

Up-Regulation of MicroRNA-21 Indicates Poor Prognosis and Promotes Cell Proliferation in Esophageal Squamous Cell Carcinoma via Upregulation of lncRNA SNHG1

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Introduction: MicroRNA-21 (miRNA-21) and lncRNA SNHG1 (small nucleolar RNA host gene 1) are known to be aberrantly upregulated and promote tumor progression in various cancers. Nevertheless, very few studies have determined the roles of tissue and circulating miRNA-21 and SNHG1 in ESCC patients. Particularly, knowledge about the characteristics of miRNA-21 and SNHG1 expression and their correlations with survival rates, as well as their interaction with each other remains inadequate in ESCC.

Methods: These expression level of miRNA-21 and SNHG1 of tissues, serum and cell lines were detected by qRT-PCR, and the characteristics of their expression and clinicopathology were analyzed. Then, the diagnostic and prognosis value of serum and tissue miRNA-21 and SNHG1 were evaluated, respectively. In addition, the interaction with each other between miRNA-21 and SNHG1, as well as the effect on ESCC cell proliferation were further clarified.

Results: The expression level of miRNA-21 and SNHG1 are significantly upregulated in tissues, serum and cell lines of ESCC, and tissue miRNA-21 and SNHG1 significantly correlates with lymph node metastasis, TNM stage, tumor size, and poor overall survival in ESCC patients. The receiver operating characteristic (ROC) curves show that areas under the ROC curve (AUC) for serum miRNA-21 and SNHG1 are 0.928 and 0.850, respectively. Pearson correlation coefficient indicated that the expression levels of miRNA-21 and SNHG1 in frozen cancerous tissues are significantly associated with their respective serum levels. Further, Cox univariate and multivariate analyses reveal that miRNA-21 and SNHG1 are independent prognostic factors for overall survival (OS) and disease-free survival (DFS) in ESCC patients. In addition, our in vitro data revealed a novel regulatory pathway, in which miRNA-21 is probably a unidirectional upstream positive regulator of SNHG1 in ESCC cells, and the interaction between miRNA-21 and SNHG1 plays an important role in the proliferation of ESCC cells.

Discussion: In summary, our data show that SNHG1 may be a novel downstream target of miRNA-21 and not vice versa in ESCC cells and contributes significantly toward the proliferation of ESCC cells. These findings suggest that miRNA-21 and SNHG1 may serve as potential diagnostic, prognostic biomarkers and therapeutic targets for ESCC patients.

Keywords: MicroRNA-21, lncRNA SNHG1, esophageal squamous cell carcinoma, diagnosis, prognostic biomarker, cell proliferation

Introduction

Esophageal cancer (EC) is the seventh most common cancer in the world and the sixth leading cause of cancer-related deaths.¹ Esophageal squamous cell carcinoma

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(ESCC) is the major histopathological form accounting for over 90% of ECs, which is also the most frequent type of histopathology in China.² ESCC has been reported to be abnormally frequent in Xinjiang, the northwestern part of China than other regions.³ Despite some recent advances in the exploration of its likely etiological mechanisms that include behaviors and environmental risk factors as well as gene alterations,⁴ the complicated mechanisms are still largely unknown; leading to a 5-year survival rate of about 10–20%.^{1,5} Therefore, a better understanding of the molecular mechanisms underlying ESCC pathogenesis and progression will substantially aid in improving the diagnosis, prognosis, and treatment of ESCC.

MicroRNAs (miRNAs) are a class of single-strand non-coding RNAs consisting of 18–24 nucleotides, which are thought to function through the inhibition of effective mRNA translation of target genes.^{6,7} An increasing number of studies have shown that various specific miRNA expression profiles are related to the development of different types of cancer implying that miRNAs can function as tumor suppressors or oncogenes.⁸ MicroRNA-21 (miRNA-21), an oncogenic miRNA, is encoded by the *MIR21* gene located on chromosome 17q23.2.⁹ Increased miRNA-21 expression during carcinogenesis and in tumor samples indicates that it functions to promote cell proliferation and migration and to inhibit cell apoptosis in majority of cancers, such as liver cancer,¹⁰ breast cancer,¹¹ non-small cell lung cancer,¹² and ESCC.¹³ Additionally, miRNA-21 is associated with poor prognosis as well as with reduced chemosensitivity of cancer cells to anticancer agents in ESCC.¹⁴ In terms of down-regulation of proteins, miRNA-21 has been reported to specifically target tumor suppressors such as PTEN,¹⁵ Bcl-2,¹⁵ Smad7,¹⁶ and PDCD4.¹⁷

Long non-coding RNAs (lncRNAs) play crucial roles in many diseases via controlling gene expression.¹⁸ One of their mechanisms of action involves the formation of regulatory networks with other RNA species, such as miRNAs and mRNAs.¹⁹ It has been well established that miRNAs can interact with lncRNAs to participate in human diseases, such as different types of cancers.²⁰ LncRNA SNHG1 (small nucleolar RNA host gene 1), located at chromosome 11q12.3, is expressed in the nucleus of most cells as well as in the cytoplasm in certain cell-types.²¹ SNHG1 is widely distributed in the body and participates in regulating proliferation, invasion, and metastasis, as well as acts as an indicator of poor survival in many types of cancer including non-small cell lung cancer,²² hepatocellular carcinoma,²³ and colorectal cancer.²⁴ More recent studies suggest that the

tumor-associated miRNAs and lncRNAs may possess the potential to be explored as sensitive and specific cancer biomarkers for diagnostic, prognostic, or monitoring of cancers.²⁰ However, no reports have yet determined the roles of tissue and circulating miRNA-21 and SNHG1 in ESCC patients. Particularly, the nature of miRNA-21 and SNHG1 expression levels and its correlation with survival rates, as well as the regulatory effect between miRNA-21 and SNHG1 still remains unexplored in ESCC. These facts thus prompted us to explore the role of miRNA-21 and SNHG1 in ESCC.

In the present study, we found that tissue miRNA-21 and SNHG1 are significantly upregulated and correlated with lymph node metastasis, TNM stage, and tumor size; and may serve as independent predictors for the survival of ESCC patients. Serum miRNA-21 and SNHG1 may serve as potential diagnostic indexes. Further, the study revealed the existence of a unidirectional regulatory interaction between miRNA-21 and SNHG1 in addition to the role of miRNA-21 overexpression in promoting ESCC cells proliferation through upregulation of SNHG1.

Materials and Methods

Tissues and Serum Specimens

60 serum ESCC samples, and 42 paired ESCC tissues and adjacent non-cancerous tissues were obtained from ESCC patients who underwent resection of the ESCC from February 2012 to December 2012 at the Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, Xinjiang, China). Tissue samples were collected from patients who provided serum samples. 6 healthy tissues (benign mass tissues with negative biopsy being from the esophageal region collected by gastroscopy) and 60 healthy serum samples came from volunteers. All tissue samples were from untreated by chemotherapy or radiotherapy patients undergoing surgery and were snap frozen in liquid nitrogen and stored at -80°C until the extraction of RNA. The diagnosis of all tissues specimens was verified by histopathological examination. For all the samples, data on patient gender, age, tumor size, location, smoking, differentiation, vessels invasion, nerve invasion, lymph node metastatic, and TNM stage were collected. Tumor stages were assessed according to the Seventh Edition of the Cancer Staging Manual of the American Joint Committee on Cancer. The use of these tissues specimens was conducted in accordance with protocols approved by the Review Board of the Affiliated Tumor

Hospital of Xinjiang Medical University. Every patient received written consent.

Cell Culture and Transfections

HET-1A, human immortalized normal esophageal epithelial cell line was obtained from American Type Culture Collection (ATCC). The ESCC cell lines TE-1, Eca-109, KYSE170, and KYSE150 were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in a humidified incubator containing 5% CO₂ at 37 °C. At the density of 50–70%. MISSION[®] microRNA Mimic hsa-miR-21 (miRNA-21 mimics) (5'-AACCAUCAGUCUGAUAGCUAUU-3'), anti-miRNA-21 (5'-UCAACAUCAGUCUGAUAAGCUA-3'), a siRNA targeting SNHG1 (Anti-SNHG1) (5'-CAGCAGTTGAGGGTTTGTGTGTAT-3') and scrambled negative control (NC) oligonucleotides (5'-CAGUACUUUUGUGUAGUACAA-3') were purchased from Sigma-Aldrich (St. Louis, MO). SNHG1-expressing vectors (SNHG1) and empty vectors were provided by GenePharma (Shanghai, China). Eca-109 and KYSE150 cells were grown to 60–70% confluence, and incubated with siRNAs at a final concentration of 0.1 μM by using Lipofectamine[™] 2000 (Invitrogen, Beijing, China) in a serum-free medium for 24 h. Cells treated with Lipofectamine 2000 reagent only were control cells. Cells transfected with empty vectors or negative control siRNA were negative control cells. When over-expression rates reached 200% and silencing rate reached 50%, the assays would be subjected.

Quantitative Real-Time PCR (qRT-PCR)

To detect the expression of miRNA-21, miRNAs were extracted using mirVana miRNA Isolation Kit (Thermo Fisher Scientific). Reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Total RNA was extracted using TRIzol reagent (Invitrogen) to detect the expression level of SNHG1. The RNA was then reverse transcribed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions to generate the first strand cDNA. Polymerase chain reaction (PCR) reaction systems were prepared using miScript SYBR Green PCR Kit (Qiagen). ABI 7500 System was used to carry out PCR reactions through the following conditions: 1 min at 95°C, and then 40 cycles of 15 s at 95°C and 35 s at 58°C. MiRNA-21 and SNHG1 expression were normalized to U6 and GAPDH endogenous control, respectively

Primers of U6, GAPDH, miRNA-21 and SNHG1 were bought from GenePharma (Shanghai, China). All normalizations were performed by using the $2^{-\Delta\Delta Ct}$ methods.

In vitro Cell Proliferation Assay

To determine cell viability, Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc (Beijing, China). All operations were performed in accordance with the manufacturer's instructions. Briefly, cells were harvested after transfection, and then were seeded at 1×10^3 cells/well in 96-well plates. Cell culture was performed in an incubator (37°C, 5% CO₂). At 24, 48, 72, and 96 hrs late, the culture medium was replaced with 100 μL of fresh medium containing 10 μL of CCK-8 solution. After that, cells were cultivated for an additional 4 h at 37°C. The optical density (OD) value of 450 nm was measured using a micro-plate reader to represent cell proliferation. Colony formation assay was conducted as followed. Five hundred cells per well were seeded in six-well plates. Two weeks later, colonies were fixed and stained by 0.05% crystal violet (Sigma, USA). Experiments were performed in quadruplicate.

Statistical Analysis

SPSS V.13.0 software was used for statistical analysis. Comparisons among the groups were subjected to Wilcoxon signed-rank test or Student's *t*-test. χ^2 or Fisher's test was used to analyze the relationship between miRNA-21 and SNHG1 expression and clinicopathological features. Survival curves were plotted by Kaplan-Meier method and compared with logarithmic rank test. The survival data were analyzed by univariate and multivariate Cox proportional risk regression analysis. The correlation analysis of Pearson was used to analyze the relationship between miRNA21 and SNHG1 between serum and tissue. The results were expressed as mean (+standard deviation). $P < 0.05$ was considered significant.

Results

miRNA-21 and SNHG1 are Up-Regulated in Tumor Tissues of ESCC Patients

To investigate the clinical significance of miRNA-21 and SNHG1 in ESCC, we measured miRNA-21 and SNHG1 expression using qRT-PCR assays in 42 paired ESCC tissues and adjacent non-cancerous tissues (ANCTs) prior to chemotherapy. The results indicated that miRNA-21 is significantly overexpressed in 30 ESCC tissues, with twice the expression in 24 ESCC tissues compared with ANCTs

($P<0.0001$) (Figure 1A). Analogously, SNHG1 is also significantly overexpressed in 29 ESCC tissues with two times greater expression in 20 ESCC tissues than in ANCTs ($P<0.0001$) (Figure 1C). In addition, compared with healthy controls and ANCTs group, the levels of miRNA-21 and SNHG1 are significantly upregulated in patients with ESCC, while there is also a slight increase in the distribution of miRNA-21 and SNHG1 in the ANCTs group compared with that in the healthy control group ($P<0.0001$) (Figure 1B and D).

miRNA-21 and SNHG1 Levels Correlate with Clinicopathologic Characteristics of ESCC Patients

Next, the correlations between expression levels of miRNA-21 and SNHG1 and clinicopathologic characteristics were

analyzed by χ^2 or Fisher test. In this study, miRNA-21 and SNHG1 expression levels of ESCC tissue over 2-fold compared with ANCTs were considered to indicate high expression levels. The results indicated that upregulated miRNA-21 expression significantly correlates with TNM stage ($P=0.010$) and lymph node metastasis ($P=0.008$); and upregulated SNHG1 expression correlates with lymph node metastasis ($P=0.016$) and tumor size ($P<0.0001$) with non-existent significant links to other clinicopathologic features such as age, gender, smoking, location, differentiation, nerve invasion, and invasion of vessels ($P>0.050$) (Table 1). Further analysis revealed that the expression level of miRNA-21 is markedly increased in the patients positive for lymph node metastasis compared with lymph node metastasis negative patients ($P<0.0001$) (Figure 2A), and also highly correlated with TNM staging (I vs II $P=0.008$; II vs III $P=0.026$; I vs III $P<0.0001$)

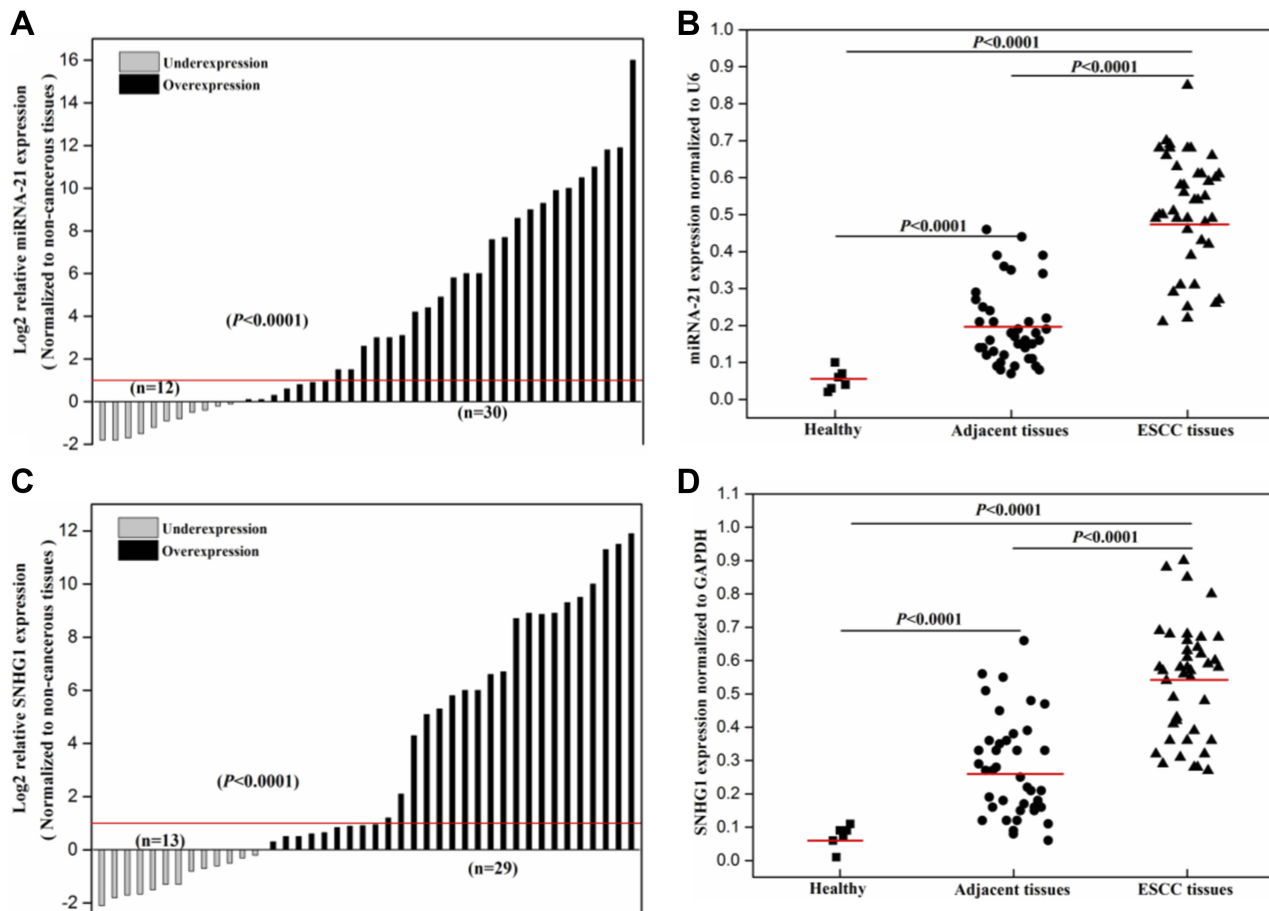


Figure 1 The expression characteristics of miRNA-21 and lncRNA SNHG1 in esophageal squamous cell carcinomas (ESCC). (A and C) miRNA-21 and lncRNA SNHG1 expression were measured by qRT-PCR in tissues and adjacent non-cancerous tissues (ANCTs) of 42 paired ESCC, respectively. The results are shown as Log2 fold change of the expression values in ESCC to that in ANCTs. Red line represent miRNA-21 or lncRNA SNHG1 expression of ESCC samples over 2-fold compared with ANCTs. $P<0.0001$ by Wilcoxon signed-rank test. (B and D) The distribution of miRNA-21 and lncRNA SNHG1 levels are evaluated by qRT-PCR in the groups of healthy ($n=6$), ESCC tissues ($n=42$), and corresponding ANCTs ($n=42$), respectively. miRNA-21 levels were normalized by U6, and lncRNA SNHG1 levels were normalized by GAPDH. $P<0.0001$ by Wilcoxon signed-rank test.

Table I The Correlation Between miRNA-21 and SNHG1 Expression and Clinicopathologic Characteristics

Characteristics	Total No.	miRNA-21 Expression		χ^2	P-Value	SNHG1 Expression		χ^2	P-Value
		Low	High			Low	High		
Age (years)				0.656	0.418			0.962	0.327
≤ 60	24	9	15			11	13		
> 60	18	9	9			11	7		
Gender				0.041	0.839			1.533	0.216
Male	31	13	18			18	13		
Female	11	5	6			4	7		
Tumor size (cm)				1.750	0.186			17.230	0.000
≤ 3	28	14	14			21	7		
> 3	14	4	10			1	13		
Smoking				3.204	0.073			0.513	0.474
Never or light	19	11	8			12	7		
Heavy	23	7	16			12	11		
Location				0.085	0.759			0.085	0.759
Upper	11	5	6			5	6		
Middle	20	8	12			8	12		
Lower	11	5	6			5	6		
Differentiation				0.125	0.527			4.192	0.123
Well	8	4	4			5	3		
Moderate	25	12	13			10	15		
Poor	9	2	7			7	2		
Nerve invasion				0.886	0.347			0.305	0.580
Negative	32	15	17			16	16		
Positive	10	3	7			4	6		
Vessels invasion				0.864	0.353			3.394	0.065
Negative	27	13	14			17	10		
Positive	15	5	10			5	10		
Lymph node metastatic				7.000	0.008			5.775	0.016
Negative	14	10	4			11	3		
Positive	28	8	20			11	17		
TNM stage				9.182	0.010			3.361	0.186
I	8	7	1			5	3		
II	11	5	6			7	4		
III	23	6	17			8	15		

(Figure 2B). Additionally, it was found that the level of SNHG1 expression is markedly increased in the patients with tumor size >3 cm compared with those with tumor sizes ≤3 ($P<0.0001$) (Figure 2C), and highly associated with lymph node metastasis ($P<0.0001$) (Figure 2D). These data suggest that miRNA-21 and SNHG1 are upregulated in ESCC tissues and correlate with lymph node metastasis, TNM stage, and tumor size.

Serum miRNA-21 and SNHG1 are Potential Diagnostic Biomarkers for ESCC Patients

To assess the diagnostic value of serum miRNA-21 and SNHG1, we detected the serum levels of miRNA-21 and SNHG1 in 60 patients with ESCC and 60 healthy controls by qRT-PCR assays. The result revealed that miRNA-21 and SNHG1 are significantly upregulated in patients with ESCC compared with healthy controls. ($P<0.0001$) (Figure 3A

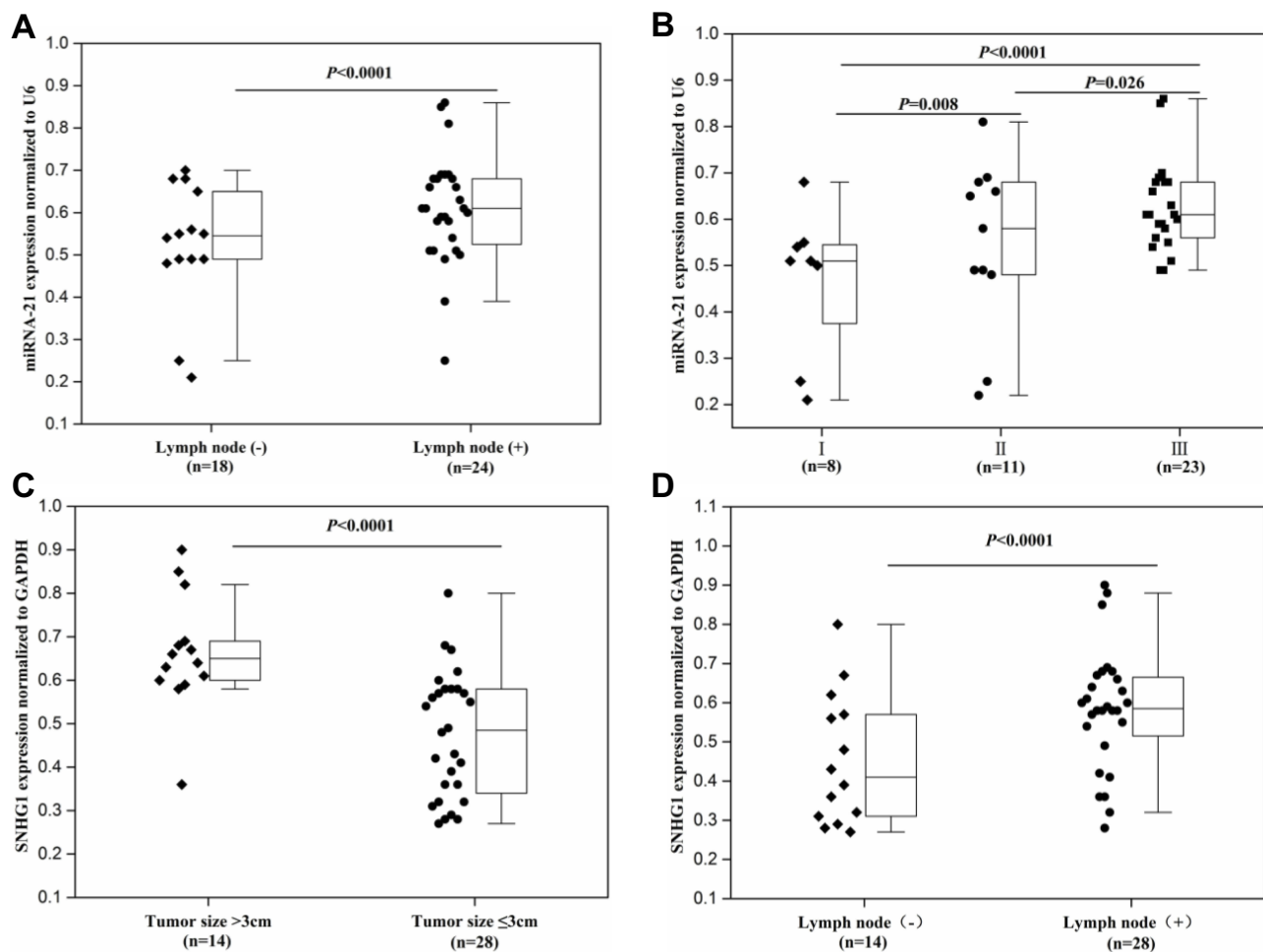


Figure 2 The correlation of miRNA-21 and SNHG1 levels with lymph node metastasis, TNM stage, and tumor size in ESCC patients. **(A)** The high expression of miRNA-21 was highly related to lymph node metastatic. $P<0.0001$ by Wilcoxon signed-rank test. **(B)** The high expression of miRNA-21 was highly related TNM stage. I vs II $P=0.008$; II vs III $P=0.026$; I vs III $P<0.0001$ by Wilcoxon signed-rank test. **(C)** The high expression of SNHG1 was highly related to tumor size. $P<0.0001$ by Wilcoxon signed-rank test. **(D)** The high expression of SNHG1 was highly related lymph node metastatic. $P<0.0001$ by Wilcoxon signed-rank test.

and D). Subsequently, we also investigated the relationship between the serum levels of miRNA-21 and SNHG1 and corresponding frozen tissues in 42 paired ESCC patients using Pearson correlation coefficient analysis. The result revealed that the relationship between serum and frozen tissue expression levels for miRNA-21 and SNHG1 is noticeably linear, with the R^2 values being 0.7290 and 0.6009 respectively ($P<0.001$) (Figure 3B and E). Additionally, the receiver operating characteristic (ROC) curve showed that area under the ROC curve (AUC) of serum miRNA-21 is 0.928, and the sensitivity and specificity of serum miRNA-21 are 88.3% and 97.3%, respectively (Figure 3C). The AUC of serum SNHG1 is 0.850, and the sensitivity and specificity of serum SNHG1 are 77.4% and 92.5%, respectively (Figure 3F). These data suggest that serum levels of miRNA-21 and SNHG1 may serve as promising diagnostic biomarkers for ESCC patients.

High Expression Levels of Tissue miRNA-21 and SNHG1 Correlate with Poor Prognosis of ESCC Patients

The prognostic data of 42 patients were used for Kaplan-Meier survival analysis and Cox regression analysis. Cox regression analyses were conducted to identify prognostic factors for overall survival (OS) and progression free survival (PFS) in ESCC patients. Univariate survival analysis indicated that OS and DFS are negatively correlated with lymph node metastasis (OS: $P<0.001$, DFS: $P<0.001$), TNM stage (I vs II, OS: $P=0.004$, DFS: $P=0.009$; I vs III, OS: $P<0.001$, DFS: $P<0.001$), miRNA-21 expression (OS: $P<0.001$, DFS: $P<0.001$), and SNHG1 expression (OS: $P<0.001$, DFS: $P<0.001$) (Table 2). Multivariate analysis confirmed that miRNA-21 and SNHG1 expression can potentially serve as independent prognostic factors for OS

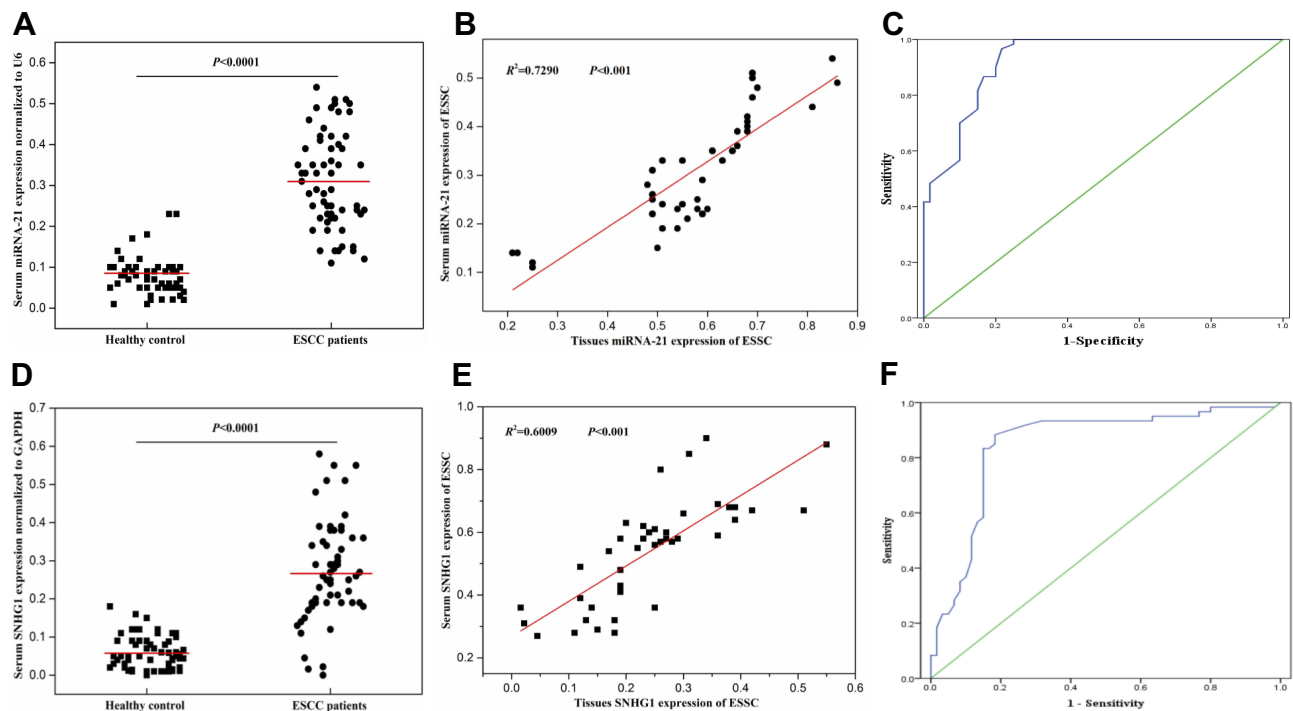


Figure 3 Serum miRNA-21 and SNHG1 are potential diagnostic biomarkers for ESCC patients. **(A and D)** Serum miRNA-21 and SNHG1 level of ESCC patients and healthy controls were detected by qRT-PCR. $P<0.0001$ by Wilcoxon signed-rank test. **(B and E)** Pearson correlation coefficient analysis was used to determine the association of miRNA-21 and SNHG1 level of 42 frozen tissues with paired serum expression level. R^2 value were 0.7290, 0.6009, respectively ($P<0.001$). **(C and F)** Receiver Operating Characteristic (ROC) analysis for the diagnostic value of serum miRNA-21 and SNHG1.

and DFS (miRNA-21: $P=0.002$ and $P=0.008$; SNHG1: $P=0.006$ and $P=0.0099$, respectively). Lymph node metastasis and TNM stage were also identified as independent predictive factors for OS and DFS in patients with ESCC ($P<0.001$, for both OS and PFS) (Table 3). Additionally, Kaplan-Meier survival analysis and log rank test revealed that the correlation between overexpression of miRNA-21 and SNHG1 is significant with shorter OS and DFS compared with downregulated levels of miRNA-21 and SNHG1 (miRNA-21 overexpression, OS: $P=0.0123$, DFS: $P=0.0146$; SNHG1 overexpression, OS: $P=0.0386$, DFS: $P=0.0378$;) (Figure 4A–D). Taken together, these data suggest the existence of potential links between miRNA-21 and SNHG1 overexpression and ESCC progression, and the expression of tissue miRNA-21 and SNHG1 as potential prognosis indices for ESCC patients.

Between miRNA-21 and SNHG1 Is a Significantly Positive Correlation of ESCC Patients

In order to further study the characteristics of miRNA-21 and SNHG1 expression in ESCC patients, we further analyzed the correlation between miRNA-21 and SNHG1 in 42 ESCC

patients from this study and 161 ESCC samples from TCGA database (<https://portal.gdc.cancer.gov/>), respectively. The results showed that the correlation between miRNA-21 and SNHG1 is significantly positive both the sample from this study and TCGA database, with the R^2 values being 0.6958 and 0.5386, respectively ($P<0.001$) (Figure 5A and B). These data suggest that miRNA-21 and SNHG1 are positively highly expressed in ESCC patients.

miRNA-21 Is a Unidirectional Upstream Regulator of SNHG1

In order to clarify the relationship of miRNA-21 and SNHG1 in ESCC cells, we analyzed the expression levels of miRNA-21 and SNHG1 and how they affect each other. According to miRNA-21 and SNHG1 expression levels in different esophageal cell lines, qRT-PCR showed that miRNA-21 and SNHG1 are highly expressed in ESCC cell lines TE-1, Eca-109, KYSE-170, and KYSE-150 compared with HET-1; with Eca-109 and KYSE-150 exhibiting respectively the highest and lowest expression of miRNA-21 and SNHG1 among these 4 ESCC cell lines (Figure 6A). To further investigate the interactions between miRNA-21 and SNHG1 in ESCC, we overexpressed miRNA-21 and SNHG1 in both Eca-109 and KYSE150 cells by transfecting miRNA-21 mimics and

Table 2 Univariate Cox Analysis of Overall and Disease-Free Survival in ESCC Patients

Variables	Total No.	OS		DFS	
		HR (95% CI)	P-Value	HR (95% CI)	P-Value
Age (years)					
≤ 60	24	1		1	
> 60	18	1.295 (0.689–2.394)	0.581	1.357 (0.714–2.501)	0.418
Gender				0.041	
Male	31	1		1	
Female	11	0.841 (0.421–1.380)	0.891	0.801 (0.389–1.199)	0.785
Tumor size (cm)					
≤ 3	28	1		1	
>3	14	1.534 (0.963–2.394)	0.088	1.460 (0.901–2.300)	0.095
Smoking					
Never or light	19	1		1	
Heavy	23	1.588 (0.899–3.011)	0.076	1.411 (0.845–2.865)	0.092
Location					
Upper	11	1		1	
Middle	20	1.894 (1.258–3.481)	0.056	1.566 (1.102–3.085)	0.089
Lower	11	0.852 (0.568–1.269)	0.851	0.824 (0.469–1.208)	0.810
Differentiation					
Well	8	1		1	
Moderate	25	1.856 (0.936–3.612)	0.086	1.628 (0.891–2.921)	0.098
Poor	9	2.091 (1.028–3.651)	0.078	1.952 (1.012–3.523)	0.082
Nerve invasion					
Negative	32	1		1	
Positive	10	2.112 (1.361–3.581)	0.066	1.953 (1.024–3.251)	0.079
Vessels invasion					
Negative	27	1		1	
Positive	15	2.281 (1.562–3.628)	0.059	2.051 (1.349–3.214)	0.062
Lymph node metastatic					
Negative	14	1		1	
Positive	28	14.891 (6.548–29.481)	<0.001	13.149 (7.186–28.191)	<0.001
TNM stage					
I	8	1		1	
II	11	5.218 (2.281–9.291)	0.004	4.813 (2.189–8.181)	0.009
III	23	23.198 (14.481–39.188)	<0.001	20.189 (12.149–35.181)	<0.001
miRNA-21 expression					
Low	18	1		1	
High	24	8.148 (5.219–16.189)	<0.001	7.654 (4.368–15.816)	<0.001
SNHG1 expression					
Low	22	1		1	
High	20	6.851 (4.356–11.867)	<0.001	6.054(3.284–9.852)	<0.001

Abbreviations: OS, overall survival; DFS, disease-free survival; CI, confidence interval.

SNHG1 expressing vectors; and downregulated the expression cell of miRNA-21 and SNHG1 by transfecting respective siRNAs. (Figure 6B and C). Compared with control (C) and

negative control (NC) groups, miRNA-21 mimics significantly elevated, while anti-miRNA-21 significantly reduced the expression level of SNHG1 in both Eca-109 and

Table 3 Multivariate Cox Regression Analysis of Overall and Disease-Free Survival in ESCC Patients

Variables	Total No.	OS		DFS	
		HR (95% CI)	P-Value	HR (95% CI)	P-Value
Lymph node metastatic					
Negative	14		<0.001		<0.001
Positive	28	8.219 (5.291–16.487)	<0.001	7.659 (5.129–15.358)	0.001
TNM stage					
I	8		<0.001		<0.001
II	11	6.818 (4.291–10.284)	0.005	6.294 (4.628–10.368)	0.015
III	23	15.659 (9.128–23.483)	<0.001	13.598 (8.354–22.357)	<0.001
miRNA-21 expression					
Low	18		<0.001		<0.001
High	24	3.813 (2.028–5.217)	0.002	3.218 (1.891–4.598)	0.008
SNHG1 expression					
Low	22		<0.001		<0.001
High	20	3.432 (1.951–5.064)	0.006	3.016 (1.294–4.645)	0.009

Abbreviations: OS, overall survival; DFS, disease-free survival; CI, confidence interval.

KYSE150 cell lines ($P<0.05$ or $P<0.01$) (Figure 6D and E). However, no significant changes in expression levels of miRNA-21 were found in these cells after either overexpressing or downregulating SNHG1 (Figure 6F and G). Thus, our data clearly suggests that miRNA-21 may be most likely an upstream unidirectional positive regulator of SNHG1 in ESCC cells.

miRNA-21 Promotes ESCC Cell Proliferation Through SNHG1

Correlation of SNHG1 expression with tumor size, as already shown, indicates the involvement of SNHG1 in tumor growth. In order to validate this hypothesis, proliferation abilities of ESCC cells of each group were assessed by the CCK-8 assay. It was found that compared with C and NC groups, miRNA-21 and SNHG1 overexpression promoted the proliferation of both Eca-109 and KYSE150 ESCC cells ($P<0.01$). On the other hand, SNHG1 siRNA silencing played an opposite role in ESCC cell proliferation ($P<0.01$). In addition, miRNA-21 overexpression partially reversed the inhibitory effects of SNHG1 downregulation on cancer cell proliferation ($P<0.05$) (Figure 7A and B). Interestingly, the clone formation assay also further confirmed the effect of miRNA-21 and SNHG1 on the proliferation of Eca-109 and KYSE150 cells (Figure 7C). Therefore, it can be inferred that miRNA-21 plays an important role in promoting ESCC cell proliferation possibly through SNHG1.

Discussion

Many miRNAs and lncRNAs are expressed in a highly tissue-specific manner and contribute to the establishment and maintenance of the characteristic tissue-related gene expression. It is well-known that the cross-talk between miRNAs and lncRNAs participates in many cellular biological processes. miRNA-21 is one of the prominent miRNAs implicated in the oncogenesis and progression of various human cancers. Not only has it been implicated in the promotion of tumor growth, anti-apoptosis, and proliferation, but it has also been shown to be associated with resistance toward radiosensitivity or/and chemotherapy.²⁵ SNHG1 is one of the most significant regulatory lncRNAs in human cancers, and it has been highlighted as a potential candidate for diagnostic, prognostic, and therapeutic purposes in malignancies.²⁶ Previously, several reports have demonstrated that miRNA-21 and SNHG1 expression plays a crucial role in the proliferation of ESCC cells by serving as accelerators of malignancy, and downregulation of miRNA-21 or SNHG1 inhibits the proliferation and invasion of ESCC cells.^{27,28}

Various studies have shown that miRNA-21 and SNHG1 are generally overexpressed in cancer tissues versus compared with ANCTs, regardless of their tumor type or origin.^{26–28} In the present study, we found that the level of miRNA-21 and SNHG1 in ESCC tissues is significantly higher than those in corresponding ANCTs. The high expression of miRNA-21 is positively correlated with advanced TNM stage and positive lymph node metastasis.

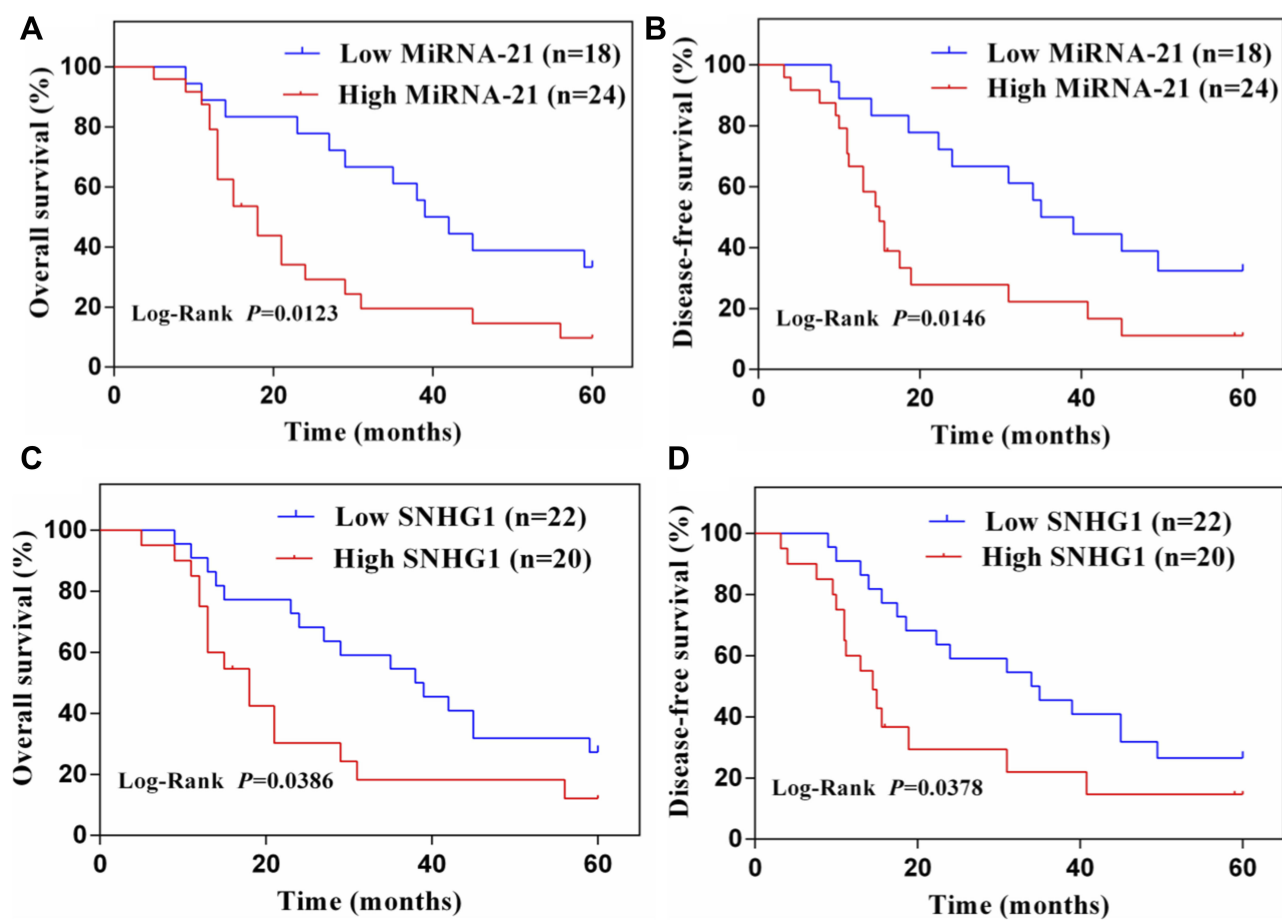


Figure 4 Survival analysis of ESCC patients with different miRNA-21 and SNHG1 expression levels. (A and B) Kaplan-Meier curves compare overall survival (OS) and disease-free survival (DFS) between ESCC patients with high and low expression of tissue miRNA-21. (OS: $P=0.0123$, DFS: $P=0.0146$, log rank test). (C and D) Kaplan-Meier curves compare OS and DFS between ESCC patients with high and low expression of tissue SNHG1 (OS: $P=0.0386$, DFS: $P=0.0378$, log rank test).

Thus, our findings indicate that miRNA-21 can be a potential biomarker for ESCC. Further, the level of SNHG1 expression is found to be markedly related to lymph node metastasis and tumor size; which is consistent with the results of Zhang et al²⁸ Overall, these data suggest that miRNA-21 and SNHG1 are upregulated in ESCC tissues and correlate with lymph node metastasis, TNM stage, and tumor size.

Traditional biomarkers used in the diagnosis of ESCC mainly include CEA, CA19-9, and SCCA. However, due to lack of high diagnostic sensitivity or specificity, the early diagnosis of ESCC still remains poor leading to early metastasis before diagnosis. Therefore, developing new diagnostic methods or new biomarkers for early diagnosis for ESCC is very essential. Nevertheless, the complex mechanisms of miRNA-21 and SNHG1 involved in cancer development are mostly tested in tissues. Thus, in order to apply this knowledge in clinical use; the critical influence of miRNA-21 and SNHG1 should be explored in

most available diagnostic samples like blood and other body fluids. We have found that serum miRNA-21 and SNHG1 expression levels are higher in patients with ESCC than in healthy controls with an AUC value of ROC curves being determined to be 0.928 and 0.850, respectively. Consequently, the Pearson correlation coefficient indicated that the expression levels of miRNA-21 and SNHG1 in ESCC tissues are significantly associated with their respective serum expression levels; suggesting that circulating levels of miRNA-21 or SNHG1 may serve as suitable biomarkers for ESCC patients for initial clinical cancer diagnosis. Nevertheless, it has been reported that miRNA-21 of serum is upregulated in several types of cancers.¹⁰⁻¹³ These findings suggest that the upregulation of serum miRNA-21 is not specific to ESCC patients. Hence, the serum level of miRNA-21 alone cannot be a marker for early detection of ESCC, and multiple biomarkers are needed to achieve high specificity. Additionally, SNHG1 is a novel oncogenic lncRNA

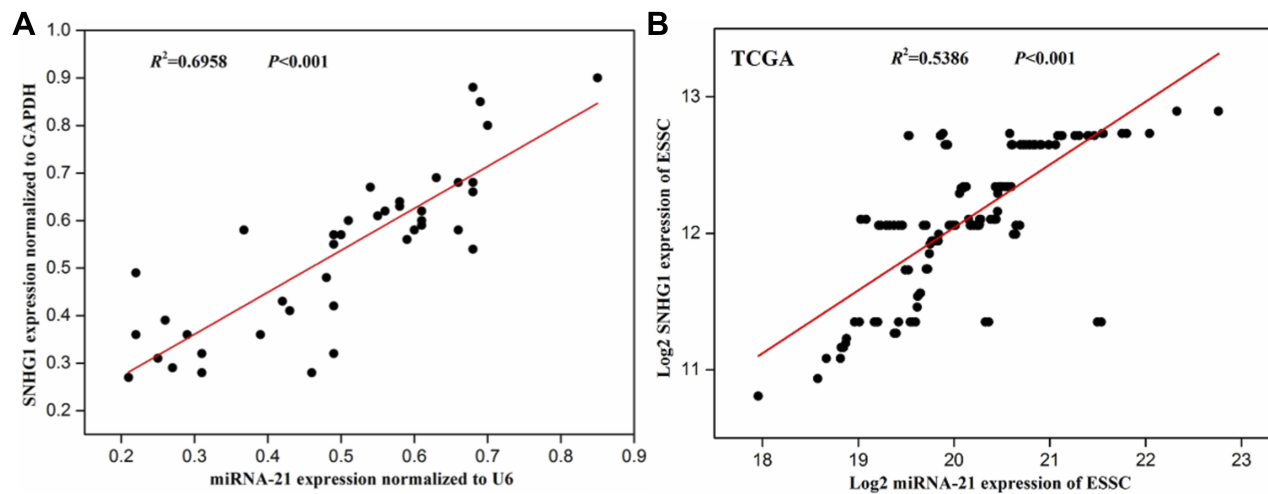


Figure 5 Between miRNA-21 and SNHG1 is a significantly positive correlation of ESCC patients. **(A)** Pearson correlation coefficient analysis was used to determine the association of miRNA-21 and SNHG1 level of 42 ESCC frozen tissues from this study, with the R^2 values being 0.6958 ($P<0.001$). **(B)** The correlation was analysed in 161 ESCC patients from TCGA database (<https://portal.gdc.cancer.gov/>), with the R^2 values being 0.5386 ($P<0.001$).

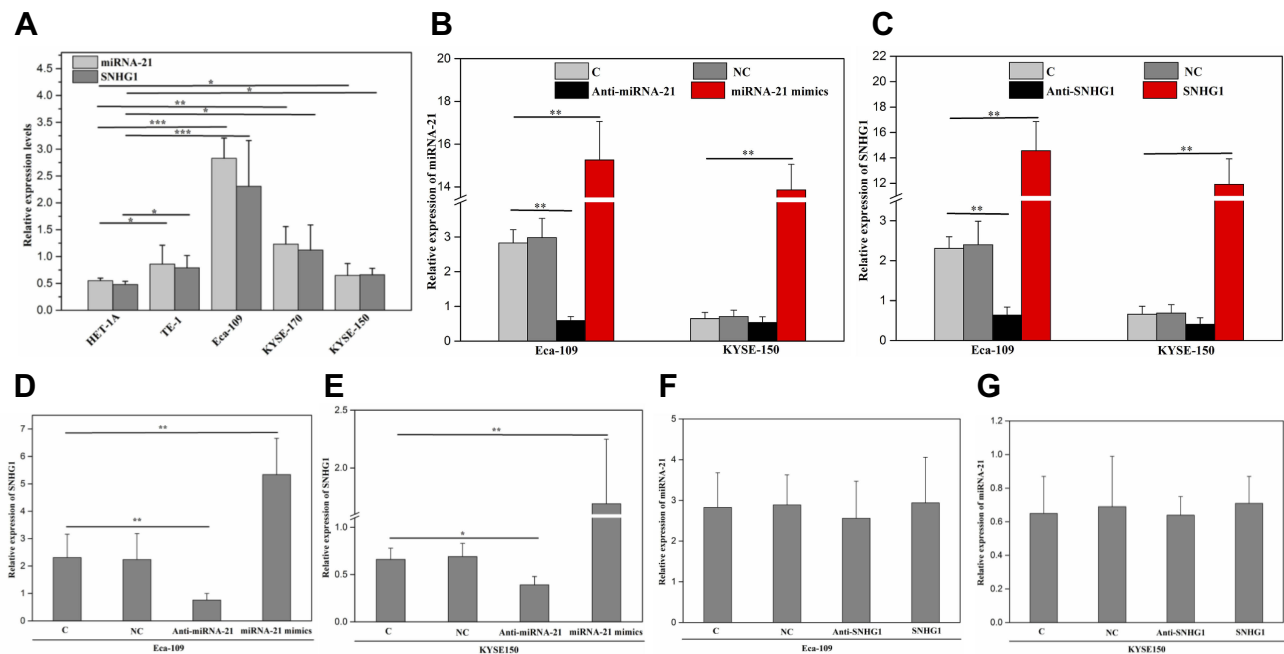


Figure 6 Overexpression of miRNA-21 induces SNHG1 expression in ESCC cells. **(A)** The expression of miRNA-21 and SNHG1 were measured by RT-qPCR in normal esophageal epithelial cell line HET-1A and ESCC cell lines TE-1, Eca-109, KYSE170, and KYSE150. **(B)** Transfection of miRNA-21 siRNA (Anti-miRNA-21), miRNA-21 mimics, and negative control oligonucleotides (scrambled sequence) into Eca-109 and KYSE150 cells. **(C)** Transfection of SNHG1 siRNA (Anti-SNHG1), SNHG1 vectors expressing (SNHG1), and negative control oligonucleotides (scrambled sequence) into Eca-109 and KYSE150 cells. **(D and E)** Overexpression of miRNA-21 lead to increase SNHG1 expression, in contrast, downregulation of miRNA-21 reduce SNHG1 expression in Eca-109 and KYSE150 cells. **(F and G)** SNHG1 overexpression or downregulation fail to significantly affect SNHG1 expression. The results are shown as mean \pm S.D. ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by Student's t-test. C, control; NC, negative control.

aberrantly expressed in different diseases including colorectal, liver, lung, prostate, gastric and ECs.²⁶ However, the sensitivity and specificity of SNHG1 still need to be further investigated. In conclusion, although the current studies indicate that high potential of miRNA-21 or SNHG1 to act as tumor biomarkers for ESCC diagnosis,

more studies are necessary to identify novel biomarkers specific to ESCC for early detection.

Next, the prognostic value of miRNA-21 and SNHG1 expression in ESCC patients is explored in this study. The upregulation of miRNA-21 and SNHG1 is found to be related to the reduction in OS and DFS, as shown by the

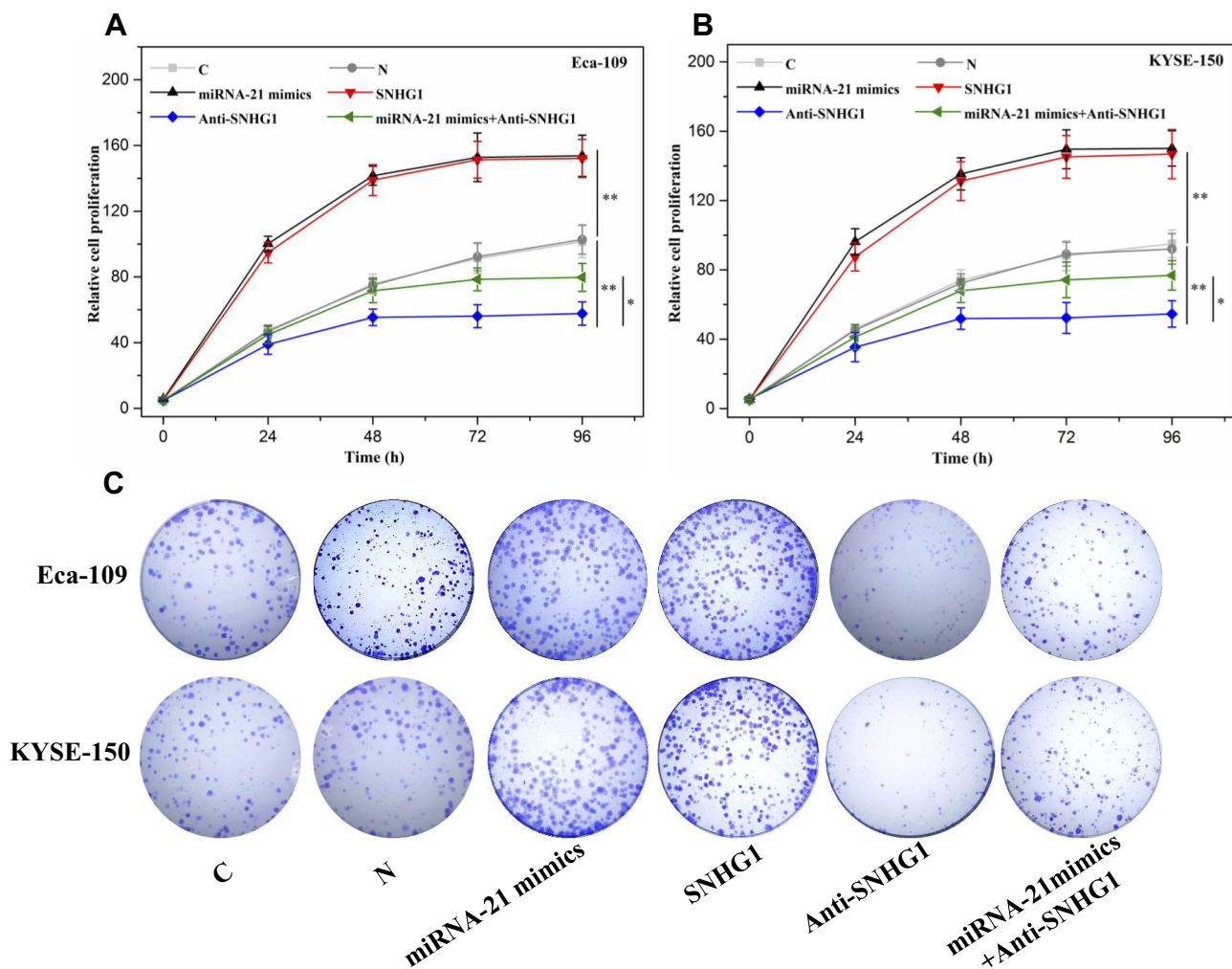


Figure 7 miRNA-21 promotes ESCC cell proliferation through SNHG1. The overexpression of miRNA-21 and SNHG1 promoted the proliferation of ESCC cells, Eca-109 and KYSE150, while SNHG1 downregulation inhibited ESCC cell proliferation. In addition, miRNA-21 overexpression partly reversed the inhibitory effects of SNHG1 downregulation on cancer cell proliferation. **(A)** In Eca-109 cell; **(B)** In KYSE150 cell. The results are shown as mean±S.D. (n=3). * $P<0.05$, ** $P<0.01$ by Student's *t*-test. **(C)** Clone formation assay about the effect of miRNA-21 and SNHG1 on Eca-109 and KYSE150 cell proliferation. C, control; NC, negative control.

Kaplan-Meier curves. Further, Cox univariate and multivariate analyses indicated that miRNA-21 and SNHG1 are independent prognostic factors for OS and PFS. Likewise, lymph node metastasis and TNM stage are relevant to the prognosis of ESCC. Most studies have shown that lncRNAs can serve as potential prognostic indicators, such as high lncRNA TUG1 expression level is associated with chemotherapy resistance and poor prognosis,²⁹ and high lncRNA SPRY4-IT1 is associated with poor prognosis in ESCC.³⁰ Although further validation is necessary, these data suggest that high expression of miRNA-21 and SNHG1 in the tumor, together with traditional risk factors such as lymph node metastasis and TNM stage, may serve as biomarkers for metastatic phenotype and survival in ESCC cases.

Interactions between lncRNAs and miRNAs have been frequently observed during the development of human cancers, such as SNHG1 exacerbated HCC cell proliferation, migration, and invasion via the inhibition of miRNA-195 in vitro³¹ In the present study, we further explored the interaction between miRNA-21 and SNHG1. Intriguingly, our in vitro data have revealed a novel regulatory pathway, in which miRNA-21 is likely an upstream positive regulator of SNHG1 in ESCC cells in a unidirectional manner. In addition, the interactions between miRNA-21 and SNHG1 aided the proliferation of ESCC cells. Therefore, our results indicate that SNHG1 may be a novel downstream target of miRNA-21 in ESCC cells and an important contributor towards proliferation abilities of ESCC cells. Yan et al³² have shown that SNHG1 acts as a sponge for miR-338-3p in ESCC progression and the

ceRNA regulatory SNHG1/miR-338-3p/CST3 axis decreases ESCC cell growth and induces apoptosis. However, the mechanism of this positive regulation between miRNA-21 and SNHG1 is yet unknown. However, our data suggest that SNHG1 may serve as a potential therapeutic target for ESCC by downregulating miRNA-21.

Taken together, the present study has identified that miRNA-21 and SNHG1 are upregulated in ESCC tissues and serum. They are associated with lymph node metastasis, TNM stage, tumor size, and poor prognosis, and promote the proliferation of ESCC cells in vitro. SNHG1 may be a novel downstream target of miRNA-21 in ESCC cells in a unidirectional manner and critically contributes toward the proliferation potential of ESCC cells. Thus, miRNA-21 and SNHG1 may serve as potential diagnostic and prognostic biomarkers and therapeutic targets for ESCC.

Ethics Approval and Informed Consent

Our study was granted ethical approval by Ethical Committee of Affiliated Tumor Hospital of Xinjiang Medical University, and all the patients and healthy volunteers provided written informed consent, which was conducted in accordance with the Declaration of Helsinki.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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