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ORIGINAL RESEARCH

Serum Circular FoxO3a Serves as a Novel Prognostic Biomarker in Squamous Cervical Cancer

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Xiaoyan Tang[1–3,*]
Songping Liu[4,*]
Yan Ding[1–3,*]
Chenyan Guo[1–3]
Jingjing Guo[1–3]
Keqin Hua[1–3]
Junjun Qiu[1–3]

[1]Department of Gynecology, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, People's Republic of China; [2]Department of Obstetrics and Gynecology of Shanghai Medical College, Fudan University, Shanghai 200032, People's Republic of China; [3]Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Shanghai 200011, People's Republic of China; [4]Department of Obstetrics and Gynecology, Zhenjiang Maternal and Child Health Hospital, Zhenjiang, Jiangsu 212001, People's Republic of China

*These authors contributed equally to this work

Purpose: Circular RNAs (circRNAs) are novel type of noncoding RNAs that play important roles and serve as noninvasive biomarkers in various cancers. In the present study, we focused on circFoxO3a and aimed to investigate its prognostic value as a novel serum biomarker for squamous cervical cancer (SCC).

Patients and Methods: Our study included 103 SCC patients from Obstetrics and Gynecology Hospital of Fudan University. Expression levels of circFoxO3a in the serum of patients with SCC were examined by reverse transcription-quantitative PCR (RT-qPCR). The correlation between serum circFoxO3a expression and clinicopathologic factors was analyzed. The Kaplan–Meier method and multivariate Cox regression analysis were applied to evaluate the independent prognostic factors for SCC. A prognostic predictive nomogram was constructed using R software.

Results: Levels of serum circFoxO3a were decreased in SCC patients compared with controls. Low expression of circFoxO3a was correlated with deeper stromal invasion and positive lymph node metastasis. Moreover, SCC patients with lower expression of serum circFoxO3a showed poorer prognosis, including both overall survival (OS) and recurrence-free survival (RFS). Multivariate Cox analysis indicated low serum circFoxO3a levels to be an unfavorable prognostic factor for both OS and RFS, independent of positive lymph node metastasis. Notably, the predictive nomogram we established further confirmed that serum circFoxO3a is a useful tool for predicting survival in SCC.

Conclusion: Altogether, our findings demonstrated that serum circFoxO3a could serve as a potential novel noninvasive predictive prognostic biomarker and therapeutic target for SCC.

Keywords: circular RNA, FoxO3a, squamous cervical cancer, biomarker

Introduction

Cervical cancer is the fourth most common and leading cause of cancer-related death in women, with an estimated 570,000 cases and 311,000 deaths worldwide in 2018.[1] Squamous cervical cancer (SCC), which is the predominant histological type, accounts for approximately 80% of all cervical cancer cases.[2] Despite the widespread implementation of screening tests and advancements in treatment for SCC, the prognosis of SCC patients remains poor.[3,4] Regarding prognostic factors, certain traditional factors such as International Federation of Gynecology and Obstetrics (FIGO) stage, lymph node metastasis, lymph-vascular space invasion (LVSI) and deep stromal infiltration are usually considered.[5] However, assessments of these clinicopathological factors are invasive because they are obtained from postoperative tissues; moreover, they cannot precisely predict prognosis

Correspondence: Junjun Qiu; Keqin Hua
Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, People's Republic of China
Tel +86-21-63455050 Ext 8261
Fax +86-21-63455090
Email qiujunjun1113@163.com; huakeqin@fudan.edu.cn

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preoperatively.[6] Therefore, noninvasive prognosis prediction is crucial, which can help decision-making and provide therapeutic guidance for SCC to a certain degree.

Currently, different types of serum biomarkers are used for the diagnosis and prognosis of various cancers because of their noninvasiveness and reliability, such as prostate-specific antigen (PSA) for prostate cancer and carcinoembryonic antigen (CEA) for colorectal cancer.[7,8] Although certain serum biomarkers are also used for CC, their sensitivity and specificity are not satisfactory.[9] For example, SCC antigen (SCC-Ag), which is currently the most commonly used biomarker for SCC, can also be elevated in squamous cell carcinoma of the lungs,[10] esophagus,[11] head and neck.[12] Moreover, SCC-Ag levels are also increased in nontumorous diseases such as psoriasis,[13] nephritic syndrome[14] and allergic asthma.[15] Hence, there is an urgent need to explore novel noninvasive serum biomarkers for a more accurate prognosis prediction of SCC.

Circular RNAs (circRNAs) are a new class of noncoding RNAs that are characterized by covalently closed loops without 3′- and 5′- ends.[16] In recent years, numerous studies have revealed that circRNAs are widely expressed in various tissues and play important roles in tumor development and progression.[17] Expression of circRNAs is tissue- and developmental stage-specific.[18] Furthermore, their closed circular structure renders circRNAs relatively resistant to RNA enzymes, and they are stable and enriched in peripheral blood and/or body fluids.[16] Therefore, circular RNAs have a distinct advantage in the development and application as novel clinical biomarkers. For example, Fan et al found that circMAN1A2 may serve as a serum biomarker for malignant tumors, providing important insights into diagnostic approaches for malignant tumors such as gastrointestinal cancer, thyroid cancer, ovarian cancer, and lung cancer.[19] In plasma and tissues obtained from patients with gastric cancer, has_circ_0001017 and has_circ_0061276 were evidently decreased and this reduction was significantly associated with distal metastasis. The combination of has_circ_0001017 and has_circ_0061276 showed a sensitivity and specificity of 95.5% and 95.7% respectively, for the diagnosis of gastric cancer.[20] Despite these findings, however, research related to circRNAs as noninvasive biomarkers in SCC is limited.

Circular Forkhead box O3a (circFoxO3a), encoded by the FoxO3 gene which is a well-known tumor suppressor, is receiving increasing attention.[21–23] For example, circFoxO3a was reported to be downregulated in various cancer cells, and is involved in cell proliferation and cell cycle progression.[24] A recent study also found that circFoxO3a has a tumor suppressive activity and may serve as a novel biomarker for the early diagnosis, treatment monitoring and prognosis of patients with non-small cell lung cancer (NSCLC).[25] However, to the best of our knowledge, the specific clinical significance of circFoxO3a and whether it can serve as a novel biomarker in SCC have not been investigated.

In this study, we first detected circFoxO3a in sera from SCC patients and then investigated the correlation between the expression of serum circFoxO3a and clinicopathological factors. Furthermore, we analyzed the prognostic value of circFoxO3a and attempted to establish a predictive model for SCC.

Materials and Methods

Patients

One hundred and three SCC patients who underwent surgery at the Obstetrics and Gynecology Hospital of Fudan University between December 2013 and October 2014 were enrolled in this study. The inclusion criteria were as follows: 1) the diagnosis of SCC was confirmed by the pathological report; 2) patients without any preoperative radiotherapy or chemotherapy; and 3) patients without any other malignancies or severe chronic disease. Thirty healthy women, as confirmed by their routine physical examination reports were enrolled as a control group. All participants were well informed and signed the written informed consent form. The study was approved by the Medical Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University. Regular follow-up was conducted with a median period of 49 months (range: 8–60 months). Preoperative blood samples were stored at −80°C in the tissue bank of our hospital. Clinical data were extracted from medical records, including FIGO stage, tumor size, depth of invasion, lymph node metastasis and LVSI status. The depth of stromal invasion was defined as the percentage of the involved cervical stroma, and the tumor size was a direct measurement of the greatest dimension. The clinicopathological characteristics of the patients are listed in Table 1.

RNA Preparation and RNase R Digestion

Total serum RNA was extracted and purified using an RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA concentration was assessed with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA (2 μg) was incubated at 37°C for 15 min with 3 U/μg

Table 1 Baseline Characteristics of 103 Patients with Squamous Cervical Cancer

Variable	Number (%)
Age, y (mean ± SD)	51.1±9.41
≤45	29 (28.2%)
>45	74 (71.8%)
Stage	
IA2	2 (1.9%)
IB1-IB2	70 (68.0%)
IIA1-IIA2	31 (30.1%)
Surgery Type	
Laparoscopy	96(93.2%)
Laparotomy	7 (6.8%)
Tumor size	
≤4cm	71(68.9%)
>4cm	32(31.3%)
Lymphovascular invasion	
Negative	44(42.7%)
Positive	59(57.3%)
Stromal invasion depth	
<1/2	38(36.9%)
≥1/2	65(63.1%)
Lymph node metastasis	
Negative	71(68.9%)
Positive	32(31.1%)
Parametrial invasion	
Negative	86(83.5%)
Positive	17(16.5%)
Margin	
Negative	98(95.1%)
Positive	5(4.9%)
HPV infection	
Negative	12(11.7%)
Positive	91(88.3%)
Ki-67	
Low (≤35%)	27(26.2%)
Median (≤70%)	64(62.1%)
High (>70%)	12(11.7%)

RNase R (Epicenter Biotechnologies, Shanghai, China) to remove linear RNA and purify the circRNAs.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

After digestion with RNase R, expression of circFoxO3a was detected by RT-qPCR. Quantitative RT-qPCR was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Germany) with the SYBR-Green PCR Master Mix kit (Takara, Dalian, China) according to the manufacturer's instructions. The primers were purchased from Sangon Biotech (Shanghai, China) and the sequences were as follows: circFoxO3a forward, 5′-ATTGTCCATGGAGA CAGGCCCGCCG-3′ and reverse, 5′-GTGGGGAACTTCA CTGGTGCTAAG-3′; and GAPDH forward, 5′-GTCTCCT CTGACTTCAACAGCG-3′ and reverse, 5′- ACCACCCTG TTGCTGTAGCCAA-3′. GAPDH was used as an internal reference, and relative expression of circFoxO3a was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analysis was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used to compare the expression of circFoxO3a between SCC patients and healthy women, and the Chi square test and Fisher's exact test were employed to analyze associations between expression of serum circFoxO3a and clinicopathological factors. Overall survival (OS) and recurrence-free survival (RFS) were defined as the time interval from the date of surgery to the date of death or recurrence, respectively, or final contact. The Kaplan–Meier method along with the Log rank test were applied for survival analysis. Multivariate Cox regression analysis was then carried out to investigate the prognostic value of the clinicopathological factors and circFoxO3a expression. Based on the results of multivariate Cox analysis, nomograms for OS and RFS were conducted in R version 3.5.3 (https://www.r-project.org/) as described in previous studies.[26] P<0.05 was considered statistically significant. The related computerized programs for the nomograms are shown in Supplementary Materials.

Results

Expression of Serum circFoxO3a Was Decreased in SCC Patients

The presence of serum circFoxO3a was detected using convergent or divergent primers by qRT-PCR and validated by gel electrophoresis (Figure 1). As shown in Figure 2, the expression of serum circFoxO3a in SCC patients was significantly lower than that of normal healthy women (P<0.01), indicating that decreased serum circFoxO3a levels might be correlated with the malignant behavior of SCC.

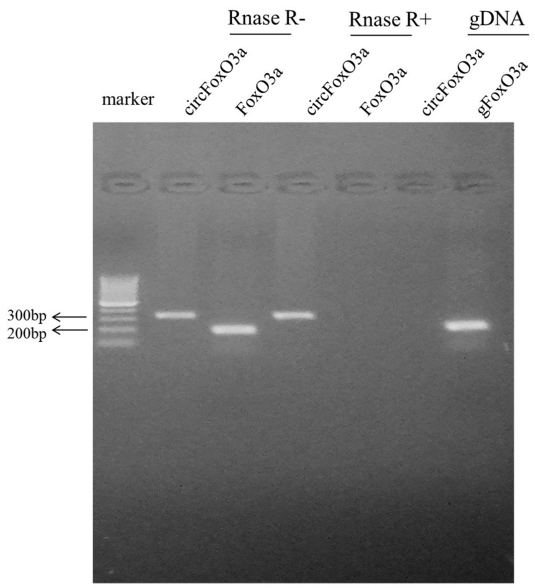

Figure 1 The existence of circFoxO3a was validated by Gel electrophoresis. The divergent primers could detect circular RNAs in cDNA with or without RNase R, which demonstrate that they are truly circular not linear, but these divergent primers could not amplify any product in genomic DNA. The convergent primers amplified PCR production in linear FoxO3a mRNA, which disappeared after RNase R treatment.

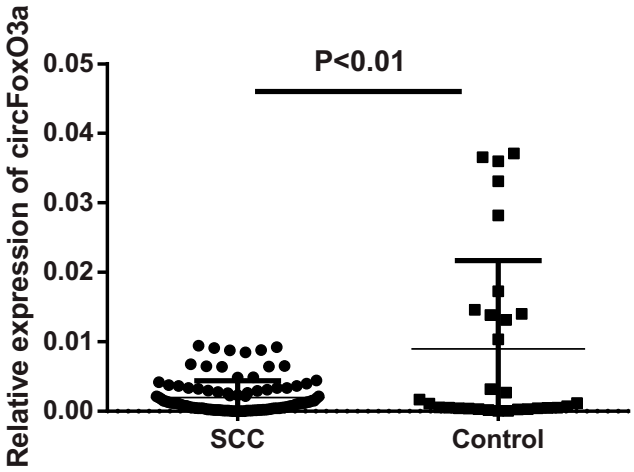

Figure 2 Relative expression of serum circFoxO3a in SCC patients (n=103) and normal controls (n=30).

Low Expression of Serum circFoxO3a Was Correlated with Deeper Stromal Invasion, and Positive Lymph Node Metastasis

Next, all 103 SCC patients were divided into two groups based on the median expression levels of circFoxO3a: high (n=52) and low (n=51) groups. We further investigated whether the expression of circFoxO3a was correlated with several clinicopathological factors of SCC (Table 2).

Table 2 The Correlation Between the Expression of Serum circFoxO3a and Clinicopathological Factors

Variable	Low Expression of circFoxO3a (n=51)	High Expression of circFoxO3a (n=52)	P
Age, y (mean ± SD)			0.105
≤45	11	18	
>45	40	34	
Stage			0.881
I	36	36	
II	15	16	
Surgery Type			0.652
Laparoscopy	48	49	
Laparotomy	3	3	
Tumor size			0.059
≤4cm	31	40	
>4cm	20	12	
Lymphovascular invasion			0.181
Negative	19	25	
Positive	32	27	
Stromal invasion depth			0.005
<1/2	12	26	
≥1/2	39	26	
Lymph node metastasis			0.008
Negative	29	42	
Positive	22	10	
Parametrial invasion			0.347
Negative	46	49	
Positive	5	3	
Margin			0.491
Negative	48	50	
Positive	3	2	
HPV infection			0.188
Negative	4	8	
Positive	47	44	
Ki-67			0.166
Low (≤35%)	13	14	
Median (≤70%)	29	35	
High (>70%)	9	3	

The results showed that a low expression of serum circFoxO3a was significantly associated with deeper stromal invasion depth (P=0.005) and positive lymph node metastasis (P=0.008). Therefore, these findings suggest

that decreased circFoxO3a levels are associated with the aggressive behaviors of SCC.

Serum circFoxO3a Was an Independent Prognostic Factor in SCC

We conducted the Kaplan-Meier method and Log rank test to evaluate the prognostic value of serum circFoxO3a in SCC. The median OS time in SCC patients with a low expression of serum cicrFoxO3a and positive lymph node metastasis was significantly shorter than that in patients with a high expression (47.47±2.68 vs 58.60±1.02, 43.44 ±3.85 vs 57.01±1.01, respectively, all P<0.05, Table 3); similar findings were obtained in the analysis of RFS. Additionally, as shown in Figure 3A and B, a low expression of serum circFoxO3a was associated with poor survival outcomes including both OS and RFS. Moreover, univariate and multivariate analyses (Table 4) identified low serum circFoxO3a, independent of positive lymph node metastasis, to be an unfavorable prognostic factor for both OS and RFS (P<0.05). Altogether, these findings suggest that serum circFoxO3a can serve as a novel prognostic biomarker for SCC.

A Nomogram for Prognosis Prediction of SCC Was Established

Based on the above findings, we established a nomogram using R version 3.5.3 (Figure 4A and B). Different scores were assigned to different statuses of circFoxO3a expression and lymph node metastasis. By summing the corresponding score, we were able to directly and easily predict the probability of survival at different time points. The c-index for the established nomogram to predict OS and RFS were both 0.808 (95% CI, 0.616 to 0.990). The calibration curves for the nomogram-predicted probability of 1- and 3-year OS/RFS showed good agreement between the actual observation and nomogram prediction (Figure 5). These results confirm that the nomogram combining expression of serum circFoxO3a and status of lymph node metastasis is a useful tool for predicting the survival of SCC patients.

Discussion

Circular RNAs, as a novel type of noncoding RNA, were originally considered nonfunctional accidental byproducts of aberrant splicing.[17] However, emerging evidence indicates that circRNAs can play vital roles via miRNA sponge-like effects or circRNA-protein interactions in tumorigenesis and metastasis, such as in breast cancer,[27]

Table 3 The Correlation Between Clinicopathological Characteristics and Overall Survival, Recurrence-Free Survival Using Kaplan–Meier Method

Variable	Overall Survival (Months) Mean ± SE	P	Recurrence-Free Survival (Months) Mean ± SE	P
Age, y (mean ± SD)		0.146		0.150
≤45	55.66±2.28		55.35±2.50	
>45	52.22±1.92		51.37±2.12	
Stage		0.124		0.134
I	56.18±1.41		55.66±1.59	
II	49.31±3.23		48.55±3.50	
Surgery Type		0.229		0.299
Laparoscopy	48.43±14.42		47.68±15.90	
Laparotomy	55.14±3.02		55.14±3.02	
Tumor size		0.086		0.094
≤4cm	54.85±1.73		54.31±1.91	
>4cm	49.59±1.54		48.53±3.29	
Lymphovascular invasion		0.342		0.352
Negative	53.80±2.20		53.27±2.42	
Positive	52.53±2.10		51.70±2.30	
Stromal invasion depth		0.314		0.323
<1/2	55.03±2.37		54.58±2.58	
≥1/2	51.71±1.96		50.86±2.16	
Lymph node metastasis		<0.001		<0.001
Negative	57.01±1.01		56.75±1,12	
Positive	43.44±3.85		41.78±4.21	
Parametrial invasion		0.066		0.073
Negative	54.13±1.54		53.52±1.69	
Positive	43.50±6.61		41.75±7.23	
Margin		0.083		0.078
Negative	54.03±1.51		53.38±1.67	
Positive	40.20±9.41		38.60±10.22	
HPV infection		0.943		0.950
Negative	52.17±3.86		51.50±4.28	
Positive	53.40±1.66		52.69±1.82	
Ki-67		0.732		0.736
Low	51.70±3.42		50.96±3.70	
Median	52.92±1.81		52.16±2.02	
High	53.45±1.54		54.50±4.31	
CircFoxO3a		0.001		0.001
Low	47.47±2.68		46.29±2.94	
High	58.60±1.02		58.37±1.17	

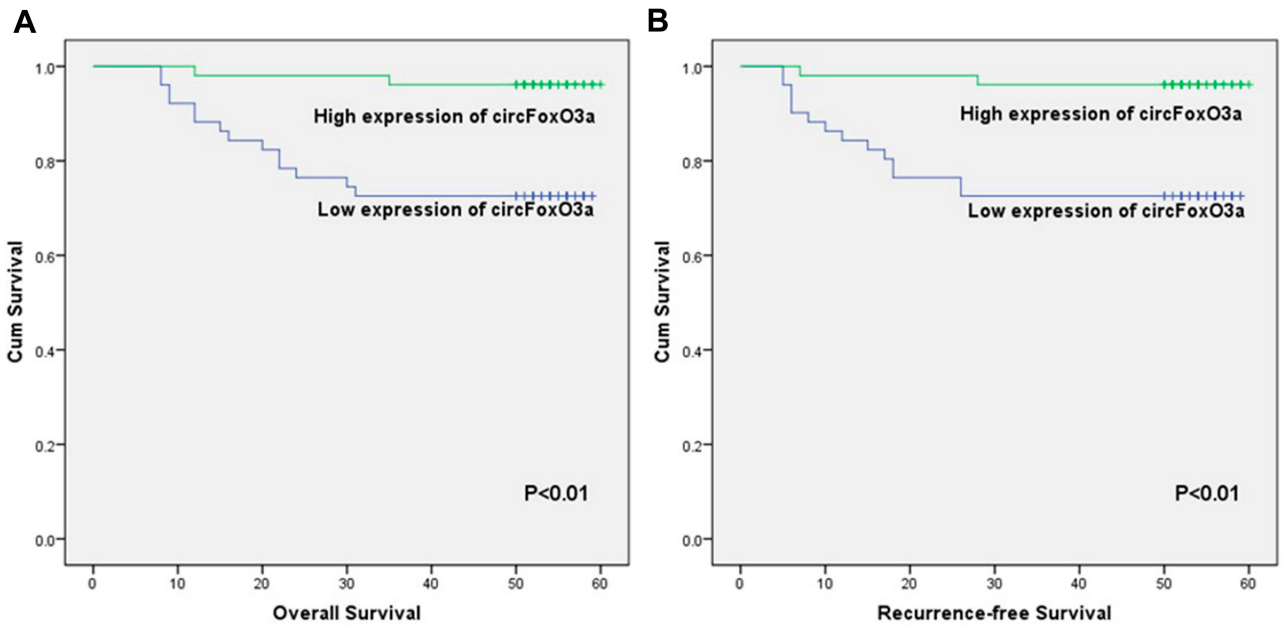

Figure 3 Kaplan–Meier survival curves for overall survival (**A**) and recurrence-free survival (**B**) of SCC patients according to expression levels of serum circFoxO3a.

hepatocellular carcinoma,[28] colorectal cancer,[29] and lung cancer.[30] Furthermore, an increasing number of studies have shown that circRNAs can serve as effective diagnostic and prognostic biomarkers for cancer.[31] Nonetheless, studies focusing on circular RNAs and SCC are limited.

Forkhead box (Fox) proteins are a well-known family of transcription factors. Among them, FoxO3a, also known as FoxO3, has been extensively investigated in various types of cancers. Most of these studies reported a tumor-suppressive function of FoxO3a. In addition, tumors with high FoxO3a expression usually indicates better patient survival.[32] With the increasing focus on circRNAs, circFoxO3a has also attracted researchers' attention. Du et al demonstrated that

Table 4 Univariate and Multivariate Cox Regression Analysis of Overall and Recurrence-Free Survival for Patients with Squamous Cervical Cancer

Variable	Recurrence-Free Survival				Overall Survival			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P	HR (95% CI)		HR (95% CI)	P	HR (95% CI)	P
Age	2.822(0.641±12.417)	0.170			2.848(0.647±12.532)	0.166		
Stage	2.242(0.753±6.675)	0.147			2.292(0.770±6.821)	0.136		
Surgery Type	0.044(0.000±179.912)	0.462			0.044(0.000±179.843)	0.462		
Tumor size	2.254(0.846±6.007)	0.104			2.295(0.861±6.117)	0.097		
Lymphovascular invasion	1.640(0.570±4.721)	0.359			1.658(0.576±4.772)	0.349		
Stromal invasion depth	1.752(0.565±5.432)	0.332			1.771(0.571±5.492)	0.322		
Lymph node metastasis	8.154(2.624±25.342)	0.000	5.940(1.883±18.744)	0.002	8.157(2.625±25.353)	0.000	5.926 (1.878±18.705)	0.002
Parametrial invasion	2.971(0.846±10.431)	0.089			3.054(0.870±10.723)	0.081		
Margin	3.472(0.788±15.297)	0.100			3.411(0.774±15.025)	0.105		
HPV infection	0.954(0.217±4.199)	0.951			0.948(0.215±4.171)	0.944		
Ki-67	0.727(0.316±1.671)	0.452			0.724(0.314±1.665)	0.447		
CircFoxO3a	0.123(0.028±0.541)	0.006	0.180(0.040±0.809)	0.025	0.122(0.028±0.539)	0.005	0.181(0.040±0.815)	0.026

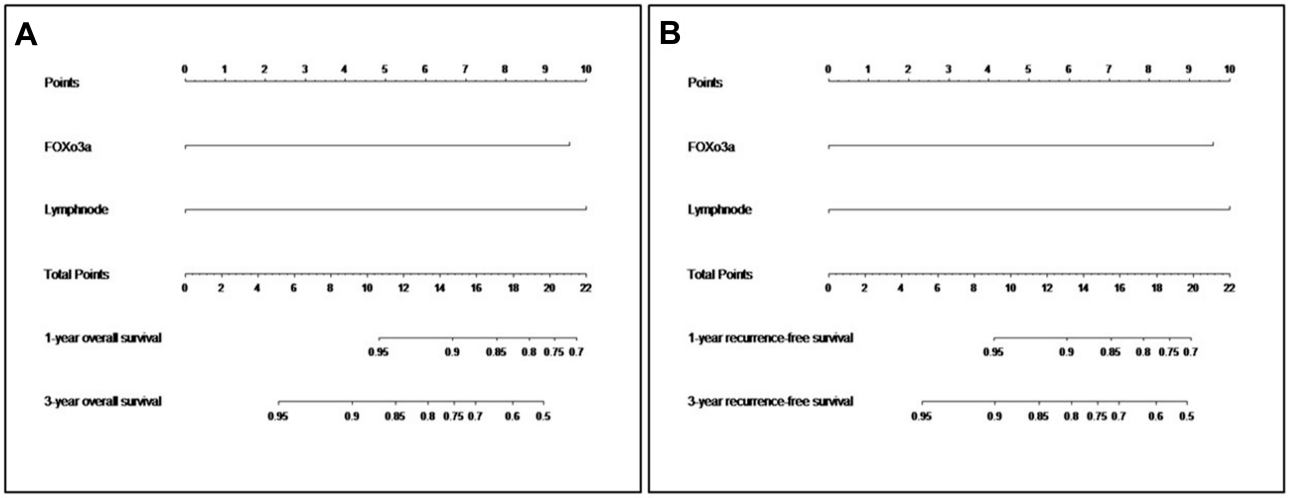

Figure 4 Nomogram for predicting overall survival (**A**) and recurrence-free survival (**B**) in SCC patients.

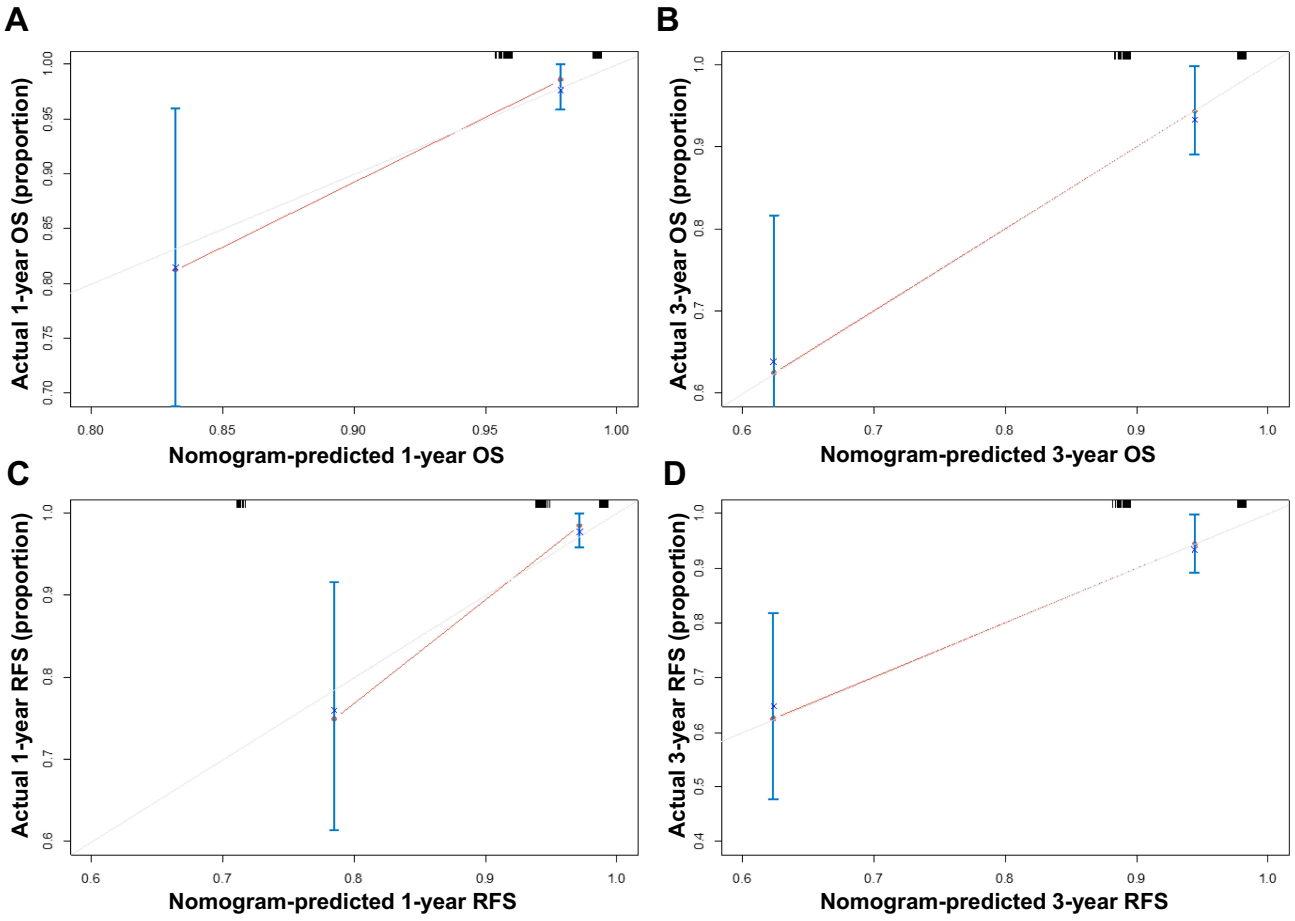

Figure 5 The calibration curves of nomogram for predicting 1-year (**A**), 3-year (**B**) overall survival and 1-year (**C**), 3-year (**D**) recurrence-free survival.

circFoxO3a is highly expressed in noncancer cells and associated with cell cycle progression.[33] Zhang et al reported that circFoxO3a appeared to have tumor-suppressive activity by sponging miR-155 in NSCLC and that it may serve as a novel biomarker for the early diagnosis and prognosis prediction of NSCLC.[25]

However, the involvement of circFoxO3a in SCC remains unclear.

In this study, we first noted that serum circFoxO3a was decreased in SCC patients compared with in normal healthy women. We next found that a low expression of serum circFoxO3a was correlated with deeper stromal invasion and positive lymph node metastasis, suggesting an association between a low expression of serum circFoxO3a and aggressive tumor behavior. Further survival analysis revealed that SCC patients with a low expression of serum circFoxO3a and positive lymph node metastasis displayed poorer outcomes, including both OS and RFS. In multivariate Cox analysis, circFoxO3a expression in addition to lymph node metastasis was an independent factor of survival in SCC patients. As all SCC patients with recurrence died due to recurrence, multivariate analysis for OS and RFS yielded similar results. Taken together, these results indicated that low expression of serum circFoxO3a is associated with the aggressive behavior of SCC and that serum circFoxO3a may serve as an independent prognostic factor for SCC.

In recent years, nomograms, as a tool for prognosis prediction, have been well developed and proven to be more accurate than conventional staging systems in some cancers.[34,35] Based on the results of multivariate Cox analysis, we successfully established a nomogram for the prognosis prediction of SCC. The nomogram performed well in predicting both OS and RFS with a c-index of 0.808. The c-index is the most widely used index to assess the predictive efficiency of nomograms. Theoretically, prognostic prediction of the nomogram is more accurate with a larger c-index. Additionally, calibration curves confirmed the optimal predictive efficiency of the nomograms. Based on this nomogram model, we conclude that serum circFoxO3a can be utilized as a novel biomarker for prognosis prediction of SCC in clinical practice.

There are several limitations of this study. First, the sample size was relatively small and all patients were from a single medical center in China, which may have led to selection bias. Second, the detailed molecular mechanism of circFoxO3a in SCC has not been explored and needs further investigation. Last, this study focused on the expression and prognostic value of circFoxO3a in SCC. In the future, we will enroll cervical cancer patients with other histology such as adenocarcinoma and adenosquamous carcinoma, and enlarge the sample size to further explore the role of the circFoxO3a in cervical cancer and enhance its clinical application.

Conclusion

In summary, we reported decreased expression of serum circFoxO3a in SCC patients for the first time. Furthermore, our study demonstrated that lower expression of circFoxO3a was associated with deeper stromal invasion and positive lymph node metastasis in SCC patients. Additionally, there was a strong correlation between expression level of serum circFoxO3a and prognosis in SCC patients. SCC patients with lower expression of serum circFoxO3a showed shorter OS and RFS. A predictive nomogram model utilizing serum circFoxO3a was successfully established and showed good predictive ability for the OS and RFS of SCC patients. Altogether, our findings indicate that serum circFoxO3a can potentially serve as a novel predictive prognostic biomarker and therapeutic target for SCC.

Abbreviations

SCC, squamous cervical cancer; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymph-vascular space invasion; CircRNAs, circular RNAs; CircFoxO3a, circular Forkhead box O3a; OS, overall survival; RFS, recurrence-free survival; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NSCLC, non-small cell lung cancer; PSA, prostate-specific antigen; CEA, carcinoembryonic antigen; SCC-Ag, squamous cell carcinoma antigen.

Consent for Publication

This manuscript has been approved by all authors for publication. I declare on behalf of my coauthors that the work described is original research that has not been published previously and is not under consideration for publication elsewhere, either in whole or in part. All of the authors have approved the enclosed manuscript.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics and Consent Statement

Written consent was obtained from each participant. The study protocol was approved by the Medical Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University and conducted in accordance with the Declaration of Helsinki.

Author Contributions

Xiaoyan Tang, Songping Liu, Yan Ding are the co-first authors to this article and contributed equally to this research project. All authors contributed to data analysis, drafting or 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 declare that there are no competing interests associated with the manuscript.

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