RETRACTED ARTICLE: Silencing circular RNA-ZNF652 represses proliferation and EMT process of renal carcinoma cells via raising miR-205

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ABSTRACT
Recently, the functions of circular RNAs (circRNAs) on cancer initiation and development arouse wide concern. Herein, we tested the influences of circ-ZNF652 on renal carcinoma cell growth and metastasis. Firstly, clinical renal carcinoma tissues and corresponding normal tissues were collected. The circ-ZNF652 expressions were tested. Then, the influences of silencing circ-ZNF652 on renal carcinoma A498 and ACHN cell proliferation, apoptosis and epithelial–mesenchymal transition (EMT) process, as well as Ras/Raf/MEK/ERK and JAK1/STAT3 pathways, were probed. Finally, whether miR-205 engaged in the influences of silencing circ-ZNF652 on A498 and ACHN cell were investigated. circ-ZNF652 had high expression level in clinical renal carcinoma tissues. Silencing circ-ZNF652 repressed A498 and ACHN cell proliferation and EMT process, but promoted cell apoptosis. Moreover, silencing circ-ZNF652 suppressed Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in A498 and ACHN cells. Besides, the miR-205 expressions in A498 and ACHN cells were raised by silencing circ-ZNF652. Knockdown of miR-205 weakened the influences of silencing circ-ZNF652 on A498 and ACHN cell proliferation, apoptosis and EMT process. Silencing circ-ZNF652 repressed proliferation and EMT process of renal carcinoma A498 and ACHN cells via suppressing Ras/Raf/MEK/ERK and JAK1/STAT3 pathways, as well as raising miR-205 expression.

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Introduction
Renal carcinoma, also named as renal cell carcinoma or renal adenocarcinoma, is a malignant tumour originated from urinary tubular epithelial cells [1]. It accounts for more than 90% of tumours in human kidney [1]. Men are more likely to suffer from renal carcinoma than women with ratio of 2:1 [2]. Surgical resection and systemic treatment with targeted agents are the main therapeutic methods for renal carcinoma [3,4]. However, due to the relatively high rate of tumour recurrence and metastasis, the curative outcomes of renal carcinoma still remain unsatisfactory [5,6]. Pathological researches verified that the molecular mechanisms of renal carcinoma growth and metastasis were very complex, which referring to a number of genetic mutations and epigenetic changes [7–9]. Thus, we believe that further comprehension of the internal mechanisms related to renal carcinoma growth and metastasis is of great important worth for renal carcinoma therapy.

Circular RNAs (circRNAs) are a specific type of non-coding RNAs in cells [10]. Unlike traditional linear RNAs (containing 5’ and 3’ ends), circRNAs have a closed circular structure and are not affected by RNA exonuclease [11]. Therefore, their expression is more stable and less prone to degradation [11]. Recent literatures have reported that circRNAs can function as critical gene expression regulatory factors taking part in the initiation and development of many human diseases, including cancers [12,13]. For renal carcinoma, circ-HIAT1 [14] and circ-0001451 [15] have been discovered to be down-regulated in renal carcinoma tissues, while circ-ABCB10 [16], circ-ZNF609 [17] and circ-PCNL2 [18] have been found to be up-regulated. circ-ZNF652 is a recently discovered circRNA in hepatocellular carcinoma [19]. It has been reported to be up-regulated in hepatocellular carcinoma and attend to the epithelial–mesenchymal transition (EMT) process of hepatocellular carcinoma cells [19]. Whether circ-ZNF652 engages in the proliferation and EMT process of renal carcinoma is still unclear.

MicroRNAs (miRNAs) are a type of linear non-coding RNAs in cells [20]. Previous researches confirmed that circRNAs exert transcriptional and post-transcriptional gene expression regulatory functions usually via modulating miRNAs expression [21,22]. miRNA-205 (miR-205) was discovered to be expressed at a low level in renal carcinoma tissues and related to the infiltration and recurrence of tumours [23]. Wang et al. reported that miR-205 overexpression could repress renal carcinoma cell proliferation, invasion and metastasis [24].
In the current research, we analysed the circ-ZNF652 expression in clinical renal carcinoma tissues and corresponding normal tissues. Subsequently, the influences of silencing circ-ZNF652 on renal carcinoma A498 and ACHN cell proliferation, apoptosis and EMT process, along with miR-205 expression were probed. The outcomes of our research will offer novel experimental evidence for comprehending the critical functions of circ-ZNF652 on renal carcinoma growth and metastasis.

Materials and methods

Clinical samples

Clinical renal carcinoma tissues and corresponding normal tissues (N = 22) were collected during surgery by Doctor Ludong Zhang and Doctor Hongbo Guo in Jining No.1 People’s Hospital (Jining, China). None of the patients obtained any therapy before surgery. This research was authorized by the Medical Ethics Committee of our hospital and informed consents were received from every patient before surgery. This procedure was conducted in the operation room of Jining No.1 People’s Hospital.

Cell lines

A498 and ACHN cells were both obtained from Procell Inc. (CL-0254 and CL-0021, Wuhan, China) and grown in Minimum Essential Medium (MEM, PM150410, Procell Inc.) including 10% fetal bovine serum (FBS, F8687, Sigma-Aldrich, MO, USA) and 1% Penicillin-Streptomycin solution (PB180120, Procell Inc.) at 37 °C with 5% CO₂ and 95% air. Transforming growth factor β1 (TGFβ1, GF346) was received from Sigma-Aldrich. A498 and ACHN cells were subjected to 10 ng/ml TGFβ1 stimulation for 12 h to induce EMT process. This procedure was conducted by Doctor Ya Guo in the lab of Jining No.1 People’s Hospital.

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was utilized for measuring circ-ZNF652 and miR-205 expressions. Total RNAs were separated using TRizol™ RNA purification Kit (12183555, Invitrogen, CA, USA). For testing circ-ZNF652 expression, cDNA was synthesized by High Capacity RNA-to-cDNA™ kit (4387406, Applied Biosystem, CA, USA). Real-time PCR was carried out using TaqMan™ Non-coding RNA Assay (4426961, Applied Biosystem) and compared to β-actin expression. For testing miR-205 expression, mirVana™ qRT-PCR miRNA Detection kit was carried out and compared to U6 snRNA expression. This procedure was conducted by Doctor Ludong Zhang in the lab of Jining No.1 People’s Hospital.

siRNA or miRNA transfection

Small interfering RNA targeting circ-ZNF652 (si-circ-ZNF652) and its negative control (si-NC), along with miR-205 inhibitor and NC inhibitor were all received from Life Technologies Corporation (CA, USA). Transfection was carried out using Lipofectamine 3000 reagent (L3000-150, Invitrogen) and transfection efficiencies were tested by qRTPCR. This procedure was conducted by Doctor Ya Guo in the lab of Jining No.1 People’s Hospital.

Bromodeoxyuridine (BrdU) incorporation assay

BrdU solution was received from MedChem Express (HY-15910, NJ, USA) and utilized for testing A498 and ACHN cell proliferation. Briefly, cells were cultivated into 6-well plate (1 × 10⁵ cells/well) for 21 h. Then, BrdU (1 mg/ml) was mixed into the culture medium for 3 h at 37 °C. Subsequently, the percentage of BrdU positive (+) cells of at least 1000 cells in each group was counted. This procedure was conducted by Doctor Ludong Zhang and Doctor Ya Guo in the lab of Jining No.1 People’s Hospital.

Cell apoptosis assay

Annexin V-FITC/PI Apoptosis kit (40302, Yeasen Biotech Co., Ltd, Shanghai, China) was utilized for testing A498 and ACHN cell apoptosis. Cells were cultivated into 6-well plate (1 × 10⁵ cells/well) for 24 h. Subsequently, cells were gathered in line with the experimental group, rinsed with kit buffer and mixed with 5 μl Annexin V-FITC and 10 μl PI solution for 20 min at 20–25 °C in the dark. The percentage of apoptotic cells was assessed by flow cytometer (Attune Nxt, Thermo Fisher Scientific, Waltham, MA, USA). This procedure was conducted by Doctor Ya Guo in the lab of Jining No.1 People’s Hospital.

Western blotting

Total proteins in A498 and ACHN cells were separated using RIPA Lysis Buffer (HY-K1001, MedChem Express) containing Protease Inhibitor Cocktail (HY-K0010, MedChem Express). Western blotting was carried out and signals of proteins were analysed as earlier described [25]. Primary antibodies, including Bax (#2774), Cleaved-caspase 3 (#9661), E-cadherin (E-cad, #14472), N-cadherin (N-cad, #4061), Vimentin (#3932), Snail (#3879), Ras (#3965), Raf (#9422), t-MEK (#4694), p-MEK (#3958), t-ERK (#9102), p-ERK (#9106), t-JAK1 (#3332), p-JAK1 (#3331), t-STAT3 (#9139), p-STAT3 (#9131) and β-actin (#3700), as well as secondary antibodies, including Anti-Rabbit (or Anti-Mouse) IgG H&L DyLight™ 680 conjugate (#5366 and #5470) were all received from Cell Signalling Technology (MA, USA). This procedure was conducted by Doctor Ludong Zhang in the lab of Jining No.1 People’s Hospital.

Statistical analysis

All experiments were repeated at least three times. Graphpad 6.0 software was utilized for statistical analysis. Results were presented as mean ± standard deviation (SD). p-Values were computed using one-way analysis of variance (ANOVA). p < .05 was regarded as significant difference. This procedure...
Results

circ-ZNF652 had high expression level in clinical renal carcinoma tissues

Firstly, the circ-ZNF652 expression in clinical renal carcinoma tissues and corresponding normal tissues were tested. As displayed in Figure 1, by contrast with corresponding normal tissues, circ-ZNF652 expression had high expression level in clinical renal carcinoma tissues ($p < .01$), which hinted that circ-ZNF652 might exhibit oncogenic activity in renal carcinoma cell proliferation and EMT process.

Silencing circ-ZNF652 repressed renal carcinoma cell proliferation, but elevated cell apoptosis

si-circ-ZNF652 was transfected into A498 and ACHN cells to probe the influences of circ-ZNF652 on renal carcinoma cell proliferation and apoptosis. Data in Figure 2(A) showed that circ-ZNF652 expressions in A498 and ACHN cells were both silenced by si-circ-ZNF652 transfection ($p < .01$). Figure 2(B) presented that silencing circ-ZNF652 notably repressed A498 and ACHN cell proliferation, as evidenced by the declined percentages of BrdU$^+$ positive cells ($p < .01$). In addition, silencing circ-ZNF652 obviously elevated A498 and ACHN cell apoptosis (Figure 2(C), $p < .01$), which were accompanied with the raised expressions of Bax and Cleaved-caspase 3 in both A498 and ACHN cells (Figure 2(D), $p < .01$). These above outcomes illustrated that silencing circ-ZNF652 could repress renal carcinoma A498 and ACHN cell proliferation, but elevate cell apoptosis.

Silencing circ-ZNF652 repressed EMT process of renal carcinoma cells

Subsequently, the influences of silencing circ-ZNF652 on EMT process of A498 and ACHN cells were tested. As shown in Figure 3, 10 ng/ml TGF$\beta$1 stimulation significantly declined the E-cad expressions and elevated the N-cad, Vimentin and Snail expressions in both A498 and ACHN cells ($p < .01$), which suggested that TGF$\beta$1 stimulation could promote EMT process of A498 and ACHN cells. Besides, by contrast with TGF$\beta$1 + si-NC group, the E-cad expressions in A498 and ACHN cells were increased, while the N-cad, Vimentin and Snail expressions were decreased in TGF$\beta$1 + si-circ-ZNF652 group ($p < .05$ or $p < .01$), which illustrated that silencing circ-ZNF652 could repress EMT process of renal carcinoma A498 and ACHN cells.

Silencing circ-ZNF652 suppressed Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in renal carcinoma cells

Followed by si-circ-ZNF652 transfection, the activities of Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in A498 and ACHN cells were assessed. Figure 4(A) pointed out that silencing circ-ZNF652 significantly suppressed Ras/Raf/MEK/ERK pathway in A498 and ACHN cells through reducing Ras, Raf, p/t-MEK and p/t-ERK expressions ($p < .05$ or $p < .01$). Similarly, silencing circ-ZNF652 also obviously inhibited JAK1/STAT3 pathway in A498 and ACHN cells through reducing the p/t-JAK1 and p/t-STAT3 expressions (Figure 4(B), $p < .05$ or $p < .01$). These outcomes illustrated that silencing circ-ZNF652 exhibited oncogenic activity in renal carcinoma cell proliferation and EMT process might be via suppressing Ras/Raf/MEK/ERK and JAK1/STAT3 pathways.

Silencing circ-ZNF652 raised miR-205 expression in renal carcinoma cells

The miR-205 expressions in A498 and ACHN cells after si-circ-ZNF652 transfection were tested. Data in Figure 5(A) displayed that silencing circ-ZNF652 dramatically raised the miR-205 expressions in both A498 and ACHN cells ($p < .01$). This outcome hinted that miR-205 might be a downstream factor of circ-ZNF652, which might attend to the influences of silencing circ-ZNF652 on renal carcinoma A498 and ACHN cell proliferation, apoptosis and EMT process.

miR-205 inhibitor transfection weakened the influences of silencing circ-ZNF652 on renal carcinoma cell proliferation and apoptosis

miR-205 inhibitor was transfected into A498 and ACHN cells to knockdown the miR-205 expression (Figure 5(B), $p < .01$). Figure 5(C) showed that miR-205 inhibitor transfection notably weakened the silencing circ-ZNF652-caused decreases of percentages of BrdU$^+$ A498 and ACHN cells ($p < .05$). In addition, silencing circ-ZNF652-caused A498 and ACHN cell apoptosis were also mitigated by miR-205 inhibitor transfection (Figure 5(D), $p < .05$). Relative to si-circ-ZNF652 + NC inhibitor group, the Bax and Cleaved-caspase 3 expressions in A498 and ACHN cells were both reduced in si-circ-ZNF652 + miR-205 inhibitor group (Figure 5(E), $p < .05$ or $p < .01$). These above outcomes confirmed that miR-205 was a downstream factor of circ-ZNF652, which engaged in the influences of silencing circ-ZNF652 on renal carcinoma A498 and ACHN cell proliferation and apoptosis.
**miR-205 inhibitor transfection weakened the influences of silencing circ-ZNF652 on renal carcinoma cell EMP process**

Finally, whether miR-205 engaged in the influences of silencing circ-ZNF652 on A498 and ACHN cell EMT process were analysed. As displayed in Figure 6, by contrast with TGFβ1 + si-circ-ZNF652 + NC inhibitor group, the E-cad expressions in both A498 and ACHN cells were reduced in TGFβ1 + si-circ-ZNF652 + miR-205 inhibitor group (p < .05). On the contrary, the N-cad, Vimentin and Snail expressions in both A498 and ACHN cells were enhanced in TGFβ1 + si-circ-ZNF652 + miR-205 inhibitor group (p < .05), relative to TGFβ1 + si-circ-ZNF652 + NC inhibitor group. These outcomes illustrated that miR-205 also engaged in the influences of silencing circ-ZNF652 on A498 and ACHN cell EMT process.

**Discussion**

The functions of circRNAs on cancer initiation and development have been attracting more and more attention in recent years [13,26]. Herein, we discovered that circ-ZNF652 had high expression level in clinical renal carcinoma tissues. Silencing circ-ZNF652 repressed renal carcinoma A498 and ACHN cell proliferation and EMT process, but raised cell apoptosis. Furthermore, silencing circ-ZNF652 suppressed Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in A498 and ACHN cells. Besides, the miR-205 expressions in A498 and ACHN cells were raised after silencing circ-ZNF652. Up-regulation of miR-205 attended to the influences of silencing circ-ZNF652 on A498 and ACHN cell proliferation, apoptosis and EMT process.

It is predicted that exceeding 90% of genes in mammalian cells are transcribed into non-coding RNAs [27]. As a class of important non-coding RNAs in cells, circRNAs have been verified to engage in the modulation of many cellular biological processes [12]. Researchers have made great effort to investigate the critical functions of circRNAs on cancer occurrence and progression [13,28]. Earlier experiment confirmed that circ-ZNF652 was expressed at a high level in hepatocellular carcinoma [19]. In this study, we discovered that circ-ZNF652 also had a high expression level in renal carcinoma tissues. Followed by silencing circ-ZNF652, the proliferation of renal carcinoma A498 and ACHN cells was reduced, while the apoptosis of A498 and ACHN cells was elevated. These outcomes illustrated that circ-ZNF652 exhibited oncogenic function on renal carcinoma growth.

Tumour metastasis is the main reason for poor prognosis of renal carcinoma [29,30]. Approximately one-third of patients with renal carcinoma exist regional or distant metastasis [31]. Renal carcinoma cells can diffuse from original tumour site to other site of the kidney or other tissues of body, such as lung, lymph nodes, liver, bone and brain [31]. The transition process between epithelium and mesenchyme is known as EMT process, by which tumour cells lose their adhesion attribute and can migrate and invade to other places [32]. E-cad is an epithelial cell adhesion factor. Reduction of E-cad expression can lead to loss of junction between cells [33]. On the contrary, the up-regulation of N-cad, Vimentin and Snail are demonstrated to contribute to EMT process [34]. It has been proved that circ-ZNF652 contributes to the vascular invasion, intrahepatic metastasis and distant metastasis of hepatocellular carcinoma by promoting EMT process [19]. Herein, we discovered that silencing circ-ZNF652 notably...
Figure 3. Silencing circ-ZNF652 repressed EMT process of renal carcinoma cells. A498 and ACHN cells were subjected to 10 ng/ml TGFβ1 stimulation and/or si-circ-ZNF652 transfection. The E-cad, N-cad, Vimentin and Snail protein levels were tested, respectively. EMT: epithelial-mesenchymal transition; TGFβ1: Transforming growth factor β1; E-cad: E-cadherin; N-cad: N-cadherin. *p < .05; **p < .01.

Figure 4. Silencing circ-ZNF652 suppressed Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in renal carcinoma cells. A498 and ACHN cells were subjected to si-circ-ZNF652 transfection. (A) The Ras, Raf, t-MEK, p-MEK, t-ERK and p-ERK protein levels, along with (B) the t-JAK1, p-JAK1, t-STAT3 and p-STAT3 protein levels were tested, respectively. *p < .05; **p < .01.
repressed the TGFβ1 stimulation-caused EMT process activation in A498 and ACHN cells through raising E-cad expression and declining N-cad, Vimentin and Snail expressions. These outcomes illustrated that circ-ZNF652 also attended to the modulation of renal carcinoma metastasis.

Ras/Raf/MEK/ERK and JAK1/STAT3 pathways are proved to be activated in renal carcinoma and take part in the growth and metastasis of renal carcinoma [35,36]. Some inhibitors targeting Ras/Raf/MEK/ERK and JAK1/STAT3 pathways have been discovered or designed to repress renal carcinoma growth and metastasis [37,38]. In this research, we discovered that silencing circ-ZNF652 suppressed Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in A498 and ACHN cells, which illustrated that circ-ZNF652 attended to the modulation of renal carcinoma growth and metastasis might also be achieved via modulating miR-205.

To sum up, this research confirmed the oncogenic function of circ-ZNF652 on renal carcinoma growth and metastasis. Silencing circ-ZNF652 repressed proliferation and EMT process of renal carcinoma A498 and ACHN cells via suppressing Ras/Raf/MEK/ERK and JAK1/STAT3 pathways, as well as raising miR-205 expression. Further researches are still needed in future to comprehensive analysis of circRNA regulatory network in renal carcinoma, which is of great important value for renal carcinoma diagnosis and treatment.

Disclosure statement

The authors declare that they have no conflict of interest.
Data availability statement
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Every patient agreed with joining the research and writing informed consent, and the present research was ratified by the Medical Ethics Committee of the Jining No.1 People's Hospital.

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