RETRACTED ARTICLE: The circular RNA hsa-circ-0072309 plays anti-tumour roles by sponging miR-100 through the deactivation of PI3K/AKT and mTOR pathways in the renal carcinoma cell lines

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ABSTRACT
Aims: To explore the roles and regulatory mechanisms of the circular RNA (circRNA)-hsa-circ-0072309 in CAKI-1 and ACHN cells.
Methods: CAKI-1 and ACHN cells were transfected with hsa-circ-0072309 overproduction vector (circRNA) and microRNA-100 (miR-100) mimic or the corresponding controls. Cell viability was detected with the CCK-8. The protein expression levels of p53, c-Myc, cleaved-caspase-3/9, matrix metalloproteinase (MMP)-2/9, vimentin, AKT, PI3K and mTOR were individually determined through western blot.
Results: The hsa-circ-0072309 was poorly expressed in tumor tissue. Abundant hsa-circ-0072309 induced the inhibitions of cell proliferation, migration and invasion, as well as the PI3K/AKT and the mTOR cascades but enhancement of apoptosis. circRNA stimulated the down-regulation of miR-100, which was low-expressed in tumour tissue and whose overproduction abolished the impacts of circRNA on these elements mentioned above.
Conclusion: The hsa-circ-0072309 played anti-tumour roles by targeting miR-100 by blocking the PI3K/AKT and mTOR cascades in the CAKI-1 and ACHN cell lines.

Introduction
Renal cell carcinoma (RCC), which is a common tumour, originated from renal tubular epithelial cells, accounts for 80–85% of kidney cancers [1,2]. The incidence and mortality of RCC are increasing annually [3].

Circular RNAs (circRNAs) are a kind of special non-coding RNAs (ncRNAs) lacking 5’ caps or 3’ poly-A tails [4]. Numerous researches have indicated that circRNAs may modulate cellular functions or tumour progression through distinct mechanisms [5–7]. circ-hippocampus abundant transcript 1 (HIAT1) acted as a metastatic inhibitor to repress the migration and invasion processes in the AR-stimulated RCC cells [8]. Zheng et al. suggested that hsa-circ-0072309 was poorly expressed in breast cancer cells and inhibited breast cancer development in vitro and in vivo [9]. Nevertheless, the exact pieces of evidence on the functions of circRNAs, especially the hsa-circ-0072309, in RCC pathogenesis remained limited.

microRNAs (miRNAs) are another group of ncRNAs, about 21–24 nt in length [10]. It regulates genes expression by silencing specific target messenger RNAs (mRNAs) at post-transcriptional levels [11]. Previous investigations have shown that circRNAs regulate biological processes through sponging miRNAs. For instance, circRNA-100269 combined with miR-630 to adjust the proliferation of GC cells [12]. circ-cirs-7 modulates the development of non-small cell lung cancer (NSCLC) by targeting miR-7 [13]. It has been reported that miR-100 plays pivotal roles in adjusting cellular processes by targeting the insulin-like growth factor 1 receptor (IGF1R)/mammalian target of rapamycin (mTOR) signalling cascades [14]. Besides, miR-100 is associated with colorectal cancer (CRC) metastasis [15]. Xue et al. pointed out that miR-100 had some connections with the proliferation and apoptosis of acute lymphoblastic leukemia (ALL) cells [16]. However, whether the miR-100 functioned in the mechanisms of hsa-circ-0072309 in the RCC cells, and the direct relationship between the hsa-circ-0072309 and the miR-100 are still waiting for further elucidation.

In our investigation, the CAKI-1 and ACHN cell lines were considered as the experiment material. We screened for the underlying roles and mechanisms of the hsa-circ-0072309 in RCC in vitro.

Materials and methods
Clinical specimens
The kidney cancer or the normal tissues were obtained from the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China).
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Hercules, CA) was introduced for absorbance measurement.

tional culture. Finally, the microplate reader (Bio-Rad, and co-incubated with the cells for 1 h, after the 24 h conven-
vention was selected as the optimum harvest time owing to the suggestions from ATCC.

The cells apoptotic rates were measured by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China) combined with flow cytometer (Beckman Coulter, CA). CAKI-1 and ACHN cells were adjusted and prepared in the 6-well plate with $1 \times 10^5$ cells/well density and cultured at $37^\circ C$ for 24 h, were washed and resuspended with PBS. Annexin V-FITC and PI were mixed under 1:1, and co-incubated with the cell suspension in the dark for 15 min to constitute the sample for flow cytometer detecting. The data were analyzed depending on FlowJo software (Stanford University, Palo Alto, CA).

Migration and invasion assay

The modified two-chamber migration assay (8 mm, Millipore, Bedford, MA) was conducted to test cells migration. The cells were re-suspended in 200 µl serum-free medium (Gibco BRL) with a concentration of $5 \times 10^4$ cells/mL. Followed by a 2 days conventional cultivation, cells suspension was then added to the upper chamber while 600 µl of serum-containing medium (Gibco BRL) was added to the lower chamber. After incubation, cells were washed twice with phosphate buffer saline (PBS) and non-traversed cells were removed with a wet cotton swab. Moreover, the methanol (Beyotime Biotechnology, Shanghai, China) (20 min) and the crystal violet (Sigma-Aldrich) (10–20 min) were then individually used for cells handling. Finally, five fields were randomly selected for cell counting and photographing under the microscope (Canon, Tokyo, Japan).

Besides, in this study, the invasive ability of cells was examined in vitro by a 24-well micelli cell hanging cell culture chamber (8 µM) (Millipore, Bedford, MA). The specific procedure of this test was also similar to the migration ability assay. The difference was that before the cell suspension was added to the upper chamber, matrigel glue (Millipore) was applied to the upper surface of the PET film, at the underside of the upper chamber. After that, it was allowed to stand at $37^\circ C$ for 4–5 h until the gel was completely solidified. We finally selected five random fields for cell counting and the average in these five fields was considered as the ultimate data.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA) and the DNasel (Promega, Madison, WI) were used for RNA extraction. The MultiScribeRTkit (Applied Biosystems) and random hexamers or oligo (dT) was consumed for the miRNA reverse transcription and qRT-PCR assessing. Experiment records were managed through $2^{-\Delta\Delta Ct}$ method and normalized with β-actin or U6.

Western blot

After cells collection, 1 × PBS buffer and 4 °C RIPA lysis buffer (Beyotime Biotechnology) containing protease inhibitors (Roche, Basel, Switzerland) were respectively used for total proteins extraction. The BCA™ Protein Assay Kit (Pierce, Appleton, WI) was applied for protein quantification and the Bio-Rad Bis-Tris Gel system (Bio-Rad, Hercules, CA) was helped to establish the western blot system. The primary antibodies were diluted with 5% blocking buffer, followed by the soak of polyvinylidene difluoride (PVDF) membrane (Millipore), which carried proteins blots at 4 °C overnight. The primary antibodies were including anti-p53 (ab131442), anti-c-Myc (ab32072), anti-cleaved-caspase 3 (ab2302), anti-cleaved-caspase 9 (ab2324), anti-matrix metalloproteinase (MMP)-2 (ab92536), (ab38898), anti-MMP-9 (ab38898), anti-vimentin (ab137321), anti-phosphatidylinositol 3-kinases (PI3K) (ab131067), anti-p-PI3K (ab138364), anti-protein kinase B (AKT) (ab219588), anti-p-AKT (ab192623), anti-mTOR...

Cell viability assessment

The CCK-8 was used to detect cell viability in the present study. Firstly, different group cells were seeded into the 96-well plate at a density of $5 \times 10^3$ cells/well, and cultured in a CO₂ incubator at $37^\circ C$ for 24 h for further assays. Besides, referring to the instruction, the CCK-8 solution was added and co-incubated with the cells for 1 h, after the 24 h conventional culture. Finally, the microplate reader (Bio-Rad, Hercules, CA) was introduced for absorbance measurement under 450 nm.

Cell transfection

The hsa-circ-0072309 sequence was cloned into the PLCDH-vector (circRNA) (Ribobio, Guangzhou, China) for hsa-circ-0072309 overproduction. Moreover, miR-100 mimic and the corresponding negative control group (NC mimic) (Life Technologies Corporation, Carlsbad, CA) were synthesized to alter the generation of miR-100 in the CAKI-1 and ACHN cell lines. The circRNA and its negative control (vector), as well as the miR-100 mimic and the NC mimic, were transfected into the cells through lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) for extra experiments. Three days after transfection was selected as the optimum harvest time owing to the highest transfection efficiency.

Western blot

After cells collection, 1 × PBS buffer and 4 °C RIPA lysis buffer (Beyotime Biotechnology) containing protease inhibitors (Roche, Basel, Switzerland) were respectively used for total proteins extraction. The BCA™ Protein Assay Kit (Pierce, Appleton, WI) was applied for protein quantification and the Bio-Rad Bis-Tris Gel system (Bio-Rad, Hercules, CA) was helped to establish the western blot system. The primary antibodies were diluted with 5% blocking buffer, followed by the soak of polyvinylidene difluoride (PVDF) membrane (Millipore), which carried proteins blots at 4 °C overnight. The primary antibodies were including anti-p53 (ab131442), anti-c-Myc (ab32072), anti-cleaved-caspase 3 (ab2302), anti-cleaved-caspase 9 (ab2324), anti-matrix metalloproteinase (MMP)-2 (ab92536), (ab38898), anti-MMP-9 (ab38898), anti-vimentin (ab137321), anti-phosphatidylinositol 3-kinases (PI3K) (ab131067), anti-p-PI3K (ab138364), anti-protein kinase B (AKT) (ab219588), anti-p-AKT (ab192623), anti-mTOR...
(ab32028), anti-p-mTOR (ab109268) and anti-β-actin (ab8227). Following 1 h attachment of horseradish peroxidase (HRP) signed secondary antibody, goat anti-rabbit (HRP) (ab7090) at 25°C, the PVDF membrane was put into the Bio-Rad ChemiDoc™ XRS system (Bio-Rad) with the addition of the Immobilon Western HRP Substrate (200 µL) (Millipore). Image Lab™ Software (Bio-Rad) was adopted to capture and quantify the protein bands.

**Statistical analysis**

The SPSS statistical software version 19.0 was the main tool for statistical analysis. The experimental data were shown as the mean ± standard deviation (SD). Analysis of variance (ANOVA) or t-test was responsible for p-values. p-Values less than .05 were statistically significant. All experiments in this study were repeated 3 times at least.

**Results**

**Hsa-circ-0072309 is poorly expressed in renal cancer tissue**

The RNA expression level of hsa-circ-0072309 was detected through qRT-PCR. First, in contrast with the normal tissue, the hsa-circ-0072309 had a lower expression level in the renal tumour tissue (p < .01, Figure 1(A)). Besides, the hsa-circ-0072309 overexpression vector and the corresponding NC were produced, and they were transfected into the CAKI-1 and ACHN cell lines. According to the qRT-PCR outcomes, the production of the hsa-circ-0072309 was highly increased by the hsa-circ-0072309 over-expression vector (circRNA) (both p < .001, Figure 1(B)). This result showed that the over-production vectors were successfully constructed and expressed in the cells.

**Abundant hsa-circ-0072309 inhibits cell proliferation but promotes apoptosis**

The cell viability was significantly decreased due to the transfection of circRNA both in CAKI-1 and ACHN cells (both p < .05, Figure 2(A)). The production of p53 (both p < .001) was evidently promoted by abundant hsa-circ-0072309, which had an opposite effect on the generating of c-Myc (both p < .05) in contrast with the corresponding group (Figure 2B–D). Additionally, the apoptotic rates and productions of apoptosis-related proteins such as cleaved-caspase 3/9 (all p < .001, Figure 2E–H) were notably stimulated by the forced hsa-circ-0072309. These data suggested that over-production of hsa-circ-0072309 induced proliferation inhibition and apoptosis in CAKI-1 and ACHN cell lines.

**miR-100 is down-regulated by the hsa-circ-0072309**

In order to investigate the functions of miR-100 in the underlying mechanisms of hsa-circ-0072309 in RCC cells, the RNA expression level of the miR-100 was assessed by qRT-PCR, firstly. The miR-100 was over-expressed in kidney tumour tissues, in contrast with the control group (p < .001, Figure 4(A)), representing a significant involvement of miR-100 in RCC. Moreover, after the transfections of circRNA and vector, the miR-100 production was evidently prevented by the enforced hsa-circ-0072309 (both p < .01, Figure 4(B)). Finally, the miR-100 mimic and the NC mimic were constructed for miR-100 alteration, and the miR-100 mimic resulted in the high expression of miR-100 (both p < .001, Figure 4(C)) in the CAKI-1 and ACHN cells. All outcomes indicated that miR-100 was negatively controlled by the hsa-circ-0072309, and the miR-100 mimic was successfully synthesized and transfected into the cells.
Figure 2. Abundant hsa-circ-0072309 prevented cell proliferation, migration and invasion but promoted apoptosis. (A) Cell viability was explored by CCK-8; (B–D) the p53 and c-Myc, as well as (F–H) the apoptosis-related proteins expression levels were tested with western blot; (E) the apoptotic rate was tested by annexin V-FITC/PI with flow cytometer. CTRL: control; CCK-8: cell counting kit-8; FITC: fluorescein isothiocyanate; PI: propidium iodide; circRNA: circularRNA hsa-circ-0072309 overproduction vector. *p < .05, ***p < .001, compared to the corresponding group.
Copious hsa-circ-0072309 constrains cell proliferation but mediates apoptosis by targeting miR-100

In contrast with the corresponding group, miR-100 mimic markedly abolished the impact of circRNA of hsa-circ-0072309. In detail, miR-100 mimic obviously accelerated the cell viability (both $p < .05$, Figure 5(A)). There was no doubt that the p53 (both $p < .05$) was down-modulated by miR-100 mimic, which induced an increase of c-Myc on the contrary (both $p < .05$) (Figure 5(B,C)). Meanwhile, the cells apoptotic rates (both $p < .05$, Figure 5(E)) and expressions of apoptosis-related
proteins (all \( p < .05 \), Figure 5(F–H)) were prohibited due to miR-100 mimic. These phenomena demonstrated that miR-100 was probably a target of the hsa-circ-0072309, and was beneficial to the survival of CAKI-1 and ACHN cells.

Over-production of hsa-circ-0072309 inhibits cell migration and invasion by down-regulating miR-100

Just like before, the migration and invasion of the CAKI-1 and ACHN cell lines were determined for the second time. As were shown in our investigation, the inhibitory influences that derived from the circRNA of hsa-circ-0072309 on the migration rates (\( p < .05 \), Figure 6(A)), invasion rates (\( p < .05 \), Figure 6(E)), as well as the productions of migration or invasion-associated proteins comprising MMP-2 and MMP-9 (all \( p < .05 \), Figure 6(B,C)), as well as vimentin (both \( p < .05 \), Figure 6(f–H)) were significantly released by the miR-100 mimic. These data revealed that hsa-circ-0072309 inhibited cell migration and invasion by down-regulating miR-100 in vitro.

Abundant hsa-circ-0072309 blocked PI3K/AKT and mTOR signalling Cascades by targeting miR-100

From the perspective of signalling pathway, we observed that over-production of hsa-circ-0072309 extremely restrained the PI3K/AKT and mTOR pathways by leading to the reductions of the protein levels of p-PI3K/AKT and the p/t-PI3K/AKT (\( p < .05 \) or \( p < .001 \)), as well as the protein levels of p-mTOR and p/t-mTOR (both \( p < .001 \)) in both the CAKI-1 and the ACHN cell lines. Moreover, the PI3K/AKT and mTOR cascades were evoked by the miR-100 mimic which had reverse effects with circRNA of hsa-circ-0072309 on p-PI3K/AKT (Figure 7(A,B)), p/t-PI3K/AKT (all \( p < .05 \), Figure 7(C,D)), p-mTOR (Figure 7(E,F)) and p/t-mTOR (both \( p < .05 \), Figure 7(G–H)). We could assume that hsa-circ-0072309 had the potential to cover the signalling pathways by targeting miR-100.

Discussion

We noticed that hsa-circ-0072309 was poorly expressed in kidney tumour tissues. Abundant hsa-circ-0072309, which markedly accelerated the apoptosis biological process, notably restrained the proliferation, migration, and invasion of the CAKI-1 and ACHN cell lines as well as the PI3K/AKT and mTOR cascades, exhibiting a tumour inhibitory effect. Furthermore, we observed that the miR-100 exerted high production in tumour tissues and was negatively modulated by the circRNA of hsa-circ-0072309. The anti-tumour impact of hsa-circ-0072309 was partly eliminated by miR-100 mimic, indicating a target relationship between the hsa-circ-0072309 and the miR-100. It could be assumed that hsa-circ-0072309 functioned in the CAKI-1 and ACHN cell lines by targeting the miR-100, which had a carcinogenic impact.

circRNAs are a type of non-coding RNA that have a stable closed-loop structure which makes them hard to be decomposed by the enzymes [17]. Many pieces of evidence have indicated that circRNAs are closely related to the progressions of tumours. For example, circ-HIAT1 acted as a metastatic inhibitor to prevent androgen receptor (AR)-accelerated RCC cell migration and invasion by de-regulating miR-195-5p [8]. circNRIP1 promoted tumour metastasis in vivo by inducing proliferation and migration as well as the activation of AKT/mTOR cascade in GC [18]. circ-ZNF609 was significantly overproduced in RCC cell lines, and thus promoted the cell proliferation and invasion through a novel network that was comprised of circ-ZNF609 and miR-138-5p [19]. Yan and colleague demonstrated that the hsa-circ-0072309 was down-regulated and played anti-tumour effects in breast cancer tissues by targeting miR-492 [9]. Similar outcomes were observed in our study, where abnormal expression of hsa-
circ-0072309 was associated with the tumourigenesis of renal carcinoma. Hsa-circ-0072309 over-production led to an evident reduction in cells survival by adjusting several cellular procedures. Among them, the apoptosis, migration and invasion are typical features of cancer cells, while apoptosis is a unique morphological response to cellular stress [20]. The migration ability is essential for the development of cancer cells and is strictly implicated in the invasion which induces

Figure 5. Copious hsa-circ-0072309 constrained cell proliferation but mediated apoptosis by targeting miR-100. (A) Cell viability was explored by CCK-8; (B–D) the p53 and c-Myc, as well as (F–H) apoptosis-related protein levels were examined by western blot; (E) the apoptotic rate was tested by annexin V-FITC/PI with flow cytometer. CTRL: control; circRNA: circularRNA hsa-circ-0072309 overproduction vector; miR-100: microRNA-100; NC: negative control; CCK-8: cell counting kit-8; FITC: fluorescein isothiocyanate; PI: propidium iodide. *p < .05, ***p < .001, compared to the corresponding group.
metastasizing [21]. Therefore, the caspase enzymes, MMPs, and vimentin are the major secreted proteinases that are individually required for the regulation of apoptosis, migration and invasion [22–24].

It is generally believed that changes in miRNAs expression have a significant correlation with the development of cancers [25]. For instance, miR-141 and miR-200c could regulate the epithelial-to-mesenchymal transition (EMT) by targeting...
E-cadherin transcriptional repressors, with a low expression in RCC [26]. Akira et al. indicated that miR-429, which was located on chromosome 1p36.33, was down-regulated in RCC and formed a cluster with the miR-200a/200b [27]. Also, miR-100 is a member of the miRNAs family and plays critical roles in adjusting cellular processes by targeting the IGF1R/mTOR signalling cascades [14]. Out of question, an ingenious phenomenon was obtained in our investigation. The miR-100 functioned as a downstream target of the hsa-circ-0072309 by having a reverse influence on cell proliferation, migration, and migration.

Figure 7. Abundant hsa-circ-0072309 blocked PI3K/AKT and mTOR signalling cascades by targeting miR-100. (A–D) the PI3K/AKT and (E–H) mTOR protein levels of were identified with western blot. CTRL: control; circRNA: circularRNA hsa-circ-0072309 overproduction vector; miR-100: microRNA-100; NC: negative control; PI3K: phosphatidylinositide 3-kinases; AKT: protein kinase B; mTOR: mammalian target of rapamycin. *p < .05, ***p < .001, compared to the corresponding group.
invasion, and apoptosis in the CAKI-1 and the ACHN cell lines.

The PI3K/AKT is one of the most distributed signalling cascades in diverse tumours and is always evoked as a cancer driver [28,29]. It has been clarified that PI3K/AKT is capable of participating in the modulation of almost every cellular processes, such as growth, metabolism and tumourigenesis, and the inhibitions of PI3K/AKT or mTOR is effectively conducive to cancers therapies [30]. As serine/threonine kinase, mTOR signalling is crucial for modifications of cell growth, angiogenesis and eukaryotic translation by regulating HIF-1α and Cyclin [31]. Abnormal productions of PI3K/AKT/mTOR proteins are illuminated to be responsible for RCC deterioration [31]. Previous studies demonstrated that the PI3K/AKT pathway was activated in clear cell RCC (ccRCC) through numerous mechanisms [32,33]. Moreover, Aysen et al. reported that everolimus prohibited the development of RCC in vitro, which was mediated through the mTOR cascade [34]. Liu et al. turned it out that long non-coding RNA (IncRNA)-TP73-AS1 modified the cell proliferation, invasion and apoptosis relying on the PI3K/AKT/mTOR signalling cascade in RCC [35]. Xie et al. pointed out that awakening of PI3K/AKT/mTOR contributed to the cell invasion and poor prognosis in RCC tumour. These conclusions indicated that the PI3K/AKT/mTOR signalling cascade occupied pivotal positions in RCC [36]. These conclusions revealed that the PI3K/AKT and the mTOR signalling cascades occupied pivotal positions in RCC. We observed in our study that in the RCC cell lines CAKI-1 and ACHN, hsa-circ-0072309 overproduction blocked the PI3K/AKT and the mTOR pathways by targeting miR-100. We could assume that hsa-circ-0072309 prevented the miR-100 to exert anti-tumour effect through blocking the PI3K/AKT and the mTOR pathways. Similar phenomena were partly revealed in others report that the miR-100, which was geared to the miR-99 family, exerted influences on the PI3K/AKT/mTOR signalling to trigger HBV replication at the post-transcription level and thus to enforce autophagy in hepatoma cells [37]. Moreover, miR-100 was connected with the cellular autophagy and apoptosis procedures through the mTOR cascade in osteosarcoma tissue [38].

There are no doubts that the hsa-circ-0072309 and the PI3K/AKT as well as the mTOR cascades are crucial for tumours modulating, albeit a little available information, which is concerned about the direct regulation relationship between the hsa-circ-0072309 and these two signalling pathways, was accessed. Referring to our achievements, we could make such a hypothesis that hsa-circ-0072309 suppressed the miR-100 to exert anti-tumour effect through blocking the PI3K/AKT and the mTOR pathways.

Conclusion

Generally, we found that the hsa-circ-0072309 might exhibit inhibitory roles on cell survival by targeting miR-100 through the PI3K/AKT and the mTOR pathways deactivation in the CAKI-1 and ACHN cell lines.

Disclosure statement

The authors declare that there is no conflict of interest.

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