CircZNF609 Promotes Bladder Cancer Progression and Inhibits Cisplatin Sensitivity via miR-1200/CDC25B Pathway

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Abstract

Circular RNAs (circRNAs) have been extensively studied in tumor development and treatment. CircZNF609 has been shown to act as an oncogene in a variety of solid tumors and may serve as a novel biomarker for tumor diagnosis and treatment. However, the underlying role and mechanism of circZNF609 in bladder cancer (BCa) development and cisplatin chemosensitivity were unknown. Quantitative real-time PCR (qRT-PCR) was applied to determine the expression of circZNF609, microRNA 1200 (miR-1200) and CDC25B in BCa cells and tissues. Western blot was used to detect the protein level of CDC25B. Functional assays *in vitro* and *in vivo* were conducted to investigate the effects of circZNF609 on tumor development and cisplatin chemosensitivity in BCa. RNA sequencing and online databases were used to predict the interactions among circZNF609, miR-1200 and CDC25B. Dual luciferase reporter assay, RNA pull-down assay and RNA fluorescence in situ hybridization (FISH) were applied to confirm the mechanism. CircZNF609 expression was significantly up-regulated in BCa cell lines and tissues. Increased expression of circZNF609 was related to a worse survival in BCa patients. In *vitro* and in *vivo*, enforced-expression of circZNF609 enhanced BCa cells proliferation, migration and cisplatin chemoresistance. Mechanistically, circZNF609 alleviated the inhibition effect on target CDC25B expression by sponging miR-1200. CircZNF609 promoted tumor growth through novel circZNF609/miR-1200/CDC25B axis, implying that circZNF609 has significant potential to serve as a new diagnostic biomarker and therapeutic target for BCa patients.

Introduction

Bladder cancer (BCa) is a common type of urological carcinoma that has a high incidence and mortality worldwide (Teoh et al., 2020). Non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) are two types of bladder cancer (Tran et al., 2021). In comparison to MIBC, which has a high mortality, around 90% of NMIBC patients have a favorable five-year survival rate (Knowles and Hurst, 2015). However, patients with NMIBC are prone to recurrence (Knowles and Hurst, 2015). For MIBC patients, cisplatin-based neoadjuvant and adjuvant chemotherapy are first-line therapeutic strategies (Plimack et al., 2014). However, resistance to cisplatin based chemotherapy results in a poor prognosis (Sagalowsky, 2013). Therefore, further research is necessary to elucidate the molecular mechanism of chemotherapy-resistance, which may offer new insights to the BCa treatment.

Circular RNAs (circRNAs), a new class of RNAs that are formed by back-splicing of exons or introns of parental pre-mRNAs (Memczak et al., 2013). To date, most of them are exon-containing circRNAs and are located in the cytoplasm (Y Dong et al., 2017). Using high-throughput sequencing and bioinformatics analysis, circRNAs were characterized as having high stability, being evolutionarily conserved, and cell-specific and tissue-specific (Z. Li et al., 2015). Since their discovery in 1970s, circRNAs has absorbed increasing attention for their potential clinical utility. Previous research has established that circRNAs could modulate the development, progression, relapse, and chemosensitivity in various malignant tumors (Xu et al., 2020). Due to possessing microRNA (miRNA) binding sites, some circRNAs develop their roles by suppressing miRNA functions via sponge adsorption (Kristensen et al., 2018; Tang et al., 2021). Our

studies have showed that circ-ITCH inhibited BCa progression by sponging miR-17/miR-224 (Yang et al., 2018) and circ-Cdr1as increased BCa susceptibility to cisplatin via miR-1270/ APAF1 axis (W. Yuan et al., 2019a). CircZNF609 has been extensively explored in a variety of tumor types but not in BCa (S Liu et al., 2021b; He et al., 2020), which makes its significance in BCa intriguing.

Cisplatin has historically been a highly effective chemotherapy regimen for individuals with BCa. However, cisplatin-based chemotherapy would be effective in less than 50% of BCa patients (Sagalowsky, 2013). As reported, cell-cycle regulation is one of the major determinants of cisplatin chemosensitivity (Yang et al., 2018; Borst et al., 2014). When more cells are enriched in the cell division phase and fewer cells are enriched in the G1 phase, BCa cells become less susceptible to cisplatin chemotherapy (Donaldson et al., 1994; Shah and Schwartz, 2001). Cell division cycle 25B (CDC25B) is a well-characterized cell cycle regulator that is required for the accelerated cell cycle transition (Boutros et al., 2007; Aressy and Ducommun, 2008). As reported, increased expression of CDC25B may promote tumor progression and predict a poor prognosis in a wide variety of human cancers, including BCa (Z Zhang et al., 2014; Sur and Agrawal, 2016). While the role of CDC25B in modulating cisplatin chemosensitivity remains unknown.

The main findings of the present research were as follows: 1) CircZNF609 was upregulated in BCa tissues and cell lines and associated with a poor prognosis in BCa patients; 2) Enforced-expression of circZNF609 could accelerate BCa cells proliferation and cisplatin resistance *in vitro* and *in vivo* via a novel circZNF609/miR-1200/CDC25B axis.

Materials And Methods

Patient samples and cell lines

Tissues used in this research were acquired from BCa patients who underwent radical surgery at the first affiliated hospital of Nanjing Medical University. 48 pairs of BCa samples and adjacent normal tissues were included between 2014 to 2019. All human-related tissues for this study were approved by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University. All patients signed the informed consent before any clinical materials were collected. Liquid nitrogen was used to preserve samples.

Cell culture and Transfection

T24 and BIU87 BCa cells were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). T24 and BIU87 cells were respectively cultured in DMEM medium (Gibco, USA) and RPMI 1640 basic medium (Gibco, USA), containing 10% fetal bovine serum (BI, Israel) and 1% penicillin–streptomycin (Gibco, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

CircZNF609 knockdown or overexpression lentiviral vectors were purchased from HANBIO (HANBIO, Shanghai, China). When T24 or BIU87 cells reached 50% or 60% confluence in six-wells plates, they were

infected with circZNF609 overexpression lentivirus (termed as circZNF609), negative control lentivirus (termed as vector), circZNF609 knockdown lentivirus (termed as si circZNF609-1, si circZNF609-2) and scramble control lentivirus (termed as si NC), respectively. Puromycin were used for 2 times to select stable transfections.

MiR-1200 mimics and controls were achieved from GenePharma (Shanghai, China). The transfection was conducted using the Lipofectamine 3000 kit (Invitrogen, USA) according to the manufacturer's guidelines.

RNA isolation, quantitative Real Time-PCR (qRT-PCR)

Total RNAs were isolated from tissues and cells using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. cDNA was synthesized using HiScript II (Vazyme, China). The circRNA, miRNA and mRNA qRT-PCR assays were conducted on StepOne Plus Real-Time PCR system (Applied Biosystems, USA) or LightCycler 480 (Roche, USA). U6 or β-actin were used as controls. Data were analyzed by comparing CT values and each experiment was repeated for three times. All PCR primers were gained from TsingKe (TsingKe, Nanjing, China) and listed in Table S1.

Protein extraction and western blot

RIPA buffer (Sigma, USA) was used to lyse the tissues or cells. Protein extractions were absorbed and then quantified by bicinchoninic acid (BCA) assessment (Beyotime, China). Protein was isolated and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) using 10% SDS-PAGE. After blocking with 5% skim milk, membranes were incubated with primary (Protech, USA) and secondary antibodies (Protech, USA). Chemiluminescence (Bio-Rad, USA) was used to identify signals , which were then evaluated by Image Lab Software.

RNase R treatment

T24 and BIU87 cells were incubated for 30 min at 37 °C with RNase R (0.2 μl/μg, Epicenter). Total RNA were then extracted, reverse transcribed and detected by qRT-PCR assay.

Actinomycin D treatment

When T24 or BIU87 cells reached 60%-70% confluence in six-wells plates, they were incubated for 2 h, 4 h, 6 h, 8 h and 10 h, respectively, with 1 μg/ml actinomycin D (Abcam, UK). The relative RNA expression of ZNF609 in the liner or circular form was then quantified by qRT-PCR.

Cell proliferation assay

To determine the proliferation ability of T24 or BIU87 cells, they were seeded in 96-well plates with starting number of 2000 or 5000 cells, respectively. Cell viability was determined every 24 h (24 h, 48 h, 72 h and 96 h) using the cell counting kit-8 (CCK-8) technique (Dojindo, Japan). The absorbance value at 450 nm was determined using a microplate reader (Tecan, Switzerland).

Cloning formation

T24 or BIU87 BCa cells were seeded at 1000 or 1500 cells per well in six-well plates. Two weeks later, cell colonies were fixed with 4% paraformaldehyde and stained by 0.1% crystal violet. Image J software was used to visualize and calculate cell colonies.

Scratch wound healing assay

When T24 or BIU87 cells reached 90%-100% confluence in six-wells plates, the monolayer cells were scraped off with the 200 μl pipette tip and cultured in serum-free medium. Cells were imaged at 24 h and 48 h, respectively, using microscope (Olympus, Japan). As a control, images of BCa cells at 0 h were also obtained. The migration rate was determined using a formula based on the distance traveled by BCa cells divided by the initial width.

Transwell assays

To examine the migration ability, 200 μl serum-free medium with 4×10^4 T24 or 6×10^4 BIU87 cells was seeded in upper chamber (Corning, USA), while 600 μl culture medium with 20% FBS was added into the lower chamber. After 24 h, the top chambers were fixed with 4% paraformaldehyde and then stained for 15 min with 0.5% crystal violet. Finally, the transwell chambers were photographed using Olympus microscope.

Evaluation of cell cycle

To detect distinct phases of cell cycle, BCa cells were fixed in 75% ethanol and dyed with the cycle test and DNA reagent kit (BD Biosciences, USA). Then using flow cytometry (Beckman Coulter, USA), cell proportion in various phases were enumerated and analyzed via ModFit software (Version 5.0).

IC50 determination

After an overnight incubation of 5000 BCa cells per well in a 96-well plate, various concentration gradients of cisplatin (128, 64, 50, 40, 32, 16, 8, 4, 2, and 1 μM, TCA, Japan) were added. After 24 h incubation, the cell viability was determined by CCK-8 method and IC50 value was obtained by the probit regression model.

RNA-fluorescence in situ hybridization (FISH) assay

Cy3-labeled circZNF609 and FAM-labeled miR-1200 probes were achieved from Genepharma (Shanghai, China). The T24 cells were stained by relative probes utilizing Fluorescent in Situ Hybridization kit (Genepharma, Shanghai, China). All images were captured from Zeiss LSM880 NLO confocal microscope system (Leica Microsystems, Germany).

Biotin-coupled probe pull-down assay

Biotinylated circZNF609 and miR-1200 (GenePharma, China) pull down assays were conducted. Oligo probe was used as control. A total of 10^7 cells were collected and lysed using lysis buffer. All lysed products were incubated at room temperature for 2 h with 3 μg biotinylated probes. Following that, streptavidin magnetic beads (50 μl) were incubated with the products generated in the previous step for 4 h. The RNA complexes attached to the beads were evaluated by qRT-PCR assay, after being washed 5 times with lysis buffer and isolated using Trizol reagent.

Luciferase reporter assay

293T cells were cultured in 96-well plates and co-transfected using Lipofectamine3000 (Invitrogen, Foster city, CA) with miR-1200 mimics and plasmids expressing wild-type or mutant fragments. After 24 h incubation, firefly and renilla luciferase activities were measured utilizing dual luciferase reporter assay system (Promega, USA).

Xenograft in nude mice

Approximately 1×10^7 T24 cells transfected with sh circZNF609, circZNF609 over expression or their negative control were subcutaneously injected into axilla of the athymic BALB/C nude mice (4-6 weeks old, 18-22 g, five mice/group). Mice in experimental groups received intraperitoneal injections of cisplatin (2.5 mg/kg) every three days for one week following inoculation, while mice in control groups received saline injections. The volume (V) was determined every week by measuring the width (W) and length (L) using calipers, and the formula $V= (W^2 \times L)/2$ was used. After 4 weeks, we euthanized the mice and quantified the tumor bulk. All animal experiments were performed according to the ethics guidelines for animal experiments, which were authorized by the animal management committee of Nanjing Medical University.

Immunohistochemistry (IHC)

Mouse tumor was fixed in paraffin and mounted on 4 μm slide each. Tumor slides were rehydrated by different concentrations of ethanol. Antigen was separated by microwave heating. The slides were soaked in 3% H_2O_2 for 10 min and then dealt with CDC25B antibody (1: 500, Proteintech, USA) in a humidified incubator at 4°C overnight. Then slides were incubated with HRP-conjugated antibody at room temperature for 30 min. The data were collected by a microscope.

Statistical analysis

Data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA) and were presented as mean ± standard deviation (means ± SD). Student's t-test and one-way ANOVA were performed to analyze differences among the groups. Survival curves were plotted using the Kaplan-Meier method, and differences were analyzed by the log-rank test. *P* values <0.05 were considered statistically significant.

Results

CircZNF609 expression was up-regulated in BCa tissues and cell lines and was related to histological grades and prognosis in BCa patients

CircZNF609 was validated as a circular product by Sanger sequencing after amplified by PCR with divergent primers (Figure 1A). We performed actinomycin D and RNase R treatment experiments to establish that circZNF609 was more stable than liner ZNF609 (Figure1B-C). CircZNF609 expression was significantly up-regulated in BCa tissues or cell lines when compared with adjacent normal tissues or SV-HUC, a normal urothelial cell line (Figure 1D-E). Furthermore, we observed a positive association between circZNF609 expression and histological grade (P=0.040). While no significant correlation was observed between circZNF609 and other clinicopathological parameters such as age, gender, tumor size and tumor node metastasis (TNM) stage (Table 1). Prognostic analysis revealed that patients with high circZNF609 expression level had a worse overall survival (Figure 1F).

CircZNF609 promoted the progression and cisplatin resistance of BCa cells *in vitro*

To investigate the function of circZNF609 in BCa cells, we constructed circZNF609 overexpression and knockdown in low malignant BCa cell line BIU87 and high malignant BCa cell line T24 separately (Figure S1A-D). Further functional assays were conducted to investigate the role of circZNF609 in BCa. CCK-8 assays indicated that circZNF609 could promote the proliferation of T24 and BIU87 BCa cells (Figure 2A-B). Results of cloning formation assays revealed a positive association between circZNF609 expression and colony numbers (Figure 2C-D). When the expression level of circZNF609 was decreased, more cells were distributed in the G1 phase and less cells were distributed in the S phase (Figure 2E-F), and vice versa (Figure 2G-H). The IC50 of cisplatin was calculated using cell viability curves of the cisplatin treatment experiments (Figure S2 A-B). The lower IC50 of cisplatin for T24 and BIU87 cells were observed when circZNF609 was knockdown (Figure 2I-J), but a higher IC50 was seen when circZNF609 was overexpressed (Figure 2K-L). Additionally, we also discovered positive correlation between circZNF609 and T24 and BIU87 cell migration during scratch wound healing and transwell assays (Figure S3A-C).

CircZNF609 acted as a molecular sponge for miR-1200

We used three publicly accessible database miRanda http://www.miranda.org/), RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) and regRNA (http://regrna2.mbc.nctu.edu.tw/) to predict 10 potential miRNAs (Figure 3A). Results of biotin-coupled probe pull-down experiments revealed that miR-1200 was most significantly associated with circZNF609 (Figure 3B). Further pull-down experiments also indicated that biotin labeled miR-1200 mimics could bind to circZNF609 abundantly (Figure 3C). Additionally, luciferase reporter assays also confirmed that circZNF609 sponged miR-1200 significantly (Figure 3D-E). The specific binding sites between circZNF609 and miR-1200 were predicted using the above database (Figure 3E). RNA FISH assays revealed that circZNF609 and miR-1200 co-localized in T24 cells (Figure 3F).

In 48 pairs of BCa tissues, miR-1200 was highly expressed in adjacent normal tissues compared with tumor tissues by qRT-PCR (Figure 3G). Pearson correlation analysis found a negative relationship

between circZNF609 and miR-1200 (Figure 3H). Patients with higher level of miR-1200 expression had a favorable prognosis (Figure 3I).

MiR-1200 inhibited the progression and cisplatin resistance of BCa cells *in vitro*

Functional experiments were used to confirm the role of miR-1200 in BCa cells. MiR-1200 could inhibit the proliferation of T24 and BIU87 cells by CCK8 assay (Figure 4A) and cloning assays (Figure 4B). When miR-1200 was overexpressed, flow cytometry results showed that more T24 or BIU87 cells were distributed in G1 phase and less T24 or BIU87 cells were distributed in S phase (Figure 4C-F). MiR-1200 could also increase the cisplatin chemosensitivity of T24 and BIU87 cells (Figure 4G-H). Additionally, we also discovered that miR-1200 could inhibit T24 and BIU87 cell migration during scratch wound healing and transwell assays (Figure S3A-C).

MiR-1200 interference rescued the intensive progression and depressed cisplatin sensitivity induced by circZNF609 in BCa cells

The rescue assays were performed by co-transfecting circZNF609 overexpression lentivirus and miR-1200 mimics into T24 and BIU87 BCa cells. CCK-8 and colony assays demonstrated that high levels of circZNF609 could promote the proliferation of BCa cells, whereas ectopic miR-1200 could repress this effect (Figure 5A-B). Flow cytometry results indicated that when circZNF609 was overexpressed, less cells were dispersed in the G1 phase and more cells were dispersed in the S phase. While co-transfection of miR-1200 mimics in cells overexpressing circZNF609, miR-1200 could both rescue the cell cycle promotion induced by circZNF609 (Figure 5C-F) and the cisplatin resistance induced by circZNF609 (Figure 5G-H). Additionally, ectopic miR-1200 was also able to inhibit circZNF609-induced increased migration rates during scratch wound healing and transwell assays (Figure S5A-B).

CircZNF609 improved the expression of CDC25B through sponging miR-1200

mRNA sequencing was performed on three pairs of circZNF609 overexpression and relative control T24 BCa cells. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to indentify related pathways and genes impacted by circZNF609. GO analysis revealed that circZNF609 expression was associated with cell population proliferation and cell cycle process functions (Figure 6A). KEGG pathway analysis revealed a correlation between circZNF609 and cancer pathways (Figure 6B). The prediction databases Targetscan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/) were used to predict the downstream genes bound with miR-1200. We found that CDC25B was screened as a downstream target gene by combining database prediction results and mRNA sequencing results (Figure 6C).

Dual luciferase reporter assay was performed to confirm the binding of miR-1200 and CDC25B. The mutant and wild type 3'-UTR of CDC25B was used to construct reporter plasmids. Co-transfection of miR-1200 mimics and wild-type CDC25B reporter plasmids dramatically suppressed luciferase activity,

whereas co-transfection of miR-1200 mimics and mutated CDC25B plasmids showed no significant effect (Figure 6D).

Next, we used qRT-PCR to determine the expression level of CDC25B in 48 pairs of BCa tissues. The results showed that CDC25B was highly expressed in tumor tissues compared to adjacent normal tissues (Figure 6E). And patients with a high level of CDC25B expression had a worse survival rate (Figure 6F). Furthermore, we found that circZNF609 could enhance CDC25B expression at both mRNA and protein levels (Figure 6G-J), while miR-1200 could inhibit CDC25B expression (Figure S6A-B). Additionally, transfection of miR-1200 mimics in BCa cells overexpressed circZNF609 rescued the circZNF609-induced high expression of CDC25B (Figure 6K-L).

Additionally, functional assays were performed to verify the role of CDC25B in BCa cells. Firstly, we transfected the short CDC25B interfering RNAs to inhibit its expression (Figure S7A-B). CCK8 and cloning formation assays indicated that CDC25B could promote the proliferation of BCa cells (Figure S7C-D). Scratch wound healing and transwell assays indicated that CDC25B could increase the migration rate of BCa cells (Figure S7E-F). Furthermore, CDC25B could also induce more BCa cells dispersed in S phase and inhibit the cisplatin sensitivity (Figure S7G-J).

CircZNF609 promoted growth and cisplatin resistance of xenografted tumor *in vivo*

In order to confirm whether circZNF609 had effects on tumor growth *in vivo*, T24 cells transfected with circZNF609 overexpression and relative control vector were subcutaneously inoculated into axilla of the nude mice. The tumors were removed after 4 weeks (Figure 7A-B). CircZNF609 overexpression had a considerable effect on tumor volume and weight growth (Figure 7C-D). IHC experiments indicated that CDC25B levels were positively related to circZNF609 (Figure 7E).

To confirm whether circZNF609 could influence the cisplatin sensitivity *in vivo,* si-circZNF609 transfected and negative control transfected T24 cells were subcutaneously inoculated into axilla of the nude mice. Mice in experimental groups received intraperitoneal injection of cisplatin (2.5 mg/kg) every three days for one week following inoculation, while mice in control groups received saline injection. The tumors were removed after 4 weeks (Figure 7F-G). Tumor weights and volume were significantly decreased in cisplatin-treated group compared to the saline-treated group (Figure 7H-I). Tumor volume and weight were lower in si-circZNF609 group treated with cisplatin than in the control group treated with cisplatin (Figure 7H-I).

Discussion

The development and progression of BCa is a complex process that has not been fully elucidated. Moreover, the emergence of chemotherapy resistance has complicated the treatment of BCa. For nearly a decade, it has been proven that an increasing number of circRNAs play a critical role in the incidence and treatment of BCa (Yang et al., 2018) (Wei Dong et al., 2019) (H. Zhang et al., 2021a). In this research, we discovered that patients with BCa who had elevated circZNF609 levels had a higher histological grade

and a worse survival. Then, we demonstrated that circZNF609 facilitated BCa cells progression and inhibited BCa cells chemosensitivity to cisplatin via a novel circZNF609/miR-1200/CDC25B axis *in vitro* and *in vivo*.

CircRNAs were characterized as single-stranded and covalently closed, which were created via back-splicing from pre-mRNAs (Capel et al., 1993). CircRNAs were found to be very stable, evolutionary conserved, and tissue-specific (Z. Li et al., 2015). Given these distinguishing features, circRNAs are increasingly being highlighted as a new candidate for regulating expression of oncogenes or tumor suppressor genes (Rybak-Wolf et al., 2015; Salzman et al., 2013).

CircZNF609 has previously been shown to promote tumor progression via sponging miRNAs (S Liu et al., 2021b; He et al., 2020; Q Liu et al., 2021a). For instance, circZNF609 accelerated the advancement of lung cancer via miR-142-3p/GNB2 axis (S Liu et al., 2021b). CircZNF609 could also enhance melanoma development via miR-138-5p/SIRT7 pathway (Q Liu et al., 2021a). In our study, we discovered that circZNF609 acted an oncogene by sponging miR-1200. MiR-1200 has been shown in previous research to suppress tumor development and enhance cisplatin sensitivity, including glioma and gastric cancer (Pan et al., 2019; Zhao et al., 2021; Z Zhang et al., 2020). Our further research revealed that miR-1200 could also enhance the sensitivity of BCa to cisplatin and repress the progression of BCa by reducing CDC25B expression. Then we demonstrated that circZNF609 acted as an oncogene via miR-1200/CDC25B pathway.

It is vital to develop novel and effective treatment targets in order to better prevent tumor progression. Numerous circRNAs with critical regulatory roles in carcinogenesis and progression are regarded to hold tremendous therapeutic potential (Xu et al., 2020). In BCa, circLIFR could attenuate cisplatin chemoresistance via synergizing with MSH2 (H Zhang et al., 2021b). A hypoxia-induced circRNA, circELP3, could also inhibit cisplatin chemosensitivity in BCa (Su et al., 2019). Our previous research also revealed that circ-Cdr1as increased BCa's susceptibility to cisplatin via miR-1270/APAF1 axis (W Yuan et al., 2019b). Therefore, the regulatory role of circRNAs in cancer therapy cannot be ignored.

Cisplatin has played an important role in chemotherapy for BCa. However, cisplatin resistance greatly harmed BCa patients' long-term survival (W Yuan et al., 2019b). We demonstrated for the first time in this study that circZNF609 could induce cisplatin resistance of BCa. Additional mechanistic investigations demonstrated that circZNF609 contributed to cisplatin resistance by up-regulating CDC25B expression via sponging miR-1200. CDC25B, as a cell cycle regulator, is capable of altering the distribution of cell cycle phases in tumor cells (Boutros et al., 2007; Aressy and Ducommun, 2008). According to studies, cisplatin is more toxic to tumor cells in the G1 phase (Donaldson et al., 1994; Shah and Schwartz, 2001). Our results showed that increasing CDC25B expression decreased the amount of BCa cells in G1 phase and decreased cisplatin chemosensitivity. The oncogenic potential of CDC25B has been thoroughly confirmed (Yan et al., 2008; Wang et al., 2020; R Li et al., 2019; Jia et al., 2021). Reduced CDC25B expression could inhibit the progression of liver cancer (Yan et al., 2008). CircRNA_102958 accelerated the carcinogenesis of colorectal cancer through increasing CDC25B expression (R Li et al., 2019). And

lncRNA FAM83A-AS1 could promote esophageal cell squamous carcinoma progression by increasing CDC25B expression (Jia et al., 2021).

According to our findings, the circZNF609-mediated function network will provide new avenues for BCa treatment. Additonally, inhibitors of this network may serve as a potential adjuvant for cisplatin-based chemotherapy in BCa.

Abbreviation

CircRNA: circular RNA; miRNA: microRNA; BCa: Bladder cancer; MIBC: muscle invasive bladder cancer; NMIBC: non-muscle invasive bladder cancer; FISH: Fluorescence in situ hybridization

Declarations

Acknowledgements:

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Authors' contributions:

QL, XY and HWY conceived and designed the study. JL, DF, QC, HY, JH, YDC, JTZ and XY performed the experiments. JL and DXF wrote the paper. QL and HWY reviewed and edited the manuscript. All authors read and approved the manuscript.

Data availability statement:

All data generated or analyzed during this study are included in this published article.

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Ethics Statement:

Utilization of tissues was granted approval by the ethics board at the first affiliated hospital of Nanjing medical university. Participants were asked to sign informed consent before using clinical resources. Animal experiments were conducted according to ethics guidelines for animal studies and granted approval through the animal ethics board of Nanjing Medical University.

Competing Interests:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1: Associations between the expression level of circZNF609 and clinicopathological features in BCa patients.

Characteristics	Case	circZNF609		*P* value
		Low	High	
All cases	48	21	27	
Age(years)				1.000
<65	24	10	14	
≥65	24	11	13	
Gender				0.095
Male	36	13	23	
Female	12	8	4	
TNM stage				1.000
pTa-pT1	12	5	7	
pT2-pT4	36	16	20	
Histological grade				**0.040***
Low	11	8	3	
High	37	13	24	
Tumor size(cm)				0.140
<3	17	10	7	
≥3	31	11	20	

**P*<0.05

Figures

Figure 1

CircZNF609 was up-regulated in BCa tissues and cell lines and was positively associated with poor survival. A Schematic illustration indicated circZNF609 was formed by circularization of ZNF609 exon 1 and sanger sequencing confirmed the junction of back splicing. B qRT-PCR confirmed the remaining RNA levels of circZNF609 and ZNF609 mRNA levels in T24 cells following actinomycin D treatment at

different time points. C qRT-PCR determined the RNA levels of circZNF609 and ZNF609 mRNA in T24 and BIU87 cells treated with or without RNase R treatment (***P<0.001, Student ’s t-test). D The expression levels of circZNF609 in 48 pairs of BCa tissues and adjacent normal tissues. E The expression levels of circZNF609 in 8 BCa cell lines compared with SV-HUC cell line (***P<0.001, Student ’s t-test). F Kaplan-Meier analysis showed the correlation between circZNF609 expression and overall survival in BCa patients.

Figure 2

CircZNF609 promoted the progression and cisplatin resistance of BCa cells in vitro A CCK8 assay showed circZNF609 knockdown could inhibit T24 and BIU87 cells proliferation (*P<0.05, ***P<0.001, Student’s t-test). B CCK8 assay showed circZNF609 overexpression could promote T24 and BIU87 cells proliferation (**P<0.01, ***P<0.001, Student’s t-test). C Colony formation assay indicated circZNF609 knockdown could inhibit the T24 and BIU87 cells colony formation (**P<0.01, Student ’s t-test). D Colony formation assay showed circZNF609 overexpression could improve the colony numbers of T24 and BIU87 cells (**P<0.01, Student's t-test). E More T24 cells were distributed in G1 phase and less T24 cells were distributed in S phase when circZNF609 was knockdown (**P<0.01, ***P<0.001, Student ’s t-test). F More BIU87 cells were distributed in G1 phase and less BIU87 cells were distributed in S phase when circZNF609 was knockdown (*P<0.05, **P<0.01, Student’ s t-test). G Less T24 cells were distributed in G1 phase and more T24 cells were distributed in S phase when circZNF609 was overexpression (*P<0.05, Student ’s t-test). H Less BIU87 cells were distributed in G1 phase and more BIU87 cells were distributed in S phase when circZNF609 was overexpression (*P<0.05, **P<0.01, Student’s t-test). I IC50 assays indicated that knockdown of circZNF609 improved sensitivity of T24 cells to cisplatin (**P<0.01, ***P<0.001, Student’s t-test). J IC50 assays showed that knockdown of circZNF609 improved sensitivity of BIU87 cells to cisplatin (*P<0.05, **P<0.01, Student’s t-test). K IC50 assays indicated that overexpression of circZNF609 inhibited sensitivity of T24 cells to cisplatin (***P<0.001, Student’s t-test). L IC50 assays showed that overexpression of circZNF609 inhibited sensitivity of BIU87 cells to cisplatin (**P<0.01, Student’s t-test). Data are mean±SD, n=3

Figure 3

CircZNF609 sponged miR-1200 mostly in BCa cells. A Schematic illustration indicated overlapping of the target miRNAs of circZNF609 predicted by regRNA, miRanda and RNAhybrid. B CircZNF609 pull down assay showed miR-1200 combined with circZNF609 significantly compared with other potential miRNAs (**P<0.01, Student’s t-test). C MiR-1200 pull down assay indicated that biotin coupled miR-1200 could captured more circZNF609 compared with biotin coupled NC (**P<0.01, Student’s t-test). D Dual luciferase reporter assays showed circZNF609 bound with miR-1200 most significantly compared with other potential miRNAs. E Dual luciferase reporter assays confirmed that miR-1200 were direct target of circZNF609 (***P<0.001, Student’s t-test). Illustration showed the binding sites of circZNF609 and miR-

1200. F RNA FISH experiment for circZNF609 and miR-1200 was performed in T24. Nuclei was stained blue (DAPI), circZNF609 was stained red, and miR-1200 was stained green. G The expression level of miR-1200 was detected in 48 pairs of BCa tissues and relative adjacent normal tissues by qRT-PCR. H Correlation between the circZNF609 and miR-1200 was investigated by Pearson correlation analysis. I The correlation of overall survival of BCa patients and miR-1200 expression was analyzed by Kaplan-Meier analysis. Data are mean±SD, n=3

Figure 4

MiR-1200 inhibits the progression and cisplatin resistance of BCa cells. A CCK8 assay showed miR-1200 could inhibit T24 and BIU87 cells proliferation (**P<0.01, ***P<0.001, Student's t-test). B Colony formation assay indicated miR-1200 could inhibit the colony numbers of T24 and BIU87 cells (**P<0.01, Student's t-test). C Flow cytometry assays were applied to investigate the function of miR-1200 in T24 cells. D More T24 cells were distributed in G1 phase and less T24 cells were distributed in S phase when miR-1200 was overexpression (*P<0.05, **P<0.01, Student's t-test). E Flow cytometry assays were applied to investigate the function of miR-1200 in BIU87 cells. F More BIU87 cells were distributed in G1 phase and less BIU87 cells were distributed in S phase when miR-1200 was overexpression (*P<0.05, Student's t-test). G IC50 assays showed that miR-1200 improved sensitivity of T24 cells to cisplatin (**P<0.01, Student's t-test). H IC50 assays showed that miR-1200 improved sensitivity of BIU87 cells to cisplatin (***P<0.001, Student's t-test). Data are mean±SD, n=3

Figure 5

Co-transfection of miR-1200 mimic eliminated the oncogenic function of circZNF609. A CCK-8 experiments indicated that co-transfection with miR-1200 mimics could reverse proliferation-promoting function of circZNF609 in T24 and BIU87 cells (***P<0.001, Student's t-test). B Cloning formation assays revealed that co-transfection with miR-1200 mimics could reverse the enhanced proliferation induced by circZNF609 in T24 and BIU87 cells (**P<0.01, ***P<0.001, Student's t-test). C-D Illustrations of rescued flow cytometry assays in T24 and BIU87 cells. E Less cells were dispersed in G1 phase and more cells were distributed in S phase when circZNF609 was overexpression in T24 cells, and miR-1200 mimic transfection could reverse this phenomenon (*P<0.05, **P<0.01, Student's t-test). F Less cells were dispersed in G1 phase and more cells were distributed in S phase when circZNF609 was overexpression in BIU87 cells, and miR-1200 mimic transfection could reverse this oncogenic function (**P<0.01, ***P<0.001, Student's t-test). G The IC50 assays indicated that circZNF609 overexpression inhibited sensitivity of T24 cells to cisplatin, while co-transfection of miR-1200 mimics could reverse it in T24 cells (*P<0.05, **P<0.01, Student's t-test). H The IC50 assays indicated that circZNF609 overexpression inhibited sensitivity of BIU87 cells to cisplatin, while co-transfection of miR-1200 mimics could reverse it in BIU87 cells (*P<0.05, **P<0.01, Student's t-test). Data are mean±SD, n=3

Figure 6

MiR-1200 inhibited BCa progression and cisplatin resistance via targeting CDC25B. A GO terms analysis of mRNA sequencing. B KEGG analysis of mRNA sequencing. C Downstream target CDC25B and binding sites to miR-1200 were predicted by overlapping of database and mRNA sequencing. D Dual luciferase reporter assays were applied to confirm the binding of miR-1200 and CDC25B (***P<0.001, Student's t-test). E The expression level of CDC25B in 48 pairs of BCa tissues was investigated by qRT-PCR. F The overall survival of BCa patients with different CDC25B expression level was investigated by Kaplan-Meier analysis. G CircZNF609 knockdown decreased the mRNA level of CDC25B in T24 and BIU87 cells (**P<0.01, Student's t-test). H CircZNF609 knockdown decreased the protein level of CDC25B in T24 and BIU87 cells. I Overexpression of circZNF609 improved the mRNA level of CDC25B in T24 and BIU87 cells (***P<0.001, Student's t-test). J Overexpression of circZNF609 improved the protein level of CDC25B in T24 and BIU87 cells. K Overexpression of circZNF609 improved the mRNA level of CDC25B in T24 and BIU87 cells, while co-transfection of miR-1200 mimic could reverse it (*P<0.05, **P<0.01, ***P<0.001, Student's t-test). L Overexpression of circZNF609 improved the protein level of CDC25B in T24 and BIU87 cells, while co-transfection of miR-1200 mimic could reverse it. Data are mean±SD, n=3

Figure 7

CircZNF609 promoted BCa cells proliferation and cisplatin resistance in nude mice. A Picture of nude mice implanted with circZNF609 overexpression or negative control T24 cells (n=5). B Image of tumor formation in nude mice implanted with circZNF609 overexpression or negative control T24 cells (n=5). C Volumes of xenograft tumors were measured by electronic scales every week (**P<0.01, Student's t-test). D Tumor weights of nude mice were measured by electronic scales (***P<0.001, Student's t-test). E Immunohistochemistry assays were applied to detect the CDC25B in two groups of xenograft tumors. F Picture of the nude mice implanted with circZNF609 knockdown or negative control T24 cells, which injected with cisplatin or saline (n=5). G Image of xenograft tumor of nude mice implanted with circZNF609 kncokdown or negative control T24 cells, which injected with cisplatin or saline (n=5). H Tumor volumes of nude mice in four groups were measured every week (**P<0.01, ***P<0.001, Student's t-test). I Tumor weights of four groups were measured by electronic scales (*P<0.05, **P<0.01, ***P<0.001, Student's t-test). Data are mean±SD, n=3

Figure 8

Mode pattern of the circZNF609-miR-1200-CDC25B regulatory and function network.

Supplementary Files

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