


Upregulation of miR-1269 Contributes to the Progression of Esophageal Squamous Cell Cancer Cells and Is Associated With Poor Prognosis

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Abstract

Background: MicroRNA-1269 (miR-1269) has been identified upregulated in several cancers, as well as in esophageal cancer. In the present study, we investigated the clinical prognostic significance and potential functional role of miR-1269 in esophageal squamous cell carcinoma (ESCC). **Methods:** A total of 107 ESCC patients who underwent surgical resection were enrolled in this study. miR-1269 expression was measured using quantitative real-time PCR (qRT-PCR). Kaplan-Meier method and multivariate Cox regression analysis were used to explore the prognostic significance of miR-1269. CCK-8 assays and Transwell assays were used to investigate the effects of miR-1269 on cell proliferation, migration, and invasion. The direct association between miR-1269 and SOX6 was evaluated using a dual-luciferase reporter assay. **Results:** The expression of miR-1269 was significantly upregulated in ESCC tissues and cell lines compared with adjacent normal tissues and esophageal epithelial cell line, respectively. What's more, the upregulation of miR-1269 was associated with positive lymph node metastasis and advanced TNM stage. ESCC patients with high miR-1269 expression had shorter overall survival than those with low miR-1269 expression levels. Compared with the control group, overexpression of miR-1269 promoted cell proliferation, migration, and invasion, while knockdown of miR-1269 inhibited cell proliferation, migration, and invasion. SOX6 was a direct target of miR-1269. **Conclusion:** These results suggest that miR-1269 plays an important role in the progression of ESCC by targeting SOX6 and may be a potential prognostic biomarker and the miR-1269/SOX6 axis may be a therapeutic target for the patient with ESCC.

Keywords

esophageal squamous cell carcinoma, miR-1269, prognosis, proliferation, migration, invasion

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Introduction

Esophageal cancer is one of the most commonly diagnosed cancers in the digestive system, with increasing incidence rates.¹ Esophageal squamous cell cancer (ESCC) is considered as the major subtype of esophageal cancer with accounting for about 90% of all cases.² Esophageal cancer is highly prevalent occurred in Asia and Africa. In China, esophageal cancer has a high incidence, accounting for about half of newly diagnosed cases worldwide in 2012.² Symptoms of ESCC at early stages are usually not obvious, and most patients are already at advanced stages when they initially diagnosed, and eventually succumb to distant metastasis.³ Although great advances in the diagnosis and treatment of ESCC approaches in recent years, the 5-year survival rate of ESCC patients remains very poor (less than 29.7%).⁴ Therefore, finding novel specific cancer-related molecular targets for prognosis and treatment is crucial to improving the survival rate of ESCC.

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It has been reported that the abnormal expression of microRNAs (miRNAs) is associated with cancer development and plays an important role in ESCC.⁵ miRNAs are a group of small non-coding endogenous RNAs that regulate their target mRNAs at the post-transcriptional level by binding to complementary sites in the 3'-UTR of mRNAs.⁶ miRNAs have been indicated that play essential roles in the initiation and progression of various diseases in humans.^{7,8} Previous studies also revealed that miRNAs control various important cell functions, including cell proliferation, migration, differentiation, and invasion.⁹⁻¹¹ Numerous studies have revealed that miRNAs may be involved in the progression of ESCC and associated with prognosis.^{12,13} miR-1269 has been reported abnormally expressed and played essential roles in various diseases, including cancers.^{14,15} A previous study by Priyanka Sharma and co-workers identified differentially expressed serum miRNAs, including miR-1269, in esophageal cancer for detection using TCGA miRNAseq and RNAseq datasets.¹⁶ However, the role of miR-1269 in ESCC remains unclear.

In the present study, we investigated the expression pattern of miR-1269 in ESCC tissues and cells. We also analyzed the association between miR-1269 expression and clinicopathological characteristics of ESCC patients, as well as the prognostic significance of miR-1269. Besides, the role of miR-1269 in ESCC cell proliferation, migration, and invasion was investigated. The interaction between miR-1269 and SOX6 was identified.

Materials and Methods

Tissue Samples

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Shandong First Medical University and in accordance with the principles outlined in the Declaration of Helsinki. All the ESCC patients signed written inform consents prior to enrollment in this study. A total of 107 histopathologically confirmed paired ESCC tissue samples and corresponding adjacent esophageal mucosa tissue samples were collected during surgical resection from May 2010 to December 2013 at The First Affiliated Hospital of Shandong First Medical University. All tissue samples were snap-frozen in liquid nitrogen until required for the RNA extraction. All patients with ESCC had not received any local or systemic therapies prior to surgery. The clinical characteristics of ESCC patients were collected and recorded in Table 1. The 5-year follow-up information was also collected by telephone.

Cell Culture and Transfection

ESCC cell lines Eca-109, TE-1, KYSE-150, and TE-10, as well as the human normal esophageal epithelial cell line Het-1A, were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). All ESCC cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% FBS (Gibco, Carlsbad, CA, USA), and Het-1A was cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented

Table 1. Comparison of miR-1269 Expression With Clinical Characteristics of Patients With ESCC.

Variables	Cases n = 107	miR-1269 expression		P-values
		Low (n = 52)	High (n = 55)	
Gender				
Male	70	34	36	0.994
Female	37	18	19	
Age (years)				0.149
< 60	55	23	32	
≥ 60	52	29	23	
Tumor size (cm)				0.520
< 4	61	28	33	
≥ 4	46	24	22	
Differentiation				0.092
Well-moderate	59	33	26	
Poor	48	19	29	
Tumor location				0.409
Up/middle	66	30	36	
Lower	41	22	19	
Lymph node metastasis				0.012
Negative	63	37	26	
Positive	44	15	29	
TNM stage				0.008
I-II	60	36	24	
III-IV	47	16	31	

with 10% FBS (Gibco). All cells were incubated at 37°C in an incubator with 5% CO₂.

The miR-1269 mimic (5'-CUGGACUGAGCCGUGCUA CUGG-3'), mimic negative control (NC; 5'-UUCUCCGAAC GUGUCACGUTT-3'), miR-1269 inhibitor (5'-CCAGUAG-CACGGCUCAGUCCAG-3'), and inhibitor NC (5'-CAGUACUUUUGUGUAGUACAA-3') were synthesized from GenePharma Co., Ltd (Shanghai, China). ESCC cells were seeded in 6-well plates and cultured at 37°C overnight. Cells were subsequently transfected with miR-1269 mimic, mimic NC, miR-1269 inhibitor, or inhibitor NC using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h transfection, cells were collected for subsequent assays. Untreated cells were used as control.

RNA Extraction and Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted from ESCC tissue samples and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) on ice. A NanoDrop spectrophotometer 1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USE) was used to measure the quantity and purity of RNA. Then RNA was reverse transcribed into cDNA using one step PrimeScript miRNA cDNA Synthesis kit (Takara, Japan) according to the manufacturer's protocol. The RT-qPCR was performed using the SYBR Premix Ex Taq II kit (Takara,

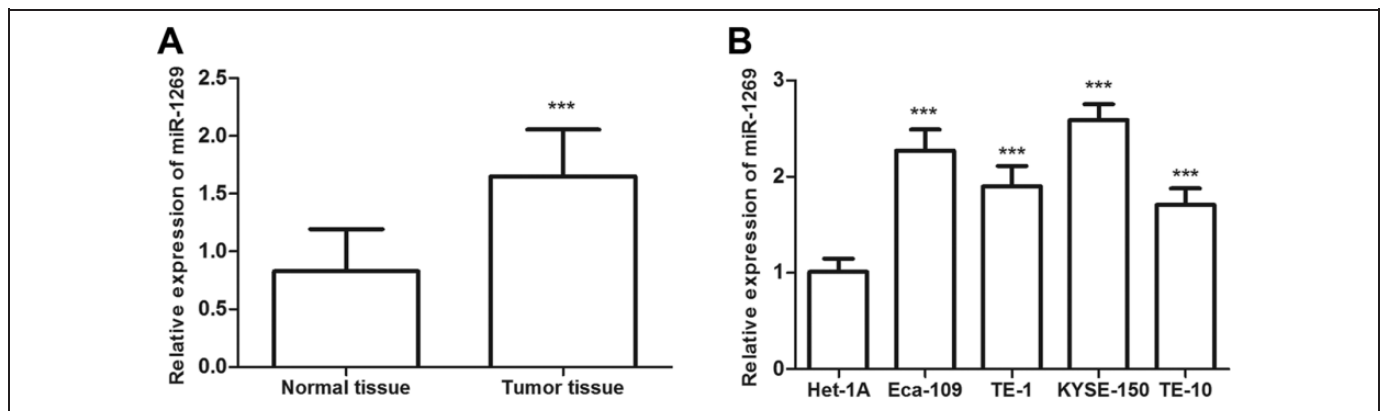


Figure 1. The expression of miR-1269 was measured using qRT-PCR in ESCC tissues and cell lines. A, The expression of miR-1269 was upregulated in ESCC tissue samples when compared with adjacent normal tissues. B, miR-1269 was upregulated in ESCC cell lines when compared with normal esophageal epithelial cell line het-1A. *** $P < 0.001$.

Japan) on the Applied Biosystems 7500HT Fast Real-Time PCR System according to the manufacturer's instructions. The RT-qPCR reaction condition was as follows: 94°C for 2 min, 35 cycles at 94°C for 30 s, 60°C for 30 s, and at 72°C for 30 s, with a final extension at 72°C for 5 min. The relative quantification of miR-1269 expression was calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to U6.

Cell Proliferation Assay

Cell proliferation abilities were evaluated using a cell counting kit-8 (CCK-8) assay with CCK-8 kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. In brief, cells were seeded into 96-well plates at a density of 3×10^3 cells/well and cultured for 0, 1, 2, and 3 days, then 10 μ l CCK-8 reagent was added to each well at each time-point. Cells were further incubated for 2 h at 37°C, and the absorbance was measured at 450 nm using a microplate reader.

Cell Migration and Invasion Assays

Cell migratory and invasive abilities were investigated using 24-well polycarbonate membrane Transwell chambers with 8 μ m pores (Corning, NY, USA). Approximately, 1×10^5 transfected cells in serum-free culture medium were seeded in the upper side membrane without (for the cell migration assay) or without (for the cell invasion assay) Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). Complete medium with 10% FBS was added to the lower chambers. After incubation for 24 h at 37°C, migrated or invaded cells were fixed and stained with 0.1% crystal violet at room temperature. The number of migrated or invaded cells in 5 randomly selected fields was counted under a light microscope.

Bioinformatic Analysis and Dual-Luciferase Reporter Assay

The potential target genes of miR-1269 and miRNA binding sites within mRNA 3'UTR were predicted using online publicly software TargetScan (www.targetscan.org/vert7.2). The 3'UTR

sequence of SOX6 containing the predicted binding site for miR-1269 was amplified and cloned into pmirGLO plasmid (Promega, Madison, WI, USA) to give the wild-type reporter plasmid SOX6 3'UTR-WT. To generate the SOX6 mutant reporter vector SOX6 3'UTR-Mut, the seed region was mutated to remove complementary nucleotides to miR-1269 and cloned into pmirGLO plasmid (Promega). Then, the WT or Mut luciferase reporter vectors of SOX6 were co-transfected with miR-1269 mimic, mimic NC, miR-1269 inhibitor, or inhibitor NC into Eca-109 cells using Lipofectamine 3000 for the luciferase reporter assay. After 48 h, the relative luciferase activity of each well was determined using a dual-luciferase reporter assay system following the manufacturer's instructions.

Statistical Analysis

All experiments were repeated at least 3 times and the data were presented as mean \pm SD. A Student's t-test was used to analyze the differences between the 2 groups. χ^2 test was used to analyze the relationship between miR-1269 expression and clinical characteristics of patients. One-way ANOVA followed by Tukey's test was used to analyze the unequal variance in multiple comparisons. The prognostic significance of miR-1269 was determined by the Kaplan-Meier survival plots and multivariate Cox regression assay. All data analyses were performed using SPSS 20.0 and GraphPad 5.0. The value of P less than 0.05 was considered statistically significant.

Results

miR-1269 Expression Was Increased in ESCC Tissues and Cells

We performed RT-qPCR to explore the expression pattern of miR-1269 in tissue samples from 107 patients with ESCC. The miR-1269 expression level was significantly increased in ESCC tissue samples compared with corresponding adjacent normal tissue samples from patients with ESCC ($P < 0.001$, Figure 1A). Similarly, the expression levels of miR-1269 were

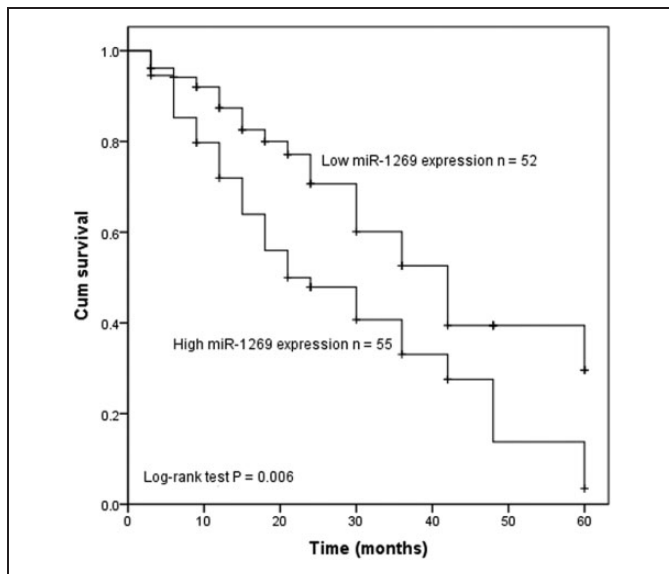


Figure 2. ESCC patients with high miR-1269 expression had shorter overall survival than those with low miR-1269 expression levels (log-rank test $P = 0.006$).

also found upregulated in ESCC cell lines Eca-109, TE-1, KYSE-150, and TE-10, compared with normal esophageal endothelial cell line Het-1A ($P < 0.001$, Figure 1B).

The Relationship Between miR-1269 Expression and Clinical Characteristics of ESCC Patients

To investigate whether miR-1269 expression was associated with the development of ESCC, we analyzed the relationship between miR-1269 expression and clinical characteristics of ESCC patients by using the χ^2 test. Using the mean expression level of miR-1269 in ESCC tissues as the cut-off point, we divided patients with ESCC into a low miR-1269 expression group and high miR-1269 expression group. As shown in Table 1, results showed that high miR-1269 expression was associated with positive lymph node metastasis ($P = 0.012$) and advanced TNM stage ($P = 0.008$). However, there is no association with gender, age, differentiation, tumor size, and tumor location (all $P > 0.05$).

The Prognostic Significance of miR-1269 in ESCC

To investigate whether the miR-1269 expression has prognostic value in ESCC, Kaplan-Meier survival curves were plotted using the clinicopathological characteristics and overall survival information of ESCC patients. The results revealed that increased miR-1269 expression level was associated with shorter overall survival (log-rank test $P = 0.006$, Figure 2). Furthermore, multivariate Cox proportional hazards regression model showed that TNM stage (HR = 1.810, 95%CI: 1.059-3.096, $P = 0.030$) and miR-1269 expression (HR = 2.027, 95%CI: 1.092-3.763, $P = 0.025$) were independent prognostic predictor for prognosis of ESCC (Table 2). These above results

Table 2. Multivariate Cox Regression Analysis of Overall Survival in ESCC Patients.

Variables	Multivariate Cox analysis		
	HR	95%CI	P -value
miR-1269	2.027	1.092-3.763	0.025
Gender	1.808	0.970-3.371	0.062
Age	0.766	0.457-1.286	0.314
Tumor size	1.554	0.901-2.680	0.113
Differentiation	1.529	0.853-2.742	0.154
Tumor location	0.860	0.494-1.496	0.593
Lymph node metastasis	1.679	0.965-2.919	0.067
TNM stage	1.810	1.059-3.096	0.030

suggested that miR-1269 expression might be an independent prognostic biomarker for ESCC.

miR-1269 Promotes ESCC Cell Proliferation, Migration, and Invasion in Vitro

To explore the potential functional role of miR-1269 in ESCC, CCK-8 and Transwell assays were used to detect the effects of miR-1269 on cell proliferation, migration, and invasion of ESCC cells. We selected Eca-109 and KYSE-150 cells to manipulate the expression levels of miR-1269 because both the cells exhibited higher miR-1269 expression levels. As indicated in Figure 3A, transfection efficiency was confirmed by RT-qPCR. The expression levels of miR-1269 were upregulated by miR-1269 mimic, while downregulated by miR-1269 inhibitor, compared with control ($P < 0.001$). The effects of miR-1269 on the proliferation of ESCC cells was investigated by CCK-8 assay. The results showed that overexpression of miR-1269 promoted cell proliferation, while downregulation of miR-1269 inhibited cell proliferation, compared with control ($P < 0.05$, Figure 3B).

As the expression of miR-1269 was associated with lymph node metastasis and poor outcome in patients with ESCC, the effects of miR-1269 on cell migration and invasion were also investigated using Transwell assays. As shown in Figure 4A and 4B, overexpression of miR-1269 significantly enhanced cell migration and invasion, while downregulation of miR-1269 inhibited migration and invasion, compared with control ($P < 0.001$).

SOX6 Is a Direct Target of miR-1269 in ESCC Cells

The target prediction tool TargetScan showed that SOX6 has the potential binding sites for miR-1269 and the putative binding sequence is revealed in Figure 5A. Then, to confirm the relationship between miR-1269 and SOX6, we performed a dual-luciferase reporter assay. As shown in Figure 5B, miR-1269 overexpression significantly inhibited the luciferase activity of the SOX6 3'UTR WT but does not affect the SOX6 3'UTR Mut. Moreover, we used RT-qPCR assay to detect the mRNA levels of SOX6 in ECA-109 cells ($P < 0.05$). The

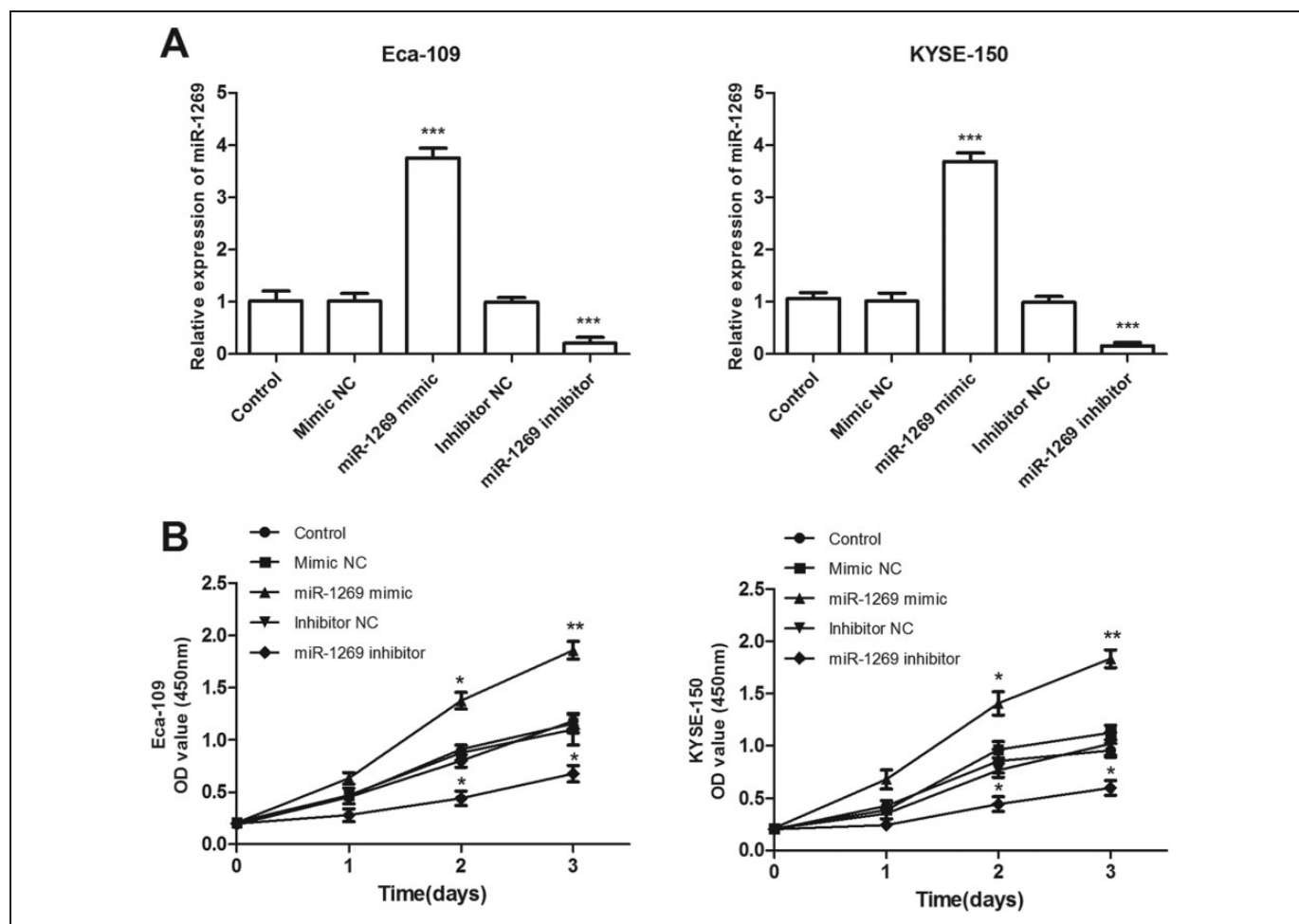


Figure 3. The effects of miR-1269 on cell proliferation in Eca-109 and KYSE-150 cells were measured by CCK-8 assay. A, Confirmation of miR-1269 overexpression or downregulation by qRT-PCR in ESCC cells transfected with miR-1269 mimic or inhibitor, respectively. B, Overexpression of miR-1269 promoted cell proliferation, while the downregulation of miR-1269 inhibited cell proliferation, compared with control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

results showed that overexpression of miR-1269 significantly reduced the mRNA levels of SOX6, while downregulation of miR-1269 increased the mRNA levels of SOX6 ($P < 0.001$, Figure 5C). These results demonstrated that SOX6 might be a direct target of miR-1269 in ESCC.

Discussion

Esophageal cancer remains one of the most common cancers, while China has the highest incidence and mortality from esophageal cancer worldwide.^{17,18} ESCC is the predominant histologic type of esophageal cancer. Despite advances in multimodal therapies, the prognosis of patients with ESCC remains poor.¹⁸ In recent years, more and more studies focused on the association between molecular genes (including miRNAs) and cancers.^{19,20} For instance, Integrin Binding Sialoprotein (IBSP) was found upregulated in ESCC patients and could promote the proliferation and metastasis of ESCC cells, suggesting that IBSP might be a prognostic marker for ESCC patients.²¹ Therefore, identifying more novel effective

therapeutic methods and prognostic markers are necessary to improve the overall survival quality of ESCC patients.

Currently, investigators have focused on the potential of miRNAs to serve as biomarkers for cancers.^{22,23} In ESCC, the expression levels of miR-30b-5p in ESCC tissues were lower than that in adjacent normal tissues and inhibited migration and invasion in Eca-109 cells, suggesting that miR-30b-5p played an important role in the occurrence and progression of ESCC and was a potential therapeutic target.²⁴ miR-490-3p expression was downregulated in ESCC and inhibited cell proliferation and stimulated apoptosis of ESCC cells by regulating MAPK1.²⁵ These studies provided potential therapeutic strategies for the treatment of ESCC.

miR-1269 was found to be upregulated in several types of cancers and to further act as an onco-miR in tumorigenesis progression.^{26,27} In the present study, we investigated the expression of miR-1269 in ESCC tissues and cell lines by using qRT-PCR, with results indicating that miR-1269 was upregulated in ESCC tissues and cell lines, compared with corresponding paired normal tissues and normal epithelial cell line

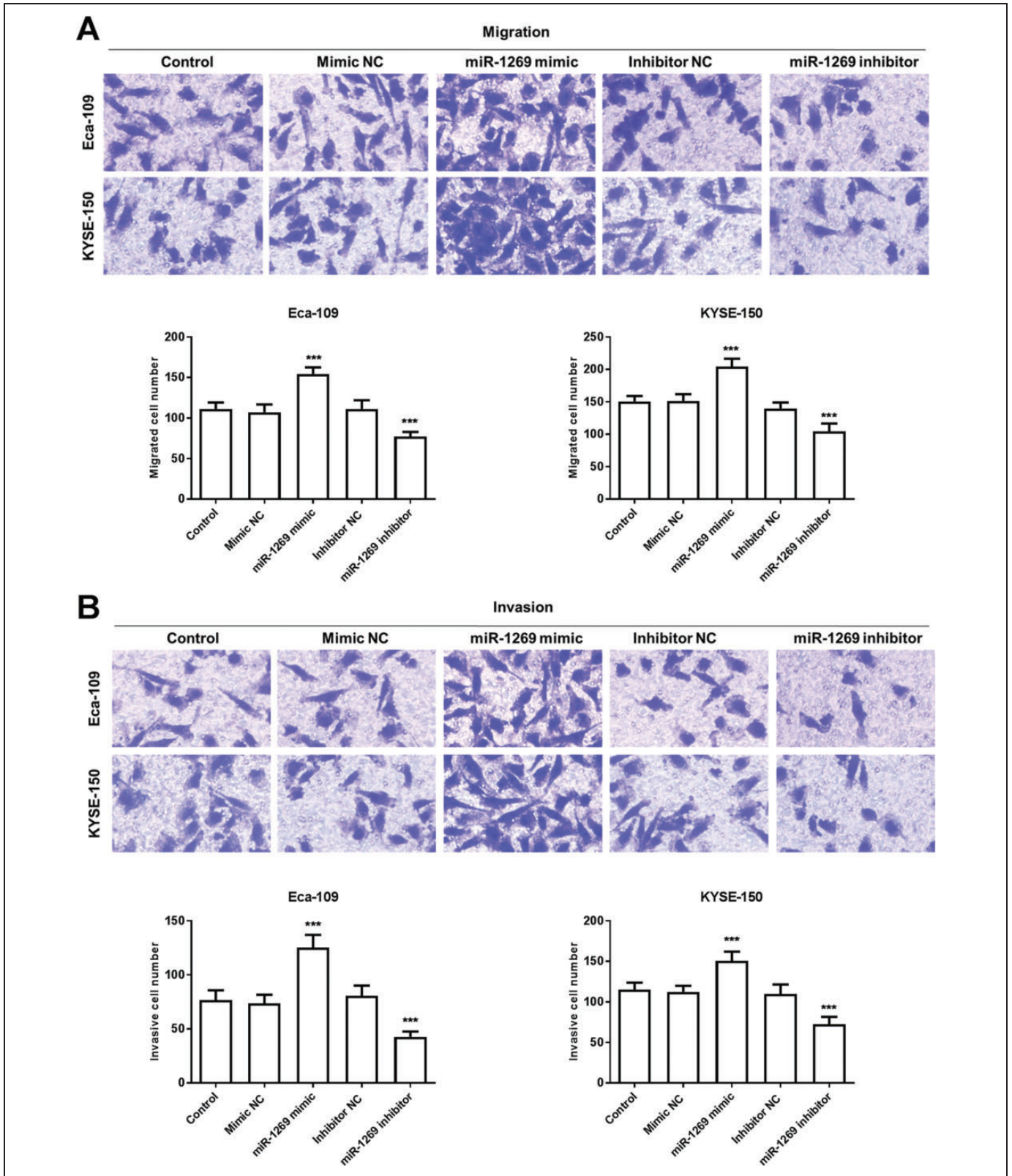


Figure 4. Enhanced miR-1269 expression promoted cell migration and invasion, while knockdown of miR-1269 suppressed cell migration and invasion of eca-109 and KYSE-150 cells, compared with control. A, Representative images of the transwell migration assay. B, Representative images of the transwell invasion assay. $\times 200$ magnification, *** $P < 0.001$.

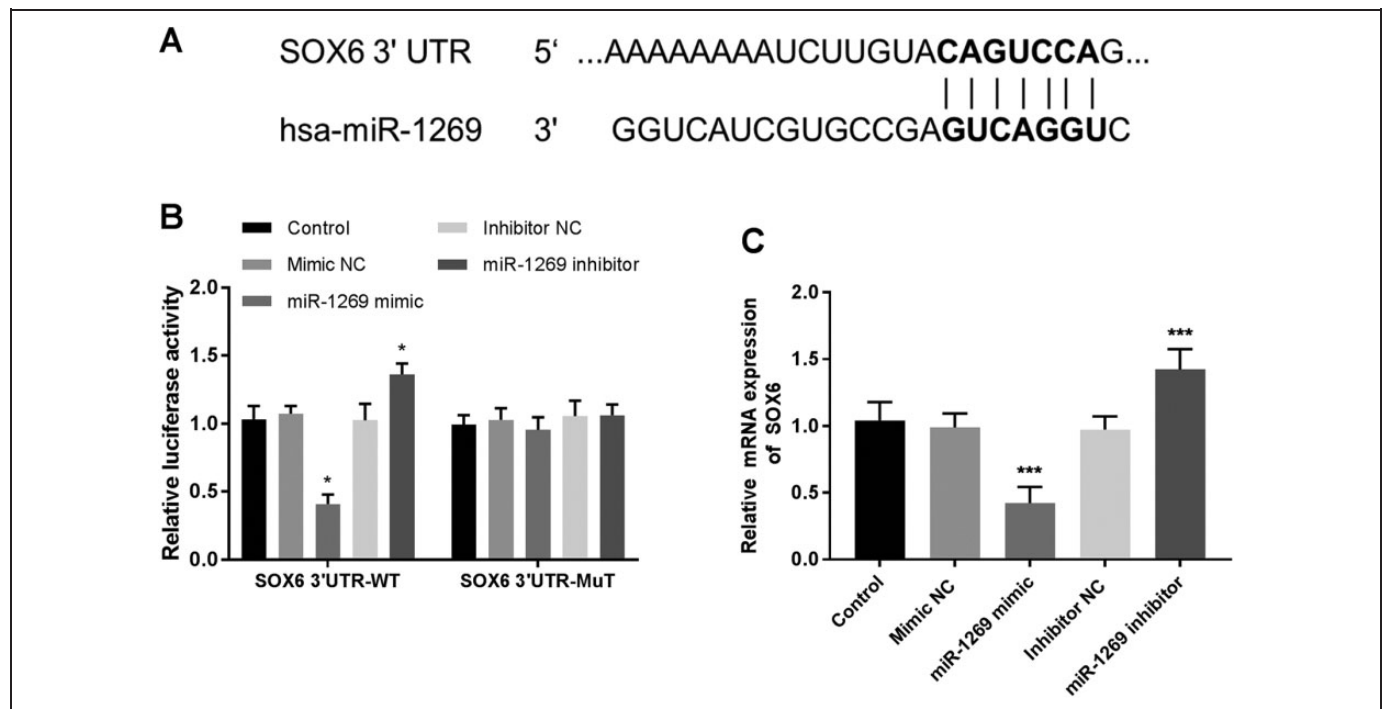


Figure 5. SOX6 may be a direct target of miR-1269. A, Bioinformatics analysis predicted interactions between miR-1269 and its binding sites in the 3'UTR of SOX6. B, Luciferase activity was measured by the dual-luciferase assay. C, SOX6 mRNA levels were suppressed by over-expression of miR-1269, while were promoted by the downregulation of miR-1269. * $P < 0.05$, *** $P < 0.001$.

Het-1A, respectively. The results were consistent with the previous study by Priyanka Sharma and co-workers.¹⁶ In addition, we further analyzed the association between miR-1269 expression and clinical characteristics of ESCC patients. The high expression level of miR-1269 was significantly associated with patients' positive lymph node metastasis and advanced TNM stages. These results suggested that miR-1269 may be an oncogene and involved in the development of ESCC.

Considering the upregulation of miR-1269 was associated with lymph node metastasis and TNM stage, we speculate that miR-1269 may be associated with the prognosis of patients with ESCC. Therefore, we further investigated the clinical prognostic significance of miR-1269 in ESCC using clinical characteristics and 5-year overall survival information of ESCC patients. The results suggested that patients with high miR-1269 expression levels showed a shorter overall survival time than those with low miR-1269 expression levels. And miR-1269 expression and TNM stage may be independent prognostic predictors for the prognosis of ESCC. These results suggested that miR-1269 may be an independent prognostic biomarker for ESCC.

Cell proliferation, migration, and invasion are basic biological processes. Numerous researches have demonstrated that miRNAs can function as either oncogenes or suppressors that participate in the development of tumors by regulating various biological processes.^{10,28} In the present study, to explore the biological role of miR-1269, we further investigated the effects of miR-1269 on cell proliferation, migration, and invasion of ESCC cells. By using Eca-109 and KYSE-150 cells that

transfected with miR-1269 mimic, mimic NC, miR-1269 inhibitor, or inhibitor NC, we observed that overexpression of miR-1269 promoted cell proliferation, migration, and invasion, while inhibition of miR-1269 suppressed these cellular activities, compared with control. These results suggested that miR-1269 may play an oncogenic role in ESCC, which was consistent with the role of miR-1269 in lung cancer,²⁹ colorectal cancer,²⁷ and hepatocellular carcinoma.³⁰ In lung cancer, miR-1269 was highly expressed in the tumors and promoted cell survival and proliferation by targeting TP53 and caspase-9.²⁹ In hepatocellular carcinoma, overexpression of miR-1269 promotes cell proliferation by directly suppressing FOXO1 and functions as an onco-miR in hepatocellular carcinoma.³¹ Another study in hepatocellular carcinoma demonstrated that miR-1269 could directly bind with the 3'UTR region of SOX6 and thereby suppresses tumor growth.³⁰ SOX6 has been confirmed to be a tumor suppressor and downregulated in various cancers.³²⁻³⁴ Subsequently, we tried to explore whether miR-1269 could target SOX6 using Eca-109 cells and found that SOX6 might be a direct target of miR-1269. In ESCC, SOX6 was found downregulated and functioned as a tumor suppressor in ESCC cells.³⁵ Thus, we speculate that miR-1269 might also be an onco-miR in ESCC by regulating ESCC tumorigenesis through targeting SOX6. In future researches, the detailed mechanism of miR-1269 regulates ESCC cellular activities by targeting SOX6 will be explored.

In conclusion, the present study indicated that miR-1269 was indicated upregulated in ESCC tissues and cell lines, as well as associated with the overall survival of ESCC patients.

In addition, miR-1269 may function as an onco-miR by affecting cell proliferation, migration, and invasion by targeting SOX6. These results suggested miR-1269 may be a potential prognostic biomarker and the miR-1269/SOX6 axis may be a therapeutic target for the treatment of ESCC.

Authors' Note

Xiuhui Bai, Qiang Wang, and Xueqi Rui contributed equally. All authors conceived and designed the project. Xiuhui Bai, Qiang Wang, and Xueqi Rui conducted the experiment and acquired the data. All authors analyzed and interpreted the data, and wrote the paper. All authors read and approved the final manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Shandong First Medical University and in accordance with the principles outlined in the Declaration of Helsinki. All the ESCC patients signed written inform consents prior to enrollment in this study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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