Exosomal circDNER enhances paclitaxel resistance and tumorigenicity of lung cancer via targeting miR-139-5p/ITGB8

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Funding information
Wuxi Taihu Lake Talent Plan Supports for Leading Talents in Medical and Health Profession and General project of Wuxi Municipal Health Commission, Grant/Award Number: M202103

Abstract
Background: Circular RNAs (circRNAs) are regarded as vital regulatory factors in various cancers. However, the biological functions of circDNER in the paclitaxel (PTX) resistance of lung cancer remain largely unexplored.

Methods: Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze circDNER, miR-139-5p, and ITGB8. Cell proliferation was assessed via colony formation and MTT assays. Cell apoptosis was evaluated by flow cytometry. Western blot was performed to assess protein expression. The targeted interaction among circDNER, miR-139-5p, and ITGB8 were validated using dual-luciferase reporter or RNA immunoprecipitation assays.

Results: Inhibition of circDNER reduced IC50 of PTX, inhibited cell proliferation, invasion and migration, as well as promoted cell apoptosis in PTX-resistant lung cancer cells. Mechanistically, circDNER sponged miR-139-5p to upregulate ITGB8 expression. Overexpression of miR-139-5p reversed the biological functions mediated by circDNER in PTX-resistant lung cancer cells. miR-139-5p overexpression suppressed PTX resistance and malignant behaviors of PTX-resistant lung cancer cells, with ITGB8 elevation rescued the impacts. Moreover, we demonstrated that circDNER was upregulated in plasma exosomes from lung cancer patients. The plasma exosomes derived from these patients are the key factors enhancing the migration and invasion potential of lung cancer cells.

Conclusion: The circDNER mediated miR-139-5p/ITGB8 axis suppresses lung cancer progression. Our findings suggest that circDNER might act as a potential prognostic biomarker and therapeutic target for lung cancer treatment.

KEYWORDS
circDNER, ITGB8, lung cancer, miR-139-5p, paclitaxel

INTRODUCTION
Lung cancer, a major malignancy, is the leading cause of cancer death worldwide. Although many therapies, such as surgery, radiotherapy and chemotherapy, have been applied to treat lung cancer, the 5-year survival rate remains low due to tumor invasion and metastasis as well as lack of effective biomarkers and targets. Hence, exploring novel approaches for the diagnosis and treatment of lung cancer are urgently required.

Circular RNAs (circRNAs) are a class of noncoding RNAs that possess a covalent closed loop. To date, it has been proven that circRNAs play a vital role and participate in proliferation, invasion, and metastasis in various malignancies, including bladder, pancreatic, and esophageal cancers. Previous studies have shown that circRNAs can regulate tumor pathological progression and may serve as potential diagnostic biomarkers of cancer; however, whether circDNER influences lung cancer remains unclear.

Exosomes are membrane vesicles produced by various cells and character with diameters ranging from 30–100 nm. The main functions of exosomes are transferring intercellular signal transduction to recipient cells. It has...
been demonstrated that exosomes are involved in modu-
larizing drug response, as well as tumorigenesis, tumor
invasion and metastasis by delivering micro (mi)RNA,
long noncoding (Inc)RNA, circular (circ) RNA and pro-
teins.\[^9\] CircRNA are exceptionally stable in all cargos car-
ried by exosomes.\[^10\] However, the mechanism of
circRNAs in the exosomes of lung cancer patients remains
to be definitively elucidated.

In this study, circDNER was first identified by circRNA
microarray analysis in exosomes from the plasma of lung
cancer patients. Our data showed that circDNER was obvi-
ously upregulated in lung cancer tissues and plasma
exosomes of lung cancer patients. Moreover, we found that
circDNER competitively binds to miR-139-5p to regulate
proliferation, invasion, metastasis and apoptosis via mediat-
ing ITGB8 expression in lung cancer. Our findings suggest
that circDNER might act as a potential prognostic bio-
marker and therapeutic target for the treatment of lung
cancer.

**METHODS**

**CircRNA microarray analysis**

A microarray assay was used to analyze the circRNA expres-
sion of lung adenocarcinoma and adjacent noncancerous tis-
ues as well as plasma exosome samples collected from the
Department of Thoracic Surgery, The Affiliated Hospital of
Jiangnan University.

**Clinical specimens**

All human lung cancer tissue samples and plasma samples
were obtained from the Department of Thoracic Surgery,
The Affiliated Hospital of Jiangnan University. The tumors
were fixed in 4% paraformaldehyde, embedded in paraffin,
and sectioned. The plasma samples were stored at \(-80^\circ\)C.
Written informed consent was obtained from all patients
and this study was approved by the ethics committee of
Affiliated Hospital of Jiangnan University.

**Exosome isolation**

All the samples were prepared by centrifuging for 5 min at
500 g. The supernatant was centrifuged at 10 000 g for 30 min
to remove shed macrovesicles. The supernatant was collected
and filtered through a 0.22 μm membrane filter. Finally, the
supernatant was centrifuged at 110 000 g for 70 min.

**Cell culture**

The human lung cancer cell lines BEAS-2B, HCC827, A549,
H1975, H1299 and H460 were purchased from GeneChem.
BEAS-2B, HCC827, A549 and H1975 cells were cultured in
RPMI-1640 containing 10% FBS and 1% penicillin–
streptomycin (Gibco). H1299 and H460 cells were cultured
in DMEM (Gibco) containing 10% FBS and 1% penicillin–
streptomycin. All cells were incubated at 37°C in an atmo-
sphere of 5% CO₂.

**Cell transfection**

Plasmids containing sh-circDNER and sh-NC, circDNER or
ITGB8 overexpression vector, miR-139-5p mimics and miR-
NC, miR-139-5p inhibitor and anti-miR-NC were designed
and transfected into lung cancer cells for up- or down-
regulating target genes according to the manufacturer’s pro-
tocol. All plasmids were synthesized by GeneChem.

**RNA isolation and qRT-PCR**

The total RNA of samples was isolated by RNAiso Plus
reagent (Takara) according to the manufacturer’s protocol.
ReverTra Ace qPCR RT Kit (TaKaRa) was used to obtain
cDNA. Quantitative reverse transcription polymerase chain
reaction (qRT-PCR) was performed using ABI 7500
StepOnePlus system (Applied Biosystems). β-actin were
used as internal controls and each reaction was performed
in triplicate. All primers are shown in Supplemental file 1.

**Colony formation, invasion and migration
assays**

The transfected cells were seeded in a 6-well plate for 14 days.
The colonies were fixed with ethanol (75%, Beyotime),
followed by staining with violet (0.1%, Beyotime). Finally, colo-
nies (>50 cells/colony) were counted and photographed.

The transfected cells were resuspended with serum-free
medium and seeded in the transwell upper chamber. Then, 500 μl
of DMEM medium supplemented with 10% FBS was
added to the transwell lower chamber. After incubation for
48 h, the chambers were stained with 0.5% crystal violet and
counted under a Nikon microscope (Minato).

**Flow cytometry analysis**

The transfected cells were collected and an apoptosis assay was
conducted using the annexin V-FITC/PI apoptosis detection
kit (Sangon Biotech) after incubation in the dark for 20 min.
Finally, flow cytometry was utilized to quantify cell apoptosis.

**MTT assay**

The transfected cells were seeded in a 96-well plate with dif-
ferent concentrations of PTX (0, 200, 400, 600, 800, 1000,
1200 nM) for 24 h. The cell proliferation-toxicity detection solution (CCK-8, Tongren Chemical Institute) was added at 10 μl per well, and then incubated at 37°C for 1 h.

**Dual-luciferase reporter assay**

The putative binding sequence of miR-139-5p and circDNER or ITGB8 was predicted by Circinteractome or TargetScan. Wild-type (WT) sequencing of circDNER or ITGB8 3′UTR with binding sequence for miR-139-5p was cloned into the pmirGLO vector (YouBia) to construct WT-circDNER or WT-ITGB8 3′UTR. At the same time, mutant reporter plasmids (MUT-circDNER or MUT-ITGB8 3′UTR) without binding sequence for miR-139-5p were generated in the same way. The indicated vector and miR-NC/miR-139-5p were then cotransfected into A549/PTX cells. After 48 h of cotransfection, a dual-luciferase reporter assay system (Promega) was used to examine luciferase activity.

**Western blot (WB)**

The samples were lysed in RIPA buffer (Beyotime), separated using 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA) for 1 h and then incubated with diluted primary antibodies to CD63 (1:1000, ab118307), HSP70 (1:1000, ab2787), TSG101 (1:1000, ab125011), ITGB8 (1:2000, ab80673) and GAPDH (1:2000, ab371685) overnight at 4°C. After washing with TBST for three times, the membrane was incubated with secondary antibodies for 35 min at room temperature. The relative levels of proteins were analyzed by calculating the ratio of the gray value.

**Tumor xenograft assay**

BALB/c (nu/nu, N = 6, male, 5 weeks) mice were selected and randomly divided into two groups. After 1 week, cells (2 × 10^6) were subcutaneously injected into the right flank
Two weeks later, one group of mice were injected with PBS and PTX, and another group of mice were injected with PTX-induced exosome and PTX. Six weeks later, the mice were euthanized and the xenograft tumors were surgically resected. The animal experiments were approved by the Institutional Animal Care and Use Committees at Affiliated Hospital of Jiangnan University.

**Statistical analysis**

GraphPad 7.0 was used for data analysis. The data are shown as mean ± standard deviation (SD). All samples were analyzed by t-test or one-way ANOVA with a significance level of $p < 0.05$.

**RESULTS**

**CircDNER is significantly upregulated in lung cancer tissues and cells, and increased circDNER expression predicts poor prognosis**

To explore the effect of circDNER in the pathogenesis of lung cancer, GSE101684 microarray analysis was performed to evaluate numerous dysregulated circRNAs in tumor tissues. The analysis showed that circDNER was significantly upregulated in lung cancer tissue (Figure 1a, b), which indicated circDNER might play a crucial role in the development of lung cancer. At the same time, using qRT-PCR, we found that circDNER was obviously increased in lung cancer tissue and A549 cells (Figure 1c,
Higher expression of circDNER resulted in increased mortality (Figure 1e). These results indicated that upregulation of circDNER may be vital in lung cancer progression.

**CircDNER could be delivered through exosomes**

CircRNA has been widely reported in exosomes which play a significant role in tumorigenesis. We isolated exosomes...
from PTX-resistant cell culture media. First, exosomes of A549/PTX cells were observed using transmission electron microscopy (TEM) (Figure 2a). Nanoparticle tracking analysis (NTA) further confirmed that the dominant size of vesicles was 100 nm, and concentrations at 100 nm multiplied by dilution factors produced concentrations of $4.5 \times 10^8$/ml for A549 and $9.6 \times 10^8$/ml for A549/PTX, respectively (Figure 2b). We examined the exosomal markers CD63, HSP70 and Tsg101 by western blotting. The data showed that higher levels of CD63, HSP70 and Tsg101 were discovered confirming the purity of the exosomes (Figure 2c). Moreover, circDNER expression in A549 cells was strikingly lower than in A549/PTX cells (Figure 2d). In addition, markedly higher expression of circDNER and IC50 values were observed after treatment with exosomes (Figure 2e,f).

To investigate the function of circDNER in vivo, a mice xenograft model was established. The data showed that A549 + PTX Exo + PTX group mice promoted tumor growth (Figures 2g–j).

**CircDNER plays a vital role in PTX-resistant lung cancer cells**

To further investigate the function of circDNER PTX-resistant lung cancer, we established the PTX-resistant A549...
cells (A549/PTX) (Figure 3a). Meanwhile, we analyzed the circDNER expression in PTX-sensitive or PTX-resistant tumor tissue, and found that circDNER expression was increased in PTX-resistant tumor tissue and cells (Figures 3b,c). To further confirm the effect of circDNER in PTX resistance, we next examined whether knockdown of circDNER would resensitize these cells to PTX treatment. We silenced circDNER expression by transfection of sh-circDNER. We found that IC50 value of PTX was decreased after knockdown of circDNER (Figure 3d,e).

To demonstrate the effect of circDNER on lung cancer cells, we examined cell proliferation using clone formation assays. Our findings suggested that silencing circDNER could obviously inhibit the proliferation of A549/PTX cells (Figure 3f). Flow cytometry data showed that silencing circDNER promoted cell apoptosis (Figure 3g). A transwell assay was utilized to detect cell invasion and motility. The results were consistent with proliferation, which indicated that downregulation of circDNER markedly restrained cell invasion and motility (Figure 3h,i). Together, these data implicated that circDNER downregulation could resensitize A549/PTX.

**CircDNER serves as a miRNA sponge for miR-139-5p**

It has previously been determined that circRNAs can serve as miRNA sponges to regulate miRNA targets. We next explored the ability of circDNER to bind miRNAs. Our results indicated that the potential interactions between miR-139-5p and circDNER by online databases starBase, circNet, and RNA22 (Figure 4a). We observed that miR-139-5p upregulation obviously decreased the luciferase activity in the WT-circDNER group but not in the MUT-circDNER group (Figure 4b). Moreover, lower expression of miR-139-5p was observed in resistant tissues and A549/PTX cells (Figure 4c,d). To assess the binding capacity of circDNER and miR-139-5p, RIP assays were performed which demonstrated that circDNER can bind with miR-139-5p (Figure 4e). As shown in Figure 4e, miR-139-5p expression was negatively correlated with circDNER expression in lung cancer tissue (Figure 4f). Furthermore, miR-139-5p expression was increased when circDNER was silenced in PTX-resistant cells. These data concluded that circDNER directly targeted miR-139-5p.

**Silencing CircDNER enhanced PTX sensitivity in A549/PTX cells by upregulating miR-139-5p**

To investigate whether miR-139-5p mediated the function of sh-circDNER, rescue experiments were performed in A549/PTX. Higher expression of miR-139-5p was observed by downregulating circDNER, which was attenuated by inhibiting miR-139-5p (Figure S1). Additionally, we found that miR-139-5p inhibition reduced the suppressive effects of knockdown circDNER colony-forming ability (Figure 5a). Moreover, miR-139-5p silencing reversed sh-circDNER-triggered cell apoptosis (Figure 5b). Meanwhile, the inhibitory effects of circDNER knockdown on migration and invasion were also counteracted by interference of miR-139-5p (Figure 5c,d). In summary, circDNER mediated PTX sensitivity by targeting miR-139-5p.

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**FIGURE 6** MiR-139-5p directly targeted ITGB8. (a) The binding sites between miR-139-5p and ITGB8 are shown. (b) The luciferase activity of A549/PTX cells cotransfected with WT-ITGB8 3'UTR/MUT-TPD52 3'UTR and miR-139-5p/miR-NC was assessed. (c and d) ITGB8 mRNA and protein expression in PTX sensitive tissues were measured via WB and qRT-PCR. (e) The RIP assay results demonstrated that ITGB8 could form silencing-induced complexes with miRNA in A549 cells. (f) The correlation between miR-139-5p expression and ITGB8 mRNA expression in PTX-resistant tissues was analyzed. (g) The abundance of ITGB8 was detected in A549/PTX cells after transfection with miR-NC or miR-139-5p. Data are presented as the mean ± SD of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001

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CircDNER regulates expression of ITGB8 via miR-139-5p

It is well known that miRNAs mediate biological functions by directly targeting mRNAs.\(^{13,14}\) We identified five genes that were screened in lung cancer cells transfected with mimic-miR-139-5p and inhibitor-miR-139-5p. The results showed that ITGB8 exhibited the most obvious change after treatment with mimic-miR-139-5p and inhibitor-miR-139-5p (Figure S2). Complementary sites between miR-139-5p and ITGB8 were observed in Figure 6a, suggesting that miR-139-5p might directly target ITGB8. Moreover, we observed that miR-139-5p upregulation obviously decreased luciferase activity in the WT-ITGB8 group but not in the MUT-TGB8 group (Figure 6b). Meanwhile, the expression of ITGB8 including mRNA and protein was higher in tumor tissues compared with normal tissues (Figure 6c,d). RIP assays demonstrated that miR-139-5p could target ITGB8 (Figure 6e). In addition, the expression of ITGB8 was negatively modulated by miR-139-5p. There was a higher expression of ITGB8 in lung cancer than in corresponding paracancerous tissues (Figure 6f). Additionally, we found that overexpression of miR-139-5p inhibited the ITGB8 protein level in A549/PTX cells. In this study, we found that miR-139-5p directly targeted ITGB8 and negatively mediated its expression in A549/PTX cells.

Overexpression of miR-139-5p improves PTX sensitivity in A549/PTX cells by targeting ITGB8

To confirm our previous results, gain-of-function and rescue experiments were performed. We found that
downregulating miR-139-5p in A549/PTX cells clearly inhibited ITGB8 expression, while ITGB8 overexpression counteracted the inhibition effect of miR-139-5p on ITGB8 expression (Figure 7a). In addition, we observed that overexpression of miR-139-5p suppressed the colony-forming ability and promoted the cell apoptosis, while addition of ITGB8 could reverse the effect (Figure 7b,c). Moreover, enforced expression miR-139-5p repressed migration and invasion of A549/PTX and the effect could be abated after overexpression of ITGB8 (Figure 7d,e).

**CircDNER mediates ITGB8 expression by binding with miR-139-5p**

Finally, we investigated whether circDNER could regulate ITGB8 expression by sponging miR-139-5p. Our data indicated that knockdown of circDNER decreased TPD52 protein expression, while silencing miR-139-5p could reverse the impact (Figure 8a), which indicated that circDNER could regulate ITGB8 expression by sponging miR-139-5p. A schematic diagram of the mechanism was created and is shown in Figure 8b.

**DISCUSSION**

Lung cancer is a common cancer characterized by high recurrence and poor 5-year survival rates. A number of therapies which have been applied for the treatment of lung cancer have failed due to lack of specific molecular targets and effective interventions. Therefore, searching for novel genetic targets and the pursuit of prognostic markers are critical for lung cancer.

In recent years, RNAs (circRNAs) have attracted great attention and have been shown to have a close relationship with various human malignant tumors. Previous studies have found that circ_0002346 and circ_0001658 are downregulated in lung cancer, which are positively correlated with tumor size, lymphatic invasion, or lymph node metastasis in patients. In our study, we found upregulated expression of circDNER in lung cancer tissues and cells, suggesting circDNER could promote lung cancer proliferation, invasion and migration in vitro and in vivo, hence downregulating the circDNER had the opposite effect. It is well accepted that circRNAs can negatively regulate miRNA activity. Our data indicated that circDNER increased lung cancer invasion and metastasis by sponging miR-139-5p which had been reported was downregulated in human tumors. Our finding is consistent with previous studies.
Integrin beta-8 (ITGB8) has been shown to be essential for tumor cell survival and serves as a target gene of miR-139-5p according to our data. As a master regulator of angiogenesis, ITGB8 has previously been reported to correlate with poor prognosis in various malignancies.\(^{20}\) In our study, we implicated for the first time that circ DNER promoted lung cancer proliferation, invasion and metastasis by modulating ITGB8 expression via sponging miR-139-5p, implying a novel regulatory axis formed by circ DNER/miR-139-5p/ITGB8 in lung cancer.

Exosomes are small endosomal-derived membrane microvesicles with a diameter of approximately 30–100 nm, which can be actively secreted by a variety of cells.\(^{21}\) A number of studies have demonstrated that exosomes are enriched in circRNAs, which may regulate tumor cell proliferation, migration and invasion.\(^{22,23}\) Previous studies have shown that exosomal circ-circRS-122 mediates PKM2 in colorectal cancer cell lines and that plasma exosomal circ-PDE8A is associated with tumor invasion.\(^{11,24}\) Plasma exosomal circular RNA circ-RanGAP1 modulates VEGFA expression by targeting miR-877-3p to facilitate invasion and metastasis.\(^{14}\) In this study, we demonstrated that plasma exosomal circ-DNER transferred from lung cancer cells increased lung cancer cell proliferation, invasion and migration, elucidating a novel mechanism of tumor metastasis in lung cancer. Moreover, our results demonstrated that plasma exosomal circ DNER can act as a tumor marker to distinguish between tumor patients and healthy people. Certainly, this conclusion needs further experimental and clinical studies to be confirmed.

ACKNOWLEDGMENTS
This work was supported by the Wuxi Taihu Lake Talent Plan, Supports for Leading Talents in Medical and Health Profession and General project of Wuxi Municipal Health Commission (M202103).

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

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