (2007) A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. BMC Genomics 8, 39
24. Tripathi, V., Ellis, J. D., Shen, Z., Song, D. Y., Pan, Q., Watt, A. T., Freier, S. M., Bennett, C. F., Sharma, A., Bubulya, P. A., Blencowe, B. J., Prasanth, S. G., and Prasanth, K. V. (2010) The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol. Cell 39, 925–938
25. Yang, L., Lin, C., Liu, W., Zhang, J., Ohgi, K. A., Grinstein, G. J., Dorrestein, P. C., and Rosenfeld, M. G. (2011) ncRNA- and P2c methylation-dependent gene relocation between nuclear structures mediates gene activation programs. Cell 147, 773–788
26. Varambally, S., Cao, Q., Mani, R. S., Shankar, S., Wang, X., Ateeq, B., Laxman, B., Cao, X., Jing, X., Ramarayanan, K., Brenner, J. C., Yu, J., Kim, J. H., Han, B., Tan, P., Kumar-Mahasak, C., Lonigro, R. J., Pal, J., Gudimal, N., Hager, C. A., and Chinnaiyan, A. M. (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322, 1695–1699
27. Wang, H. J., Ruan, H. J., He, X. J., Ma, Y. Y., Jiang, X. T., Xie, J. Y., Ye, Z. Y., and Tao, H. Q. (2010) MicroRNA-101 is down-regulated in gastric cancer and involved in cell migration and invasion. Eur. J. Cancer 46, 2295–2303
28. Su, H., Yang, J. R., Xu, T., Huang, J., Xu, L., Yuan, Y., and Zhuang, S. M. (2009) MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res. 69, 1135–1142
29. Zhao, W. G., Yu, S. N., Lu, Z. H., Ma, Y. H., Gu, Y. M., and Chen, J. (2010) The miR-217 microRNA functions as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS. Carcinogenesis 31, 1726–1733
30. Li, H., Zhao, J., Zhang, J. W., Huang, Q. Y., Huang, J. Z., Chi, L. S., Tang, H. J., Liu, G. Q., Zhu, D. J., and Ma, W. M. (2013) MicroRNA-217, down-regulated in clear cell renal cell carcinoma and associated with lower survival, suppresses cell proliferation, and migration. Neoplasma 60, 511–515