Serum Exosomal lncRNA DLX6-AS1 Is a Promising Biomarker for Prognosis Prediction of Cervical Cancer

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Abstract

Objective: Deregulation of long noncoding RNAs (lncRNAs) is involved in the initiation and progression of cancer. LncRNA DLX6-AS1 is regarded as an oncogene in many cancer types. However, the clinical role of serum exosomal lncRNA DLX6-AS1 in cervical cancer (CC) is poorly known. This study aimed to analyze the diagnostic and prognostic value of serum exosomal lncRNA DLX6-AS1 in CC. **Methods:** A total of 114 patients with CC, 60 patients with CIN (cervical intraepithelial neoplasia), and 110 healthy women were enrolled in this study. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to measure the serum exosomal lncRNA DLX6-AS1 levels in all participants. **Results:** Serum exosomal lncRNA DLX6-AS1 level was significantly elevated in CC patients compared with CIN patients and normal controls. In addition, high serum exosomal lncRNA DLX6-AS1 expression was positively associated with lymph node metastasis, differentiation, FIGO stage, and shortened survival. Patients with high serum exosomal lncRNA DLX6-AS1 expression were more prone to have a relapse. Furthermore, univariate and multivariate analyses suggested that serum exosomal lncRNA DLX6-AS1 was a potential prognostic indicator for overall survival of CC patients. **Conclusions:** These findings demonstrated that serum lncRNA DLX6-AS1 might serve as a promising marker for the diagnosis and prognosis prediction of CC.

Keywords

exosomes, DLX6-AS1, cervical cancer, biomarker, diagnosis

Introduction

Cervical cancer (CC) is one of the most frequent cancers among women.[1,2] CC is mainly caused by the infection of high-risk human papillomavirus (HR-HPV).[3] Though the progresses in the treatment of CC have been made, it remains a major health problem in China with an increasing incidence trends.[4] Early diagnosis of cervical dysplasia and cancer can reduce the incidence and mortality. Serum squamous cell carcinoma antigen (SCC-Ag) is a widely used biomarker for CC detection. However, its sensitivity is relatively low.[5] Therefore, identification of novel biomarkers for the early detection of CC is urgently required.

Exosomes (40-100 nm) are key mediators of cell-to-cell communication by transmitting biomolecules such as mRNAs, microRNA (miRNAs), and long noncoding RNAs (lncRNAs).[6] LncRNAs are a class of RNA molecules with more than 200 nucleotides in length.[7] Accumulating evidence has demonstrated that lncRNAs participate in various cellular and biological processes, such as cell proliferation, migration, invasion and apoptosis.[8,9] The aberrantly expressed lncRNAs, acting as oncogenes or tumor suppressors, have been found to play important roles in diagnosis and progression of cancers.[10] Exosomes can be detected in the blood with a high degree of stability, which makes serum exosomal lncRNAs as promising candidate biomarkers in many types of cancer including CC.

LncRNA distal-less homeobox 6 antisense 1 (DLX6-AS1) is located on human chromosomal region 7q21.3.[11] Recently, lncRNA DLX6-AS1 was reported to play an oncogenic role in multiple cancer types, such as bladder cancer,[12] breast cancer,[13,14] esophageal squamous cell carcinoma,[15,16]

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non-small cell lung cancer,[17,18] and gastric cancer.[19,20] However, the diagnostic and prognostic value of serum exosomal lncRNA DLX6-AS1 has been not determined in CC yet. Therefore, this study explored the clinical significance of serum exosomal LncRNA DLX6-AS1 in CC.

Material and Methods

Study Subjects

In the study, a total of 114 CC patients, 60 cervical intraepithelial neoplasia (CIN) patients and 110 healthy volunteers were recruited. The average age of CC patients, CIN patients and healthy controls was 46 (range 33.4-61.3), 43 (range 35.6-59.7) and 45 (range 30.2-63.2) years, respectively. All CC patients were staged in accordance to the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging standard, and the pathologic diagnosis was confirmed by pathological examination. No patient had received any chemotherapy or radiotherapy before blood collection. In addition, paired blood samples were withdrawn from all CC subjects 90 days after the surgery. Informed consent was obtained from all individual participants, and this study was approved by the Ethics Committees of Wuxi Xishan Hospital.

Clinical information of all CC patients was presented in Table 1, including age, tumor size, HPV infection, lymph node metastasis, differentiation and FIGO stage. Serum was isolated from blood specimen at 1600 g for 10 min with another 16,000 g for 10 min at 4°C, and then stored at −80°C until exosome extraction.

Exosome Isolation

The isolation of exosomes from serum was performed using the Total Exosome Isolation Kit (Invitrogen, CA, USA). The serum samples were thawed in a 25°C water bath and centrifuged at 2000 g for 30 minutes to remove cells and debris. Next, 100 μL Total Exosome Isolation reagent was added to 500 μL serum, and the mixture was vortexed until it was homogenous. After incubation at 4°C for 30 minutes, the sample was centrifuged at 10000 g for 10 minutes at room temperature. Exosome pellets were resuspended in phosphate-buffered saline (PBS).

RNA Extraction and qRT-PCR Detection

Total exosomal RNA was isolated with the miRNeasy Micro Kit (QIAGEN, Valencia, CA). The quality of total RNAs was assayed by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). In the RNA isolation step, 25 fmol of synthetic Caenorhabditis elegans cel-miR-39 (RiboBio, Guangzhou, the People's Republic of China) was added to each sample as a spike-in control. Then, cDNA was synthesized from total RNAs with a PrimeScriptTM RT reagent Kit (Takara, Tianjin, China). Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using LightCycler480 (Roche Applied Science, Basel, Switzerland) and Taqman Universal PCR Master Mix (Applied

Table 1. Association Between Clinicopathological Factors and Serum Exosomal lncRNA DLX6-AS1 Expression.

| | | LncRNA DLX6-AS1 | | |
Characteristics	Total	Low expression	High expression	*P*-value
Age				
<50	56	30	26	0.4536
≥50	58	27	31	
Tumor size (cm)				
<4	73	40	33	0.1719
≥4	41	17	24	
HPV infection				
No	17	12	5	0.0657
Yes	97	45	52	
Lymph node metastasis				
Negative	70	42	28	0.0071
Positive	44	15	29	
Differentiation				
Well/moderate	81	49	32	0.0004
Poor	33	8	25	
FIGO stage				
I-II	79	52	27	<0.0001
III-IV	35	5	30	

Biosystems). Each sample was analyzed in duplicate. The relative serum exosomal lncRNA DLX6-AS1 levels were normalized against cel-miR-39 using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers were as follows: DLX6-AS1 forward: 5'-AGTTTCTCTCTAGATTGCCTT-3', DLX6-AS1 reverse: 5'-ATTGACATGTTAGTGCCCTT-3', Cel-miR-39: 5'-UCACCGGGUGUAA-AUCAGCUUG-3'.

Western Blotting

Exosomes were separated by SDS-PAGE and proteins were transferred onto PVDF membrane. Membranes were blocked with 5% skim milk in TBST for 1 h at room temperature. Then they were probed with the following primary antibodies at 4°C overnight: CD63 (Abcam, Cambridge, UK; 1:800 diluted), CD9 (Abcam, 1:1000 diluted) and GM130 (Abcam, 1:500 diluted). Followed by incubation with HRP-conjugated secondary antibodies, the bands were visualized with ECL Detection Reagent.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc, CA). The difference of serum exosomal lncRNA DLX6-AS1 levels between different groups were determined by the Kruskal-Wallis test. Associations of serum exosomal lncRNA DLX6-AS1 with clinical parameters were examined by the Chi-square test. Receiver operating characteristic (ROC) curves and the area under the curves (AUC) were established to assess the predictive value of serum exosomal lncRNA DLX6-AS1 in CC. Overall survival (OS) and relapse free survival (RFS) rate were calculated with the
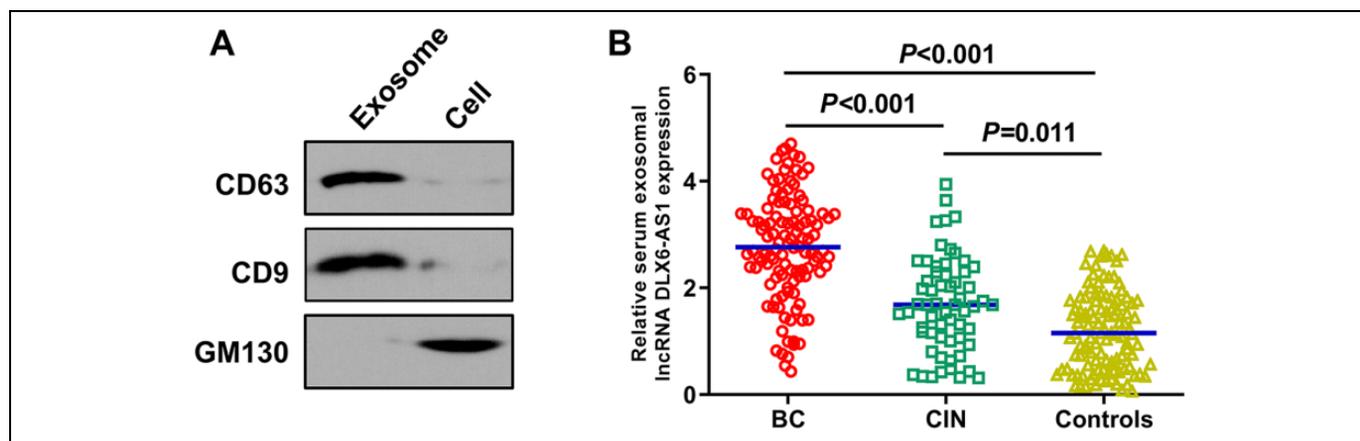
Figure 1. The serum derived exosomes were positive for CD63 and CD9, while negative for GM130 (A). serum exosomal lncRNA DLX6-AS1 levels were significantly higher in CC patients compared to CIN patients and healthy controls (B).
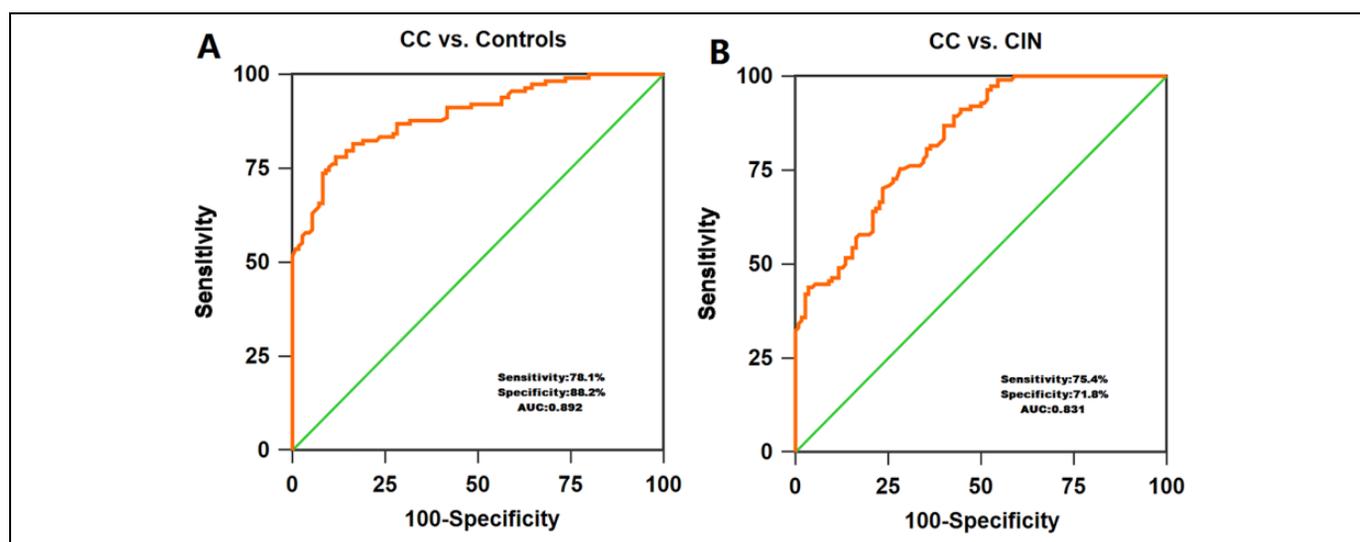
Figure 2. The diagnostic value of serum exosomal lncRNA DLX6-AS1 for the differentiation between CC patients and controls (A) aa well as CC patients and CIN patients (B).

Kaplan–Meier method and further compared with the log-rank test. Univariate and multivariate Cox proportional hazards models were used to analyze the correlations of prognostic factors and serum exosomal lncRNA DLX6-AS1 on OS. Results were considered statistically significant at $P < 0.05$.

Results

Expression Levels of Serum Exosomal lncRNA DLX6-AS1 in CC Patients

Our results showed that the exosomes isolated from the serum samples were positive for CD9 and CD63. However, no or weak signal was detected in the cellular samples. GM130 is a tethering factor associated with giantin in the cis-Golgi compartment commonly used as a negative control for exosome. It was only detected in the cells but not in serum derived

exosomes (Figure 1A). The expression levels of serum exosomal lncRNA DLX6-AS1 were measured in all participants using qRT-PCR. Serum exosomal lncRNA DLX6-AS1 levels were significantly higher in CC patients than in CIN and healthy controls (both $P < 0.001$, Figure 1). In addition, higher serum exosomal lncRNA DLX6-AS1 levels was observed in CIN patients compared with controls ($P = 0.011$, Figure 1B).

Diagnostic Values of Serum Exosomal lncRNA DLX6-AS1 for CC

The ROC analysis was performed to evaluate the diagnostic accuracy of serum exosomal lncRNA DLX6-AS1 as a potential tumor marker of CC. As shown in Figure 2A, serum exosomal lncRNA DLX6-AS1 discriminated CC patients from normal controls with an AUC of 0.892 (95% CI: 0.844-0.929; specificity: 88.2%; sensitivity: 78.1%). The AUC value of serum
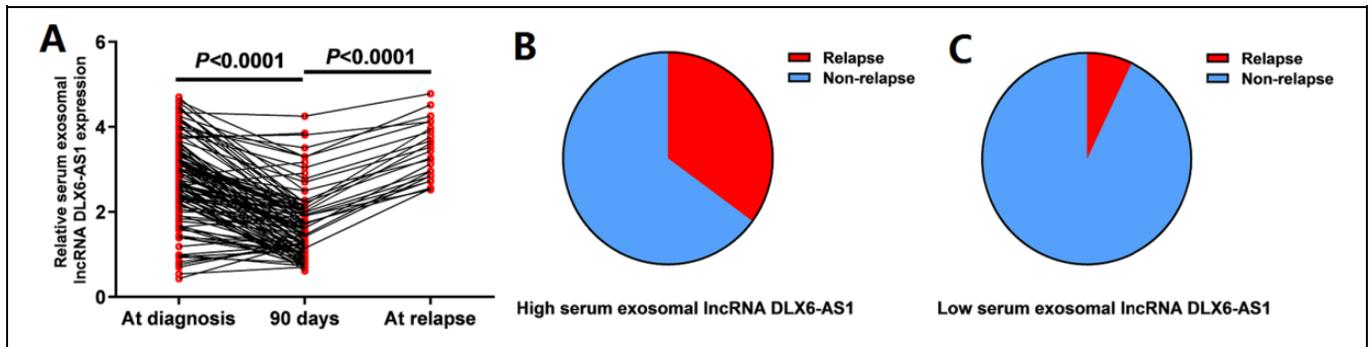
Figure 3. Serum exosomal lncRNA DLX6-AS1 levels in all CC patients were significantly decreased 90 days after treatment. Increased serum exosomal lncRNA DLX6-AS1 levels in relapsed patients was observed (A). Patients in high serum exosomal lncRNA DLX6-AS1 expression group were more prone to have a relapse (B, C).
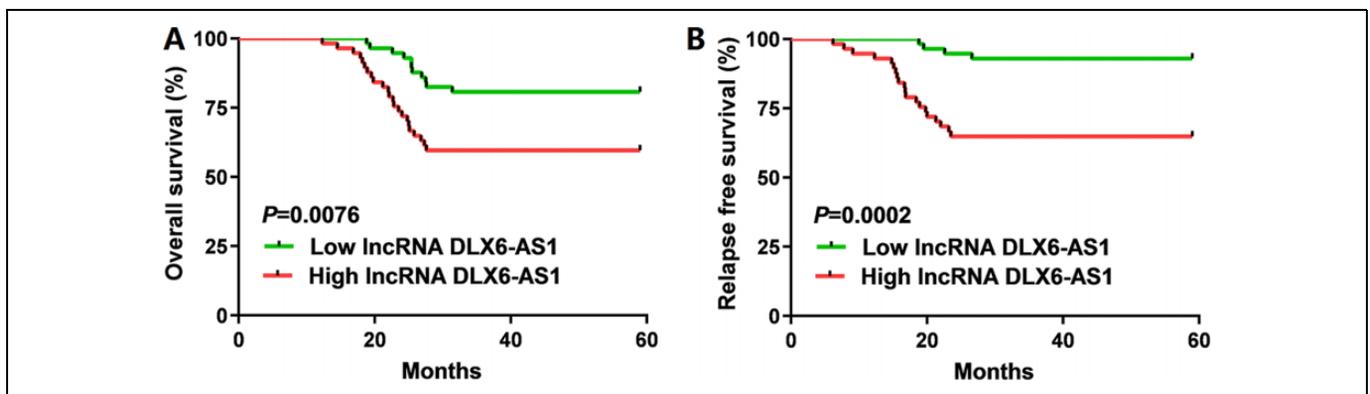
Figure 4. Kaplan-Meier survival curves of OS (A) and RFS (B) in CC patients.

exosomal lncRNA DLX6-AS1 for distinguishing cervical cancer from CIN was 0.831 (95% CI: 0.775-0.877). The sensitivity and specificity were 75.4% and 71.8%, respectively (Figure 2B).

Expression of Serum Exosomal lncRNA DLX6-AS1 and Its Relationship With Clinical Variables in CC Patients

Based on the median serum exosomal lncRNA DLX6-AS1 expression level, all 114 CC subjects were divided into high serum exosomal lncRNA DLX6-AS1 expression group (n = 57) and low serum exosomal lncRNA DLX6-AS1 expression group (n = 57). As shown in Table 1, high serum exosomal lncRNA DLX6-AS1 levels were positively correlated with lymph node metastasis ($P = 0.0071$), differentiation ($P = 0.0004$) and FIGO stage ($P < 0.0001$). However, no significant correlation was found between serum exosomal lncRNA DLX6-AS1 expression with other clinical features, such as age, tumor size, and HPV infection (all $P > 0.05$).

Dynamic Changes in Serum Exosomal lncRNA DLX6-AS1 of CC Patients

The changes in serum exosomal lncRNA DLX6-AS1 levels in pre- and post-operative blood samples from all CC patients

were evaluated. Compared to pre-operative blood samples, serum exosomal lncRNA DLX6-AS1 levels were significantly decreased in blood samples 90 days after treatment ($P < 0.0001$, Figure 3A). During the post-operative follow ups, 24 patients had a relapse. Then the blood samples were obtained from these relapsed cases, and the serum exosomal lncRNA DLX6-AS1 levels were detected. Compared to the blood samples 90 days after treatment, the elevation of serum exosomal lncRNA DLX6-AS1 at the timepoint of relapse was observed ($P < 0.0001$, Figure 3A). Interestingly, as shown in Figure 3B and Figure 3C, CC patients with high serum exosomal lncRNA DLX6-AS1 expression (20 out of 57) had a higher risk of tumor relapse than those with low serum exosomal lncRNA DLX6-AS1 expression (4 out of 57).

Upregulation of Serum Exosomal lncRNA DLX6-AS1 Conferred Poor Prognosis in CC Patients

Kaplan-Meier analyses were used to analyze the association between serum exosomal lncRNA DLX6-AS1 expression and the prognosis of CC patients. The patients in the high serum exosomal lncRNA DLX6-AS1 expression group had shorter OS ($P = 0.0076$, Figure 4A) and RFS ($P = 0.0002$, Figure 4B) compared with those in the low serum exosomal lncRNA DLX6-AS1 expression group. Furthermore, univariate Cox

Table 2. Univariate and Multivariate Analyses of the Prognostic Factors for OS in All CC Patients.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P	HR	95% CI	P
Age	1.21	0.633-2.745	0.312	-	-	-
Tumor size	1.45	0.722-3.176	0.243	-	-	-
HPV infection	1.82	0.843-3.482	0.134	-	-	
Lymph node metastasis	2.85	1.342-5.468	0.026	2.06	1.0153.214	1.137
Differentiation	3.13	1.563-5.873	0.015	2.35	1.2153.612	0.063
FIGO stage	4.17	2.332-7.545	<0.001	3.73	1.935-6.869	0.004
LncRNA DLX6-AS1	3.54	1.852-6.422	0.008	3.38	1.742-6.178	0.009

regression analysis showed that lymph node metastasis (HR = 2.85, 95% CI = 1.342-5.468, P = 0.026), differentiation (HR = 3.13, 95% CI = 1.563-5.873, P = 0.015), FIGO stage (HR = 4.17, 95% CI = 2.332-7.545, P < 0.001) and serum exosomal lncRNA DLX6-AS1 (HR = 3.54, 95% CI = 1.852-6.422, P = 0.008) were independent poor prognostic factors for OS of CC patients. In multivariate Cox regression analysis, FIGO stage (HR = 3.73, 95% CI = 1.935-6.869, P = 0.004) and serum exosomal lncRNA DLX6-AS1 (HR = 3.38, 95% CI = 1.742-6.178, P = 0.009) were independent prognostic markers for OS (Table 2).

Discussion

In this study, we quantified the serum exosomal lncRNA DLX6-AS1 levels in a cohort of 114 CC patients, 60 CIN patients and 110 healthy women. Serum exosomal lncRNA DLX6-AS1 levels were markedly higher in CC patients than those in CIN patients and controls. In addition, serum exosomal lncRNA DLX6-AS1 was sensitive for monitoring treatment response. Moreover, high serum exosomal lncRNA DLX6-AS1 expression was significantly correlated with shorter 5-year OS/RFS, and demonstrated as an independent prognostic factor in CC patients. Our findings were in line with previous studies. Xie et al found that lncRNA DLX6-AS1 overexpression occurred more frequently in CC cells. LncRNA DLX6-AS1 knockdown markedly inhibited CC cell proliferation, migration, and epithelial-mesenchymal transition via modulating the miR-16-5p/ARPP19 axis or degrading FUS. In addition, upregulation of lncRNA DLX6-AS1 greatly enhanced tumor growth in vivo.[21,22]

Besides CC, there have been many studies on the oncogenic role of lncRNA DLX6-AS1 in cancer. In bladder cancer, lncRNA DLX6-AS1 was dramatically upregulated in both cancerous tissues and cell lines. High lncRNA DLX6-AS1 expression was closely associated with lymph node metastasis and advanced TNM stage. LncRNA DLX6-AS1 upregulation significantly promoted cancer cell growth, invasion and migration in vitro, and lncRNA DLX6-AS1 inhibition suppressed the carcinogenesis in vitro and reduced tumor growth in vivo.[12] In breast cancer (BC), Zhao et al reported that lncRNA DLX6-AS1 was markedly upregulated in BC tissues compared to normal tissues. Downregulation of lncRNA DLX6-AS1

significantly inhibited BC cell viability, growth and stimulated cell apoptosis by inversely regulating miR-505-3p expression, and its upregulation significantly accelerated carcinogenesis and metastasis of BC through regulating FUS.[13,14] In esophageal squamous cell carcinoma (ESCC), lncRNA DLX6-AS1 expression was dramatically elevated in cancerous tissues compared to normal adjacent tissues. Ectopic lncRNA DLX6-AS1 expression was positively associated with more aggressive phenotypes, such as TNM stage, distant metastasis, and lymph node metastasis. LncRNA DLX6-AS1 knockdown exhibited the anti-tumorigenic activities in ESCC cells.[15,16] In non-small cell lung cancer (NSCLC), lncRNA DLX6-AS1 was markedly overexpressed both in tumor tissues and cell lines. Downregulation of lncRNA DLX6-AS1 significantly inhibited cancer cell proliferation, migration, invasion, and promoted apoptosis through upregulating miR-27b-3p or miR-144 expression.[17,18] Moreover, upregulation of lncRNA DLX6-AS1 was observed in gastric cancer (GC) tissues in comparison with matched normal tissues. GC patients with higher lncRNA DLX6-AS1 expression was associated with advanced clinical stage, lymph node metastasis, distant metastasis, and shorter survival. In vitro analysis showed lncRNA DLX6-AS1 inhibition markedly decreased GC cell proliferation, migration and invasion.[19,20] The above findings showed that serum exosomal lncRNA DLX6-AS1 functioned as an oncogene in different cancer types.

There are several limitations for our study. Firstly, the sample size of our patient cohort relatively small. Thus, the clinical application value of serum exosomal lncRNA DLX6-AS1 for CC needs further validation in larger independent cohorts. Secondly, using serum exosomal lncRNA DLX6-AS1 alone for the diagnosis and prognosis prediction of CC might lead to high false positive and negative results. Therefore, combining serum exosomal lncRNA DLX6-AS1 with other known tumor biomarkers and clinicopathological parameters is highly needed to accurately predict the clinical outcome of CC.

To the best of our knowledge, this is the first report demonstrating that serum exosomal lncRNA DLX6-AS1 overexpression was closely associated with aggressive clinical features and poor prognosis of CC. Collectively, serum exosomal lncRNA DLX6-AS1 might serve as a promising marker for the diagnosis of CC and an indicator of its progression.

Authors' Note

Xian-zhen Ding, Shi-qiang Zhang, Xiao-lan Deng, Jin-hu Qiang designed the study, conducted the experiments, analyzed the data and wrote the manuscript. All authors have read and approved the final version of the manuscript. Our study was approved by Ethics Committees of Wuxi Xishan Hospital (approval no. 201805046). All patients provided written informed consent prior to enrollment in the study.

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