

Retrospective Study

Development of a prognostic prediction model based on microRNA-1269a in esophageal cancer

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Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract**BACKGROUND**

Esophageal cancer (ESCA) is a heterogeneous cancer with variable outcomes that are challenging to predict. MicroRNA (miR)-1269a is a newly discovered non-coding RNA that shows promising prognostic prediction in other cancers, but its clinical value in ESCA remains unclear.

AIM

To explore the relationship between miR-1269a and its clinical value and to develop a nomogram to succinctly display this relationship.

METHODS

We analyzed the expression of miR-1269a in 125 ESCA tissue samples with complete clinical data and 52 normal tissue samples. We determined the prognostic value of miR-1269a for overall survival (OS) and cancer-specific survival (CSS) and evaluated the association between miR-1269a and clinical variables including tumor location, histologic grade, metastatic stage, and American Joint Committee on Cancer (AJCC) stage using multivariate Cox analysis. Additionally, we developed a nomogram for OS and CSS based on miR-1269a expression using age and AJCC stage and assessed its prognostic performance. Using Gene Ontology and Kyoto Encyclopedia of Gene and Genomes analyses, we predicted the target genes of miR-1269a and analyzed their potential function in cancer development.

RESULTS

The expression of miR-1269a was significantly higher in ESCA patients than healthy controls. Patients with high expression of miR-1269a showed poor prognosis in OS and CSS, suffered increased rates of low differentiation and meta-

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stasis, and exhibited tumor stage T3 + T4, positive lymph stage, and AJCC stage III + IV. The area under the receiver operating characteristic curve of miR-1269a was 0.716 for OS and 0.764 for CSS. Multivariate Cox analysis revealed that AJCC stage and miR-1269a were independent factors for OS and CSS. Combining with age, we constructed a nomogram for prognostic prediction. Additionally, our nomogram showed excellent predictive performance for OS and CSS after 3 years and 5 years and was easy to use. Ultimately, the functional analysis suggested that miR-1269a was mostly involved in the PI3K-AKT signaling pathway.

CONCLUSION

miR-1269a can be used as a potential indicator for the prognosis of ESCA patients. We developed an easy-to-use nomogram with excellent ESCA prognostic prediction for clinical use.

Key Words: MicroRNA-1269a; Esophageal cancer; Prognosis; Overall survival; Cancer-specific survival

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Core Tip: MicroRNA-1269a expression levels, along with cancer stage and age, were shown to have significant predictive capacity for overall survival and cancer-specific survival in esophageal cancer. Using these results, we developed an easy-to-use nomogram for clinical use.

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INTRODUCTION

Esophageal cancer (ESCA) is one of the common gastrointestinal cancers in China, ranking eighth in terms of morbidity and sixth with regard to mortality among all cancers[1]. According to cancer statistics, China has 477900 new cases of ESCA and 375000 deaths each year[2]. The high mortality rate of ESCA brings a heavy burden on patients and health care system. Esophagogastroduodenoscopy and endoscopic ultrasound as the main assessment methods of esophageal lesions have made remarkable progress[3-7]. However, most patients are still diagnosed in advanced stages because of misleading and unreliable symptoms in early stages, which reduces the overall survival (OS) in ESCA[8]. Despite advances in surgery, chemotherapy, and radiotherapy that have improved OS, the outcomes remain unsatisfactory with a 5-year OS rate of barely 20%[9]. Therefore, it is critical to discover early-stage prognostic markers for ESCA, providing a therapeutic guide for ESCA treatment.

Non-coding RNAs, which do not encode proteins, have attracted interest in cancer. Long non-coding RNA and microRNAs (miR) have aroused much attention because they regulate protein expression at the post transcriptional level[10-12]. MicroRNAs are short-chain non-coding, highly conserved RNAs approximately 21-25 nt in length. They play a powerful role in regulating various cellular activities, including cell growth, development, proliferation and apoptosis[13,14]. Accumulating evidence suggests that microRNAs not only act as regulators in cancer progression but also have great potential as biomarkers because they are stable, easily detected and highly tissue-specific[15,16]. Among them, miR-1269a has been found to be involved in multiple types of cancer.

Studies have revealed that late-stage colorectal cancers (CRCs) have higher miR-1269a expression levels than early-stage CRCs, and high miR-1269a expression is associated with relapse and metastasis in stage II CRC patients[17]. The high miR-1269a levels of low-grade glioma are significantly correlated with poorer OS[18]. Moreover, miR-1269a from serum exosomes can be detected as a diagnostic biomarker to differ patients with lung cancer from healthy subjects, with a diagnostic efficacy of

0.878[19]. Using The Cancer Genome Atlas databases, Jang *et al*[20] established a risk score model for assessing the OS and recurrence of ESCA with three miRNAs (miR-223, miR-1269a and miR-886) whose expression is significantly associated with OS and recurrence-free survival. Furthermore, this model is an independent risk prediction for OS and cancer recurrence in multivariable analysis[20]. However, the role of miR-1269a in ESCA has never been investigated. Therefore, there is a need to clarify the correlation between miR-1269a expression and prognosis in ESCA.

Against this background, we determined miR-1269a expression in ESCA, evaluated its prognostic value in OS and cancer-specific survival (CSS), and assessed the association between miR-1269a levels and clinical variables. Univariate and multivariate Cox analyses were used to detect the risk performance of miR-1269a in OS and CSS. Additionally, we developed a nomogram model for OS and CSS, based on miR-1269a expression with age and American Joint Committee on Cancer (AJCC) stage, and assessed the prognostic performance of our nomogram. Finally, the downstream genes of miR-1269a were predicted. Functional analysis was performed to discover the potential cancer processes and pathways related to miR-1269a.

Generally, we determined the expression value and prognostic roles of miR-1269a and developed an easy-to-use nomogram for prognostic prediction, which can be used as promising prognostic markers for ESCA treatment.

MATERIALS AND METHODS

Patient eligibility

We recruited 125 patients diagnosed with ESCA and treated in the Shengjing Hospital of China Medical University between January 2012 and January 2015. They were enrolled as the patient group. Additionally, a total of 52 healthy subjects were recruited as the normal group at the same time. The inclusion criteria were as follows: (1) Patients diagnosed by pathology or endoscopy as ESCA patients; (2) Patients who underwent complete esophagectomy without preoperative cancer therapy; and (3) Patients whose complete clinicopathological data were available and agreed to be followed-up. The exclusion criteria were as follows: (1) Patients showing comorbidity with other tumors; (2) Patients who survived less than 1 mo; and (3) Patients unwilling to cooperate with follow-up. All patients met the 8th edition of ESCA tumor node metastasis stage standard released by the AJCC[21]. All specimens were collected after surgical resection and immediately frozen at -80 °C until use. Patients were not required to give informed consent because our clinical data from ESCA patients were obtained anonymously with written consent. And our project was reviewed and approved by the ethics committee of the Shengjing Hospital of China Medical University.

miRNA isolation and quantitative real-time polymerase chain reaction

Total RNA from frozen tissue was extracted using RNAiso Plus reagent (TaKaRa, Japan) and was soluble in 10 µL RNase-free water. We determined the concentration and quality of total RNA in each samples using NanoPhotometer 50 (Thermo Fisher Scientific, USA). For reverse transcription, we synthesize cDNA with 1.0 µg total RNA according to the manufacturer's instructions of miRNA-specific stem-loop RT primer and the PrimeScript RT Reagent Kit (TaKaRa). Briefly, the condition of 20 µL reactions were shown in the following order: 15 min at 42 °C, 5 s at 85 °C, and cooled to 4 °C. The resulting cDNA were stored at -20 °C for next assays. Then, we amplified miR-1269a expression through quantitative real-time polymerase chain reaction (qRT-PCR). The amplification system included 2 µL cDNA, 1 µL each of forward and reverse primers, 10 µL SYBR® Premix Ex TaqII (2×), and 0.4 µL ROX Reference DyeII (50×); ddH₂O was added to make the volume 20 µL in total (TaKaRa). The ABI 7500 fast RT-PCR system (Thermo Fisher Scientific, USA) was applied to run the reactions with the following amplification conditions. In briefly, the initial denaturation process started at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melt curve was automatically drawn to determine the specificity of our primers. U6 was used as an endogenous reference, and the expression levels were quantified through the 2^{-ΔΔCt} method. All samples were tested in triplicate and repeated three times. The primers sequences were synthesized as follows (Sangon Biotech): miR-1269a: 5'-GACTGAGC-CGTGCTACTGG-3' (Forward), 5'-TGT CGT GGA GTC GGC AAT TG-3' (Reverse); U6: 5'-CGC AAG GAT GAC ACG CAA AT-3' (Forward), 5'-CGG CAA TTG CAC TGG ATA CG -3' (Reverse).

Clinicopathological data collection

Complete clinicopathological data were extracted for each patient, including age at diagnosis, gender, smoking history, alcohol consumption, tumor location, histologic grade, tumor stage, lymph stage, metastatic stage, and AJCC stage. The primary endpoints of our research are OS and CSS. OS refers to the period from the date of diagnosis until the date of death. CSS is defined as death from the diagnosis until the date of death for ESCA and has a much greater relevance to cancer biology and therapeutic impact. All patients were followed-up mainly by outpatient visits or telephone as well as re-examination at 3, 6, 9, 12 mo in the 1st year, every 4 mo from the 2nd to 3rd years, every 6 mo from the 4th to the last day of follow-up.

Prognostic analysis and nomogram construction

To detect the role of miR-1269a in ESCA, we assessed its expression and clinical correlation statistically and evaluated its prognostic value through Kaplan-Meier analysis for OS and CSS. We constructed a receiver operating characteristic (ROC) curves to evaluate the diagnostic value of miR-1269a in ESCA. Area under curve (AUC) was applied to determine its diagnostic performance. The closer the AUC was to 1.0, the better was the diagnostic performance of miR-1269a. Additionally, we estimated independent risk factors for OS and CSS using univariate and multivariate Cox analysis, respectively and compared the diagnostic performance of miR-1269a with clinical variables including age, histologic grade, and AJCC stage both in OS and CSS. Furthermore, a nomogram was established to succinctly display the predictive relationships among age, AJCC stage, and miR-1269a expression both in OS and CSS. Kaplan-Meier analysis was applied to assess the prognostic ability of our nomogram for both OS and CSS. ROC analysis was performed to evaluate the 3- and 5-year diagnostic ability of our nomogram for both OS and CSS.

Function enrichment analysis

Target genes of miR-1269a were predicted using three databases including miRTarBase, Tarbase v8, and TargetScan[22-24]. Next, a Venn diagram was drawn to obtain the common genes from the three databases for functional analysis. Gene Ontology (GO) term analysis and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis were applied to detect the potential functions of those common genes through the DAVID database and the Kobasdatabase[25,26]. Moreover, the top 10 most enriched GO terms and KEGG pathways were displayed with the threshold of *P* value < 0.05.

Statistical analysis

In this study, GraphPad 7 software and R software (version 3.6.1) were applied to analyze and draw figures. Briefly, we analyzed the expression level of miR-1269a through a two-tailed Student *t*-test, and expressed the results by mean ± SD. The χ^2 test was performed to detect the connection between clinicopathological features and miR-1269a expression levels. We calculated the statistic difference between groups through the Student *t*-test and compared the statistic difference among multiple using one-way ANOVA. A Kaplan-Meier test and a log-rank *T*-test were applied to calculate the statistical significance of OS and CSS. We used univariate and multivariate Cox analyses to test the independent risk factors of prognosis through R software with "Survival" packages[27]. We constructed a nomogram using R software with "Survival" and "rms" packages[28]. We carried out a time-dependent ROC analysis to evaluate the diagnostic performance of our nomogram using R software with the "survival ROC" package[29]. We repeated all experiments at least three times. A *P* value less than 0.05 was considered statistically significant.

RESULTS**MiR-1269a roles in expression and prognosis**

A total of 125 ESCA patients and 52 healthy subjects were enrolled in our research. There is no statistical significance between two groups including age, gender, smoking history, and alcohol consumption (Table 1). As shown in Figure 1, miR-1269a expression in the ESCA group was significantly higher than in healthy subjects (Figure 1A). High miR-1269a expression was significantly related to poor survival compared with low miR-1269a expression for both OS (Figure 1B) and CSS (Figure 1C). Additionally, we evaluated the prognostic ability of miR-1269a for OS and CSS through ROC curves.

Table 1 Clinical characteristics of esophageal cancer patients in this study, *n* (%)

Characteristics	Normal group, <i>n</i> = 52	ESCA group, <i>n</i> = 125	<i>P</i> value
Age in yr	58.7 ± 4.8	60 ± 5.4	0.857
Gender			0.962
Female	21 (40.4)	50 (53.6)	
Male	31 (59.6)	75 (46.4)	
Smoking history			0.383
Yes	32 (61.5)	68 (54.4)	
No	20 (38.5)	57 (45.6)	
Alcohol consumption			0.567
Yes	18 (34.6)	49 (39.2)	
No	34 (65.4)	76 (60.8)	
Location			
Upper area		16 (12.8)	
Middle area		41 (32.8)	
Lower area		68 (54.4)	
Histologic grade			
G 1		38 (30.4)	
G 2 + G 3		87 (69.6)	
AJCC stage			
Stage I + II		74 (59.2)	
Stage III + IV		51 (40.8)	
Tumor stage			
T1 + T2		52 (41.6)	
T3 + T4		73 (58.4)	
Lymph stage			
N0		59 (48.8)	
N1 + N2 + N3		66 (51.2)	
Metastatic stage			
M0		119 (95.2)	
M1		6 (4.8)	

AJCC: American Joint Committee on Cancer; ESCA: Esophageal cancer; G 1: Grade 1, high differentiation; G 2: Grade 2, moderate differentiation; G 3: Grade 3, low differentiation.

The results from AUC indicated that miR-1269a had excellent predictive ability for ESCA prognosis with 0.716 for OS and 0.764 for CSS (Figure 1D-E). This evidence indicates that miR-1269a has a potential application in clinical prognostic assessments.

Correlation between miR-1269a expression and clinicopathological data

We investigated the relationship between miR-1269a expression and clinicopathological data (Table 2). As depicted in Figure 2, there are no statistical differences with age, gender, or tumor location (Figure 2A-C). High miR-1269a expression levels were significantly associated with lower histologic grade, higher tumor stage, positive lymph stage, and higher AJCC stage (Figure 2D-G). Notably, there was high miR-1269a expression levels in patients with poorer prognosis (Figure 2H and I), which suggests a close correlation between miR-1269a expression and OS and CSS. Therefore, univariate and multivariate Cox analyses were applied to detect the relationship between miR-1269a expression and OS and CSS. As displayed in Figure 3A and B,

Table 2 Relationship between microRNA-1269a and pathological data

Characteristics	High expression, n = 62	Low expression, n = 63	P value
Age in yr			0.531
< 60	33 (53.2)	30 (47.6)	
≥ 60	29 (46.7)	33 (52.3)	
Gender			0.77
Female	24 (38.7)	26 (41.3)	
Male	38 (61.2)	37 (58.7)	
Location			0.8
Upper + middle area	24 (38.7)	23 (36.5)	
Lower area	38 (61.2)	40 (63.5)	
Histologic grade			0.003
G 1	27 (38.7)	12 (38.7)	
G 2 + G 3	35 (38.7)	51 (38.7)	
AJCC stage			0.001
Stage I + II	21 (33.9)	53 (84.1)	
Stage III + IV	41 (66.1)	10 (15.9)	
Tumor stage			0.004
T1 + T2	18 (29.0)	34 (54.0)	
T3 + T4	44 (70.0)	29 (46.0)	
Lymph stage			0.009
N0	22 (35.5)	37 (58.7)	
N1 + N2 + N3	40 (64.5)	26 (41.3)	
Metastatic stage			0.011
M0	56 (0.0)	63 (100.0)	
M1	6 (100.0)	0 (0.0)	

AJCC: American Joint Committee on Cancer; ESCA: Esophageal cancer; G 1: Grade 1, high differentiation; G 2: Grade 2, moderate differentiation; G 3: Grade 3, low differentiation.

high expression of miR-1269a showed a significantly lower OS rate than those with low expression by univariate analysis [hazard ratio (HR)[30] in 2.379; $P < 0.001$] and multivariate analysis (HR = 2.177; $P < 0.001$) and a significantly lower CSS rate by univariate analysis (HR = 3.073; $P < 0.001$) and multivariate analysis (HR = 2.496; $P < 0.001$). Furthermore, we performed univariate and multivariate Cox analysis to investigate the association between clinical parameters and OS and CSS. The results indicate that only AJCC stage is an independent survival factor for both OS and CSS (Figure 3A and B). In addition, we evaluated the prognostic ability for age, histologic grade, and AJCC stage compared with miR-1269a expression. Combined with AUC results above (Figure 1D and E), miR-1269a expression had better prognostic predictive ability than clinical variables for both OS and CSS (Figure 3E and F). Therefore, miR-1269a has a strong association with clinical prognosis, which suggests a high prognostic value.

Nomogram construction and evaluation

According to the results above, miR-1269a and AJCC stage had strong predictive capacity for prognosis. Based on our clinical practice, age also plays a central role in a patient's prognosis. Therefore, a nomogram was established to display the predictive relationships among age, AJCC stage, and miR-1269a with OS and CSS (Figure 4A and Figure 5A). Additionally, we developed a risk assessment model for OS and CSS prediction. As shown in Figure 4B, our nomogram had distinguished differences in OS between the high risk and low-risk models ($P < 0.001$). The AUC values indicated the

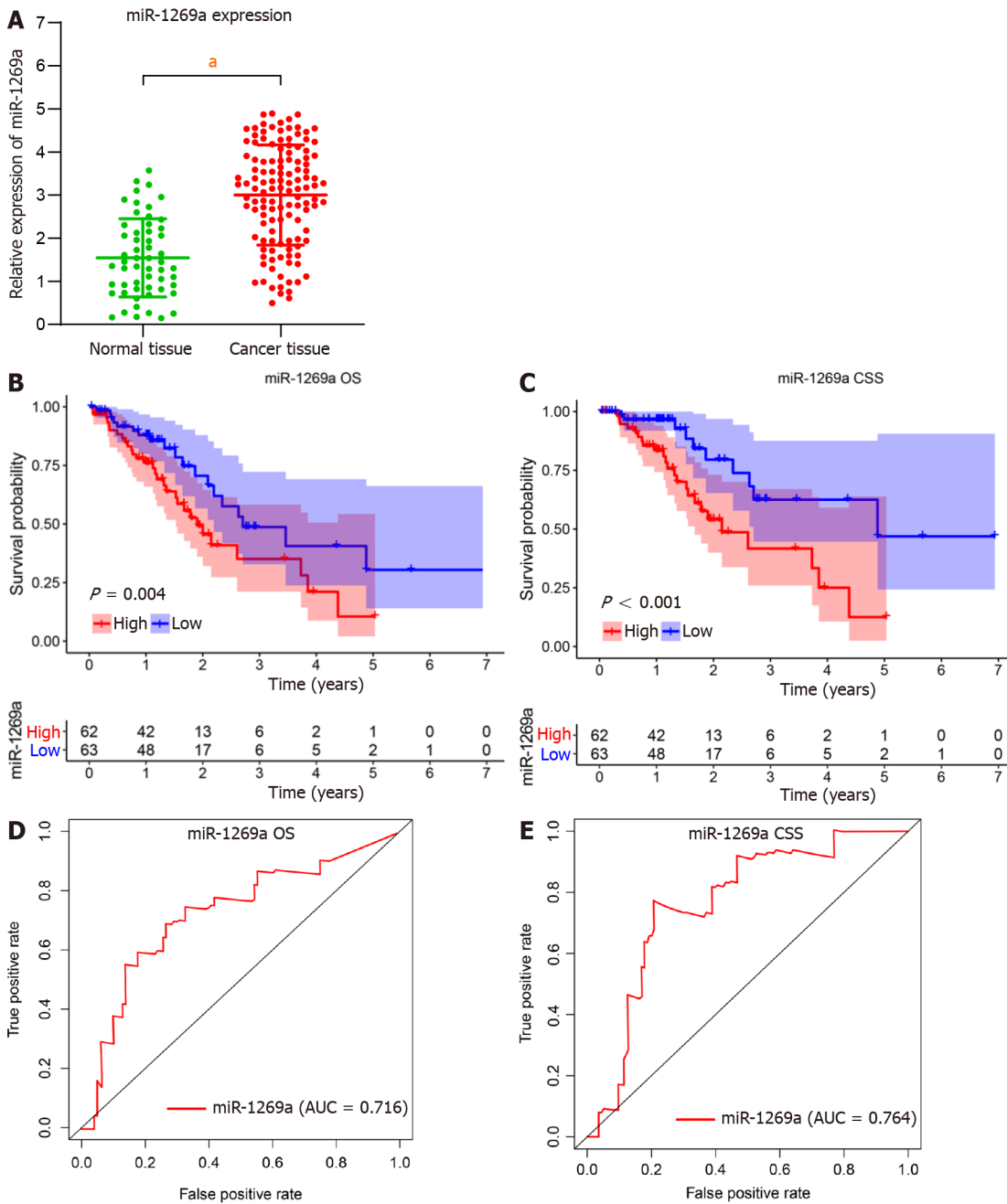


Figure 1 Expression and prognostic roles of microRNA-1269a in esophagus cancer. A: Expression of microRNA (miR)-1269a in esophagus cancer tissue was higher than that in normal tissue; B, C: High expression of miR-1269a was correlated to poor overall survival (OS) and cancer specific survival (CSS), as compared to low expression group; D, E: miR-1269a had significant predictive capacity for prognosis with 0.716 in OS and 0.764 in CSS. * $P < 0.05$. AUC: Area under curve.

1-, 3-, and 5-year OS of our nomogram were 0.633, 0.763, and 0.756, respectively, which is better than miR-1269a and AJCC stage alone (Figure 4C). The same or slightly better results were found for CSS predictions. The AUC values indicated the 1-, 3-, and 5-year CSS of our nomogram were 0.836, 0.821, and 0.931, respectively. As depicted in Figure 5C, our nomogram had a significant difference for CSS ($P < 0.001$). Generally, our nomogram including age had a higher discriminative capacity for predicting OS and CSS as compared to miR-1269a with AJCC stage.

Function enrichment analysis

There are 937 target genes for miR-1269a in Targetscan, 440 target genes in miRTarBase, and 291 target genes in Tarbase. However, 34 common target genes predicted from all three databases were selected for subsequent analysis (Figure 6A). We performed GO term and KEGG pathway analyses to determine the functional roles of these 34 target genes. A total of 25 GO terms and 14 KEGG pathways were primarily

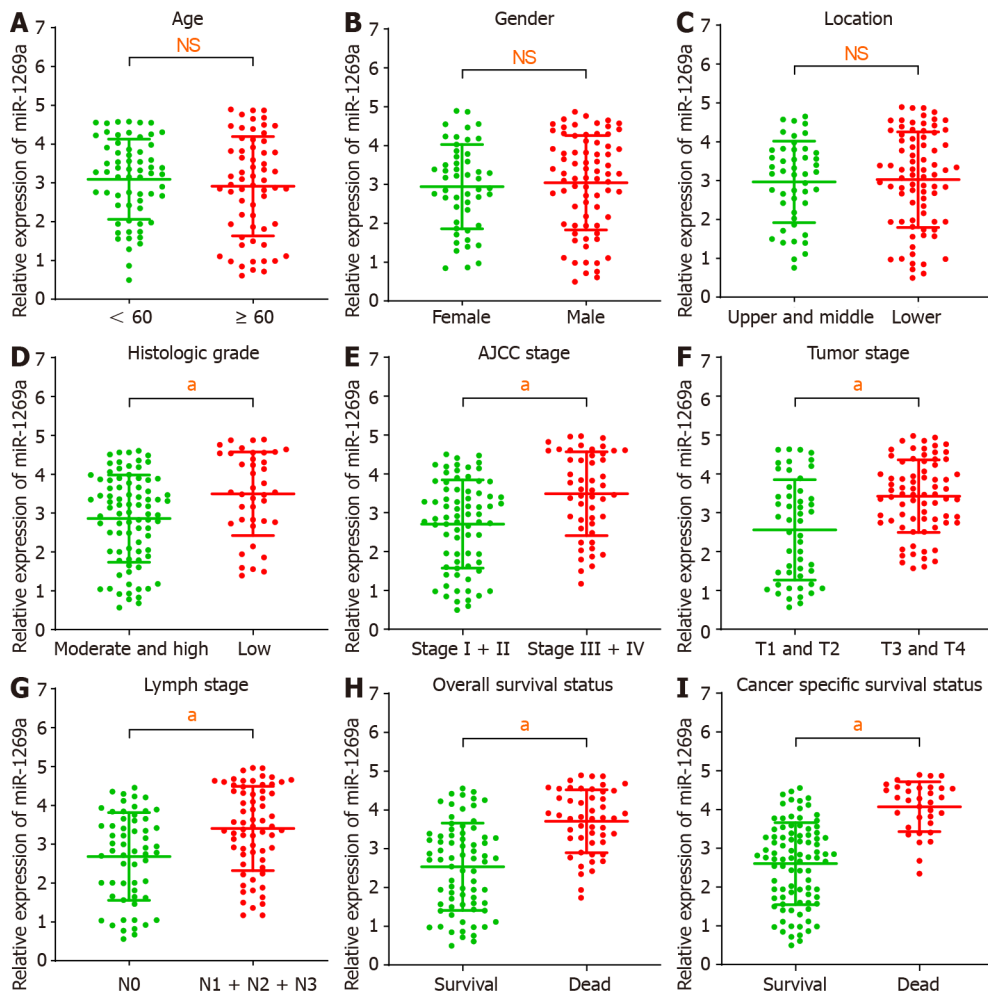


Figure 2 Correlation between microRNA-1269a expression and clinicopathological data. A-C: There are no statistical differences between microRNA (miR)-1269a expression and age, gender, or tumor location; D-I: High miR-1269a expression had a significantly high correlation with lower histologic grade, higher tumor stage, positive lymph stage, higher American Joint Committee on Cancer (AJCC) stage, worse overall survival, and poor cancer specific survival. ^a*P* < 0.05. NS: Not significant.

enriched for target genes with a *P* < 0.05 (Table 3 and Table 4). We present the top ten GO terms and KEGG pathways in Figure 6B and C. Briefly, the GO terms indicated that the miR-1269a was most enriched in protein binding, cytosol, and nucleus. Additionally, several cancer pathways were detected in the KEGG pathway analysis, for example, PI3K-AKT signaling pathway, pathway in cancer, and cell cycle pathway. Overall, the results from the functional enrichment analysis indicated that the miR-1269a was closely involved in the cancer development.

DISCUSSION

Currently, we mainly estimate the survival outcome of tumor patients through AJCC stage[31,32]. However, some limitations can be found in our clinical practice when we exclusively use AJCC stage. For instance, some patients with similar AJCC staging and clinical characteristics may differ in response to treatment and survival outcomes. This difference may arise from cancer heterogeneity, which partly root in genetic mutations [33,34]. Therefore, we attempted to establish a comprehensive staging system that combines clinical characteristics with genetic mutations. Moreover, recent studies have demonstrated that microRNAs play have great potential as biomarkers[30,35,36]. For example, low miR-335 expression is an independent prognostic factor and indicates a favorable prognosis in ESCA[37]; miR-1304 can be used as a powerful indicator for the diagnosis and recurrence of ESCA[38]; and miR-21 and miR-93 can be adopted as effective biomarkers for predicting radiotherapy and chemotherapy efficacy in ESCA [39]. Although some research has explored the diagnostic and prognostic roles in colorectal cancer, glioma, and lung cancer, the roles of miR-1269a in ESCA remain to

Table 3 Gene Ontology enrichment analysis of microRNA-1269a target genes

ID	Term	Gene	Count	P value
GO:0005515	Protein binding	<i>CLTC, WDR6, PUM2, MATR3, AKAP11, SMARCA4, UBN1, YWHAZ, BSDC1, SMG1, ZNF146, BACH2, KHSRP, TPM3, TAGLN2, PTBP1, PHKG2, TNPO2, MAP1A, ZBTB4, HMGCR, AP3D1, FN1, PTMA, TBP, CCND2, NCL, CDK6, SEC61A1</i>	29	3.58E-11
GO:0005829	Cytosol	<i>YWHAZ, MAP1A, CCND2, SMG1, WDR1, PTMA, ZBTB4, BACH2, KHSRP, TPM3, CLTC, SEC61A1, PUM2, WDR6, CDK6, AKAP11, TAGLN2, PHKG2, ZNF146</i>	19	3.79E-09
GO:0005634	Nucleus	<i>YWHAZ, ZNF146, CCND2, SMG1, MATR3, PTMA, ZBTB4, BACH2, SLC25A1, KHSRP, NCL, CDK6, TBP, PTBP1, SMARCA4, UBN1, TNPO2</i>	17	3.02E-07
GO:0005654	Nucleoplasm	<i>YWHAZ, CCND2, SMG1, PTMA, ZBTB4, BACH2, KHSRP, NCL, CDK6, TBP, PTBP1, SMARCA4, UBN1</i>	13	5.25E-06
GO:0005737	Cytoplasm	<i>YWHAZ, MAP1A, CCND2, SMG1, WDR6, TBP, KHSRP, PUM2, CDK6, AKAP11, TNPO2</i>	11	0.0013
GO:0070062	Extracellular exosome	<i>YWHAZ, FN1, WDR1, SLC25A1, KHSRP, TPM3, NCL, CLTC, PGAM1, PTBP1, TAGLN2</i>	11	8.89E-07
GO:0003723	RNA binding	<i>YWHAZ, WDR6, SMG1, CLTC, KHSRP, PUM2, NCL, MATR3, PTBP1, SMARCA4</i>	10	1.64E-07
GO:0016020	Membrane	<i>CLTC, KHSRP, SEC61A1, NCL, MATR3, PTBP1, SMARCA4, AP3D1</i>	8	0.00032
GO:0042802	Identical protein binding	<i>YWHAZ, HMGCR, FN1, KHSRP, NCL, MATR3</i>	6	0.00144
GO:0005730	Nucleolus	<i>ZNF146, KHSRP, SMARCA4, UBN1, ZBTB4</i>	5	7.83E-05
GO:0003677	DNA binding	<i>ZNF146, CCND2, NCL, AKAP11, PTBP1, SMARCA4</i>	6	0.00369
GO:0000122	Negative regulation of transcription by RNA polymerase II	<i>BACH2, SMARCA4, CDK6, ZBTB4</i>	4	0.00569
GO:0019901	Protein kinase binding	<i>YWHAZ, CCND2, CLTC, ZBTB4</i>	4	0.00067
GO:0003729	mRNA binding	<i>PUM2, PTBP1, KHSRP, MATR3</i>	4	2.90E-05
GO:0032991	Protein-containing complex	<i>TBP, SMARCA4, CLTC</i>	3	0.01671
GO:0043066	Negative regulation of apoptotic process	<i>YWHAZ, CCND2, PTMA</i>	3	0.00866
GO:0006468	Protein phosphorylation	<i>YWHAZ, PHKG2, CDK6</i>	3	0.00703
GO:0098978	Glutamatergic synapse	<i>YWHAZ, AP3D1, NPTX1</i>	3	0.00361
GO:0019899	Enzyme binding	<i>TBP, PHKG2, FN1</i>	3	0.00358
GO:0051301	Cell division	<i>CCND2, CLTC, CDK6</i>	3	0.00339
GO:0008134	Transcription factor binding	<i>YWHAZ, TBP, SMARCA4</i>	3	0.00285
GO:0001525	Angiogenesis	<i>YWHAZ, NCL, FN1</i>	3	0.00118
GO:0030054	Cell junction	<i>KHSRP, SLC7A2, WDR1</i>	3	0.00062
GO:0005198	Structural molecule activity	<i>MAP1A, CLTC, MATR3</i>	3	0.00043
GO:0043488	Regulation of mRNA stability	<i>YWHAZ, PUM2, KHSRP</i>	3	0.00013

be elucidated. Therefore, it is essential to detect miR-1269a prognosis in ESCA.

Here, we identified miR-1269a expression in ESCA and evaluated its association with clinical variables. Additionally, we performed a Kaplan-Meier analysis assessing its relationship with OS and CSS. ROC curves were drawn for diagnostic value. We estimated independent risk factors for OS and CSS using univariate and multivariate Cox analysis. Ultimately, we successfully confirmed the prognostic ability of miR-1269a for ESCA. Furthermore, a nomogram was created to evaluate the relationship between risk factors and OS and CSS, and a risk model including age, AJCC stage, and miR-1269a expression constructed. Kaplan-Meier analysis and ROC curves indicated that our risk model has substantial predictive value. Moreover, some advantages can be found in our nomogram such as simplicity, accuracy, easy to use and understand. These features enable the surgeon to quickly assess and make treatment decisions, which indicated that our nomogram was an effective tool for clinical applications.

Table 4 Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of microRNA-1269a target genes

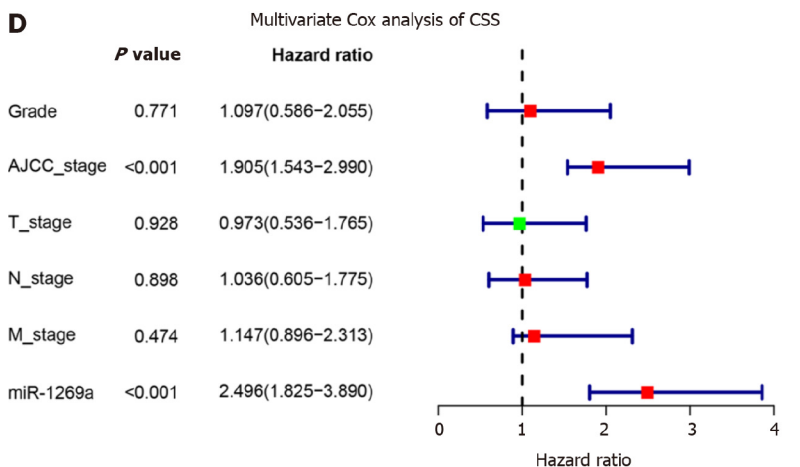
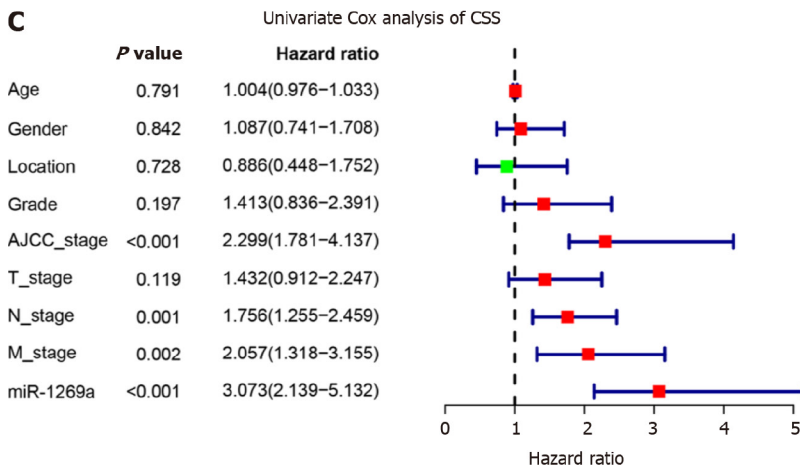
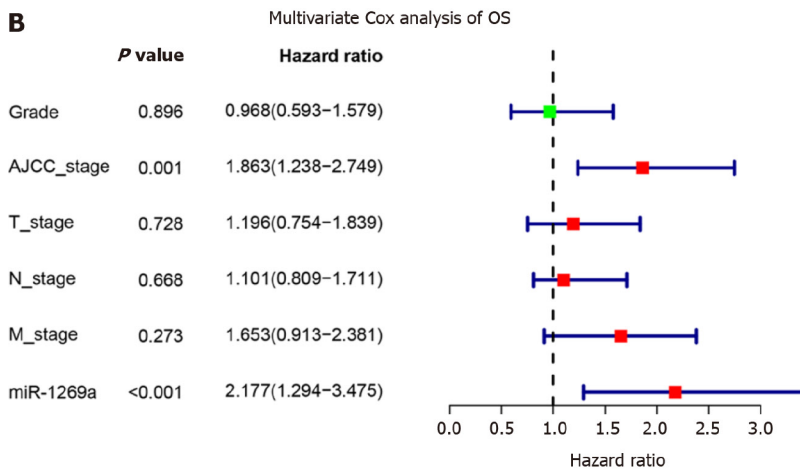
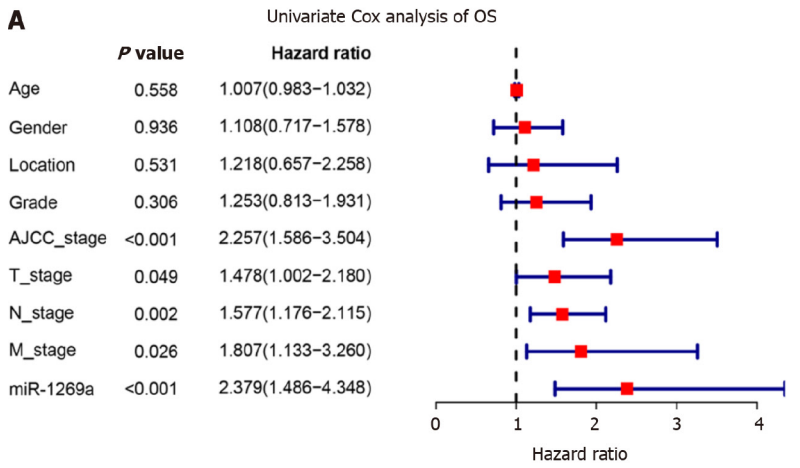
ID	Term	Gene	Count	P value
hsa04151	PI3K-Akt signaling pathway	<i>YWHAZ, CCND2, FN1, CDK6, IGF1, MET, PRLR, MAGI2, CREB5</i>	9	0.00863
hsa05200	Pathways in cancer	<i>CCND2, TPM3, FN1, CDK6, IGF2, NOTCH3, MET, CDK6</i>	8	0.02116
hsa04110	Cell cycle	<i>YWHAZ, CCND2, CDK6, E2F5, YWHAQ, CCND1, STAG2</i>	7	0.00751
hsa04115	p53 signaling pathway	<i>CCND2, CDK6, CCND1, WIG1, CDK6, IGF1</i>	6	0.03033
hsa04510	Focal adhesion	<i>CCND2, FN1, ITGB3, IGF1, MET</i>	5	0.02116
hsa05203	Viral carcinogenesis	<i>YWHAZ, TBP, CCND2, CDK6</i>	4	0.00190
hsa05165	Human papillomavirus infection	<i>TBP, CCND2, FN1, CDK6</i>	4	0.00751
hsa05100	Bacterial invasion of epithelial cells	<i>CLTC, FN1</i>	2	0.03033
hsa05222	Small cell lung cancer	<i>FN1, CDK6</i>	2	0.03757
hsa04922	Glucagon signaling pathway	<i>PHKG2, PGAM1</i>	2	0.03757
hsa04142	Lysosome	<i>AP3D1, CLTC</i>	2	0.03757
hsa04390	Hippo signaling pathway	<i>YWHAZ, CCND2</i>	2	0.03757
hsa04218	Cellular senescence	<i>CCND2, CDK6</i>	2	0.03757
hsa05130	Pathogenic Escherichia coli infection	<i>YWHAZ, NCL</i>	2	0.04294

In addition, the result from functional analysis revealed that miR-1269a might play important roles in cancer pathways including cell cycle pathway, pathway in cancer and PI3K/AKT pathway. Moreover, some studies also supported our prediction. For instance, miR-1269a overexpression in gastric cancer cell could stimulate the activation of PI3K/AKT pathway, thereby inducing cell proliferation and invasion; Meanwhile, the overexpression of miR-1269a inhibited RASSF9 expression, thereby impeding the cell apoptosis of AGS/MKN-45 cells through Bax/Bcl-2 signaling pathway[40]. miR-1269a contributes to down-regulation of FOXO1 and affects the dysregulation of cyclin D1, CDK2 and Bcl-2, thereby inducing cell proliferation, migration, and invasion[41]. The low expression of ATRX can be controlled by miR-1269a, also promoting proliferation and progression *in vitro*[18]. And this suppressive effects can be rescue by regulating ATRX overexpression in glioma cells. Those studies indicated that miR-1269a could serve as a potential therapeutic target for cancer.

Although our miR-1269a-related nomogram suggested a good performance in prognostic prediction of ESCA, there are some limitations. First, we only compared miR-1269a expression in tissue from ESCA and normal subjects. Further investigations are needed to detect the plasma difference of miR-1269a in ESCA. Additionally, retrospective study might weaken the validity of our research. Randomized controlled trials with larger populations are required to confirm our results. Lastly, the relevant mechanisms of miR-1269a *in vivo* remain to be elucidated. Therefore, we hope to carry out basic experiments in the future that could provide more evidence for the clinical application of miR-1269a in ESCA.

CONCLUSION

In conclusion, our findings suggest that miR-1269a expression could serve as a prognostic indicator for ESCA. Moreover, the simplicity and accuracy of our nomogram enable the surgeon to quickly assess and make treatment decisions, which suggested that our nomogram was an effective tool for clinical applications.



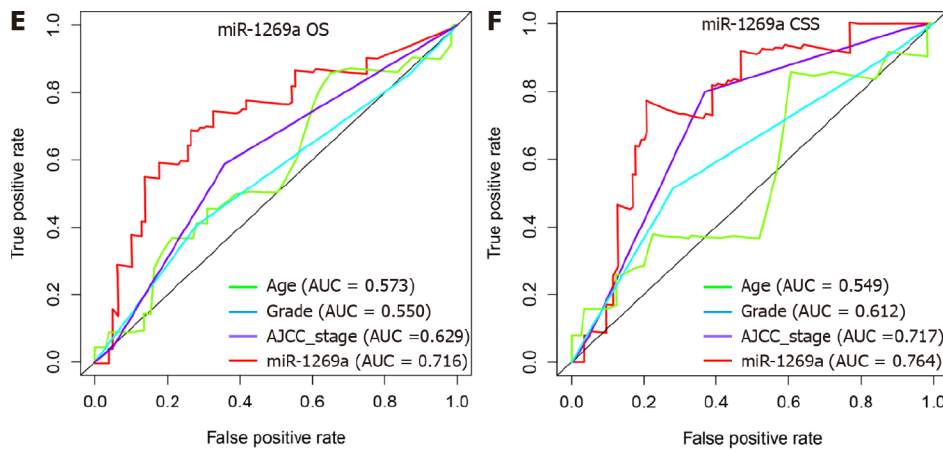


Figure 3 Identification risk factors for overall survival and cancer specific survival by Cox analysis. A, B: Univariate and multivariate Cox analyses recognized microRNA (miR)-1269a expression and American Joint Committee on Cancer (AJCC) stage as independent risk factors for overall survival (OS); C, D: Univariate and multivariate Cox analyses recognized miR-1269a expression and AJCC stage as independent risk factors for cancer specific survival (CSS); E, F: miR-1269a expression had better prognostic prediction capacity than age, histologic grade, or AJCC stage for both OS and CSS. AUC: Area under curve.

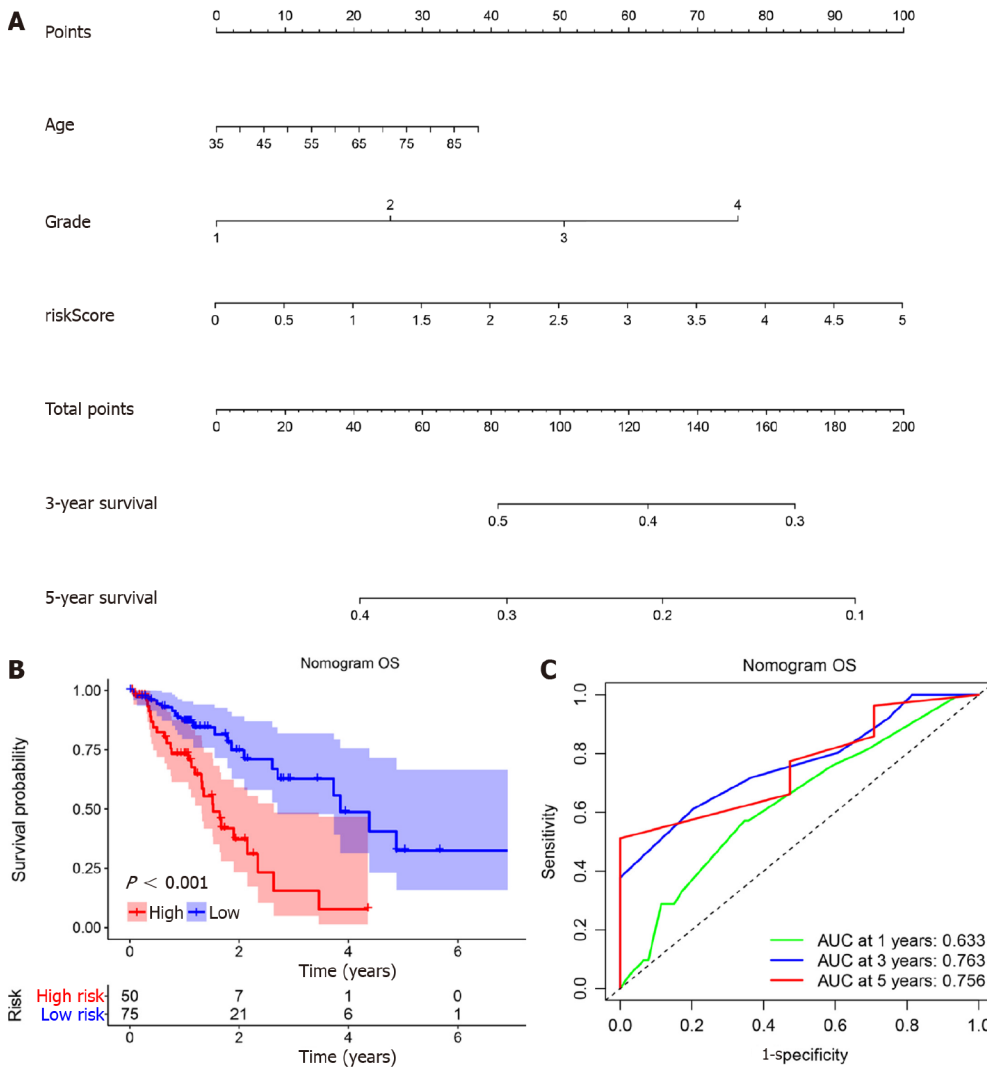


Figure 4 Nomogram construction and evaluation for overall survival. A: A nomogram was developed to predict overall survival (OS) of patients with esophageal cancer; B: The nomogram showed excellent discrimination of prognosis for OS between high risk and low risk models ($P < 0.001$); C: The area under curve (AUC) values of the nomogram for the 1-, 3-, and 5-year OS were 0.633, 0.763, and 0.756, respectively, which were better than the prediction using miR-1269a and American Joint Committee on Cancer stage alone.

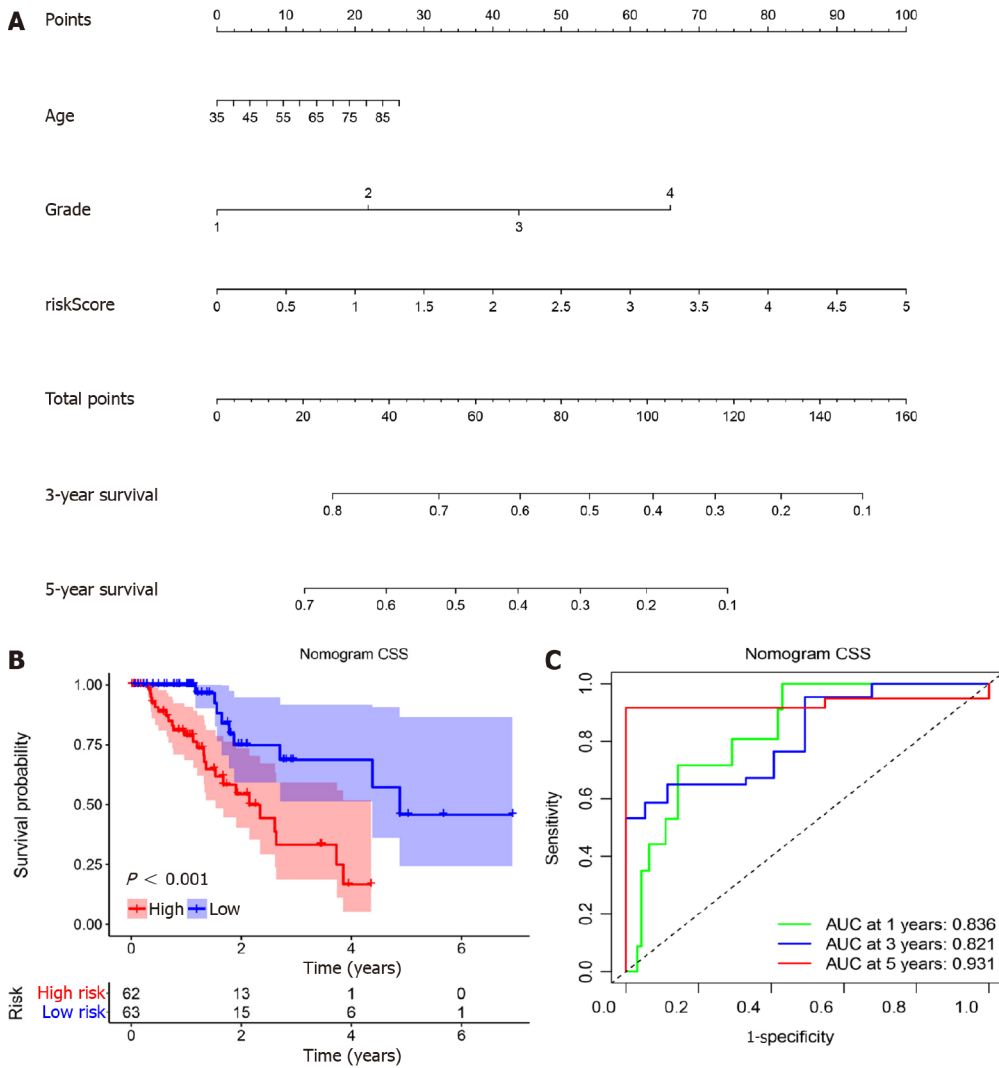


Figure 5 Nomogram construction and evaluation for cancer specific survival. A: A nomogram was developed to predict cancer specific survival (CSS) of patients with esophageal cancer; B: The nomogram showed excellent discrimination of prognosis for CSS between high risk and low risk models ($P < 0.001$); C: The area under curve (AUC) values of nomogram for the 1-, 3-, and 5-year CSS were 0.633, 0.763, and 0.756, respectively, which were better than the prediction using miR-1269a and American Joint Committee on Cancer stage alone.

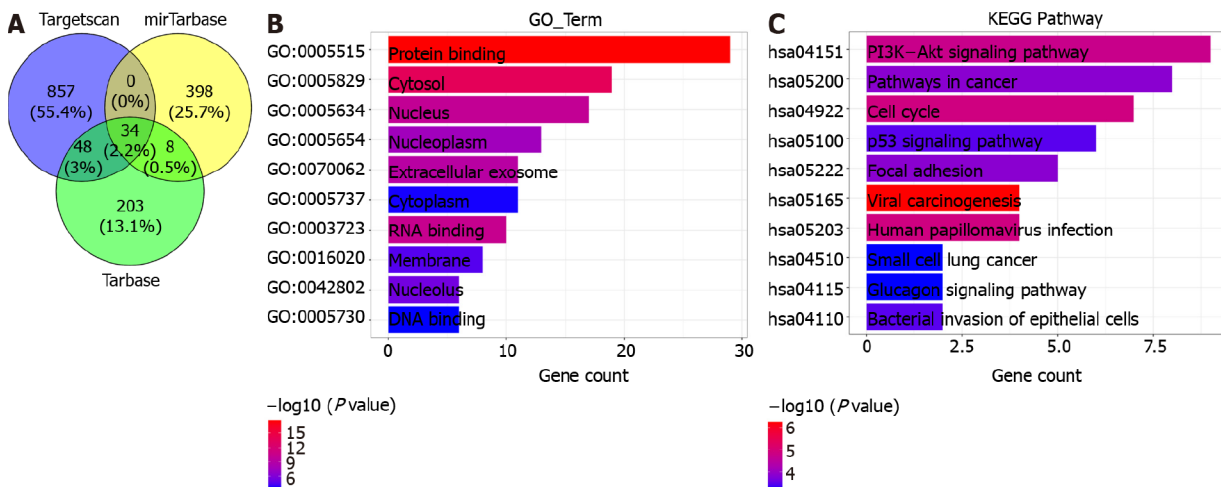


Figure 6 Function enrichment analysis of microRNA-1269a. A: Venn diagram shows the common target genes in Targetscan, miRTarBase, and Tarbase; B: The top ten enriched Gene Ontology (GO) terms of common target genes in esophageal cancer (ESCA); C: The top ten enriched Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways of common target genes in ESCA.

ARTICLE HIGHLIGHTS

Research background

Esophageal cancer (ESCA) is a heterogeneous cancer with variable outcomes that are challenging to predict. MicroRNA (miR)-1269a is a newly discovered non-coding RNA that shows promising prognostic prediction in other cancers, but its clinical value in ESCA remains unclear.

Research motivation

This study established a comprehensive staging system that combines clinical characteristics with genetic mutations in ESCA.

Research objectives

This study aimed to determine the prognostic value of miR-1269a, and to develop a nomogram to succinctly predict the prognosis in esophageal carcinoma.

Research methods

miR-1269a expression in ESCA were detected using quantitative real-time polymerase chain reaction. Then we determined its prognostic value with clinical variables through multivariate Cox analysis. A nomogram based on miR-1269a expression using age and American Joint Committee on Cancer (AJCC) stage was developed and assessed its prognostic performance. Finally, we predicted the target genes of miR-1269a and analyzed their potential function in cancer development using Gene Ontology and Kyoto Encyclopedia of Gene and Genomes analyses.

Research results

High expression of miR-1269a in ESCA showed poor prognosis in overall survival (OS) and cancer-specific survival (CSS), suffered increased rates of low differentiation and metastasis, and exhibited tumor stage T3 + T4, positive lymph stage, and AJCC stage III + IV. The area under the receiver operating characteristic curve of miR-1269a was 0.716 for OS and 0.764 for CSS. Multivariate Cox analysis revealed that AJCC stage and miR-1269a were independent factors for OS and CSS. Combining with age, we constructed a nomogram for prognostic prediction, which showed excellent performance for OS and CSS after 3 years and 5 years and was easy to use. And functional analysis indicated that miR-1269a was closely related to PI3K-AKT signaling pathway.

Research conclusions

miR-1269a can be used as a potential indicator for the prognosis of esophageal cancer. We developed an easy-to-use nomogram with excellent prognostic prediction for clinical use.

Research perspectives

In further research, the molecular mechanism of miR-1304 in esophageal cancer will be elucidated.

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