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Enhanced expression of circular RNA hsa_circ_000984 promotes cells proliferation and metastasis in non-small cell lung cancer by modulating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: In recent years, several circular RNAs (circRNAs) have been identified to play important roles in human cancers. However, the exact effects and molecular mechanisms of circRNAs in non-small cell lung cancer (NSCLC) progression are still unknown. In this study, we aimed to investigate the effect of circular hsa_circ_000984 on NSCLC.

PATIENTS AND METHODS: Quantitative real-time PCR was performed to detect the expression levels of hsa_circ_000984 in NSCLC tissues and cell lines. The associations between the expression of hsa_circ_000984 and clinicopathological parameters and patients' survival were analyzed. The cell growth was detected by CCK-8 assay and colony formation assay. Cell migration and invasion assays were used to study the changes in cell migration and invasion capacity. Western blot was performed to analyze the possible relationship between hsa_circ_000984 and the genes downstream of the Wnt/β-catenin pathway.

RESULTS: We found that hsa_circ_000984 was highly expressed in NSCLC tissues and cell lines and correlated with advanced TNM stage and lymph nodes metastasis. The clinical assays indicated that patients with high hsa_circ_000984 expression had shorter overall survival and disease-free survival. The functional investigations showed that the knockdown of hsa_circ_000984 suppressed NSCLC cells proliferation, migration, invasion, and EMT. Moreover, hsa_circ_000984 displayed its oncogenic roles by modulating the activation of Wnt/β-catenin pathway, which was demonstrated by measuring the expression levels of, β-catenin, c-myc, and cyclin D1.

CONCLUSIONS: Our findings may facilitate a better understanding of hsa_circ_000984, and it might be a potential prognostic biomarker and treatment target for NSCLC patients.

Key Words:
Circular RNA, Hsa_circ_000984, NSCLC, Prognosis, Metastasis, Wnt/β-catenin pathway.

Introduction

Lung cancer has become one of the most serious malignant types of tumor, accounting for more than 1 million deaths annually[1]. This disease is divided into two major categories, according to histological features and response to conventional therapies[2]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases[3]. Despite the significant advances in medication and surgery, the prognosis of patients with advanced stages who have a little prospect of effective and curative treatment is still poor, with a 5-year survival rate of around 15%[4,5]. A lack of sensitive biomarkers for early diagnosis and tumor metastasis is still one of the most critical challenges for clinical management of NSCLC patients[6]. Therefore, it is urgent to explore the potential novel molecular mechanism of NSCLC pathogenesis and progression. Circular RNAs (circRNAs), previously misconstrued as "splicing rubbish", represent a large class of endogenous RNAs with a covalently closed continuous loop that regulates the gene expression at the transcriptional or post-transcriptional level[7,8]. Circular RNAs are novel RNA molecules that are formed by back-splicing covalently joined 3'- and 5'-ends. With recent advancement in high-throughput sequencing and novel computational approaches, more and more circRNAs were confirmed to be dysregulated in various diseases, especially in tumors[9-11]. Growing studies provided evidence that circRNAs may be involved in the regulation of tumor progression. For instance, in colorectal cancer, it was reported that high circRNA_001569 expression was associated with advanced clinical progression and its knockdown

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could suppress the proliferation and invasion of colorectal cancer by targeting miR-145[12]. In lung cancer, circFADS2 was reported to be highly expressed, and its inhibition suppressed lung cancer cells proliferation and invasion ability by targeting miR-498[13]. However, the expressions and functions of most circRNAs in NSCLC are largely unknown. Recently, a newly identified circRNA hsa_circ_000984, encoded by the CDK6 gene, attracted our attention. Previously, CDK6 has been observed to be dysregulated in various tumors, including NSCLC and involved in the development and progression of tumors, suggesting that hsa_circ_000984 may act as a potential regulator in tumors[14,15]. Recently, it was reported that hsa_circ_000984 was highly expressed in colon cancer and served as a tumor promoter by sponging miR-106b[16]. However, whether hsa_circ_000984 played a functional role in other tumors had not been investigated. In this study, for the first time, we reported that hsa_circ_000984 expression was upregulated in NSCLC and predicted poor prognosis of NSCLC patients. Furthermore, we found that hsa_circ_000984 acted as an oncogene by regulating Wnt/β-catenin pathway in NSCLC. This study adds to our understanding of NSCLC pathogenesis.

Patients and Methods

Patients and Tissue Samples

A total of 155 paired primary NSCLC tissues and corresponding non-tumor tissues were collected from patients with NSCLC in Jining No.1 People's Hospital, Jining Medical University. This study was approved by the Ethics Committee of Jining No. 1 People's Hospital, Jining Medical University, and the written informed consents were obtained by all patients enrolled in this study from May 2009 to March 2012. None of the patients had undergone radiotherapy or chemotherapy before the surgery. The comprehensive pathological and clinical data including age, tumor size, TNM stage, were summarized in Table I. All the tissue samples were immediately put in liquid nitrogen and preserved at -80°C after removal from patients.

Cell Lines and Cell Transfection

The six human lung cancer cell types (H1975, SPC-A1, H1299, HCC827, PC 9, A549) and one immortalized epithelial cell type, BEAS-2B, were all purchased from Jennio Biotechnology Co., Ltd. (Guangzhou, Guangdong, China), and cultured using Roswell Park Memorial Institute (RPMI-1640) medium (Eallbio, Daxing, Beijing, China) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. In addition, a GoldenTran-DR transfection kit (Genetransfection Biotech, Changchun, Jilin, China) was applied to transfect small interfering RNAs (siRNAs) into A549 and H1299 cells. Briefly, after plating cells into the 6-well plates, siRNAs were mixed with appropriate volumes of GoldenTran-DR transfection reagent for 15 min. Then, the mixture was added into the cells, and the cells were cultured until use. The siRNAs targeting hsa_circ_000984 (si-circ_000984#1 and si-circ_000984#2) were purchased from General Biosystems Co., Ltd. (Chuzhou, Anhui, China).

Real-Time PCR Assay

Total RNA from lung cancer cells was extracted using TRIzol reagent (Labgene Biotech, Guangzhou, Guangdong, China). Afterward, 2 μg RNA was reversely transcribed to cDNA using a Golden 1st cDNA Synthesis kit (HaiGene, Haerbin, Heilongjiang, China), and a Hi-SYBR Green qPCR Mix kit (HaiGene, Haerbin, Heilongjiang, China) was applied to conduct the qRT-PCR assays to examine the mRNA levels of β-catenin, cyclin D1, and c-myc. Besides, the expression levels of hsa_circ_000984 were examined by KAPA SYBR FAST qPCR kit (BioLeaf, Fengxian, Shanghai, China). The relative expression of β-catenin, cyclin D1, c-myc, and hsa_circ_000984 were calculated using $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as the internal control. The following primers were used as follow: hsa_circ_000984, 5'- CGCCTATGGGAAGGTGTTCA-3' (forward) and 5'- CCCCTCCTCCTCCTTTACGA-3' (reverse); and GAPDH, 5'-ACCCAGAAGACT GTGGATGG-3' (forward) and 5'-CAGTGAGCTTCCCGTTCAG-3' (reverse).

Western Blot Assay

The A549 and H1299 cells after hsa_circ_000984 siRNAs treatment were firstly rinsed twice with phosphate-buffered saline (PBS) and sequentially lysed with an EpiQuik Whole Cell Extraction kit (AmyJet, Wuhan, Hubei, China). Thereafter, dodecyl sulfate, Sodium Salt (SDS)-Polyacrylamide Gel Electrophoresis (8-12% polyacrylamide gel) was utilized to separate the total proteins. The separated proteins were then transferred onto polyvinylidene diflu-

Table I. Expression of miR-889 and DAB2IP according to patients' clinical features.

Variable	Number	Hsa_circ_000984 expression		p-value
		Low	High	
Age (years)				NS
< 60	75	35	40	
≥ 60	80	45	35	
Gender				NS
Male	94	50	44	
Female	61	30	31	
Tumor size (cm)				NS
< 3	95	46	49	
≥ 3	60	34	26	
TNM stage				0.004
I-II	109	48	61	
III	46	32	14	
Lymph nodes metastasis				0.005
No	112	50	52	
Yes	43	30	13	

oride (PVDF) membranes. After blocking with 5% Bull Serum Albumin (BSA), the membranes were then incubated with primary antibodies against β-catenin, Cyclin D1, c-Myc, vimentin, GAPDH, N-cadherin, caspase 3, and caspase 9 at 4°C overnight. After washing with TBST for three times, the membranes were incubated with corresponding secondary antibodies (BOSTER, Wuhan, Hubei, China), followed by visualization using a SuperDura ECL luminescence reagent kit (Yeason, Pudong, Shanghai, China). The primary antibodies against Cyclin D1, c-Myc, vimentin, caspase 3, and caspase 9 were purchased from Protein Tech Group Co., Ltd. (Wuhan, Hubei, China). The primary antibodies against β-catenin, N-cadherin, and GAPDH were obtained from BOSTER Co., Ltd. (Wuhan, Hubei, China).

Cell Counting Kit-8 (CCK-8) Assay

In short, hsa_circ_000984 siRNAs or control siRNAs were separately transfected into A549 and H1299 cells. Then, the treated NSCLC cells were seeded at 1×10^4 cells per well in 96-well culture plates. At the indicated time points such as 48 h, 72 h, and 96 h, the cells were incubated with 10 µl CCK-8 reagents (MedChemExpress, Pudong, Shanghai, China). Finally, a microplate reader (Flash Spectrum, Minhang, Shanghai, China) was employed to measure the absorbance at 450 nm wavelength.

Colony Formation Assay

Briefly, A549 and H1299 cells were transfected with hsa_circ_000984 siRNAs or control siRNAs. Subsequently, a certain number of treated A549 and H1299 cells were seeded in 6-well plates and continued to be cultured in complete medium at 37°C with 5% CO_2 until the cell colonies were clearly observed. After the cells were fixed with 95% methanol for 20 min, the cells were stained with crystal violet (0.1% concentration; JissKang, Qingdao, Shandong, China), and observed using a microscope (37XF-PC; Shanghai Optical Instrument Factory, Yangpu, Shanghai, China).

Cell Apoptosis Analysis

The apoptosis of A549 and H1299 cells after treatment with hsa_circ_000984 or control siRNAs were detected by an Attune NxT flow cytometer (Thermo, Pudong, Shanghai, China) using a MultiSciences Annexin V Apoptosis assay kit (Hangzhou, Zhejiang, China). In brief, the treated cells were collected, washed with ice-cold PBS, and incubated with annexin V-FITC and PI (propidium iodide) for 10-15 min, after being analyzed by the flow cytometer.

Wound Healing Assays

In brief, A549 and H1299 cells after treatment with hsa_circ_000984 siRNAs or control siRNAs were seeded into 6-well plates, and the cells were cultured in complete medium (with 10% FBS) until the confluence reached 100%. Then, the wounds were created in the cell monolayers by scratching with a 200 µl sterilized pipette tip. The cellular debris were washed by PBS, and the cells were continued to be cultured for 24 h. The photographs were taken using a microscope

(37XF-PC; Shanghai Optical Instrument Factory, Yangpu, Shanghai, China) at 0 h and 24 h.

Transwell Invasion Assay

A549 or H1299 cells transfected with hsa_circ_000984 siRNAs or control siRNAs were re-suspended in culture medium (100 µl; without FBS). Subsequently, the 70 µl Matrigel pre-coated topsides of the transwell inserts (KaiTaiBio, Hangzhou, Zhejiang, China) were added with the treated cells (1.5×10^5 cells per well). Besides, the lower chambers of the transwell inserts were added with culture medium with 15% FBS. Twenty-four hours later, crystal violet (0.1% concentration; JissKang, Qingdao, Shandong, China) was employed to stain the invasive cells on the lower membrane surface. Finally, the images were taken using a microscope (37XF-PC; Shanghai Optical Instrument Factory, Yangpu, Shanghai, China).

Statistical Analysis

The statistical analysis in this study was carried out using the SPSS program (version 17.0, SPSS Statistics for Windows, Chicago, IL, USA). The survival rates were calculated using the Kaplan-Meier method with the log-rank test for comparison. The influence of each variable on survival was examined by the Cox multivariate regression analysis. A paired two-tailed *t*-test or one-way ANOVA was applied to evaluate the measurement data. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between the groups. A $p < 0.05$ was considered significant.

Results

Up-Regulation of hsa_circ_000984 Predicted Poor Prognosis in NSCLC

In order to explore the expression pattern of hsa_circ_000984 in NSCLC, we performed RT-PCR to detect its expression in NSCLC tissues and matched normal tissues from 155 NSCLC patients. As shown in Figure 1A, we found that hsa_circ_000984 expression was significantly up-regulated in NSCLC tissues compared to matched normal lung tissues ($p < 0.01$). Furthermore, we also observed that hsa_circ_000984 was overexpressed in six NSCLC cell lines compared to BEAS-2B (Figure 1B). Then, we divided the patients into two groups (High and Low) using the median value of hsa_circ_000984 expression levels in NSCLC patients and performed Chi-square test to explore its clinical significance. As shown in Table I, we found that high hsa_circ_000984 expression was significantly associated with advanced TNM stage ($p = 0.004$) and lymph nodes metastasis ($p = 0.005$), suggesting that hsa_circ_000984 may influence the clinical prognosis of NSCLC patients. More-

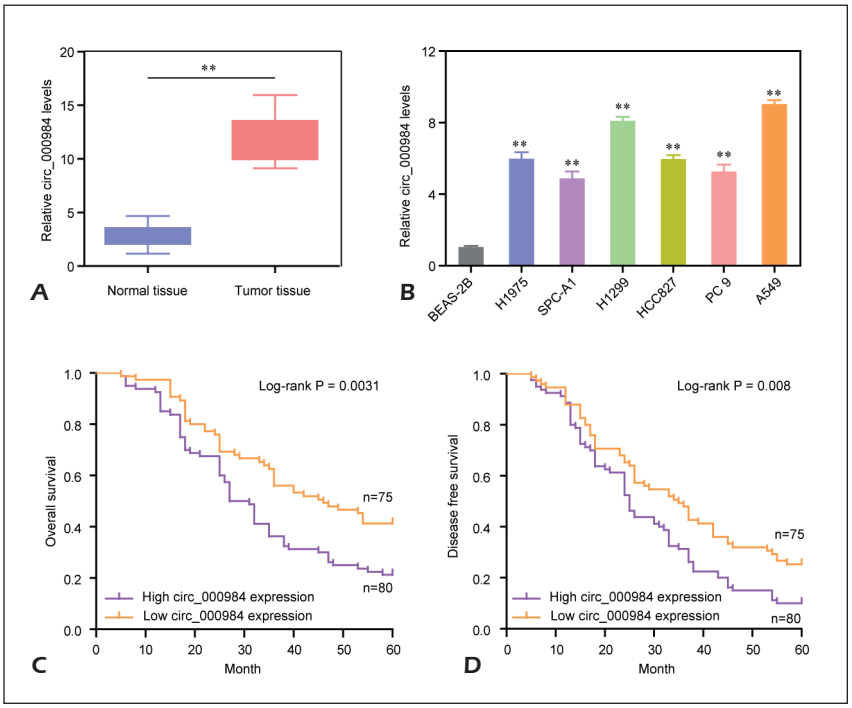

Figure 1. Expression of hsa_circ_000984 in human NSCLC tissues and its prognosis of NSCLC patients. **A**, Hsa_circ_000984 expression was increased in NSCLC tissues compared with adjacent normal tissues. **B**, Real-time RT-PCR was conducted to examine the expression levels of hsa_circ_000984 in six human NSCLC cell lines and in a normal human lung epithelial cell line BEAS-2B. **C-D**, Kaplan-Meier analyses of correlations between the hsa_circ_000984 expression level and overall survival **C** and disease-free survival **D** of 155 NSCLC patients are shown. *p<0.05, **p<0.01.

Table II. Univariate analysis of prognostic parameters in patients with NSCLC by Cox regression analysis.

Variable	Overall survival			Disease-free survival		
	RR	95% CI	p	RR	95% CI	p
Age	1.326	0.671-2.445	0.215	1.452	0.842-2.231	0.133
Gender	1.426	0.832-2.644	0.139	1.548	1.121-2.323	0.114
Tumor size	1.218	0.944-2.031	0.114	1.326	1.137-2.332	0.089
TNM stage	3.426	1.532-4.889	0.001	3.256	1.378-4.461	0.003
Lymph nodes metastasis	3.655	1.437-5.137	0.001	3.437	1.228-5.462	0.001
Hsa_circ_000984 expression	3.326	1.257-4.452	0.004	3.427	1.306-4.778	0.002

Table III. Multivariate analysis of prognostic parameters in patients with NSCLC by Cox regression analysis.

Variable	Overall survival			Disease-free survival		
	RR	95% CI	p	RR	95% CI	p
TNM stage	3.123	1.246-4.356	0.007	3.205	1.337-4.643	0.002
Lymph nodes metastasis	3.045	1.317-4.664	0.003	3.278	1.652-5.231	0.001
Hsa_circ_000984 expression	2.985	1.342-4.231	0.008	3.138	1.224-4.427	0.004

over, the results of Kaplan-Meier assays indicated that the overall survival and disease-free survival were both significantly lower in the hsa_circ_000984-high group compared to those in the hsa_circ_000984-low group ($p < 0.01$, Figure 1C and 1E). Finally, in Cox model, we observed that hsa_circ_000984 expression was an independent prognostic indicator for overall survival ($p < 0.05$) and disease-free survival ($p < 0.05$) in patients with NSCLC (Table II and III). Taken together, our data firstly reported hsa_circ_000984, an upregulated circRNA, which was associated with advanced clinical stages and poor prognosis of NSCLC patients.

Effects of hsa_circ_000984 on Cellular Proliferation and Apoptosis of NSCLC Cells

Given that hsa_circ_000984 was up-regulated in NSCLC tissues and cells, we thus hypothesized that hsa_circ_000984 might play essential roles in modulating the development and progression of NSCLC. To achieve that, siRNAs specific targeting hsa_circ_000984 (si-circ_000984#1 and si-circ_000984#2) were synthesized, and sequentially transfected into A549 and H1299 cells. The data of qRT-PCR assays indicated that the silencing efficiency of hsa_circ_000984 siRNAs was high (Figure 2A). In addition, the results of the CCK-8 assays showed that the transfection of hsa_circ_000984 siRNAs led to a significant decrease of proliferative rates of A549 and H1299 cells at 48 h, 72 h, and 96 h (Figure 2B). Moreover, cell colony formation assays demonstrated that knockdown of hsa_circ_000984 remarkably impaired the clonogenic capacities of A549 and H1299 cells (Figure 2C). Besides, we performed flow cytometry to analyze the alteration of cell apoptosis when they were transfected with hsa_circ_000984 siRNAs. The data revealed that repressing the expression of hsa_circ_000984 markedly elevated the cell apoptotic rates of A549 and H1299 cells (Figure 2D). We next analyzed the expression of two apoptosis-associated molecules, caspase 3, and caspase 9 in A549 and H1299 cells after transfecting hsa_circ_000984 siRNAs. The results suggested that the silence of hsa_circ_000984 notably reduced the protein levels of caspase 3 and caspase 9 (Figure 2E). Overall, our data indicated that hsa_circ_000984 played crucial roles in regulating the cellular growth and apoptosis of NSCLC.

Depression of hsa_circ_000984 Inhibited the Metastatic Potentials of NSCLC Cells

The effects of hsa_circ_000984 on the migration and invasion of NSCLC cells were also evaluated using wound healing and transwell invasion assays. After transfection of hsa_circ_000984 siRNAs, the migratory capabilities of A549 and H1299 cells were significantly decreased (Figure 3A and B). Additionally, the transwell invasion

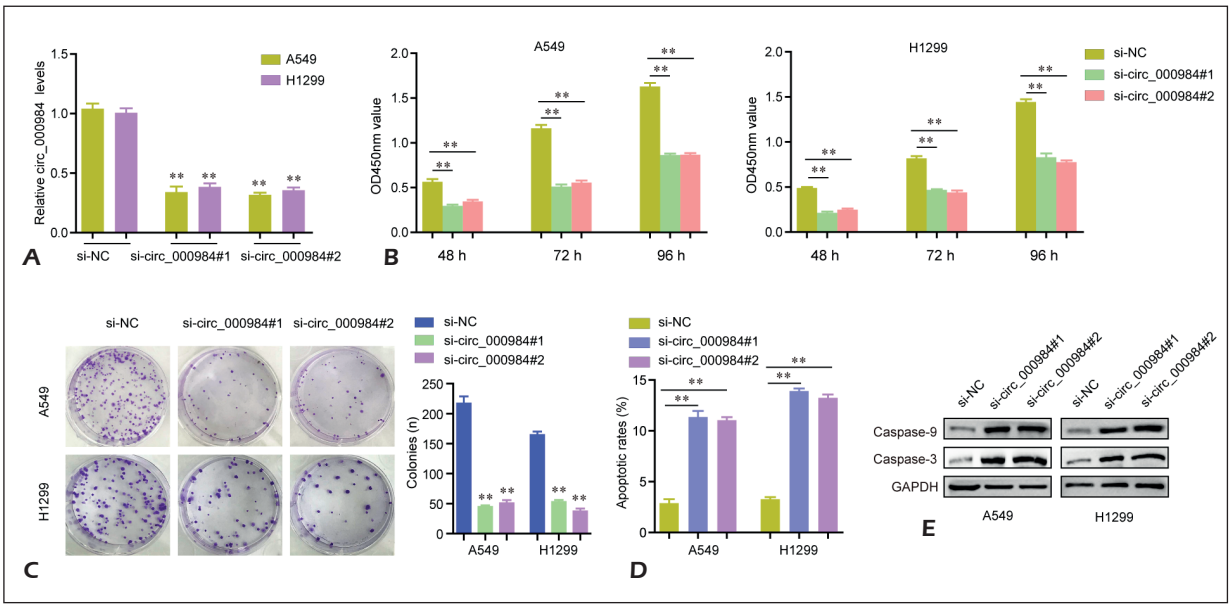

Figure 2. The silencing of hsa_circ_000984 inhibited the proliferation and induced the apoptosis of A549 and H1299 cells. **A**, Targeted siRNAs (si-circ_000984#1 and si-circ_000984#2) efficiently repressed the expression levels of hsa_circ_000984 in the A549 and H1299 cells. **B**, Hsa_circ_000984 knockdown inhibited cell proliferation determined by CCK-8 assay. **C**, After transfection of hsa_circ_000984 siRNAs, the number of A549 and H1299 cell colonies was decreased (magnification: 10×). **D**, Flow cytometry detected the cell apoptosis. **E**, Silencing of hsa_circ_000984 elevated the protein levels of caspase 3 and caspase 9. *p<0.05, **p<0.01.

assays validated that the cell invasive abilities of A549 and H1299 cells were remarkably impeded when hsa_circ_000984 was knocked down (Figure 3C). Furthermore, the underlying mechanisms of hsa_circ_000984 that affected the metastatic potentials of NSCLC cells were also investigated using Western blot assays. The data demonstrated that the knockdown of hsa_circ_000984 significantly reduced the relative protein expression of N-cadherin and vimentin in A549 and H1299 cells (Figure 4D and E). Therefore, these data indicated that hsa_circ_000984 modulated the migration and invasion of NSCLC cells via affecting epithelial-mesenchymal transition associated molecules, such as N-cadherin and vimentin.

Silence of hsa_circ_000984 Depressed the Activation of the Wnt/β-catenin Signaling in NSCLC Cells

We next aimed to discover the molecular mechanisms that hsa_circ_000984 orchestrated for the cell growth, apoptosis, and metastatic potentials of NSCLC cells. As Wnt/β-catenin signaling played extensively regulatory roles in multiple cancer types, we next utilized qRT-PCR and Western blot assays to evaluate the changes of Wnt/β-catenin signaling associated molecules in A549 and H1299 cells after transfecting hsa_circ_000984 siRNAs. As shown in the data of Figure 4A and B, the repression of hsa_circ_000984 resulted in a remarkable decline of the mRNA levels of several Wnt/β-catenin signaling-related molecules, including β-catenin, cyclin D1, and c-myc, in A549 and H1299 cells. Similar results were also revealed by Western blot assays, and the data suggested that the transfection of hsa_circ_000984 siRNAs notably reduced the protein levels of β-catenin, cyclin D1, and c-myc in A549 and H1299 cells (Figure 4C and D). Taken together, these data proved that hsa_circ_000984 affected the development and progression of NSCLC via modulating Wnt/β-catenin signaling.

Discussion

NSCLC is the leading cause of cancer-related mortality in China. Despite advances in treatment modalities, the prognosis for NSCLC patients remains poor[17]. A major challenge for improving clinical outcomes is to identify novel and sensitive biomarkers for individualized treatment[18]. Recently, the potential of noncoding RNA as a novel biomarker attracted growing attention[19]. Among them, circRNAs became the

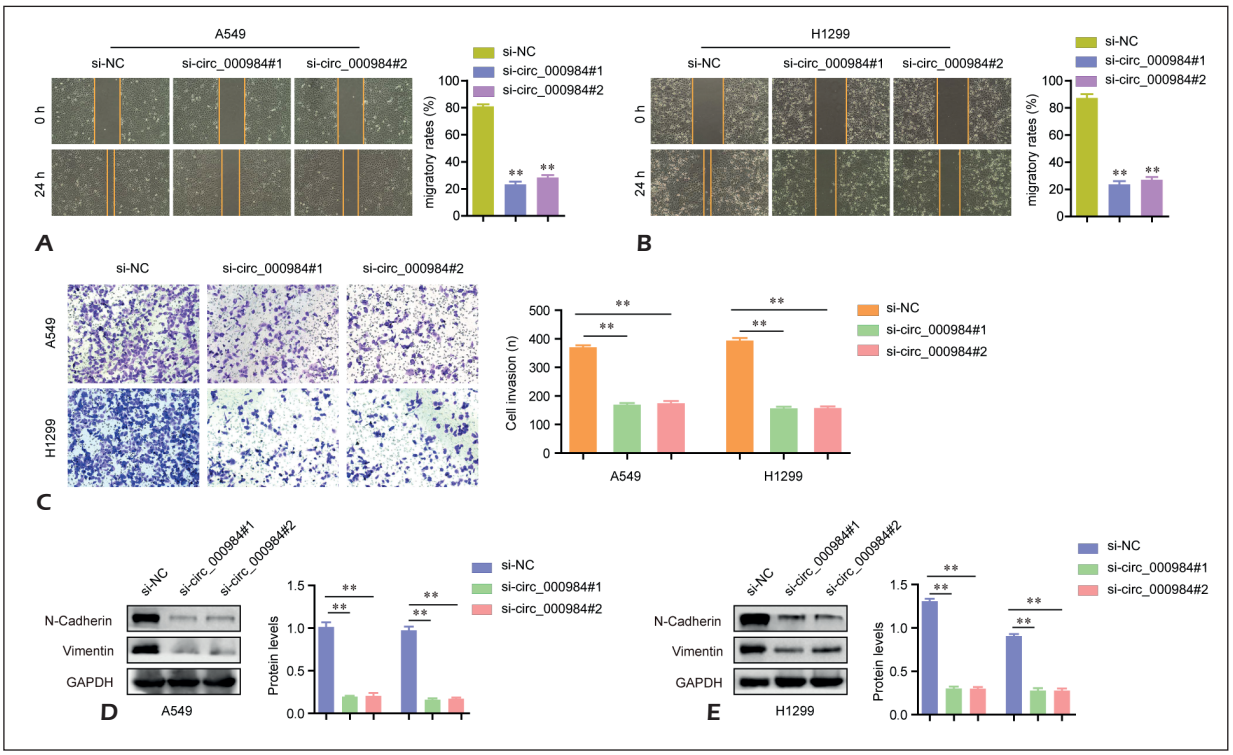

Figure 3. Knockdown of hsa_circ_000984 suppressed the invasion and migration of A549 and H1299 cells. **A**, and **B**, Silencing of hsa_circ_000984 reduced the migration of A549 and H1299 cells. **C**, The invasive number of A549 and H1299 cells was decreased after transfection of hsa_circ_000984 siRNAs (magnification: 40×). **D**, and **E**, Knockdown of hsa_circ_000984 reduced the N-cadherin and vimentin protein levels. *p<0.05, **p<0.01.

nearest candidates because of its relative stability compared to other noncoding RNA[20]. In this study, we focused on a newly identified circRNA hsa_circ_000984 which has been reported to be up-regulated in colon cancer. In line with previous results, we also found that hsa_circ_000984 expression was up-regulated in NSCLC tissues and cell lines. In addition, the Chi-square analysis showed that high hsa_circ_000984 expression was significantly associated with advanced TNM stage and positively lymph nodes metastasis, suggesting its oncogenic roles in clinical progression of NSCLC. Furthermore, the Kaplan-Meier assays revealed that patients with high hsa_circ_000984 exhibited shorter overall survival and disease-free survival, indicating its negative roles in long-term survival. More importantly, in a Cox model, we detected that hsa_circ_000984 expression was an independent poor prognostic factor for both 5-year overall survival and 5-year disease-free survival. Overall, our present data provided the first evidence that high hsa_circ_000984 might be a prognostic predictor for NSCLC. Whether this tendency could be demonstrated in more cancer types need to be further elucidated. Growing evidence strongly indicates the importance of circRNAs in the essential regulation of tumor progression. Several circRNAs were identified to be functional regulators in NSCLC by acting as a tumor suppressor or oncogenes[21,22]. For instance, Yu et al[23] reported that hsa_circ_0003998 expression was up-regulated in NSCLC and its overexpression promotes cell proliferation and invasion by targeting miR-326. Chen et al[24] showed that hsa_circ_100395 expression was decreased in both NSCLC tissues and cell lines and associated with advanced TNM stage and poor prognosis of NSCLC patients. Furthermore, in their functional assays, they discovered that hsa_circ_100395 overexpression could inhibit NSCLC cells proliferation and metastasis by regulating miR-1228/TCF21 pathway. In addition, Li et al[25] found that a higher expression of circ_0016760 indicates an unfavorable prognosis of NSCLC patients, and its knockdown suppresses cells metastasis through the miR-1287/GAGE1 axis. These findings develop our understanding of NSCLC progression. Xu et al[16] firstly reported that hsa_circ_000984 may act as a tumor promoter in colon cancer progression and they provided a series of functional assays to

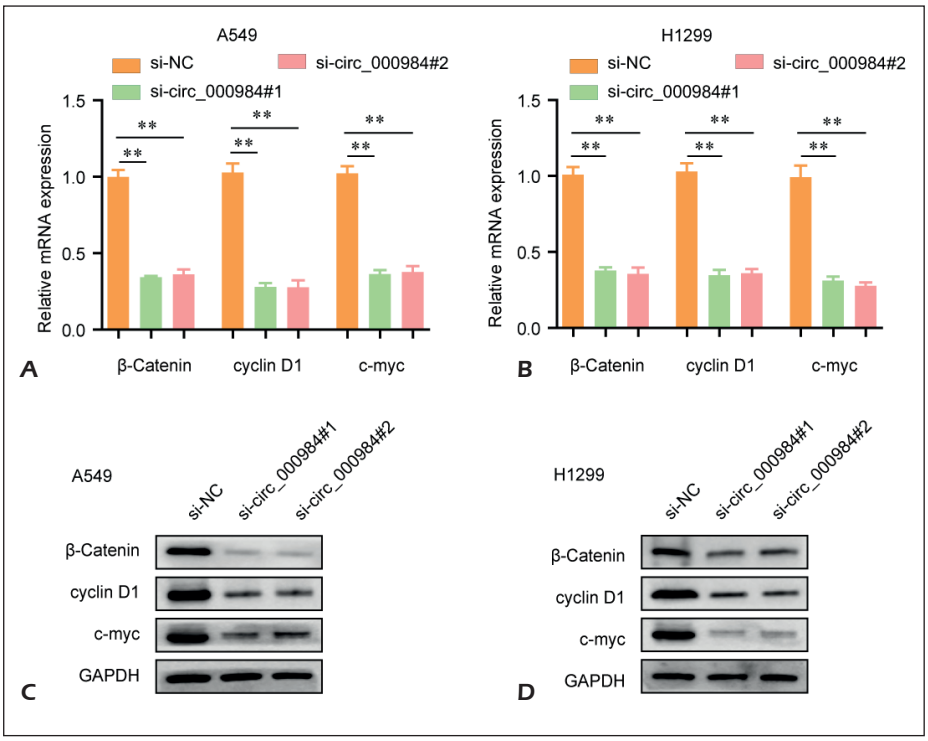

Figure 4. Depression of hsa_circ_000984 impeded the activity of Wnt/β-catenin signaling in A549 and H1299 cells. **A**, and **B**, Transfection of hsa_circ_000984 siRNAs reduced the mRNA levels of β-catenin, cyclin D1, and c-myc in A549 and H1299 cells. **C**, and **D**, The protein levels of β-catenin, cyclin D1, and c-myc in A549 and H1299 cells were decreased after the cells were transfected with hsa_circ_000984 siRNAs. *p<0.05, **p<0.01.

confirm their conclusion. However, the expression and potential function of hsa_circ_000984 in NSCLC have not been investigated. In this study, given the up-regulation of hsa_circ_000984 in NSCLC, we assumed that hsa_circ_000984 may function as a positive regulator in NSCLC, and thus we used si-hsa_circ_000984 to down-regulate the levels of hsa_circ_000984 in NSCLC cell lines. Our functional assays elucidated that the knockdown of hsa_circ_000984 suppressed NSCLC cells proliferation, migration, and invasion, which confirmed our suggestion. In addition, we detected the effects of hsa_circ_000984 knockdown on the expression of EMT-related proteins, finding that the down-regulation of hsa_circ_000984 inhibited EMT progression. Overall, our data firstly showed that hsa_circ_000984 acted as a tumor promoter in NSCLC by promoting NSCLC cells proliferation, metastasis, and EMT pathway. The Wnt/β-catenin signaling pathway, also called canonical Wnt pathway, acted as a critical functional regulator cellular processes involved in development, differentiation, and adult tissue homeostasis[26]. It has been reported that the aberrant activation of the Wnt pathway is highly associated with tumorigenesis and tumor metastasis, and Wnt/β-catenin pathway is involved in the regulation of EMT, which is the main cause of tumor metastasis[27,28]. Growing evidence has pointed out that miRNAs modulated the EMT by interacting with certain target mRNAs of the Wnt/β-catenin pathway in tumors[29]. Interestingly, circRNAs contain the miRNA targeting site, which can bind to miRNAs[30]. Thus, circRNAs may exhibit their roles by modulating Wnt/β-catenin pathway. In this study, we found that the knockdown of hsa_circ_000984 inhibited the transcriptional activity of β-catenin, cyclin D1, and c-myc, suggesting that hsa_circ_000984 may display its oncogenic roles by modulating Wnt/β-catenin pathway. However, the potential mechanism by which hsa_circ_000984 modulated Wnt/β-catenin pathway needs to be further studied.

Conclusions

We firstly reported that hsa_circ_000984 was highly expressed in NSCLC and could lead to poor prognosis. We further found that hsa_circ_000984 through activation of Wnt/β-catenin signaling could promote NSCLC cell proliferation and metastasis. This provides a novel biomarker for prognosis prediction and a better understanding of the molecular therapeutic strategy for NSCLC patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
2) Dutkowska AE, Antczak A. Comorbidities in lung cancer. Pneumonol Alergol Pol 2016; 84: 186-192.
3) Brody H. Lung cancer. Nature 2014; 513: S1.
4) Wahab A, Kesari K, Chaudhary S, Khan M, Khan H, Smith S, Boumber Y. Sequential occurrence of small cell and non-small lung cancer in a male patient: is it a transformation? Cancer Biol Ther 2017; 18: 940-943.
5) Lanuti M. Surgical management of lung cancer involving the chest wall. Thorac Surg Clin 2017; 27: 195-199.
6) Johnson DH, Schiller JH, Bunn PA, Jr. Recent clinical advances in lung cancer management. J Clin Oncol 2014; 32: 973-982.
7) Haque S, Harries LW. Circular RNAs (circRNAs) in health and disease. Genes (Basel) 2017; 8. pii: E353.
8) Chen LL, Yang L. Regulation of circRNA biogenesis. RNA Biol 2015; 12: 381-388.
9) Fan X, Weng X, Zhao Y, Chen W, Gan T, Xu D. Circular RNAs in cardiovascular disease: an overview. Biomed Res Int 2017; 2017: 5135781.
10) Szabo L, Salzman J. Detecting circular RNAs: bioinformatic and experimental challenges. Nat Rev Genet 2016; 17: 679-692.
11) 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.
12) Xie H, Ren X, Xin S, Lan X, Lu G, Lin Y, Yang S, Zeng Z, Liao W, Ding YQ, Liang L. Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer. Oncotarget 2016; 7: 26680-26691.
13) Zhao F, Han Y, Liu Z, Zhao Z, Li Z, Jia K. CircFADS2 regulates lung cancer cells proliferation and invasion via acting as a sponge of miR-498. Biosci Rep 2018; 38. pii: BSR20180570.
14) Sherr CJ, Beach D, Shapiro GI. Targeting CDK4 and CDK6: from discovery to therapy. Cancer Discov 2016; 6: 353-367.
15) Tadesse S, Yu M, Kumarasiri M, Le BT, Wang S. Targeting CDK6 in cancer: state of the art and new insights. Cell Cycle 2015; 14: 3220-3230.
16) 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.
17) Yu N, Zhou J, Cui F, Tang X. Circulating tumor cells in lung cancer: detection methods and clinical applications. Lung 2015; 193: 157-171.
18) Wu B, Zhang XJ, Li XG, Jiang LS, He F. Long non-coding RNA Loc344887 is a potential prognostic biomarker in non-small cell lung cancer. Eur Rev Med Pharmacol Sci 2017; 21: 3808-3812.
19) Martignano F, Rossi L, Maugeri A, Galla V, Conteduca V, De Giorgi U, Casadio V, Schepisi G. Urinary RNA-based biomarkers for prostate cancer detection. Clin Chim Acta 2017; 473: 96-105.
20) Zhang HD, Jiang LH, Sun DW, Hou JC, Ji ZL. CircRNA: a novel type of biomarker for cancer. Breast Cancer 2018; 25: 1-7.
21) Wang J, Li H. CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC. Eur Rev Med Pharmacol Sci 2018; 22: 3053-3060.
22) Hu W, Bi ZY, Chen ZL, Liu C, Li LL, Zhang F, Zhou Q, Zhu W, Song YY, Zhan BT, Zhang Q, Bi YY, Sun CC, Li DJ. Emerging landscape of circular RNAs in lung cancer. Cancer Lett 2018; 427: 18-27.
23) Yu W, Jiang H, Zhang H, Li J. Hsa_circ_0003998 promotes cell proliferation and invasion by targeting miR-326 in non-small cell lung cancer. Onco Targets Ther 2018; 11: 5569-5577.
24) Chen D, Ma W, Ke Z, Xie F. CircRNA hsa_circ_100395 regulates miR-1228/TCF21 pathway to inhibit lung cancer progression. Cell Cycle 2018; 17: 2080-2090.
25) Li Y, Hu J, Li L, Cai S, Zhang H, Zhu X, Guan G, Dong X. Upregulated circular RNA circ_0016760 indicates unfavorable prognosis in NSCLC and promotes cell progression through miR-1287/GAGE1 axis. Biochem Biophys Res Commun 2018; 503: 2089-2094.
26) Janovska P, Bryja V. Wnt signalling pathways in chronic lymphocytic leukaemia and B-cell lymphomas. Br J Pharmacol 2017; 174: 4701-4715.
27) Smith BN, Bhowmick NA. Role of EMT in metastasis and therapy resistance. J Clin Med 2016; 5. pii: E17.
28) Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell 2012; 149: 1192-1205.
29) Song JL, Nigam P, Tektas SS, Selva E. MicroRNA regulation of Wnt signaling pathways in development and disease. Cell Signal 2015; 27: 1380-1391.
30) Rong D, Sun H, Li Z, Liu S, Dong C, Fu K, Tang W, Cao H. An emerging function of circRNA-miRNAs-mRNA axis in human diseases. Oncotarget 2017; 8: 73271-73281.