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Circular RNA ciRS-7 correlates with advance disease and poor prognosis, and its down-regulation inhibits cells proliferation while induces cells apoptosis in non-small cell lung cancer

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Abstract. – OBJECTIVE: This study aimed to assess the association of circular RNA (circRNA) ciRS-7 expression with clinicopathological characteristics and prognosis of non-small cell lung cancer (NSCLC) patients, and to investigate its effect on cells proliferation as well as apoptosis in NSCLC.

PATIENTS AND METHODS: 132 patients with primary NSCLC who received surgical resection were recruited in this retrospective study. All patients' tumor tissue and paired adjacent tissue were collected for circRNA ciRS-7 expression detection by RT-qPCR. Disease-free survival (DFS) and overall survival (OS) were calculated. CCK-8 and Annexin-V/propidium iodide (AV/PI) assays were performed to detect cells proliferation and apoptosis in A549 cells after circRNA ciRS-7 inhibition plasmid transfection.

RESULTS: CircRNA ciRS-7 expression in tumor tissue was elevated compared to paired adjacent tissue, and positively correlated with tumor size, lymph node metastasis and tumor node metastasis (TNM) stages. K-M curves illustrated that circRNA ciRS-7 high expression was correlated with both shorter DFS and OS, and multivariate Cox's proportional hazards regression analysis showed that circRNA ciRS-7 high expression was an independent factor for predicting unfavorable DFS and OS. Cells methods revealed that circRNA ciRS-7 expression was elevated in NSCLC cell lines (A549, PC9, NCI-H1299 and NCI-H1650) compared to normal lung epithelial cells (DEAS-2B), and the inhibition of circRNA ciRS-7 expression reduced cells proliferation and promoted cells apoptosis in A549 cells.

CONCLUSIONS: CircRNA ciRS-7 overexpression is associated with advanced disease and poor prognosis in NSCLC patients, and the down-regulation of circRNA ciRS-7 inhibits tumor cells proliferation as well as improves cells apoptosis.

Key Words

Circular RNA ciRS-7, Non-small cell lung cancer (NSCLC), Prognosis, Proliferation, Apoptosis.

Introduction

Lung cancer, one of the most commonly diagnosed cancers worldwide, is associated with a high rate of mortality[1]. 2015 Global Cancer Statistics Report discloses that nearly 1.8 million new cases of lung cancer and 1.6 million deaths caused by lung cancer were discovered in 2012, accounting for approximately 13% of the total number of diagnosed cancers and 19% of total cancer deaths worldwide[2]. Lung cancer is comprised of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) according to histological type, among which NSCLC accounts for nearly 85% of all lung cancers[3]. Although great improvements have been achieved in NSCLC such as early diagnosis, individual treatment, novel drugs and medical care, the prognosis is still unsatisfactory due to the high occurrence of relapse and metastasis[2,4]. Therefore, for the purpose of improving patients' prognosis, it's essential to investigate the underlying mechanism in NSCLC development and progression as well as explore the novel treatment target. Circular RNA (circRNA), a kind of noncoding RNA molecule with a circular configuration, is different from traditional linear RNA with 5' or 3' terminus[5]. Due to its special closed-loop structure, which is unaffected by excision enzyme, its expression is more stable and not easily degraded in cells[5]. According to previous reports[6-9], circRNAs is involved in the pathogenesis of a variety of cancers by regulating cancer cells proliferation, migration and invasion by sponging multiple miRNAs. CircRNA ciRS-7, also known as cerebellar-degeneration-related protein 1 antisense RNA (CDR1as), is an oncogene which contributes to cancer development in gastric cancer (GC), colorectal carcinoma (CRC), cholangiocarcinoma (CCA) and

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hepatocellular carcinoma (HCC)[10-14]. However, the correlation of circRNA ciRS-7 with prognosis in NSCLC patients and its effects on NSCLC cells are seldom reported. Thus, the aim of this study was to evaluate the correlation of circRNA ciRS-7 expression with clinicopathological features and prognosis in NSCLC patients, and to investigate its effects on cells proliferation as well as apoptosis in NSCLC cells.

Patients and Methods

Patients

132 patients who received surgical resection for primary NSCLC at the Department of Respiratory Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University from July 2009 to June 2012 were enrolled in this retrospective study. Inclusion criteria were as follows: 1) Patients who were diagnosed with primary NSCLC based on clinical features, radiological examination and pathological confirmation; 2) Age $\geq$ 18 years; 3) Fresh tumor tissue and paired adjacent tissue were obtained from surgical resection, snap-freezed in liquid nitrogen and stored at -80°C in the hospital storage room. Exclusion criteria were as follows: 1) Baseline data and follow-up information were insufficient; 2) Received neoadjuvant therapy before surgery; 3) Had a history of other solid tumors, hematologic malignancy, serious infection, severe hepatic or renal dysfunction. The present study has been approved by the Ethics Committee of Shanghai Chest Hospital, Shanghai Jiao Tong University and written informed consents were obtained from all patients. The number of Ethics Committee approval was KS1725.

Information Collection and Follow Up

Demographic, pathological and clinical information of patients was obtained from the hospital electronic medical record system, including age, gender, pathological grade, tumor size, lymph node metastasis, distant metastasis and tumor node metastasis (TNM) stage (according to the 7th edition of the American Joint Committee on Cancer (ATCC) cancer staging manual). The median value of follow-up duration was 46 months (range 1-94 months), and the last follow-up month was in June 2017. For further analyses, OS and disease-free survival (DFS) were calculated from the surgery to time of death from any cause and time of documented local or distant recurrence of initial cancer, respectively.

Tissue Samples and CircRNA ciRS-7 Determination

All patients' tumor tissue samples and paired adjacent tissue samples were obtained from the hospital storage room, which were resected during the surgery, snap-freezed in liquid nitrogen, and stored at -80°. Tissue samples were removed from the liquid nitrogen tank and were cut as much as possible with scissors. Next, 200 μl of TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) was added, and the tissue pieces were ground with a grinding rod. Finally, 800 μl of TRIzol Reagents was added and the total RNA was extracted according to the manufacturer's instructions. Then, the total RNA was used to detect the circRNA ciRS-7 expression by quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR). (Detailed methods were described in subsection "RT-qPCR assay").

Cells Culture

A-549, NCI-H1650, NCI-H1299 and PC9 NCSLC cell lines as well as DEAS-2B cell line (normal lung epithelial cells) were purchased from Shanghai Institutes for Biological Science (Shanghai, China) or kindly given from Shanghai Bio-Technology (Shanghai, China). After cells resuscitation, A-549 cells were cultured in F12K medium (Gibco, Grand Island, NY, USA), NCI-H1650 and NCI-H1299 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA), PC9 and DEAS-2B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) and Minimum Essential Medium (MEM; Gibco, Grand Island, NY, USA), respectively. 10% (V/V) fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Corning, Lowell, NY, USA) were added in all the cell lines. All cell lines were incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C. After the cells grown into a compact monolayer in a culture bottle, they were passaged. The total RNA was extracted from all the cell lines by TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and the expression of circRNA ciRS-7 in all cell lines was detected by RT-qPCR. (Detailed methods were described in subsection "RT-qPCR assay").

Cells Transfection

A549 cells were transfected with circRNA ciRS-7 inhibitor plasmid (Shanghai Gemma, Shanghai, China) as circRNA ciRS-7 inhibitor

group and the blank plasmid (Shanghai Gemma, Shanghai, China) were transfected in A549 cells as NC inhibitor group. RT-qPCR assay was applied to detect the expression of circRNA ciRS-7 in the two groups at 24 h after transfection. Cells proliferation was measured by Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) at 0 h, 24 h, 48 h and 72 h after transfection. Additionally, after 72 h of transfection, apoptotic cell rate was quantified by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) by the fluorescein isothiocyanate (FITC) Annexin-V (AV) apoptosis detection kit with propidium iodide (PI; Invitrogen, Carlsbad, CA, USA).

RT-qPCR Assay

CircRNA ciRS-7 expression was evaluated by RT-qPCR. First, after the total RNA in tissue or cells was extracted, 1 μg of total RNA was reversed-transcribed with random primer using iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Then, cDNA products were subscribed to qPCR with SYBR Green kit (KAPA, Wilmington, MA, USA). The PCR amplification was performed as follows: initial heat activation at 95°C for 5 min, followed by 40 cycles of amplification, each cycle includes denaturation at 95°C for 5 s, annealing and extension at 61°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. All experiments were triplicated. The qPCR results were calculated with the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: circRNA ciRS-7 forward: 5'-TCAACTGGCTCAATATCCATGTC-3', reverse: 5'-ACCTTGACACAGGTGCCAT-3'; GAPDH forward: 5'-TGACCACAGTCCATGCCATCAC-3', reverse: 5'-GCCTGCTTCACCACCTTCTTGA-3'.

CCK8 Assay

10 ul CCK-8 and 90 ul medium were added to each plate of A549 cells, and the A549 cells were incubated under 5% CO_2 at 37°C. Optical density (OD) value was determined by a microplate reader (BioTek, Winooski, VT, USA) at 0 h, 24 h, 48 h, and 72 h after transfection. The OD values were collected and analyzed to create a proliferation curve.

AV/PI Assay

A549 cells were digested by pancreatin and subsequently washed by Phosphate-Buffered Solution (PBS). After the cells were suspended in 100 ul Blinding Buffer, 2 ul Annexin V (AV; Invitrogen, Carlsbad, CA, USA) was added and the cells which were stood in the dark for 15 min on the ice. Subsequently, 1 μl PI (Invitrogen, Carlsbad, CA, USA) was added just before flow cytometry assay and the results were analyzed by flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical Analysis

Statistical analysis was performed by SPSS Software 22.0 (IBM, Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). Data were presented as mean ± standard deviation, median (25^{th}-75^{th}) or count (%). The comparison between the two groups was determined by *t*-test, Wilcoxon rank sum test or Wilcoxon signed-rank sum test; the comparison among three or above groups was detected by Kruskal-Wallis H rank sum test. The association of circRNA ciRS-7 expression with DFS and OS were evaluated by Kaplan-Meier (K-M) curves and compared by log-rank test. Univariate Cox's proportional hazards regression was conducted to investigate the predicting values of baseline factors for DFS and OS, and all factors with $p \leq 0.1$ were further included in the multivariate Cox's proportional hazards regression analysis. $p \leq 0.05$ was considered significant.

Results

Characteristics of NSCLC Patients

132 NSCLC patients with a mean age of 62.04 ± 10.11 years were enrolled in this study, including 72 males and 60 females. The numbers of NSCLC patients in Pathological G1, G2, and G3 were 20 (15.2%), 83 (62.9%) and 29 (22.0%), respectively. In addition, there were 55 patients (41.7%) with tumor size >5 cm, 49 patients (37.1%) with lymph node metastasis positive and 1 patient (0.8%) with distant metastases positive. The other clinical and pathological characteristics of NSCLC patients were shown in Table I.

CircRNA CiRS-7 Expression in Tumor Tissue and Paired Adjacent Tissue

Wilcoxon signed-rank sum test was used to analyze circRNA ciRS-7 expression in cancer tissue and paired adjacent tissue. As shown in Figure 1, the expression level of circRNA ciRS-7 in tumor tissue (2.974 (1.441-5.060)) was higher than paired adjacent tissue (1.041 (0.529-1.731)) ($p<0.001$).

Table I. Patients' characteristics.

	NSCLC patients (N=132)
Age (years)	62.04±10.11
Gender (male/female)	72/60
Pathological grade	
G1 (n/%)	20 (15.2)
G2 (n/%)	83 (62.9)
G3 (n/%)	29 (22.0)
Tumor size	
≤5 cm (n/%)	77 (58.3)
>5 cm (n/%)	55 (41.7)
Lymph node metastasis	
Negative (n/%)	83 (62.9)
Positive (n/%)	49 (37.1)
Distant metastases	
Negative (n/%)	131 (99.8)
Positive (n/%)	1 (0.8)
TNM stage	
I	45 (34.1)
II	44 (33.3)
III	42 (31.8)
IV	1 (0.8)

Data were presented as mean ± standard deviation or count (%). NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

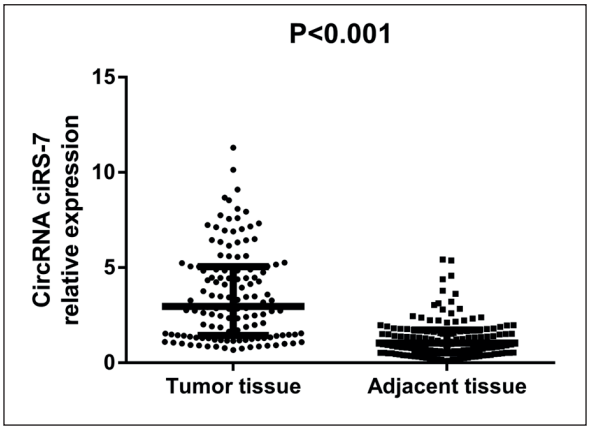

Figure 1. The circRNA ciRS-7 expression in NSCLC tumor tissue and adjacent tissue. The expression of circRNA ciRS-7 was increased in NSCLC tumor tissue compared to adjacent tissue. The comparison between the two groups was determined by Wilcoxon signed-rank sum test. $p<0.05$ was considered significant. NSCLC, non-small cell lung cancer.

The Correlation of CircRNA CiRS-7 Expression With Clinicopathologic Features

Compared to patients with tumor size ≤5 cm (2.552 (1.301-4.235)), circRNA ciRS-7 expression in patients with tumor size >5 cm was increased (4.461 (2.342-6.329)) (p=0.001). Besides, circRNA ciRS-7 expression in patients with positive lymph node metastasis (4.440 (2.595-5.906)) was higher than patients with negative lymph node metastasis (2.475 (1.307-4.753)) (p=0.003). The circRNA ciRS-7 expression in TNM stage I, II, III and IV was 2.475 (1.204-3.529), 3.386 (1.628-5.505), 4.368 (1.854-5.720) and 1.552, respectively. Moreover, the circRNA ciRS-7 overexpression was correlated with the poor TNM stage (p=0.024). However, circRNA ciRS-7 expression was not associated with age (p=0.823), gender (p=0.197), pathological grade (p=0.105) or distant metastases (p=0.606) (Table II).

DFS and OS Comparison Between CircRNA CiRS-7 Low and High Expression Patients

NSCLC patients were divided into circRNA ciRS-7 high expression and low expression groups by circRNA ciRS-7 expression median value as cut off point. The Kaplan-Meier curve disclosed that NSCLC patients with circRNA ciRS-7 high expression in tissue had a shorter DFS (p<0.001, Figure 2A) and OS (p=0.001, Figure 2B) compared to low expression.

Factors Affecting DFS

Univariate Cox's proportional hazards regression model analysis was applied to analyze circRNA ciRS-7 expression and clinical pathological characteristics in predicting DFS. The results were shown in Table III, circRNA ciRS-7 high expression (p<0.001), gender (male) (p=0.045), higher pathological grade (p=0.003) and lymph node metastasis positive (p=0.014) were proved to be associated with worse DFS. All the factors with p<0.1 were further detected by multivariate Cox's proportional hazards regression analysis, which suggested that circRNA ciRS-7 high expression (p=0.005) was an independent factor for predicting shorter DFS, as well as higher pathological grade (p=0.041).

Factors Affecting OS

Univariate Cox's proportional hazards regression model analysis was performed to analyze circRNA ciRS-7 expression and clinical pathological characteristics on predicting OS and the results revealed that circRNA ciRS-7 high expression (p=0.001), high pathological grade (p=0.003) and lymph node metastasis positive (p=0.001) were correlated with worse OS (Table IV). All the factors with $p \leq 0.1$ were subsequently analyzed by multivariate Cox's proportional

Table II. CircRNA ciRS-7 expression in subgroups.

	CircRNA ciRS-7 relative expression	*p*-value
Age		0.823
≤60 years	2.897 (1.431-5.447)	
>60 years	3.226 (1.441-4.907)	
Gender		0.197
Male	3.681 (1.458-5.226)	
Female	2.754 (1.360-4.866)	
Pathological grade		0.105
G1	2.221 (1.291-3.102)	
G2	3.549 (1.492-5.069)	
G3	3.439 (1.442-5.258)	
Tumor size		**0.001**
≤5 cm	2.552 (1.301-4.235)	
>5 cm	4.461 (2.342-6.329)	
Lymph node metastasis		**0.003**
Negative	2.475 (1.307-4.753)	
Positive	4.440 (2.595-5.906)	
Distant metastases		**0.606**
Negative	2.948 (1.436-5.062)	
Positive	1.552	
TNM stage		0.024
I	2.475 (1.204-3.529)	
II	3.386 (1.628-5.505)	
III	4.368 (1.854-5.720)	
IV	1.552	

Data were presented as median (1/4-3/4 quartile). The comparison was determined by Wilcoxon ran sum test or Kruskal-Wallis H rank sum test. p<0.05 was considered significant. TNM, tumor node metastasis.

hazards regression, which disclosed that circRNA ciRS-7 high expression (p=0.042), high pathological grade (p=0.004) and lymph node metastasis positive (p=0.004) remained independent factors for predicting shorter OS (Table IV).

CircRNA CiRS-7 Expression in NSCLC Cell Lines

The comparison of circRNA ciRS-7 expression between NSCLC cell lines, including A-549, PC9, NCI-H1299, NCI-H1650 and DEAS-2B cell line was determined by the *t*-test. The results showed that circRNA ciRS-7 expression was elevated in A-549 (p<0.001), PC9 (p<0.001), NCI-H1299 (p<0.01) and NCI-H1650 (p<0.001) cell lines compared to DEAS-2B cell line, and the elevation was significant in A-549 cells (Figure 3).

CircRNA CiRS-7 Expression After Transfection

After the transfection for 24 h, the expression of circRNA ciRS-7 was detected by RT-qPCR and the results showed that circRNA ciRS-7 was decreased in the circRNA ciRS-7 inhibitor group compared to the NC inhibitor group (p<0.001, Figure 4A).

Downregulation of CircRNA CiRS-7 Inhibited Proliferation and Promoted Apoptosis of A549 Cells

The results of cells proliferation were presented in Figure 4B, at 48 h (p<0.05) and 72 h (p<0.01) after transfection; the OD value in the circRNA ciRS-7 inhibitor group was lower than that in the NC inhibitor group. Additionally, 72 h after the transfection, the total cells apoptosis rate in the circRNA ciRS-7 inhibitor group was higher than that in the NC inhibitor group (p<0.01, Figure 4C, D). The aforementioned results suggested that the inhibition of circRNA ciRS-7 expression suppressed cells proliferation and promoted cells apoptosis of A549 cells.

Discussion

The results of our study showed that (1) circRNA ciRS-7 expression was increased in tumor tissue compared to paired adjacent tissue and was positively correlated with tumor size, lymph node metastasis and TNM stage. (2) CircRNA ciRS-7 high expression is associated with worse DFS and OS, and multivariate Cox's analysis showed that

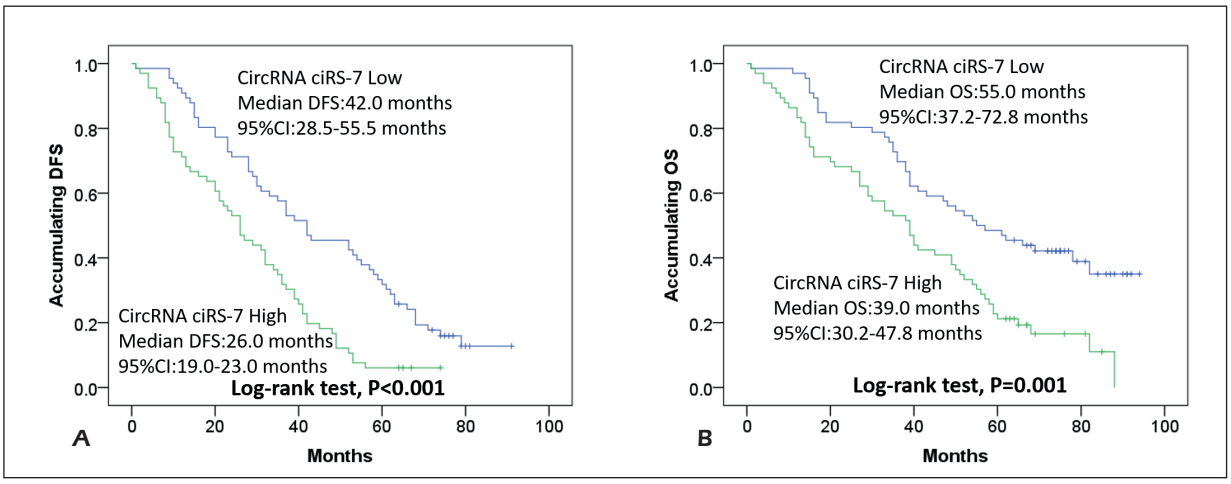

Figure 2. The association of circRNA ciRS-7 expression with DFS and OS in NSCLC patients. NSCLC patients with high tumor tissue expression of circRNA ciRS-7 had poor DFS **A**, and OS **B**, compared with patients with low tumor tissue circRNA ciRS-7 expression. K-M curve and log-rank test were performed to evaluate the DFS and OS between patients with high and low circRNA ciRS-7 expression. $p<0.05$ was considered statistically significant. DFS, disease-free survival; OS, overall survival; NSCLC, non-small cell lung cancer; K-M, Kaplan-Meier.

circRNA ciRS-7 high expression was an independent predictive factor for both shorter DFS and OS. (3) *In vitro*, circRNA ciRS-7 expression was elevated in NSCLC cell lines compared to normal lung epithelial cells, and the down-regulation of circRNA ciRS-7 expression reduced cells proliferation and promoted cells apoptosis in A549 cells. CircRNAs, as a novel type of non-coding RNAs, is a class of RNAs with closed loop structure existing in a large number of eukaryotic transcriptomes[15]. The stability of circRNAs is better than linear RNAs, which might result from the insensitivity of circRNAs to nuclease, a protein that is capable of degrading the nucleotides by identifying the end of linear RNAs; thereby circRNA maintains high abundance and is more easily detected[16,17]. Since circRNAs are unable to encode proteins, they cannot perform biological functions by coding function proteins[18]. However, similar to the long non-coding RNAs (lncRNA) and messenger RNA (mRNA), circRNAs are rich in miRNA binding sites, which enable circRNAs

Table III. Factors affecting DFS.

	Univariate Cox's proportional hazard regression				Multivariate Cox's proportional hazard regression			
	p-value	HR	95% CI		*p*-value	HR	95% CI	
			Lower	Higher			Lower	Higher
CircRNA ciRS-7 High expression	**<0.001**	2.126	1.455	3.106	**0.005**	1.774	1.185	2.655
Age >60 years	0.210	1.268	0.875	1.837	–	–	–	–
Gender (male vs. female)	**0.045**	1.459	1.008	2.110	0.523	1.137	0.766	1.688
Pathological grade	**0.003**	1.534	1.152	2.042	**0.041**	1.398	1.014	1.928
Tumor size >5 cm	0.443	1.155	0.799	1.669	––	–	–	–
Lymph node metastasis (positive)	**0.014**	1.609	1.101	2.350	0.116	1.374	0.925	2.042
Distant metastases (positive)	0.207	3.603	0.492	26.374	–	–	–	–
TNM stage	0.205	1.152	0.926	1.433	–	–	–	–

Data were presented as p-value, HR (hazard ratio), 95%CI (confidence interval). Factors affecting DFS (disease-free survival) were determined by univariate Cox's proportional hazards regression model analysis, while all factors with p-value no more than 0.1 were further detected by multivariate Cox's proportional hazards regression analysis. $p<0.05$ was considered significant. Pathological grade was scored as 1-G1, 2-G2, 3-G3. TNM stage was scored as 1-I, 2-II, 3-III, 4-IV. TNM, tumor node metastasis.

Table IV. Factors affecting OS.

	Univariate Cox's proportional hazard regression				Multivariate Cox's proportional hazard regression			
	p-value	HR	95% CI		*p*-value	HR	95% CI	
			Lower	Higher			Lower	Higher
CircRNA ciRS-7 High expression	**0.001**	2.028	1.345	3.060	**0.042**	1.575	1.016	2.440
Age >60 years	0.696	1.084	0.724	1.622	–	–	–	–
Gender (male vs. female)	0.210	1.296	0.864	1.944	–	–	–	–
Pathological grade	**0.003**	1.631	1.184	2.246	**0.004**	1.678	1.176	2.396
Tumor size >5 cm	0.125	1.371	0.916	2.051	–	–	–	–
Lymph node metastasis (positive)	**0.001**	2.050	1.362	3.087	**0.004**	2.380	1.316	4.303
Distant metastases (positive)	0.126	4.758	0.645	35.121	–	–	–	–
TNM stage	0.082	1.242	0.973	1.587	**0.148**	0.775	0.548	1.095

Data were presented as p-value, HR (hazard ratio), 95% CI (confidence interval). OS (disease-free survival) were determined by univariate Cox's proportional hazards regression model analysis, while all factors with p-value no more than 0.1 were further detected by multivariate Cox's proportional hazards regression analysis. $p<0.05$ was considered significant. Pathological grade was scored as 1-G1, 2-G2, 3-G3. TNM stage was scored as 1-I, 2-II, 3-III, 4-IV. TNM, tumor node metastasis.

to regulate mRNAs by sponging miRNAs[15,19]. A growing number of researches suggest that circRNAs are closely involved in tumor progression and prognosis. For example, hsa_circ_000984, hsa_circ_0018289 and hsa_circ_000322 are associated with the progression of colon cancer, cervical cancer and bladder cancer, respectively[6,20,21]. A study of lung adenocarcinoma (LAC) shows that circRNA circ_0013958 is elevated in tumor tissue, cells and plasma, and the tumor tissue circRNA circ_0013958 expression level positively correlates with TNM stage and lymphatic metastasis[22]. In addition, the expression of circRNA circ_100876 is increased in NSCLC tumor tissue compared to adjacent tissue, which is also positively correlated with TNM stage and lymphatic metastasis. Moreover, patients with circ_100876 high expression have poor OS[23]. Thus, the above studies show that circRNAs might play essential roles in the development and prognosis of various cancers, including NSCLC.

CircRNA ciRS-7, located on human chromosome Xq27.1, is reported to be an oncogene in a variety of cancers[10-13]. Yu et al[13] illustrate that circRNA ciRS-7 expression is increased in HCC tissue compared with adjacent non-tumor tissue, and the up-regulation of circRNA ciRS-7 inhibits miR-7 expression to promote tumor cells proliferation and invasion in HCC. Tang et al[11] also observed that circRNA ciRS-7 is overexpressed in tumor tissue compared with adjacent tissue and normal mucosa in CRC; it is positively associated with tumor size, T stage and lymph node metastasis, and predicts poor OS. Additionally, they illustrate that the downregulation of circRNA ciRS-7 suppresses CRC cells proliferation, invasion and increases microRNA-7 (miR-7) expression[11]. Furthermore, circRNA ciRS-7 is up-regulated in GC tumor tissue compared to adjacent tissue and is an independent risk factor for shorter OS[10]. Besides, *in vitro* and *in vivo* experiments exhibit that the upregulation of circRNA ciRS-7 blocks the miR-7-induced tumor suppression in GC cell lines (MGC-803 and HGC-27) and leads to a

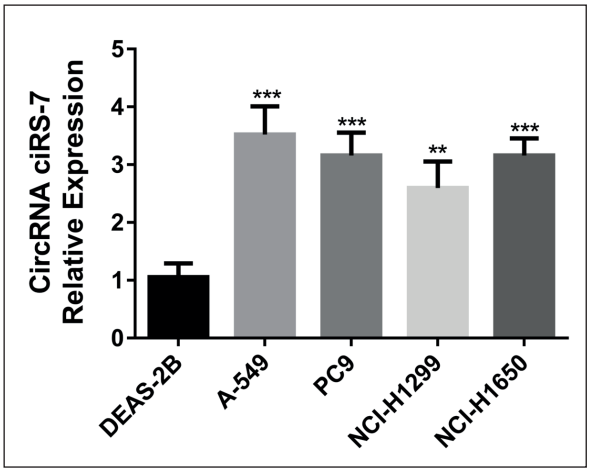

Figure 3. The expression of circRNA ciRS-7 in cell lines. Compared with DEAS-2B cell line, circRNA ciRS-7 expression was increased in A-549, PC9, NCI-H1299 and NCI-H1650 cell lines. The comparison between the two groups was determined by *t*-test. $p<0.05$ was considered significant. *$p<0.01$, **$p<0.01$, ***$p<0.001$.

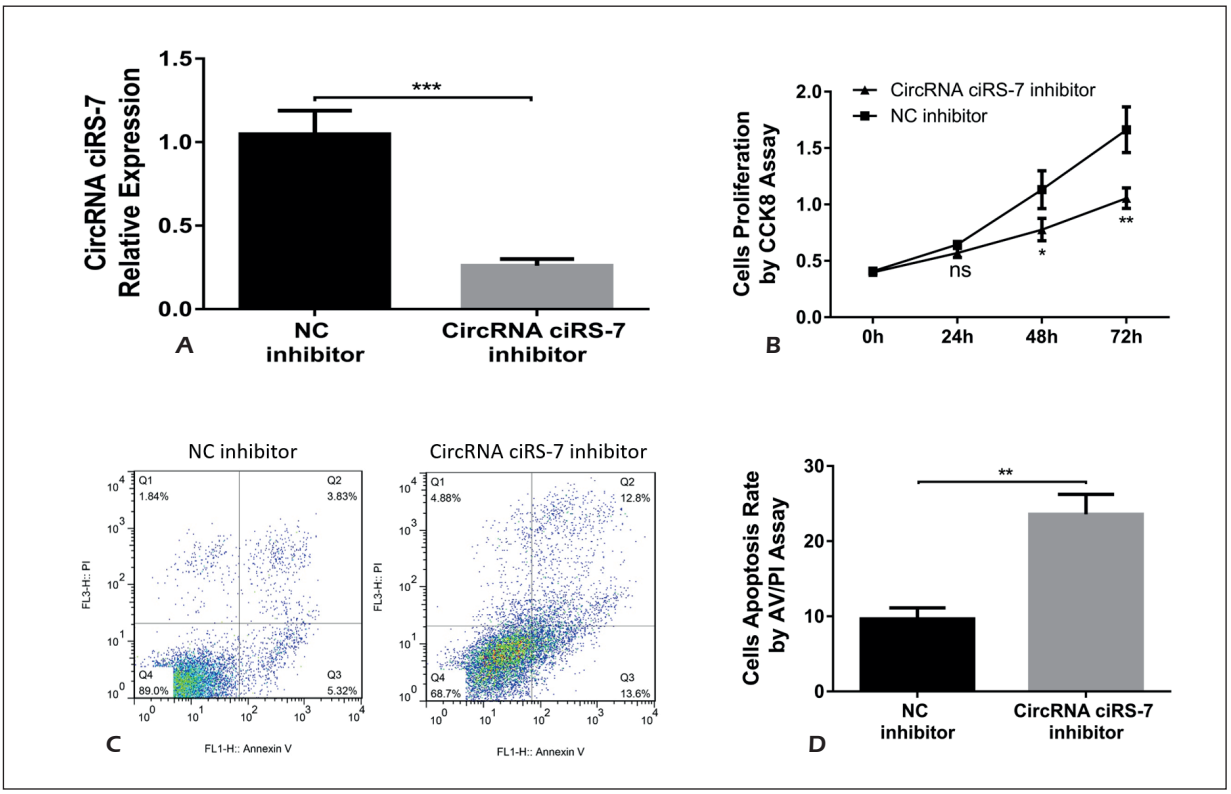

Figure 4. The effect of circRNA ciRS-7 down-regulation on proliferation and apoptosis in A549 cells. 24 h after transfection, the expression of circRNA ciRS-7 was blocked in circRNA ciRS-7 inhibitor group compared with the NC inhibitor group **A**, Compared with the NC inhibitor group, the cells proliferation in circRNA ciRS-7inhibitor group was decreased at 48 h and 72 h after transfection **B**, and higher cells apoptosis rate was found in circRNAs ciRS-7 inhibitor group compared with NC inhibitor group at 72 h after transfection **C-D.** The comparison between the two groups was determined by *t*-test. $p<0.05$ was considered statistically significant. *$p<0.01$, **$p<0.01$, ***$p<0.001$.

more aggressive oncogenic phenotype via antagonizing miR-7-mediated phosphatase and tensin homolog (PTEN)/phosphoinositide-3-kinase regulatory subunit 1 (PI3K)/serine/threonine kinase 1 (AKT) pathway in nude mice[10]. These previous studies suggest that circRNA ciRS-7 functions as an oncogene in multiple cancers, but the role of circRNAs ciRS-7 in NSCLC has not been validated yet. Therefore, the expression of circRNA ciRS-7 in tumor tissue and its association with clinicopathological characteristics and prognosis in NSCLC patients have been evaluated in our study. Partially in line with the previous studies in other cancers, our results elucidated that ciRS-7 expression was increased in tumor tissue compared with paired adjacent tissue, and the up-regulation of ciRS-7 correlated with tumor size, lymph node metastasis and TNM stages in NSCLC patients. Additionally, K-M curve revealed that NSCLC patients with circRNA ciRS-7 high tumor tissue expression presented worse DFS and OS, and multivariate Cox's proportional hazards regression model analysis indicated that circRNA ciRS-7 high tumor tissue expression independently predicts shorter DFS and OS. These results are probably derived from the fact that circRNA ciRS-7 promotes the cells proliferation, migration and invasion while inhibits cells apoptosis of tumor cells by regulating the several miRNAs and cancer-related genes or pathways. It is highly expressed in tumor tissues and positively correlates with advanced disease, including larger tumor size, lymph node metastasis and higher TNM stages, and the advanced disease results in the unfavorable prognosis[10,11,13,24]. To further investigate the effects of circRNA ciRS-7 on cells proliferation and apoptosis in NSCLC, the expression of circRNA ciRS-7 was down-regulated by siRNA-mediated inhibition *in vitro*, and the results disclosed that after repression of circRNA

ciRS-7 expression, the cells proliferation was declined, and cells apoptosis was increased in A549 cells. Scholars[10,11,13] indicated that circRNA ciRS-7 contributes to the development of various carcinomas *via* modulating cells proliferation and invasion by regulating the downstream target genes of miR-7. CircRNA ciRS-7 overexpression was observed to enhance the progression of GC by antagonizing the miR-7-mediated the PTEN/PI3K/AKT pathway or by inhibiting the expression of miR-7 and activating epidermal growth factor receptor (EGFR)/serine/threonine kinase (RAF1) oncogenes[10,25]. Furthermore, the inhibition of circRNA ciRS-7 expression promotes miR-7 overexpression and inhibits CRC cells proliferation and invasion[11]. These data indicate that circRNA ciRS-7 presents potential for treatment target in NSCLC.

Conclusions

We found that circRNA ciRS-7 overexpression correlated with advanced disease and poor prognosis in NSCLC patients, and the down-regulation of circRNA ciRS-7 inhibited tumor cells proliferation as well as improved cells apoptosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ, Jr., Wu YL, Paz-Ares L. Lung cancer: current therapies and new targeted treatments. Lancet 2017; 389: 299-311.
2) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics 2012. CA Cancer J Clin 2015; 65: 87-108.
3) Chan BA, Coward JI. Chemotherapy advances in small-cell lung cancer. J Thorac Dis 2013; 5 Suppl 5: S565-578.
4) Reck M, Heigener DF, Mok T, Soria JC, Rabe KF. Management of non-small-cell lung cancer: recent developments. Lancet 2013; 382: 709-719.
5) Chen LL. The biogenesis and emerging roles of circular RNAs. Nat Rev Mol Cell Biol 2016; 17: 205-211.
6) Xu ZQ, Yang MG, Liu HJ, Su CQ. Circular RNA hsa_circ_0003221 (circPTK2) promotes the proliferation and migration of bladder cancer cells. J Cell Biochem 2018; 119: 3317-3325.
7) Tang YY, Zhao P, Zou TN, Duan JJ, Zhi R, Yang SY, Yang DC, Wang XL. Circular RNA hsa_circ_0001982 promotes breast cancer cell carcinogenesis through decreasing miR-143. DNA Cell Biol 2017; 36: 901-908.
8) Zhang J, Liu H, Hou L, Wang G, Zhang R, Huang Y, Chen X, Zhu J. Circular RNA_LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging miR-424-5p and regulating LATS1 expression. Mol Cancer 2017; 16: 151.
9) Zhang R, Xu J, Zhao J, Wang X. Silencing of hsa_circ_0007534 suppresses proliferation and induces apoptosis in colorectal cancer cells. Eur Rev Med Pharmacol Sci 2018; 22: 118-126.
10) Pan H, Li T, Jiang Y, Pan C, Ding Y, Huang Z, Yu H, Kong D. Overexpression of circular RNA ciRS-7 abrogates the tumor suppressive effect of miR-7 on gastric cancer via PTEN/PI3K/AKT signaling pathway. J Cell Biochem 2018; 119: 440-446.
11) Tang W, Ji M, He G, Yang L, Niu Z, Jian M, Wei Y, Ren L, Xu J. Silencing CDR1as inhibits colorectal cancer progression through regulating microRNA-7. Onco Targets Ther 2017; 10: 2045-2056.
12) Xu L, Zhang M, Zheng X, Yi P, Lan C, Xu M. The circular RNA ciRS-7 (Cdr1as) acts as a risk factor of hepatic microvascular invasion in hepatocellular carcinoma. J Cancer Res Clin Oncol 2017; 143: 17-27.
13) Yu L, Gong X, Sun L, Zhou Q, Lu B, Zhu L. The circular RNA Cdr1as act as an oncogene in hepatocellular carcinoma through targeting miR-7 expression. PLoS One 2016; 11: e0158347.
14) Jiang XM, Li ZL, Li JL, Xu Y, Leng KM, Cui YF, Sun DJ. A novel prognostic biomarker for cholangiocarcinoma: circRNA Cdr1as. Eur Rev Med Pharmacol Sci 2018; 22: 365-371.
15) Salzman J. Circular RNA expression: its potential regulation and function. Trends Genet 2016; 32: 309-316.
16) Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA 2013; 19: 141-157.
17) Yang W, Du WW, Li X, Yee AJ, Yang BB. Foxo3 activity promoted by non-coding effects of circular RNA and Foxo3 pseudogene in the inhibition of tumor growth and angiogenesis. Oncogene 2016; 35: 3919-3931.
18) Holdt LM, Kohlmaier A, Teupser D. Molecular roles and function of circular RNAs in eukaryotic cells. Cell Mol Life Sci 2018; 75: 1071-1098.
19) Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature 2013; 495: 384-388.
20) Xu XW, Zheng BA, Hu ZM, Qian ZY, Huang CJ, Liu XQ, Wu WD. Circular RNA hsa_circ_000984 promotes colon cancer growth and metastasis by sponging miR-106b. Oncotarget 2017; 8: 91674-91683.

21) Gao YL, Zhang MY, Xu B, Han LJ, Lan SF, Chen J, Dong YJ, Cao LL. Circular RNA expression profiles reveal that hsa_circ_0018289 is up-regulated in cervical cancer and promotes the tumorigenesis. Oncotarget 2017; 8: 86625-86633.

22) Zhu X, Wang X, Wei S, Chen Y, Chen Y, Fan X, Han S, Wu G. hsa_circ_0013958: a circular RNA and potential novel biomarker for lung adenocarcinoma. FEBS J 2017; 284: 2170-2182.

23) Yao JT, Zhao SH, Liu QP, Lv MQ, Zhou DX, Liao ZJ, Nan KJ. Over-expression of CircRNA_100876 in non-small cell lung cancer and its prognostic value. Pathol Res Pract 2017; 213: 453-456.

24) Hsiao KY, Sun HS, Tsai SJ. Circular RNA - New member of noncoding RNA with novel functions. Exp Biol Med (Maywood) 2017; 242: 1136-1141.

25) Weng W, Wei Q, Toden S, Yoshida K, Nagasaka T, Fujiwara T, Cai S, Qin H, Ma Y, Goel A. Circular RNA ciRS-7-a promising prognostic biomarker and a potential therapeutic target in colorectal cancer. Clin Cancer Res 2017; 23: 3918-3928.