Overexpressed circ-CEP128, a potential new circular RNA biomarker, promotes cisplatin resistance of bladder cancer cells by regulating necroptosis

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Primary research

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Abstract

Background

Bladder cancer (BC) is the most common malignancy of urinary system and cisplatin (DDP) remains the only chemotherapy option for treatment of BC at the advanced stage. The critical molecules involved in the regulation of cisplatin resistance are still largely unknown. CircRNAs has been demonstrated to be involved in tumorigenesis and development and drug resistance of various cancer cells. CircCEP128 contributed to BC progression by regulating miR-145-5p/MYD88/MAKP axis. However, functions and molecular mechanisms of circCEP128 in DDP resistance of bladder cancer cells still remain largely unclear.

Methods

Bladder cancer tissue and the corresponding adjacent normal tissue as well as serum samples were obtained from a total of 60 BC patients who received the same cisplatin-based chemotherapy. The expression level of circCEP128 in tissues and serums was measured using qRT-PCR. WB was utilized to detect expression level of PCNA, Cyclin D1, RIPK3, p-RIPK3, MLKL or p-MLKL. Functionally, BC cell viability and proliferation are measured through relevant experiments, including CCK8 assay and cell colony formation assay.

Results

In the current study, we demonstrated that circCEP128 expression was distinctly elevated in the BC tissues and serums, especially in the chemoresistant BC tissues or cell lines, correlated with poor prognosis of BC patients. In addition, ROC curve suggested that circCEP128 might serve as an effective diagnostic biomarker for BC and treatment. Furthermore, cell function assays showed that circCEP128 silencing by siRNA could reverse the drug-resistance of BC cells to cisplatin by inducing necroptosis through regulation of RIPK3/MLKL signaling pathway.

Conclusions

Our findings indicated that circCEP128 may serve as a valuable diagnostic biomarker in BC and contribute to cisplatin resistance of bladder cancer cells by repressing necroptosis through RIPK3/MLKL signaling pathway. These findings provide novel insights into the role of circCEP128 as a biomarker for the diagnosis and treatment target of BC.

Background
Bladder cancer (BC) is the most common malignancy of urinary system and leads to 165,000 deaths per year. An estimated 400,000 new cases of BC were diagnosed globally each year. Furthermore, it typically shows a high postoperative recurrence ratio at approximately 61% within 2 years in high-risk BC [1].

Cisplatin (DDP), one of platinum-based broad spectrum anti-tumor drugs, was considered as the only chemotherapy option for treatment of bladder cancer at the advanced stage [2, 3]. However, the occurrence of intrinsic or acquired resistance to cisplatin remains an urgent challenge in BC therapy. The critical molecules involved in the regulation of cisplatin resistance are still largely unknown. Hence, further understanding of the molecular mechanisms responsible for the cisplatin resistance in BC would be helpful for developing novel approaches to ameliorate drug resistance and improve the efficacy of BC treatment.

Circular RNAs (circRNAs) are a category of non-coding endogenous transcripts that are generated by back-splicing or exon skipping events. CircRNAs were originally considered as transcription artifacts or “splicing noises” [4]. Previously studies showed that circRNAs are involved in tumorigenesis and development and drug resistance of various cancer cells [5]. CircRNA FECR1 was demonstrated to facilitate tumor invasive and metastatic potential in breast cancer [6]. Jian et al. revealed that the proliferation and metastasis of hepatocellular carcinoma could be suppressed by overexpression of circRNA cSMARCA5 [7]. CircPVT1 was found to promote the chemoresistance of osteosarcoma cells to cisplatin and doxorubicin [8]. Moreover, Huang et al. revealed that circAKT3 participated in the emergence of cisplatin resistance in gastric cancer [9]. As for bladder cancer, circ-ITCH has been reported to promote tumor progression [10]. Hypoxia-induced circELP3 could facilitate bladder cancer development and DDP resistance [11]. Our previous study revealed that upregulation of circCEP128 contributed to BC progression by regulating miR-145-5p/MYD88/MAKP axis [12]. However, functions and molecular mechanisms of circCEP128 in DDP resistance of bladder cancer cells still remain unknown and need further investigations.

Necroptosis is considered as a variant of necrosis and typically mediated by receptor-interacting kinase 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL) signaling pathway [13]. A couple of previous studies have demonstrated that induction of necroptosis in target cancer cells could be an effective treatment approach for tumor [14, 15]. In the current study, through functional loss experiments, we confirmed that silencing of circCEP128 was capable of inducing RIPK3/MLKL-mediated necroptosis through increasing phosphorylation level of RIPK3 and MLKL in BC cells, so as to ameliorate the drug resistance of bladder cancer cells to cisplatin.

**Methods**

**Human tissue sample preparation**

Bladder cancer tissue and the corresponding adjacent normal tissue were obtained from a total of 60 BC patients who received the same cisplatin-based chemotherapy (two cycles of adjuvant chemotherapy with cisplatin and gemcitabine after radical cystectomy) at Shengjing Hospital of China Medical
University from 2016 Apr 5th to 2019 Jun 1st. This study gained the approval of the Ethics Committee of Shengjing Hospital of China Medical University, and a written informed consent was signed by each patient. Besides, the blood samples from above 60 BC patients were collected ahead of surgery, and 30 sex- and age-matched healthy individuals as the control group. All tissue samples were placed immediately into liquid nitrogen and stored at -80 °C. The serum samples were extracted from the collected blood samples following the standard protocols. The clinicopathological features of BC patients enrolled in this research are listed in Table 1.

**Cell culture**

Human bladder cancer cell lines (BIU-87 and T24) and immortalized cells of normal human bladder epithelium (SV-HUC-1) were obtained from American Type Culture Collection and cultured in 1640 medium containing 10% FBS and 100 U/ml penicillin and streptomycin (Sigma, USA). Cells were cultivated at 37°C in a humidified CO₂ (5%) atmosphere. The chemosensitive BIU-87 and T24 cells were subjected to increasing doses of cisplatin in a stepwise way to establish cisplatin-resistant BC cell lines BIU-87R and T24R. The drug-resistant phenotype of BIU-87R and T24R were retained by maintaining the surviving cells in conditioned medium supplemented with 1 μg/mL cisplatin (Sigma).

**Quantitative real-time PCR (qRT-PCR) assay**

Total RNA was extracted from cells, tissues or serums using an RNAiso Plus kit (TaKaRa, Japan) according to manufacturer’s protocol. Quantitative real-time PCR assay was conducted to assess the expression of circCEP128 using SYBR green kit (TaKaRa, Japan) on the Light Cycler 480 (Roche, Switzerland) according to the manufacturer’s recommendations. The primers were synthesized by Shanghai Sangon Biotech (China). GAPDH was used as an internal control. Relative gene expression levels were calculated using the 2^△△CT method. The primers used are listed as follows: circCEP128 forward primers: 5’-ACCCACATCGCTGGTTAGC-3′, reverse primers: 5’-TCGATCACCTTCTGCTTTTCGT-3′; GAPDH forward primers: 5’- GGAAAGCTGTGGCGTGAT-3′, reverse primers: 5’-AAGGTGGAAGAATGGGAGTT-3′.

**Cell transfection**

The siRNAs for circCEP128 and NC were ordered from Genepharm (Shanghai, China) and transfected into BIU-87R or T24R cells using Lipofectamine 3000 in accordance with the instructions. 48h after transfection, cells were harvested for further experiments. Expression level of circCEP128 was evaluated by qRT-PCR. Three siRNAs were designed with the following sequences for circCEP128: si-circCEP128-1: 5’-CUGUCAGCUGCAUGGAGCUUCGU-3′, si-circCEP128-2: 5’-GAGAGCUUGAACGAAUU-3′, si-circCEP128-3: 5’-GCACTGAGCCATTGTGAAT-3′ (si-circCEP128-2 was demonstrated to be the most effective inhibitory sequence and si-circCEP128 mentioned in the following results refers to si-circCEP128-2) and the corresponding si-NC sequence was 5’-AAUUCUCCGAACGUGUCACGU-3′.

**CCK8 assay**
BIU-87R or T24R cells were collected and inoculated in 96-well plates (1000 cells per well) 24h before transfection. Cells were cultured in 1640 medium supplemented with different concentrations of DDP (0, 1, 2, 4, 8, 16μg/mL) 48h after transfection of siRNAs in 96-well plates, with three replicate wells for each concentration. Cell viability was detected using Cell Counting Kit-8 (Beyotime, China) after 48h of incubation, based upon provided directions. A microplate reader was employed to determine the absorbance at 450 nm for each well.

Cell colony formation assay

BIU-87R or T24R cells transfected with siRNAs were collected and plated in 6-well plates (200 cells per well), and then cultured in medium supplemented with 3μg/mL cisplatin at 37 °C for 2 weeks. Afterwards, cells were fixed and subjected to 0.1% staining of crystal violet, the numbers of colonies were then counted for each well. Triplicate independent experiments were conducted.

Western blot analysis

The standard procedures were followed in the western blot analysis. In brief, the isolated protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. Then the separated proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) by electrotransfer. The membrane was then blotted with primary antibodies against PCNA, Cyclin D1, RIPK3, p-RIPK3, MLKL or p-MLKL (1:1000, Abcam, UK), followed by horseradish peroxidase (HRP)-labeled secondary antibody (BIBC, China) incubation. All bands were detected by an ECL Western blot kit (Thermo Fisher Scientific, USA) and analyzed by Lab Works (TM ver4.6, UVP, Bio Imaging Systems, NY, USA). GAPDH was used as control.

Statistical analysis

Statistical analysis was conducted using SPSS 22.0 software (IBM) and Graph pad Prism 5.0. Differences between BC tissues and its corresponding adjacent normal tissues were analyzed using the Student’s t test, one-way ANOVA was used to evaluate the significance of differences between circRNA expression levels and clinicopathological features. The Kaplan-Meier survival analysis was performed to establish the overall survival. A receiver operating characteristic (ROC) curve was generated to assess its diagnostic value. p values < 0.05 were considered statistically significant.

Results

CircCEP128 is up-regulated and correlated with poor clinical outcomes in BC

The difference of circCEP128 expression level in 60 paired BC and adjacent paracancerous tissues was measured using qRT-PCR. It was showed that circCEP128 expression was significantly increased in BC tissues in comparison with the adjacent counterpart control (Fig. 1a). Then the 60 patients were classified
into lymph node negative and lymph node positive groups or chemosensitive and chemoresistant groups in accordance with the clinical information. The results indicated that circCEP128 expression level was remarkably up-regulated in the lymph node positive or cisplatin-resistant group compared with the control group (Fig. 1b, c). Moreover, the 60 patients were divided into low or high circCEP128 expression groups according to the average circCEP128 expression level. Results of Kaplan-Meier survival analysis suggested that patients with relatively low circCEP128 expression have longer overall survival lifetime than those with high circCEP128 expression (Figure.1d). Furthermore, the relationship between clinicopathological parameters and circ-CEP128 expression in BC patients was analyzed. Our findings indicated that up-regulation of circCEP128 was distinctly correlated with advanced pathological stage, lymph node metastasis and drugresistance (Table 1). These data suggested the potential tumorigenic role of circCEP128 in BC.

CircCEP128 level in serum was a valuable biomarker for diagnosis in BC and chemoresistant phenotype

CircCEP128 expression level was detected in serum samples of 60 BC patients and 30 sex- and age-matched healthy individuals. The results indicated that expression of circCEP128 in serum was notably up-regulated in BC relative to the healthy control group (Fig. 2a). In addition, ROC curve was established to demonstrate the diagnostic value of circCEP128 in distinguishing BC patients from the healthy individuals, the data indicated that the area under the ROC curve (AUC) was 0.760 (Fig. 2b). Moreover, we evaluated the effectiveness of circCEP128 in distinguishing the chemoresistant patients from the chemosensitive cases in BC. Our findings showed that circCEP128 expression in serum was markedly increased in chemoresistant cases compared with the chemosensitive group (Fig. 2c), and the results of ROC curve analysis indicated that the area under the ROC curve (AUC) was 0.779 (Fig. 2d). These results together corroborated the potential valuable role of circCEP128 as a diagnostic biomarker in BC.

Inhibition on circCEP128 partly ameliorated the drug-resistance in cisplatin resistant BC cell lines

To further validate the role of circCEP128 in malignant phenotype of BC, the expression levels of circCEP128 in two paired chemosensitive and chemoresistant BC cell lines (BIU-87 vs BIU-87R, T24 vs T24R), and the immortalized cells of normal human bladder epithelium (SV-HUC-1) were detected. The results showed that expression of circCEP128 was increased in the BC cell lines compare with the SV-HUC-1 and dramatically up-regulated in the chemoresistant BC cell lines relative to the corresponding chemosensitive cell lines, which further demonstrated the potential function of circCEP128 in BC progression and chemoresistance (Fig. 3a). SiRNAs specifically targeted at circCEP128 junction site were introduced into the BIU-87R and T24R cell lines to further evaluate the function of circCEP128 in BC cisplatin resistance. Inhibitory effect of siRNAs was examined using qRT-PCR. Based on the results of qRT-PCR, si-circCEP128-2 (named si-circCEP128) with highest knockdown efficiency was selected for the following experiments (Fig. 3b). CCK-8 and clone formation assays were conducted to investigate the role of circCEP128 in cell activity and drug-resistance to cisplatin. IC50 values of BIU-87R (or T24R) cells for cisplatin in response to inhibition of circCEP128 were examined. Our results indicated that the IC50 values of cisplatin in BIU-87R (or T24R) cells treated with si-circCEP128 were decreased by 45.5% (or...
48.6%) in comparison with BIU-87R (or T24R) cells treated with si-NC (Fig. 3c-e). Similarly, colony formation assays revealed that cell proliferation rate was significantly suppressed in si-circCEP128 BIU-87R (or T24R) cells relative to si-NC transfected cells exposed to 3 µg/mL cisplatin for two weeks (Fig. 3f, g). Therefore, these results indicated that knockdown of circCEP128 by siRNA could partly ameliorate BC cells resistance to cisplatin in vitro.

**CircCEP128 knockdown induces necroptosis through modulating RIPK3/MLKL signaling pathway in BC cells**

It has been demonstrated that induction of necroptosis in cancer cells might be an alternative approach for tumor therapy. Therefore, we speculated whether circCEP128 could affect BC chemoresistance through regulating necroptosis. Then the protein expression levels of cell proliferation biomarker (PCNA, cyclin D1) and genes involved in the necroptosis signaling pathway (RIPK3, p-RIPK3, MLKL and p-MLKL) in the si-NC or si-circCEP128 transfected BIU-87R (or T24R) cells were examined using WB assays. And the results indicated that PCNA or cyclin D1 expression was markedly reduced in the si-circCEP128 transfected cells compared with the si-NC, conversely, the phosphorylation level of RIPK3 or MLKL was distinctly elevated in the si-circCEP128 transfected cells, and comparable to the chemosensitive BIU-87 (or T24) cells, which suggested that circCEP128 knockdown may resensitize BC cells to cisplatin through inducing RIPK3/MLKL-mediated necroptosis by increasing phosphorylation level of RIPK3 and MLKL (Fig. 4a, b)

**Discussion**

Chemoresistance has always been a challenging issue in cancer research. As to the current situation of bladder cancer, cisplatin-based chemotherapy is considered as the only way of therapy once patients have progressed to advanced stage [16]. Therefore, for patients with advanced bladder cancer, cisplatin resistance is vital to determine the prognosis of patients. To resolve this major problem, various of combined treatments based on cisplatin are adopted in clinical practice including cisplatin chemotherapy combined with a PD-L1 inhibitor [17]. Although improved effectiveness of treatment have been seen in some cases, drug resistance still remains the major obstacle for BC therapy.

CircRNA is considered as a class of stable non-coding transcripts in tissues and serum due to its special closed loop structure [18]. In addition, the relatively higher expression level also makes it an potential biomarker candidate for cancer diagnosis and treatment [19–22]. For example, Zhu et al reported that hsa_circ_0013958 could be used as an effective non-invasive biomarker for the early screening and detection of lung adenocarcinoma [23]. It was also reported that circRNA_100876 is deeply involved in the carcinogenesis of non-small cell lung cancer (NSCLC) and is expected to served as a valuable prognostic biomarker and therapeutic target for NSCLC [24]. There have been several reports exploring the correlation between BC progression and circRNA. It was reported that circRIP2 accelerates bladder cancer progression via miR-1305/Tgf-β2/smcd3 pathway [25]. Feng et al. reported that circ_0061140 promotes metastasis of bladder cancer through adsorbing microRNA-1236 [26]. Chen et al. reported that
circ_0008532 promotes bladder cancer progression by regulation of the miR-155-5p/miR-330-5p/MTGR1 axis [27]. Nevertheless, seldom study has been focusing on drug resistance and circRNA in BC, and effort is still need to be made to uncover reliable circRNA biomarker for BC diagnosis and treatment.

Results from our previous study suggested that circCEP128 might promote BC progression through modulating miR-145-5p/MYD88/MAKP axis. However, its role and underlying mechanism in the occurrence and progress of chemoresistance of BC cells has not been elucidated. In the current study, circCEP128 expression in 60 BC tissues were determined using qRT-PCR and found to be significantly increased in the BC tissues relative to the adjacent normal tissues. Furthermore, we identified that circCEP128 expression in the lymph node metastasis positive or chemoresistant patients was much higher than that of lymph node metastasis negative or chemosensitive group. Multivariable analysis also suggested that up-regulation of circCEP128 was markedly correlated with advanced pathological stage, lymph node metastasis and chemoresistance. Additionally, K-M survival analysis verified that patients with higher circCEP128 expression have shorter overall survival lifetime than those with lower circCEP128 expression.

To evaluate the possibility of circCEP128 to be a biomarker of BC diagnosis and treatment, the expression levels of circCEP128 in serum from BC patients and the healthy control were further detected. Our findings suggested that expression level of circCEP128 was distinctly elevated in BC in comparision with the controlled. In addition, circCEP128 expression in serum was demonstrated to be much higher in chemoresistant group compared with the chemosensitive group. ROC curve analysis further demonstrated that circCEP128 might serve as a effective biomarker to diagnose bladder cancer and predict the prognosis of chemotherapy. Furthermore, circCEP128 expression was found to be increased in the BC cell lines compare with the normal human bladder epithelium (SV-HUC-1), especially remarkably up-regulated in the drug resistant BC cell lines. Results of in vitro cell function assays further testified that silencing of circCEP128 expression by siRNA could partly alleviate the drug resistance of BC cells to cisplatin.

Necroptosis has been recognized as an alternative to apoptotic cell death. A few studies have demonstrated that induction of necroptosis by certain drugs or molecules in target cancer might resensitize the drug-resistant tumor cells to chemotherapy [28]. In the current study, we attempted to illustrate the relationship between necroptosis and chemoresistance of BC. We speculated whether the circCEP128 could modulate the cisplatin resistance of BC cells by regulating the level of necroptosis. Results of WB assays showed that circCEP128 silencing dramatically increases the phosphorylation level of RIPK3 and MLKL and induced necroptosis level. However, the underlying regulatory mechanism between circCEP128 and necroptosis is still needed to be illustrated in the following study.

Collectively, in the current study, we demonstrated that circCEP128 expression is up-regulated in tissues and serums, correlated with adverse outcomes and may be a valuable diagnostic biomarker in BC. Additionally, silencing of circCEP128 results in partial reversement of cisplatin resistance in BC cells by elevating the level of necroptosis through regulation of RIPK3/MLKL signaling pathway. Our findings
provide novel insights into the role of circCEP128 as a biomarker for the diagnosis and treatment target of BC.

**Abbreviations**

BC: Bladder cancer; DDP: cisplatin; HRP: horseradish peroxidase; NSCLC: non-small cell lung cancer; PVDF: polyvinylidene fluoride; ROC: receiver operating characteristic

**Declarations**

**Ethics approval and consent to participate**

The study protocol was approved by Shengjing Hospital of China Medical University on human research.

**Consent for publication**

All patients have provided written informed consent for the publication of any associated data and accompanying images.

**Availability of data and materials**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors have no conflicts of interest to declare.

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**Authors’ Contributions**

Ming Sun, Wenyan Zhao, Bin Zhang and Donghua Geng have given substantial contributions to the conception and the design of the manuscript, Ming Sun, Wenyan Zhao, Shuqiang Li and Renge Bu to acquisition, analysis and interpretation of the data. All authors have participated to drafting the manuscript, Xuefeng Liu revised it critically. All authors read and approved the final version of the manuscript.

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Table

Table 1. Relationship between circ-CEP128 expression and clinicopathological parameters of BC. *P<0.05
| Clinicopathological feature | Cases (n) | circ-CEP128 expression | p value |
|-----------------------------|-----------|------------------------|---------|
|                             | Low(28)   | High(32)               |
| Gender                      |           |                        |         |
| ≥ 65                        | 44(73.3%) | 19(67.9%)              | 0.753   |
| < 65                        | 16(26.7%) | 9(32.1%)               |         |
| Male                        | 51(85%)   | 22(78.6%)              | 0.002*  |
| Female                      | 9(15%)    | 6(21.4%)               |         |
| Pathological grade          |           |                        |         |
| Low                         | 22(36.7%) | 17(60.7%)              | 0.003*  |
| High                        | 38(63.3%) | 11(39.3%)              |         |
| Stage                       |           |                        |         |
| T2                          | 31(51.7%) | 15(53.6%)              | 0.001*  |
| T3                          | 16(26.7%) | 9(32.1%)               |         |
| T4                          | 13(21.6%) | 4(14.3%)               |         |
| Lymph node                  |           |                        |         |
| Positive                    | 20(33.3%) | 3(10.7%)               | 0.022*  |
| Negative                    | 40(66.7%) | 25(89.3%)              |         |
| Chemoresistant              |           |                        |         |
| Yes                         | 23(38.3%) | 5(17.9%)               | 0.029*  |
| No                          | 37(61.7%) | 23(82.1%)              |         |

**Figures**
CircCEP128 is up-regulated and correlated with poor clinical outcomes in BC. (A) Expression level of circCEP128 in 60 paired BC and paracancerous tissues. (B) Expression level of circCEP128 in BC tissues of lymph node positive and lymph node negative group. (C) Expression level of circCEP128 in BC tissues of chemosensitive and chemoresistant group. (D) Patients with relatively low circCEP128 expression have longer overall survival lifetime than those with high circCEP128 expression. *P < 0.05.
Figure 2

Serum circCEP128 may be a valuable biomarker for diagnosis in BC and chemoresistant phenotype. (A) Expression level of circCEP128 in serum from 60 BC patients and 30 sex- and age-matched healthy individuals. (B) ROC curves of the serum circCEP128 in 60 BC patients and 30 healthy donors. (C) Expression level of circCEP128 in serum from chemoresistant cases as well as chemosensitive cases. (D) ROC curves of the serum circCEP128 in 23 chemoresistant BC patients and 37 chemosensitive BC patients. *P < 0.05.
Figure 3

Inhibition on circCEP128 partly reversed the drug-resistance of cisplatin resistant BC cells. (A) Expression level of circCEP128 in two paired chemosensitive and chemoresistant BC cell lines (BIU-87 vs BIU-87R, T24 vs T24R), and the immortalized cells of normal human bladder epithelium (SV-HUC-1). (B) RT-qPCR...
analysis of the effect on knockdown of circCEP128 expression by siRNA in the BIU-87R and T24R cell lines. si-circCEP128-2 was selected for the siRNA used in the study due to the highest knockdown efficiency. (C-E) CCK-8 assay showed that the viability and IC50 value of BIU-87R (T24R) cells in the si-circCEP128 group when exposed to cisplatin were reduced compared with the control groups. (F, G) Cell colony formation assay showed that the clone numbers of BIU-87R (T24R) cells in the si-circCEP128 group were reduced compared with the si-NC group when exposed to 3ug/ml cisplatin for 14 days. *P < 0.05.
Figure 4

CircCEP128 knockdown induces necroptosis through modulating RIPK3/MLKL signaling pathway in BC cells. (A) The protein expression levels of cell proliferation biomarker (PCNA, cyclin D1) and genes involved in the necroptosis signaling pathway (RIPK3, p-RIPK3, MLKL and p-MLKL) in the si-NC or si-circCEP128 transfected BIU-87R cells and controlled BIU-87 cells were examined using WB assays. (B) The protein expression levels of cell proliferation biomarker (PCNA, cyclin D1) and genes involved in the necroptosis signaling pathway (RIPK3, p-RIPK3, MLKL and p-MLKL) in the si-NC or si-circCEP128 transfected T24R cells and controlled T24 cells were examined using WB assays. *P < 0.05.