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CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC

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Abstract. – OBJECTIVE: Circular RNAs are a subgroup of non-coding RNAs and generated by a mammalian genome. The purpose of this study was to investigate the clinical significance, biological function and molecular mechanism of circ_0067934 in human non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: We assayed expression level of circ_0067934 in NSCLC tissues and cell lines by Real-time PCR. The associations of circ_0067934 expression with clinicopathologic features and overall survival of patients with NSCLC were statistically analyzed. Biological functions of circ_0067934 were analyzed using MTT and transwell migration and invasion assays *in vitro*. The expression of EMT-related mRNAs and proteins was assayed using Real-time PCR and Western blot.

RESULTS: We found that circ_0067934 expression was significantly increased in NSCLC tissues and cell lines. High circ_0067934 expression was significantly associated with TNM stage (p=0.003), lymph node status (p=0.000), and distant metastasis (p=0.017). Moreover, Kaplan-Meier curves showed that higher expression of circ_0067934 led to a significantly poorer survival. Multivariate Cox proportional hazards analysis suggested that circ_0067934 was an independent poor prognostic factor for patients with NSCLC. *In vitro* assay indicated that down-regulation of circ_0067934 could suppress NSCLC cells proliferation, migration, and invasion. Mechanistic analysis showed that aberrant circ_0067934 expression could modulate the expression levels of markers of epithelial-to-mesenchymal transition.

CONCLUSIONS: Our data suggested that circ_0067934 functioned as an oncogenic circular RNA in NSCLC, which provided a potential prognostic biomarker and therapeutic target for NSCLC.

Key Words
Circ_0067934, EMT, NSCLC, Metastasis, Growth.

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide[1]. More than 1.8 million patients were diagnosed with lung cancer every year, accounting for 13% of overall cancer incidence[2]. An approximate 85% of the lung cancer cases belong to the non-small cell lung cancer (NSCLC)[3]. Over the past few years, more and more progress has been achieved in treatment technologies for NSCLC; however, the 5-year survival rate of NSCLC is only lower than 15%[4,5]. Long-term survival after surgical resection remains poor owing to the high rate of recurrence and metastasis[6]. A deeper understanding of the potential mechanism underlying metastasis is imperative for developing effective therapy and exploring novel diagnostic methods.

Circular RNAs (circRNAs) are a class of non-coding RNAs which form a covalently closed continuous loop without 5' caps and 3' tails and exist extensively in mammalian cells[7]. Since the first circRNA was identified in a RNA virus in 1976, circRNAs have long been regarded as an accidental byproduct, resulting from errors during post-transcriptional processing[8,9]. Huang and Shan[10] have found that circRNAs are involved in the RNR-RNA regulation network or RNA-protein complex formation, suggesting that circRNAs play an important role in cellular biological function. Importantly, previous studies[11,12] reported that circRNAs harbored microRNA binding sites and function as miRNA sponges. It has known to us that miRNAs serve as tumor suppressors or oncogenes in tumorigenesis and progression of tumors[13,14]. The association between circRNAs and miRNAs highlighted circRNAs as useful biomarkers for diagnosis and treatment of tumors. However, up to date, little is known about their roles in human NSCLC.

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Table I. Primers used in our study.

Primer name	Primers for qPCR (5′–3′)	
	Forward	Reverse
Circ_0067934	TAGCAGTTCCCCAATCCTTG	CACAAATTCCCATCATTCCC
U6	CGCTTCGGCAGCACATATA	TTCACGAATTTGCGTGTCAT
E-cadherin	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG
N-cadherin	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
Vimentin	TGGCACGTCTTGACCTTGAA	GGTCATCGTGATGCTGAGAA
GAPDH	CACCATCTTCCAGGAGCGAG	TCACGCCACAGTTTCCCGGA

Circ_0067934 is a circular RNA molecule of 170 nt which is generated from chromosomal region 3q26.2. Recently, dysregulation of circ_0067934 expression has been reported[15,16] in two cancers, including hepatocellular carcinoma and esophageal squamous cell carcinoma. Both the two studies suggested circ_0067934 as a tumor promoter. However, whether circ_0067934 expression was dysregulated, and its potential function in NSCLC has not been reported. Our present work firstly provided evidence that circ_0067934 played a positive regulator in the progression of NSCLC.

Patients and Methods

Patients and Tissue Samples

We obtained 159 paired NSCLC and adjacent normal tissues from NSCLC patients who underwent surgery at the Department of Thoracic Surgery, Beijing Friendship Hospital between 2009 and 2013. All patients were diagnosed with NSCLC based on the histopathological evaluation. Before surgical therapy, none of the patients had received neoadjuvant chemotherapy, radiation therapy, or immunotherapy. All the samples were snap-frozen in liquid nitrogen and then stored at -80°C for qRT-PCR. Clinicopathological characteristics in this study are summarized in Table II. Tissue sample use was approved by the Ethics Committees of our hospital and written informed consent was obtained from all study participants.

Cell Culture and siRNA Transfection

NSCLC cell lines (A549, H1299, H358, SPCA1) and a normal cell line (MRC-5). were obtained from Type Culture Collection of the Chinese Academy of Sciences (Xuhui, Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (GIBCO, Pudong, Shanghai, China) supplemented with 10% Gibco fetal calf serum (FCS) (Gibco; Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin (GenePharma, Pudong, Shanghai, China) and 100 μg/ml streptomycin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

The si-circ_0067934 and si-NC were synthesized by RiBoBio (Haidian, Beijing, China). The sequences of si-circ_0067934 were as follows: sense: 5'- UGUUGAUUGGGAUAUGUUAUU-3'; antisense: 5'-UAACAUAUCCCAAUCAACAUU-3'. Transient cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

RNA Extract and Quantitative Real-Time PCR

Total RNA from tissues and the cultured cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNA was synthesized with the PrimeScript Reagent Kit (Promega, Madison, WI, USA). Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the ABI 7500 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). The fold change of circ_0067934 expression was determined with the $2^{-\Delta\Delta Ct}$ method by using GAPDH as a reference gene. The primers used for polymerase chain reaction (PCR) amplification are listed in Table I.

Cell Proliferation Assays

H1299 cells were seeded into 96-well plates (2×10^4/well) and incubated in RPMI-1640 at 37°C and 5% CO_2 atmosphere for 96 hours. Subsequently, MTT solution was added to each well, and the cells were incubated for an additional 4 h. The blue dye taken up by cells was dissolved with dimethyl sulfoxide (100 ml /well), and absorbance was measured using a microtiter plate reader at 450 nm.

Table II. Association of circ_0067934 expression levels with clinical factors in NSCLC patients.

Characteristics	All patients	Circ_0067934 expression		*p*-value
		High	Low	
Age (years)				0.379
<60	74	34	40	
≥60	85	45	40	
Gender				0.288
Male	94	50	44	
Female	65	29	36	
Smoking index				0.385
<400	75	40	35	
≥400	84	39	45	
Histological type				0.298
Adenocarcinoma	73	33	40	
Squamous carcinoma	86	46	40	
TNM stage				0.003
I+II	97	39	58	
III+IV	62	40	22	
Lymph node status				0.000
Yes	57	37	20	
No	102	42	60	
Distant metastasis				0.017
Yes	56	35	21	
No	103	44	59	

Transwell Assay

Cell migration and invasion were detected using transwell chambers with (invasion assay) or without (migration assay) Matrigel (BD Biosciences, Chengdu, Sichuan, China) matrix. In brief, medium containing 10% FBS was added into the lower chamber serving as the chemoattractant. After incubation, the upper surface of the membrane was wiped with a cotton tip and cells attached to the lower surface were stained with crystal violet for 5 min. The number of cells on the lower surface was counted under a light microscope in five random fields. Every experiment was select at least three visions.

Western Blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haidian, Beijing, China), and protein concentrations were detected using the BCA Assay Kit (ThermoFisher Scientific, MA, Waltham, USA). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes (Millipore, Billerica, MA, USA) at 80 V for 2 h at 4°C. The membranes were blocked with 5% non-fat milk and then incubated with the primary antibody. The membrane was washed and then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:1,000, Sigma-Aldrich, St. Louis, MO, USA China) at room temperature for 2 h. After three washes by PBST, immune-reactive protein bands were detected with an Odyssey scanning system (Li-Cor, Lincoln, NE, USA). Primary antibody used in this study include anti- E-cadherin, anti-N-cadherin, anti-Vimentin and anti-GAPDH antibodies. We repeated every experiment at least three times.

Statistical Analysis

SPSS software 16.0 (SPSS Inc., Chicago, IL, USA) was used to carry out all computations. The significance of differences between groups was estimated by Student's *t*-test and x^2-test. Overall survival curves were plotted according to the Kaplan-Meier method. Survival data were further estimated using the univariate and multivariate Cox proportional hazards model. Differences were considered to be statistically significant at values of $p<0.05$.

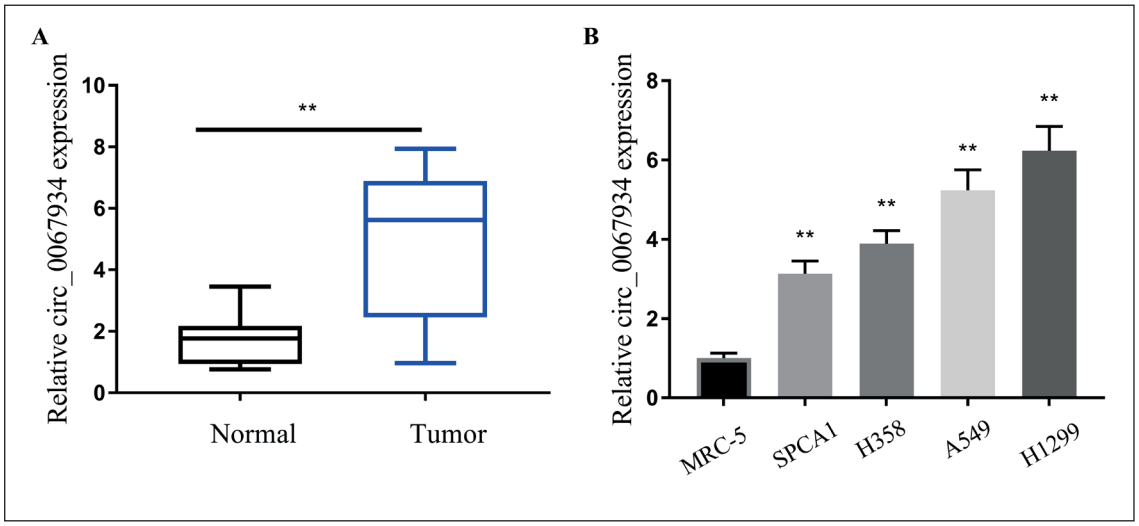

Figure 1. Circ_0067934 are overexpressed in NSCLC tissues and cell lines. **A**, Circ_0067934 was analyzed by qRT-PCR assays in NSCLC tissues and adjacent normal tissues. **B**, Average relative circ_0067934 expression was detected in four NSCLC cell lines (H1299, H358, A549, and SPCA1) and a normal cell line, MRC-5. Circ_0067934 expression levels were normalized to GAPDH. *p<0.05, **p<0.01.

Results

Circ_0067934 Expression Was Upregulated in NSCLC Tissues and Cell Lines

To study the role of circ_0067934 in NSCLC, we first examined the expression levels of circ_0067934 in NSCLC tissues and matched normal lung tissues by RT-PCR. As shown in Figure 1A, we found that the expression of circ_0067934 was significantly elevated in NSCLC tissues compared to matched normal tissues (p<0.01). Furthermore, we detected the expression levels of circ_0067934 in NSCLC cell lines and a normal cell line (MRC-5). It was observed that the expression of circ_0067934 significantly increased in four NSCLC cell lines compared with MRC-5. The H1299 cell line, which possessed the highest levels of circ_0067934 expression among all tested NSCLC cell lines, was selected for *in vitro* assay.

Circ_0067934 Expression and Clinicopathological Features in NSCLC

To explore the effect of circ_0067934 in clinical progression of NSCLC patients, we divided patients into a high circ_0067934 expression group and a low circ_0067934 expression group using the median circ_0067934 expression as a cutoff. Chi-square test was used to analyze clinicopathological factors. As shown in Table II, we observed that higher expression of circ_0067934 was positively associated with high TNM stage (p=0.003) and positive lymph node status (p=0.000) and distant metastasis (p=0.017). However, statistical analysis revealed no significant correlation between expression levels of circ_0067934 and age, gender, smoking index, and histological type (All p>0.05).

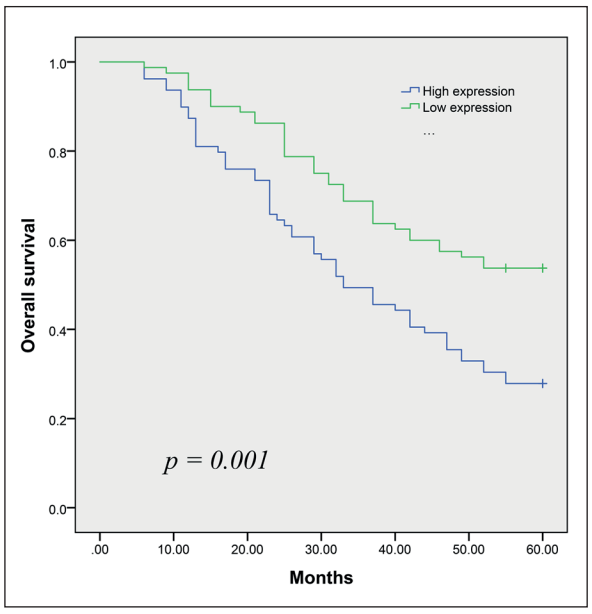

Figure 2. Kaplan-Meier overall survival curves of NSCLC patients according to the level of circ_0067934 expression.

Table III. Prognostic factors in Cox proportional hazards model.

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	p	RR	95% CI	p
Age	1.324	0.783-2.667	0.218	—	—	—
Gender	1.783	0.822-2.231	0.415	—	—	—
Smoking index	1.317	0.633-1.973	0.519	—	—	—
Histological type	2.144	0.865-2.784	0.133	—	—	—
TNM stage	3.452	1.669-5.732	0.005	3.127	1.382-4.893	0.008
Lymph node status	4.821	2.133-8.379	0.001	3.983	1.783-7.231	0.001
Distant metastasis	3.431	1.362-5.012	0.012	2.673	1.139-3.988	0.015
Circ_0067934 expression	3.774	1.498-6.673	0.001	3.198	1.293-5.673	0.004

Association Between circ_0067934 Expression and Survival in NSCLC Patients

Kaplan-Meier survival analysis was performed to determine the association between the circ_0067934 expression and the prognosis of NSCLC. As shown in Figure 2, the results showed that patients with high circ_0067934 expression had a significantly shorter overall survival than those with low expression (p=0.001). In addition, univariate Cox regression analysis showed that TNM stage, lymph node status, distant metastasis and circ_0067934 expression were also significantly correlated with overall survival (Table III). Furthermore, we performed multivariate analysis to determine whether circ_0067934 expression level is an independent prognostic factor for outcomes. Our results confirmed that circ_0067934 expression was an independent factor that affected overall survival (RR: 3.774, 95% CI: 1.498-6.673, p=0.001, Table III).

Circ_0067934 Down-Regulation Inhibited Cell Proliferation, Migration, and Invasion in NSCLC

To investigate whether circ_0067934 has an effect on proliferation, migration, and invasion in H1299 cells, we performed a knockdown experiment, and the transfection efficiency was measured by qRT-PCR (Figure 3A). The results of MTT showed that down-regulation of circ_0067934 suppressed the H1299 cells proliferation (Figure 3B). Moreover, transwell assay was used to evaluate the migrative and invasive ability of H1299 cells. As shown in Figure 3C and 3D, we found that down-regulation of circ_0067934 in H1299 cells resulted in a significant reduction of cells passed through the chambers, suggesting a promotive function of circ_0067934 on cell migration and invasion of H1299 cells. Together, these findings suggest that circ_0067934 acts as a tumor promoter in NSCLC.

Decreased circ_0067934 Expression Inhibited EMT

It was known to us that EMT was critical for cancer cell metastasis. To determine whether circ_0067934 affected the EMT process, we performed qRT-PCR and Western blot to detect the expression of EMT-related genes. As shown in Figure 4A, the results indicated that down-regulation of circ_0067934 increased E-cadherin protein expression and decreased N-cadherin and Vimentin proteins expression. We also detected an up-regulation of E-cadherin mRNA and a down-regulation of N-cadherin and Vimentin mRNA in H1299 cells transfected with si-circ_0067934 (Figure 4B). These results indicated that circ_0067934 promoted NSCLC cells metastasis by modulating EMT.

Discussion

NSCLC is becoming one of the most lethal threats to human health and life. Although various novel treatment strategies have been developed for NSCLC, the patients diagnosed at an advanced stage still have a high mortality rate and a poor 5-year survival rate[17]. Therefore, identification of novel molecular biomarkers which could be used for NSCLC screening and prognosis are in urgent needed in clinical practice. In the present investigation, we found that circ_0067934 expression was significantly up-regulated in both NSCLC tissues and cell lines. Then, elevated circ_0067934 expression was associated with advanced TNM stage, lymph node status, and distant metastasis, suggesting that circ_0067934 contributed to the progression

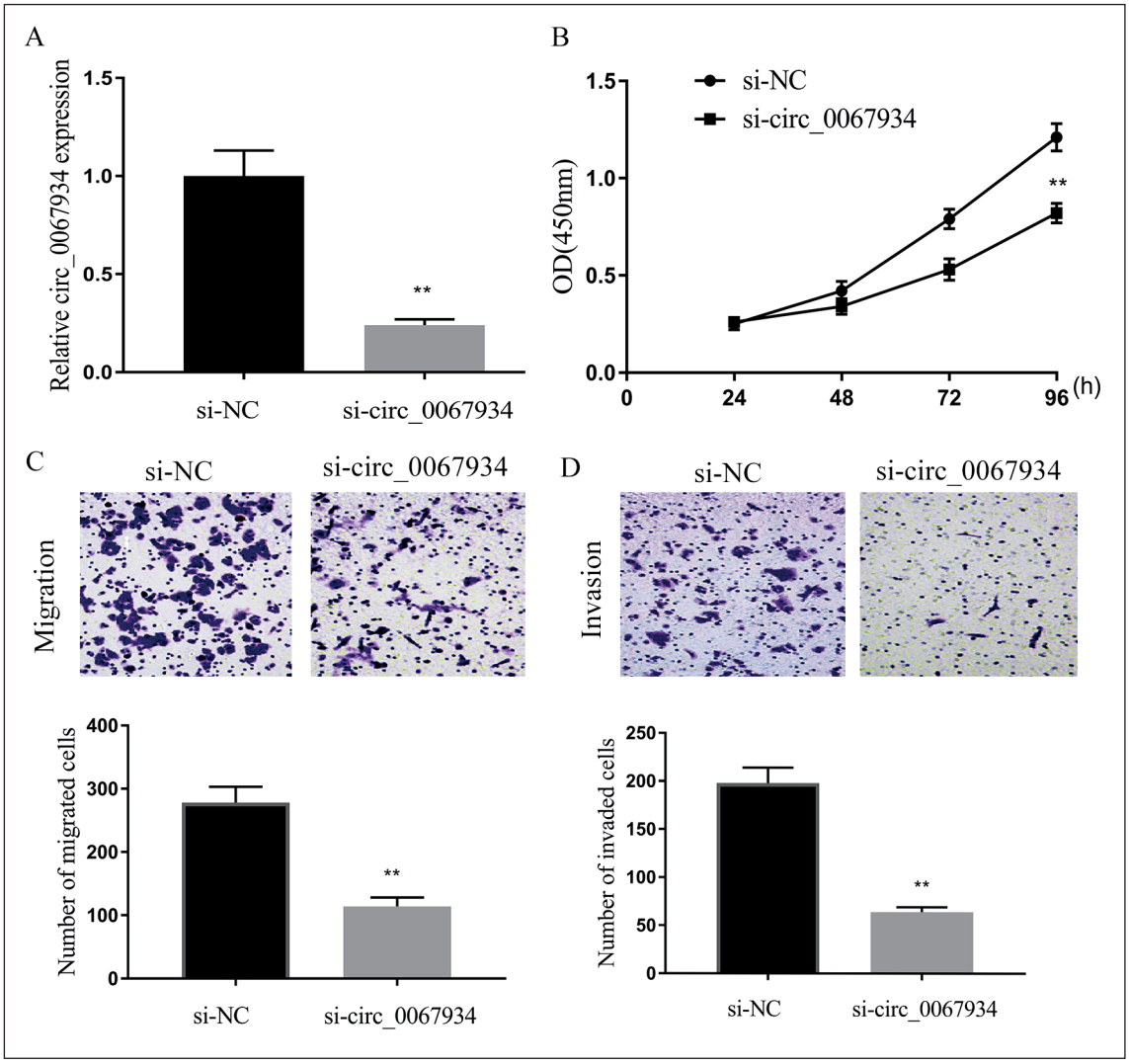

Figure 3. Down-regulation of circ_0067934 suppressed NSCLC cell growth and metastasis. **A**, qRT-PCR analysis confirmed increased circ_0067934 expression in H1299 cells transfected with si-circ_0067934. **B**, MTT assay was performed to examine H1299 cells proliferation. **C**, Transwell-migration assay was performed in H1299 cells. **D**, Transwell-invasion assay was performed in H1299 cells. All data are shown as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$.

of NSCLC. Furthermore, Kaplan-Meier analysis indicated that patients with a high level of circ_0067934 expression had significantly shorter overall survival compared to those with a low level of circ_0067934 expression. In addition, high levels of circ_0067934 expression could be an independent prognostic indicator for NSCLC patients by univariate and multivariate COX regression analyses. Our findings suggested, for the first time, that circ_0067934 holds great promise as a diagnostic and prognostic marker for NSCLC.

Recently, several studies have revealed that circular RNA play important roles in tumor development. For instance, Zhuo et al[18] reported that circ_0003906 was significantly lowly expressed in colorectal cancer and its high levels were associated differentiation and lymphatic metastasis. They also found that circ_0003906 may be used as a potential biomarker in the diagnosis of CRC. Li et al[19] reported circular RNA ITCH as a tumor suppressor because it overexpression could inhibit esophageal squamous cell carcinoma growth by suppressing the Wnt/β-catenin pathway. In addition, Yao et al[20] reported that circ_100876 expression was correlated with tumor stage and lymphatic node metastasis and its high expression was associated with poor prognosis in NSCLC patients. Those findings encouraged us to identify

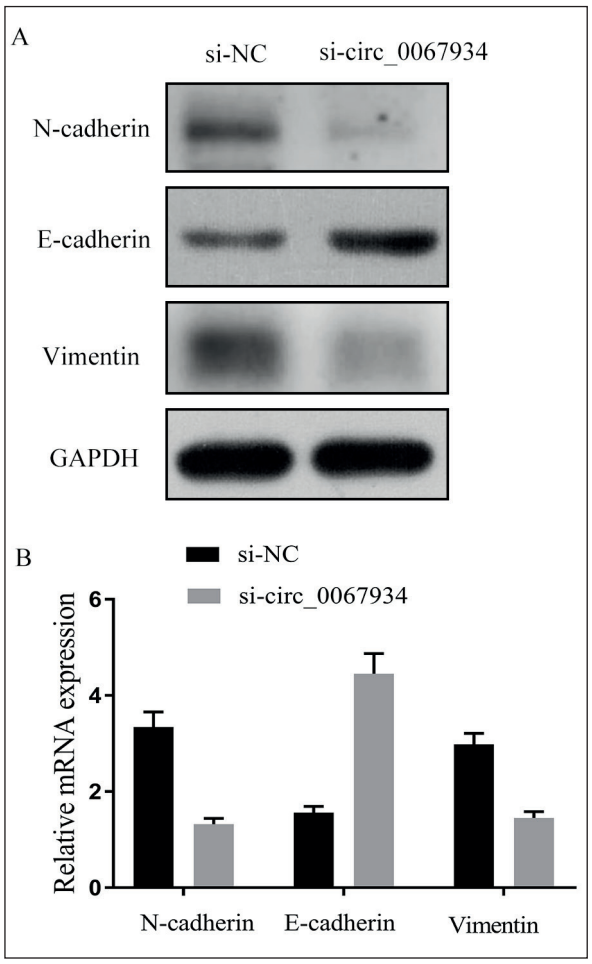

Figure 4. Inhibition of circ_0067934 suppressed epithelial-mesenchymal transition (EMT) program. **A**, Western blot was used to determine the protein expression of E-cadherin, N-cadherin, Vimentin. **B**, qRT-PCR was used to determine the mRNA expression of E-cadherin, N-cadherin, Vimentin. *$p<0.05$, **$p<0.01$.

the function and potential mechanism of other circular RNA. Recently, Xia et al[16] reported that circ_0067934 was up-regulated in esophageal squamous cell carcinoma and its knockdown cells proliferation, migration, and invasion in esophageal squamous cell carcinoma. Subsequently, Zhu et al[15] showed that circ_0067934 served as a tumor promoter by promoting tumor growth and metastasis in hepatocellular carcinoma via regulation of miR-1324/FZD5/ Wnt/β-catenin axis. However, the function of circ_0067934 in NSCLC remains unclear. On line with the previous study, our *in vitro* assay also confirmed down-regulation of circ_0067934 inhibited NSCLC cells proliferation, migration, and invasion, suggesting that circ_0067934 acted as an oncogene in NSCLC progression. To explore the potential mechanism by which circ_0067934 promoted the proliferation and metastasis of NSCLC cells, our attention focused on epithelial-mesenchymal transition (EMT). EMT is an essential mechanism in embryonic development and tissue repair[21]. It has been confirmed that EMT process leads to invasion and metastasis of cancer cells[22]. On the other hand, EMT process could be modulated by genes, long noncoding RNA, and miRNA[23-25]. In our study, the results of RT-PCR and Western blot indicated that down-regulation of circ_0067934 inhibited the EMT in H1299 cells by increasing expression of the epithelial marker E-cadherin and reducing expression of the mesenchymal marker N-cadherin and Vimentin. Thus, circ_0067934 may exhibit its anti-cancer role by modulating EMT progression.

Conclusions

We demonstrated that the expression of circ_0067934 was increased in NSCLC tissues and may be a negative prognostic factor. Moreover, circ_0067934 was implicated in NSCLC tumorigenesis, EMT, and metastasis, suggesting that circ_0067934 could be an effective therapeutic target for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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