

Correlation Analysis of circRNA Circ_0071662 in Diagnosis and Prognosis of Esophageal Squamous Cell Carcinoma

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Background: The role of circRNA circ_0071662 has been studied in bladder cancer. The present study aimed to analyze its involvement in esophageal squamous cell carcinoma (ESCC).

Methods: Patients with ESCC (n = 66), esophageal ulcer (EU, n = 66), or gastroesophageal reflux disease (GERD, n = 66) and healthy controls (n = 66) were enrolled in this study. Plasma samples were collected from all patients and controls. ESCC and paired non-tumor tissue samples were collected from ESCC patients. Circ_0071662 levels in these samples were determined by RT-qPCRs. Diagnostic and prognostic values of circ_0071662 for ESCC were analyzed with ROC curve and survival curve analyses.

Results: Circ_0071662 level was decreased in ESCC, but not in GERN and EU compared to the controls and in ESCC tissues compared to the non-tumor tissues. Plasma circ_0071662 was closely correlated with patients' tumor size but not with other clinical features. Decreased plasma circ_0071662 levels separated ESCC patients from GERN patients, EU patients, and healthy controls. Low plasma circ_0071662 levels were closely correlated with worse survival outcomes of ESCC patients.

Conclusion: Circ_0071662 is lowly expressed in ESCC and may serve as a potential diagnostic and prognostic biomarker for ESCC.

Keywords: esophageal squamous cell carcinoma, circ_0071662, diagnosis, prognosis

Introduction

Esophageal squamous cell carcinoma (ESCC) originates from the flat and thin cells lining the inside of the esophagus.¹ ESCC accounts for more than 90% of esophageal cancer, the sixth most common cancer worldwide.¹ ESCC is most frequently observed in the middle and upper part of the esophagus but can also affect other parts of the esophagus.^{2,3} ESCC patients, in general, have poor survival, although their overall survival is significantly affected by stages.⁴ It is reported that about 47% of patients with ESCC detected at the local stage can survive longer than 5 years, while only 25% of ESCC patients with regional metastasis and 5% of ESCC patients with distant metastasis can survive 5 years.^{5,6} Therefore, the key for the survival of ESCC patients is still early diagnosis.⁷

One of the major causes of delayed diagnosis of ESCC is misdiagnosis.^{8,9} Several clinical disorders, such as esophageal ulcer (EU) and gastroesophageal reflux disease (GERD) can mimic the symptoms of ESCC,^{10,11} leading to common misdiagnosis. Although different clinical disorders may share similar clinical

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presentations, they may require the involvement of different molecular factors. Therefore, certain differentially expressed factors may serve as biomarkers to distinguish ESCC from other clinical disorders.^{12,13} CircRNAs encode no protein products, but they are involved in the regulation of protein synthesis and degradation, thereby exerting oncogenic or tumor-suppressive functions.¹⁴ Therefore, circRNAs may be applied as the diagnostic biomarkers for ESCC. The role of circRNA circ_0071662 has been studied in bladder cancer.¹⁵ The present study aimed to analyze its involvement in ESCC.

Materials and Methods

Participants and a 5-Year Follow-Up

The study was approved by the Ethics Committee of the Third Affiliated Hospital of Chongqing Medical University and included 66 ESCC patients (43 males and 23 females, 55.9±7.5 years), 66 esophageal ulcer (EU) patients (43 males and 23 females, 55.7±7.1 years), 66 gastroesophageal reflux disease (GERD) patients (43 males and 23 females, 55.8±7.4 years) and 66 healthy controls (43 males and 23 females, 55.8±8.0 years) who admitted to the hospital. All ESCC patients were diagnosed by multiple approaches, including imaging techniques and biopsies. EU and GERD patients were diagnosed according to the standard method. The inclusion criteria were 1) newly diagnosed cases and 2) no therapies had been initiated. The exclusion criteria were 1) patients with blood relationships, 2) patients complicated with severe infections and other severe diseases, and 3) pregnant women. All healthy controls were enrolled if they completed systemic physiological examination including height, weight, vision, blood routine, urine routine, stool routine, lung function test, liver and kidney function test, blood pressure test, blood sugar and blood lipid test and electrocardiography and had normal physiological functions. All participants signed informed consent. From the day of admission, the 66 ESCC patients were visited monthly via telephone or outpatients visit to monitor their survival. All 66 patients completed the follow-up or died of ESCC during the follow-up. None of these patients died of other causes.

Preparation of Tissue and Plasma Samples

After admission, paired non-tumor and ESCC tumor specimens were collected from all 66 ESCC patients either through biopsy or dissecting the resected primary tumors. Blood (3mL) samples were extracted from the elbow vein

of both patients (ESCC, EU, and GERD) and healthy controls after fasted overnight and used to isolate plasma samples using conventional methods.

RNA Extraction and Purification

RNA samples were immediately isolated from the collected samples using Monarch Total RNA Miniprep Kit (T2010, NEB). In brief, samples were lysed, loaded onto gDNA removal columns, and centrifugation at 12000g for 10 min. The follow-through RNA samples were then loaded onto RNA purification columns, treated with DNase I, washed with washing buffer, and eluted with RNase-free water. Finally, RNA samples were subjected to the analysis of quality and quantity using 2100 Bioanalyzer.

RT-qPCRs

RNA samples with a RIN value higher than 8.5 were diluted to about 1500 ng/μL with RNase-free water. After that, 4 μL RNA samples were used to prepare cDNA samples through reverse transcriptions using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Circ_0071662 levels were determined by qPCRs with 18S rRNA as the internal control using Fast Start Universal SYBR Green Master (Roche). Relative gene expression levels were normalized using the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

SPSS software program (15.0) was applied to analyze data and plot images. With ESCC patients as the true positive cases, and EU, GERD or controls as the true negative cases, ROC curve analysis was carried out to analyze the role of plasma circ_0071662 in the diagnosis of ESCC. The 66 ESCC patients were grouped into high and low plasma circ_0071662 level groups (n = 33) with the median plasma circ_0071662 level in ESCC patients as the cutoff value, and chi-squared test was applied to explore the associations between plasma circ_0071662 and patients' clinical factors. The survival curves of patients in both circ_0071662 level groups were plotted based on the 5-year survival data and compared using Log rank test. P < 0.05 was statistically significant.

Results

RT-qPCR Analysis of Circ_0071662 Levels in Tissues and Plasma

Circ_0071662 levels in paired ESCC and non-tumor tissue samples from 66 ESCC patients and in plasma from ESCC

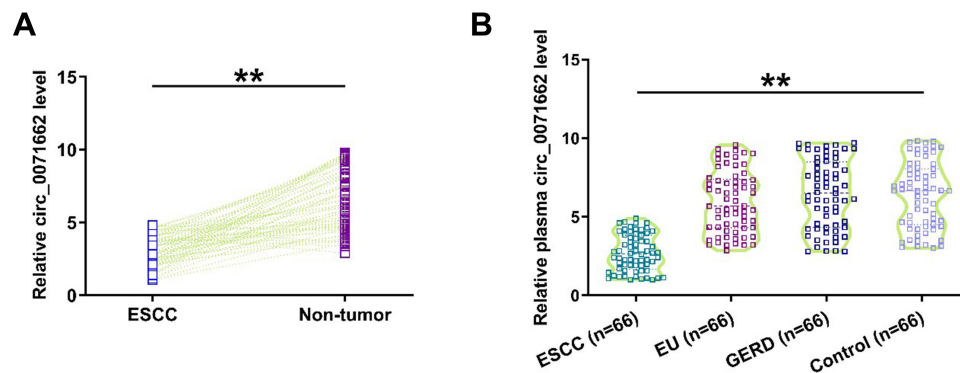


Figure 1 RT-qPCR analysis of t circ_0071662 levels in tissues and plasma. RNA samples were isolated from paired ESCC and non-tumor tissue samples from 66 ESCC patients (**A**) and plasma samples from ESCC patients (n = 66), EU patients (n = 66), GERD patients (n = 66), and controls (n = 66) (**B**), and subjected to cDNA synthesis and subsequent qPCR to determine the expression of circ_0071662. Circ_0071662 levels. **p < 0.01.

patients (n = 66), EU patients (n = 66), GERD patients (n = 66) and controls (n = 66) were determined using RT-qPCR. The results showed circ_0071662 levels were decreased in ESCC tissues than in non-tumor tissues (Figure 1A, p < 0.01), and the plasma circ_0071662 levels also decreased in ESCC patients, but not in GERD and EU patients compared with the healthy controls (Figure 1B, p < 0.01).

Associations Between Plasma Circ_0071662 and Patients' Clinical Factors

The 66 ESCC patients were assigned into high and low plasma circ_0071662 level groups, and chi-squared test was

applied to explore the associations between plasma circ_0071662 and patients' clinical factors. Interestingly, plasma circ_0071662 was closely correlated with patients' tumor size but not other clinical features (Table 1). Therefore, it is reasonable to hypothesize that circ_0071662 is mainly involved in the growth of ESCC tumors.

Diagnostic Values of Plasma Circulating Circ_0071662 for ESCC

With ESCC patients as the true positive cases and other participants (EU, GERD, or controls) as the true negative cases, ROC curve analysis was carried out to analyze the role of plasma circulating circ_0071662 in the diagnosis of

Table 1 Associations Between Plasma Circ_0071662 and Patients' Clinical Factors

Clinical Parameters		Cases	Low (n = 33)	High (n = 33)	P value
Age	<55y	32	15	17	0.62
	>55y	34	18	16	
Gender	Male	43	20	23	0.44
	Female	23	13	10	
Differentiation grade	Well/Moderate	48	22	26	0.27
	Poor	18	11	7	
Tumor size	>5cm	38	13	25	0.62
	≤5cm	28	20	8	
Primary tumor invasion depth	T1/T2	36	20	16	0.003
	T3/T4	30	13	17	
Lymph node	Negative	43	20	23	0.44
	Positive	23	13	10	
TNM stage	I/II	44	24	20	0.30
	III	22	9	13	
Smoking	Yes	40	22	18	0.31
	No	26	11	15	
Drinking	Yes	43	20	23	0.44
	No	23	13	10	

ESCC. Our data analysis revealed that decreased circ_0071662 plasma levels could be used to effectively separate ESCC patients from GERN patients (Figure 2A), EU patients (Figure 2B) and healthy controls (Figure 2C). Therefore, decreased plasma circ_0071662 may be applied in clinical practice to increase the diagnostic accuracy of ESCC.

Analysis of the Role of Plasma Circulating Circ_0071662 in Predicting the Survival Outcomes of ESCC Patients

The 66 ESCC patients were grouped into high and low plasma circ_0071662 level groups. The survival curves of both circ_0071662 level groups were plotted based on the 5-year survival data and compared using Log rank test. Low plasma circ_0071662 levels were closely correlated with worse survival outcomes (Figure 3).

Discussion

The present study mainly analyzed the role of circ_0071662 in ESCC. We showed that circ_0071662 was lowly expressed in ESCC but not in EU and GERD. Moreover, decreased plasma circ_0071662 levels could be used to assist the diagnosis and prognosis of ESCC.

A recent study characterized circ_0071662 as a novel tumor suppressor in bladder cancer, in which circ_0071662 was found to be lowly expressed and could sponge miR-146b-3p to suppress its role, thereby decreasing tumor growth and metastasis.¹⁵ To our best knowledge, the involvement of circ_0071662 in other cancers is unclear. The present study showed that circ_0071662 is specifically downregulated in ESCC but not in EC and GERD, compared to the healthy controls. Therefore, circ_0071662 may be specifically involved in ESCC. Although we did not analyze the role of circ_0071662 in the development and progression of ESCC, we showed that plasma circ_0071662 level is only closely correlated with ESCC tumor size, but not ESCC tumor differentiation and invasion. Therefore, circ_0071662 is likely only involved in the growth, but not metastasis, of ESCC tumors. However, our speculation is purely based on statistical analysis. Future studies with in vitro cell and/or in vivo animal studies are needed to further confirm our conclusions.

In clinical practices, ESCC is frequently misdiagnosed as EU and GERD, which can mimic the symptoms of ESCC, leading to delayed diagnosis and the development of tumor metastasis. The present study showed that decreased plasma circ_0071662 levels

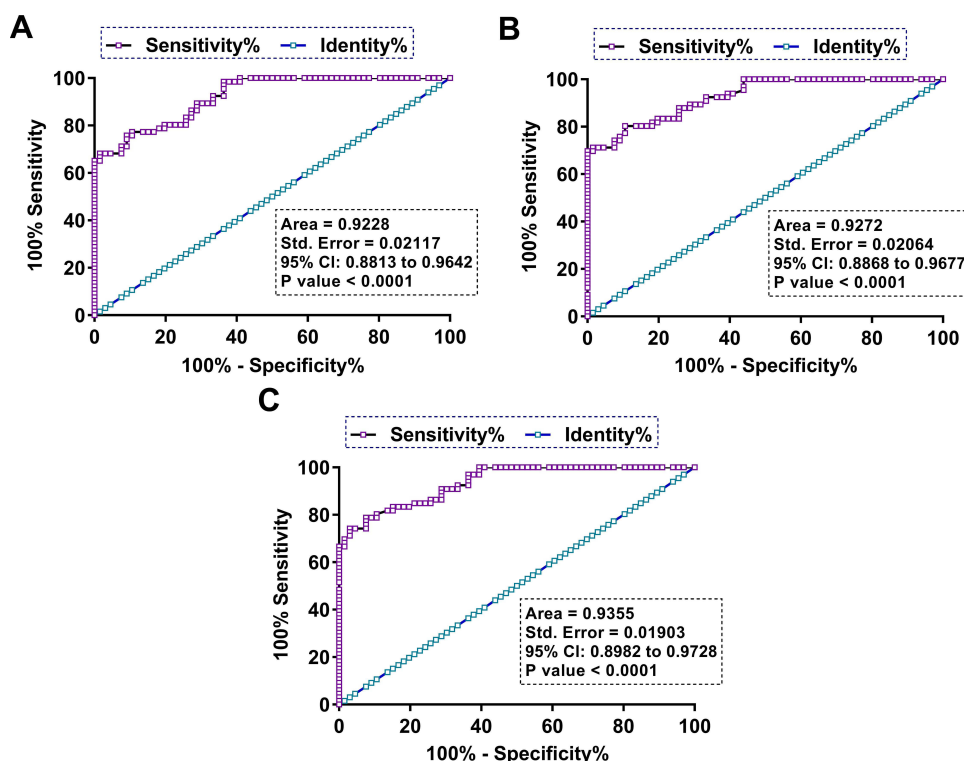


Figure 2 Diagnostic values of plasma circulating circ_0071662 for ESCC. ROC curve analysis was carried out with ESCC patients as the true positive cases and EU (A), GERD (B), or controls (C) as the true negative cases to analyze the role of plasma circulating circ_0071662 in the diagnosis of ESCC.

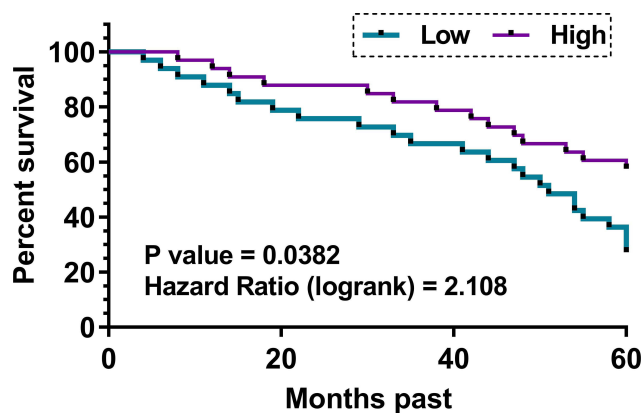


Figure 3 Analysis of the role of plasma circulating circ_0071662 in predicting the survival outcomes of ESCC patients. The 66 ESCC patients were grouped into high and low plasma circ_0071662 level groups. The survival curves of both circ_0071662 level groups were plotted using the 5-year survival data and compared using the Log rank test.

could effectively separate ESCC patients from EU and GERD patients. Therefore, measuring plasma circ_0071662 levels prior to treatment may increase the diagnostic accuracy of ESCC. Moreover, plasma circ_0071662 was found to be closely correlated with the worse survival outcomes of ESCC patients. Our data suggested that plasma circ_0071662 could be used in clinical practices to identify patients with a high risk of death, thereby designing personalized treatment approaches to prolong survival. Although previous studies have reported the role of circRNAs as biomarkers for ESCC, these studies failed to include other diseases that can mimic the symptoms of ESCC.^{16,17} Our study is the first to report the application of a circRNA to distinguish ESCC from EU and GERD.

This study is limited by the small sample size. Our conclusions should be verified by future studies. In addition, functional assays are needed to explore the function of circ_0071662 in ESCC.

Conclusion

Circ_0071662 is lowly expressed in ESCC and decreased plasma circ_0071662 may serve as a potential diagnostic biomarker to separate ESCC patients from EU and GERD patients. In addition, patients with low circ_0071662 levels may have poor prognoses.

Data Sharing Statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to Participate

All patients signed the written informed consent. All procedures were approved by the Ethics Committee of the Third Affiliated Hospital of Chongqing Medical University and completed in keeping with the standards set out in the Announcement of Helsinki and the Laboratory Guidelines of Research in China.

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Disclosure

The authors declare that they have no conflicts of interest.

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