The circular RNA NRIP1 plays oncogenic roles by targeting miR-505 through the activation of AMPK and PI3K/AKT/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) nuclear receptor-interacting protein 1 (NRIP1) (circNRIP1) in ACHN and CAKI-1 cells. Methods ACHN and CAKI-1 cells were transfected with si-circNRIP1 and microRNA-505 (miR-505) inhibitor or the corresponding controls. Cell viability was detected with the CCK-8. The protein expression levels of Bcl-2, Bax, cleaved-caspase-3, matrix metalloproteinase (MMP)-2, MMP-9, adenosine AMPK, AKT, PI3K, and mTOR were individually determined via western blot. qRT-PCR was used to examine the expressions of circNRIP1 and miR-505. The apoptotic rate, the colony numbers and the migration rate were separately determined by the annexin v-FITC/PI with flow cytometer, colony formation assay and modified two-chamber migration assay. Results circNRIP1 was overexpressed in tumor tissue. Silencing circZNF292 induced the inhibitions of cell viability, colony formation and migration, as well as the AMPK and PI3K/AKT/mTOR cascades but enhancement of apoptosis. si-circNRIP1 stimulated the up-regulation of miR-505, whose silence abolished the effects of si-circNRIP1 on these elements mentioned above. Conclusion The circNRIP1 played oncogenic roles by targeting miR-505 via stimulating AMPK and PI3K/AKT/mTOR cascades in the ACHN and the CAKI-1 cell lines.

Background

Renal cell carcinoma (RCC), which is responsible for 3% of adult cancer incidences worldwide, is one of the most common tumors originated from renal tubular epithelial cells [1] with a high mortality rate [2]. However, the exact pathogenesis of renal carcinoma is not fully clear.

circRNAs are specific non-coding RNAs that are structurally characterized by a continuous closed loop, which is a key component that distinguishes from the circRNAs and linear RNAs [3]. Numerous evidences have indicated that circRNAs are capable of regulating genes expression at different transcription levels [4, 5]. This provided possibilities for circRNAs to get involved in the development of diseases. It has been found that the circRNAs play key roles in neurological disorders, diabetes and diverse cancers such as colorectal cancer, breast cancer and pancreatic ductal adenocarcinoma [6]. circ-hippocampus abundant transcript 1 (HIAT1) acted as a metastatic inhibitor to repress the migration and invasion processes in the AR-stimulated RCC cells [7]. What's more, as a regulator of the miR-149-5p, circNRIP1 induced inhibitions of proliferation and migration, as well as the deactivation of AKT/mTOR cascade in gastric cancer (GC) cells [8]. This conclusion represented a compelling connection between the circNRIP1 and tumor development. Nevertheless, the functional mechanisms of circNRIP1 in RCC cell lines were needed to be investigated more detailed.

miRNAs are a class of endogenous short non-coding RNA molecules, found in eukaryotic cells, and regulate a variety of biological processes [9]. Abnormal expression of miR-505 has been observed in various cancers. Dang *et al.* found that miR-505 had some connection with GC cell proliferation and invasion [10]. Zhong *et al.* pointed out that miR-505 functioned as a tumor suppressor in RCC by down-regulating high mobility group box-1 protein (HMGB1) [11]. What's interesting, circRNAs always exert

dedicated regulatory functions in carcinoma via acting as a sponge of the miRNAs [12]. For instance, circRNA-100269 combined with miR-630 to adjust the proliferation of GC cells [13]. circ-PCNXL2 was extremely implicated in the proliferation and invasion of RCC cells by binding to miR-153 to modulate the expression of ZEB2 [14]. However, until nowadays, it has been incompletely investigated that how does the circNRIP1 function in RCC cells. The direct relationship between the circNRIP1 and the miR-505 was still undefined.

Here, the ACHN and CAKI-1 cell lines were considered as the experiment material. We screened for the underlying roles and mechanisms of the circNRIP1 in RCC *in vitro*.

Methods

Clinical specimens

The kidney cancer or the normal tissues were obtained from The Affiliated Hospital of Qingdao University Hospital (The Affiliated Hospital of Qingdao University, China). None therapies were administrated on the patients prior to the surgery. We obtained informed consents from all patients, and the present study was with the permission of the Medical Ethics Committee of the The Affiliated Hospital of Qingdao University Hospital.

Cell cultivation

The kidney cancer cells ACHN and CAKI-1 (ATCC, Manassas, Virginia, USA) were considered as the main study materials. The cells were cultured with RPMI-1640 (Gibco BRL, Grand Island, New York, USA) plus 10% fetal bovine serum (FBS, Gibco, Waltham, Massachusetts, USA), at 37°C with the humidity of 95% air and 5% CO_2, in line with the suggestions from ATCC.

Cell transfection

Small-interfering RNA (si)-circNRIP1 (si-circNRIP1) and miR-505 inhibitor as well as their corresponding negative controls (NC) (Life Technologies Corporation, Carlsbad, California, USA) were synthesized to alter the productions of the circNRIP1 and the miR-505 in ACHN and CAKI-1 cell lines. They were transfected into the cells via lipofectamine 3000 reagent (Invitrogen, Carlsbad, California, USA) for further experiment. Three days after transfection was selected as the optimum harvest time owing the highest transfection efficiency.

Cell viability assessing

The CCK-8 was introduced to detect cells viability in the present study. Firstly, different group cells were seeded into the 96-well plate with 5×10^3 cells/well, and cultivated in a CO_2 incubator, for 24 h, at 37°C for further assays. Besides, referring to the instruction, the CCK-8 solution was injected and co-incubated with the cells for 1 h, after the 24 h conventional culture. Finally, the microplate reader (Bio-Rad, Hercules, California, USA) was introduced for absorbance measurement under 450 nm.

Colony formation assay

The 6-well plates, which contained 500 cells/well, were put into the carbon dioxide incubator and the cells were cultured for 2 weeks. After that, cells were fixed with paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) and stained by crystal violet (Sigma-Aldrich). The colony formation ability was measured with a microscope (Olympus IX81, Tokyo, Japan).

Cell migration assay

A modified two-chamber migration assay (Millipore, Bedford, Massachusetts, USA) was conducted to test cells migration. The cells suspension were prepared with 200 μL serum-free medium (Gibco BRL) with a concentration of 5×10^4 cells/ml. Followed by a two days conventional cultivation, the suspension was then injected in the upper chamber while 600 μL of serum-containing medium (Gibco BRL) was complemented into the lower chamber. After incubation, cells were rinsed 1-2 times with phosphate buffer saline (PBS), and the 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).

Apoptosis assay

The cells apoptotic rate was measured by Annexin V-FITC/PI detection kit (Beijing Biosea Biotechnology, China) and the flow cytometer (Beckman Coulter, California, USA). Totally, these treated cells, which were adjusted and prepared in the 6-well plate with 1×10^5 cells/well density and cultured at 37°C for 24 h, were washed and re-suspended 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.

Western blot

After cells collection, 1 × PBS buffer and 4°C RIPA buffer (Beyotime Biotechnology) containing protease inhibitors (Roche, Basel, Switzerland) was respectively used for total proteins extraction. The BCA™ Protein Assay Kit (Pierce, Appleton, Wisconsin, USA) was applied for protein quantification and the Bio-Rad Bis-Tris Gel system (Bio-Rad, Hercules, California, USA) was helped to form 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-Bcl-2 (ab32124), anti-Bax (ab53154), anti-cleaved-caspase-3 (ab2302), anti-matrix metalloproteinase (MMP)-2 (ab92536), anti-MMP-9 (ab38898), anti-AMPK (ab131512), anti-p-AMPK (ab240058), anti-PI3K (ab40776), anti-p-PI3K (ab191606), anti-p-AKT (ab192623), anti-AKT (ab106693), 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.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, California, USA) and the DNaseI (Promega, Madison, wisconsin, USA) were used for RNA extracting. 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 $2^{-\triangle\triangle Ct}$ method and normalized with β-actin or U6.

Statistical analysis

The SPSS statistical software version 19.0 was the main tool for statistical analysis. The experimental data were showed as the mean + standard deviation (SD). Analysis of variance (ANOVA) or *t*-test was responsible for *P* values. $P < 0.05$ was considered to be statistically significant. All experiments in this study were repeated 3 times at least.

Results

circNRIP1 is over-expressed in renal cancer tissue

The RNA expression level of circNRIP1 was detected through qRT-PCR. As was shown in our study, high expression of circNRIP1 was observed in the renal tumor tissues, in contrast with the control grouop ($P < 0.001$, Figure 1). This phenomenon showed that the circNRIP1 was closely related to renal cancer development.

Knockdown of circNRIP1 induces apoptosis and inhibitions of cell viability, colony formation and migration

In order to explore the functions of the circNRIP1 in ACHN and CAKI-1 cell lines, the si-circNRIP1 and si-NC were individually transfected into ACHN and CAKI-1 cell lines. Comparing with the corresponding group, si-circNRIP1 significantly led to a poor-expression of the circNRIP1 in both of the cells (both $P < 0.001$, Figure 2). It displayed that the si-circNRIP1 and si-NC were successfully constructed and transfected into cells. What's more, the viability (both $P < 0.05$, Figure 3A) and the colony formation ($P < 0.01$ or $P < 0.001$, Figure 3B) of the ACHN and the CAKI-1 cells were notably restrained by the si-circNRIP1. However, the apoptotic rate (both $P < 0.001$, Figure 3C), and the productions of pro-apoptosis proteins such as Bax, cleaved-caspase 3 (all $P < 0.001$) were markedly increased by si-circNRIP1, which prevented the generation of the Bcl-2 on the contrary (both $P < 0.001$) (Figure 3D-3F). Meanwhile, the migration of the ACHN and CAKI-1 cells was detected as well. Generally, the migration rate ($P < 0.05$ or $P < 0.01$, Figure 3G), as well as the expressions of the MMP-2 (both $P < 0.05$) and MMP-9 (both $P < 0.01$) were evidently prevented by the si-circNRIP1 (Figure 3H-3J). All data indicated that circNRIP1 silence induced inhibitions of the proliferation, colony formation and migration, as well as the promotion of the apoptosis in both the

ACHN and the CAKI-1 cells. circNRIP1 might be beneficial to the survival of the ACHN and the CAKI-1 cells.

circNRIP1 silence promotes the production of miR-505

To further study the potential mechanisms of the circNRIP1, we measured the expression level of the miR-505 relying on the qRT-PCR. In contrast with the relative group, the miR-505 was drastically up-modulated by the si-circNRIP1 ($P < 0.001$ or $P < 0.01$, Figure 4A). This outcome suggested that miR-505 was negatively controlled by the circNRIP1 in the ACHN and the CAKI-1 cells. Besides, the miR-505 inhibitor was successfully synthesized and transfected into the cell lines to restrain the miR-505 expression (both $P < 0.001$, Figure 4B), in contrast with the NC group.

circNRIP1 silence constrains cell viability, colony formation and cell migration but accelerates apoptosis via up-regulating miR-505

miR-505 inhibitor apparently elevated the cell viability (both $P < 0.05$, Figure 5A) and the colonies numbers (both $P < 0.05$, Figure 5B). The apoptotic rate (both $P < 0.05$, Figure 5C), and productions of apoptosis-related proteins (all $P < 0.05$), excluding the Bcl-2 (both $P < 0.001$) were down-regulated by the miR-505 inhibitor (Figure 5D-5F). Additionally, the migration rate (both $P < 0.05$, Figure 5G) of the ACHN and the CAKI-1 cells, as well as these migration-related proteins (all $P < 0.05$, Figure 5H-5J) were significantly increased by miR-505 inhibitor. These results demonstrated that the miR-505 inhibitor significantly abolished the effects of the si-circNRIP1 by promoting cell viability, colony formation and migration but preventing cell apoptosis. The miR-505, which was probably a target of the circNRIP1, was damage to the survival of the ACHN and the CAKI-1 cells.

circNRIP1 silence blocks AMPK and PI3K/AKT/mTOR signaling pathways by up-regulating miR-505

According to the western blot outcomes, the protein expression levels of the p-AMPK (Figure 6A) and the p/t-AMPK ($P < 0.01$ or $P < 0.001$) were notably decreased by the si-circNRIP1 in both of the ACHN and the CAKI-1 cells. Moreover, the influences of the si-circNRIP1 on the p-AMPK and the p/t-AMPK (both $P < 0.001$, Figure 6B) were disturbed by the miR-505 inhibitor. In addition, the protein expression levels of p-PI3K, p-AKT and p-mTOR (Figure 6D or Figure 6F) as well as the p/t-PI3K ($P < 0.001$ or $P < 0.05$), p/t-AKT (both $P < 0.001$) and p/t-mTOR ($P < 0.01$ or $P < 0.05$) were notably reduced by si-circNRIP1 in the ACHN and CAKI-1 cell lines. Likely, these phenomena were reversed by the miR-505 inhibitor, which mediated the increase of p-PI3K, p-AKT and p-mTOR, as well as the p/t-PI3K ($P < 0.001$ or $P < 0.05$), p/t-AKT (both $P < 0.01$) and p/t-mTOR ($P < 0.05$ or $P < 0.01$) (Figure 6E or Figure 6G). These data displayed that si-circNRIP1 deactivated the AMPK and PI3K/AKT/mTOR signaling cascades by promoting miR-505 in the ACHN and CAKI-1 cell lines. We could assume that circNRIP1 had the potential to trigger the signaling pathways by targeting miR-505.

Discussion

We observed that the circNRIP1 was overexpressed in tumor tissues. Besides, the cell viability, the colony formation and migration were evidently reduced by si-circNRIP1 in both of the ACHN and CAKI-1 cell lines. On the contrary, the apoptosis was markedly promoted by si-circNRIP1. What's more, circNRIP1 silence extremely induced the up-regulation of miR-505, and the effects of si-circNRIP1 were disturbed by the miR-505 inhibitor, showing a negative regulatory relationship between circNRIP1 and miR-505. Additionally, si-circNRIP1 deactivated the AMPK and PI3K/AKT/mTOR pathways, which was mediated via miR-505.

circRNAs are a type of non-coding RNA that have a stable closed-loop structure which make them hard to be decomposed by the enzymes [15]. A large number of evidences have indicated that circRNAs are closely related to the development of tumors [16]. For instance, circ-HIAT1 acted as a metastatic inhibitor to prevent androgen receptor (AR)-accelerated RCC cell migration and invasion by de-regulating miR-195-5p [7]. circ-ZNF609 was significantly overproduced in RCC cell lines, and thus promoted the cell proliferation and invasion through a novel network that was comprised by circ-ZNF609 and miR-138-5p [17]. What's important, Zhang *et al.* demonstrated that circNRIP1 promoted tumor metastasis *in vivo* by inducing proliferation and migration as well as the activation of AKT/mTOR cascade in GC [8]. Similar outcomes were observed in our study that abnormal expression of circNRIP1 was associated with the tumorigenesis of renal carcinoma. circNRIP1 silence led to an inhibition of proliferation, colony formation and migration but promoted apoptosis in ACHN and CAKI-1 cell lines. This result exhibited a tumor promoting effect of the circNRIP1. Apoptosis and migration are typical features of cancer cells, while apoptosis is a unique morphological response to cellular stress [18]. The pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 are geared to the Bcl-2 family, which occupy important positions in the regulation of apoptosis [19, 20]. The migration ability is essential for development of cancer cells [21]. MMPs are the major secreted proteinases that are required for extracellular matrix (ECM) degradation to regulate tumor migration [22]. It is generally believed that changes in miRNAs expression have a significant correlation with the development of cancers [23]. For example, miRNA-7 acted as a tumor suppressor by targeting epidermal growth factor receptor (EGFR) [24]. miR-505 is known as a tumor suppressor and participated in the regulations of various cancers. miR-505 induces cell proliferation inhibition and apoptosis in breast cancer [25]. It was turned out by Chen *et al.* that miR-505 was overproduced in EC cells, leading to the reduction of TGF-α, MMP-2 and MMP-9, but enforced the productions of Bax and cleaved-PARP [26]. Additionally, Zhong *et al.* revealed that miR-505 functioned as a tumor suppressor in RCC by down-regulating HMGB1 [11]. Out of question, an ingenious phenomenon was obtained in this present investigation. The tumor inhibitory effect of miR-505 was completely eliminated by the miR-505 inhibitor, which resulted in an increase of cell viability, colony formation and migration, but a weak apoptosis. Besides, miR-505 inhibitor released the influences that derived from the si-circNRIP1, exhibiting a target relationship between the circNRIP1 and the miR-505. It could be concluded that circNRIP1 functioned in the ACHN and CAKI-1 cell lines by targeting the miR-505. Just like what was clarified by Zhang's team that circNRIP1 functioned as a sponge of miRNA-149-5p to promote GC metastasis by inducing proliferation and migration as well as the AKT/mTOR cascade [8].

AMPK is the primary sensor and central regulator of cellular metabolism and energy status in mammalian tissues [27]. According to that, the AMPK pathway is capable of participating in the regulation of metabolic diseases or cancers [28, 29]. Generally, AMPK has potential capabilities cancers inhibition. Nonetheless, recent studies have shown that AMPK can play an oncogenic or anti-tumor effect in cancers depending on the specific situation [30, 31]. Brandon and colleagues found that AMPK activation could be conducive to the growth of tumor cells by modulating the metabolic plasticity and the flexibility [29]. What's more, AMPK pathway was implicated in the functional effect of metformin in renal cancer [32]. Another investigation revealed that compound C modulated tumor apoptosis relying on AMPK pathway in human renal cancer cells [33]. In addition, PI3K/AKT/mTOR signaling pathway has direct connections with many cellular processes such as growth, motility and apoptosis [34]. Recent researches observed that PI3K/AKT/mTOR cascade is often irregular in various tumors, including breast cancer, colorectal cancer and RCC [35]. Xie *et al.* pointed out that awakening of PI3K/AKT/mTOR contributed to the cell invasion and poor prognosis in RCC tumors [36]. Besides, PI3K/AKT/mTOR signaling pathway, which led to an increase of proliferation and apoptosis reduction, had some connections with the inhibitory influences on RCC cell derived from the chlorogenic acid (CA) [37]. These conclusions indicated that the AMPK and the PI3K/AKT/mTOR signaling cascades occupied pivotal positions in RCC. We observed in our study that in the RCC cell lines ACHN and CAKI-1, knockdown of circNRIP1 blocked the AMPK and the PI3K/AKT/mTOR pathways by up-regulating miR-505. We could assume that si-circNRIP1 stimulated the miR-505 to exert anti-tumor effect through blocking the AMPK and the PI3K/AKT/mTOR pathways. The circNRIP1 is crucial for controlling pathway target expression in tumors, albeit a little available information, which is concerned about the direct regulation relationship between the circNRIP1 and these two signaling pathways, was accessed. Our achievement represented an undoubtly direct relationship between the circNRIP1, miR-505 and the AMPK and the PI3K/AKT/mTOR pathways. This result was partly confirmed by Zhang *et al.* who demonstrated that circNRIP1 promoted tumor metastasis by evoking the AKT/mTOR cascade in GC cells [8].

Conclusions

Above all, we found that the circNRIP1 might exhibit oncogenic roles by targeting miR-505 through the AMPK and the PI3K/AKT/mTOR pathways activation in the ACHN and the CAKI-1 cell lines.

Abbreviations

circular RNA circRNA

nuclear receptor-interacting protein 1.....NRIP1

circular RNA nuclear receptor-interacting protein 1 circNRIP1

microRNA-505.....miR-505

matrix metalloproteinase .....MMP

Renal cell carcinoma …..RCC

circ-hippocampus abundant transcript 1…..HIAT1

gastric cancer…..GC

high mobility group box-1 protein…..HMGB1

Small-interfering RNA -circNRIP1…..si-circNRIP1

negative controls …..NC

phosphate buffer saline …..PBS

Quantitative reverse transcription polymerase chain reaction …..qRT-PCR

standard deviation…..SD

Analysis of variance …..ANOVA

extracellular matrix …..ECM

androgen receptor…..AR

epidermal growth factor receptor …..EGFR

chlorogenic acid …..CA

American Type Culture Collection ATCC

Declarations

Ethics approval

We obtained informed consents from all patients, and the present study was with the permission of the Medical Ethics Committee of the The Affiliated Hospital of Qingdao University Hospital.

Consent for publication

Not applicable

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: ZD and QHW; Performed the experiments and analyzed the data: HYW and JLJ; Contributed reagents/materials/analysis tools: TH and AK; Wrote and revised the manuscript: HN and YWC. All authors read and approved the final version of the manuscript and ensure this is the case.

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Figures

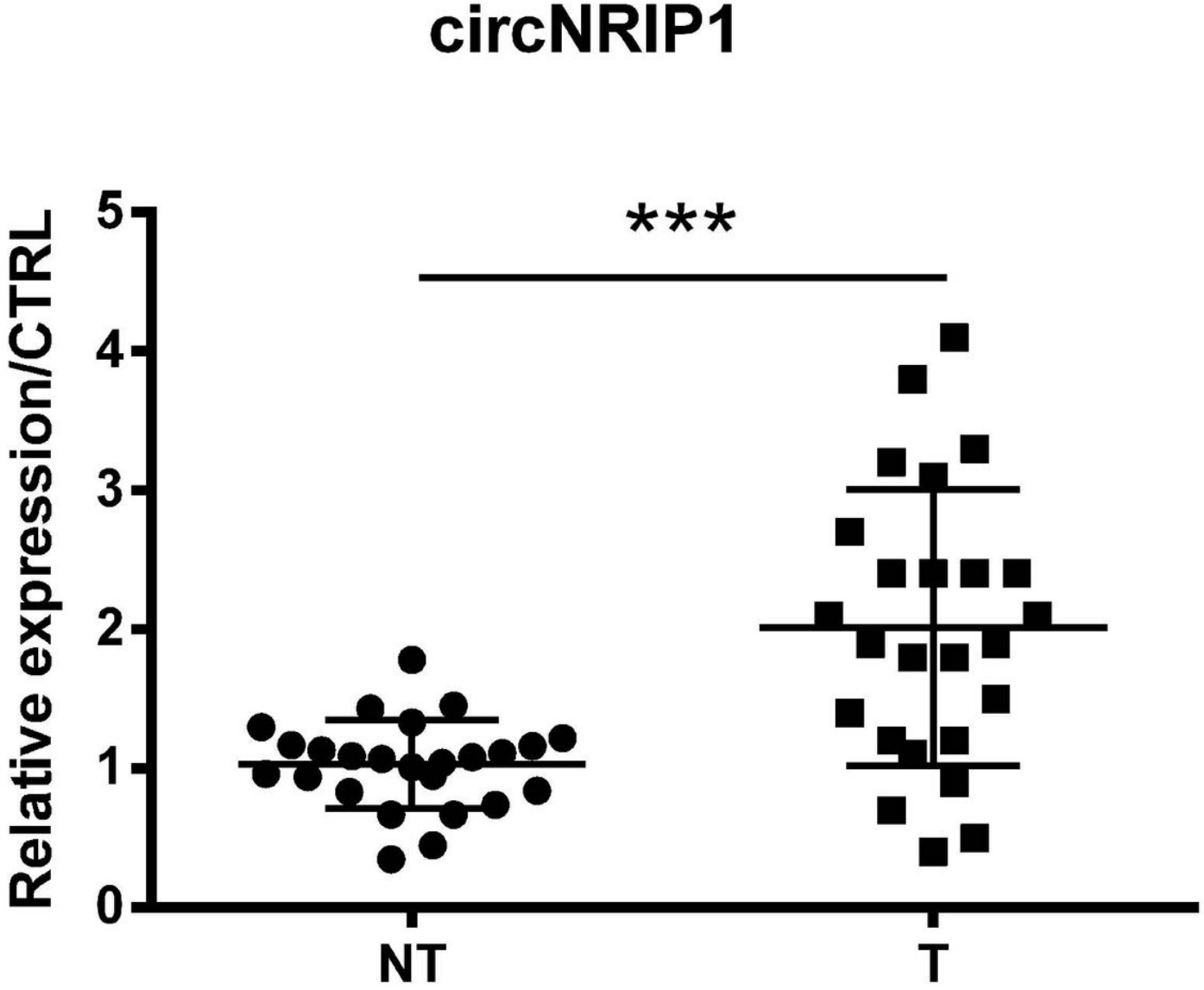

Figure 1

circNRIP1 was over-expressed in renal cancer tissue. After the preparation, the production level of circNRIP1 was detected by qRT-PCR. CTRL, control; NT, none tumor; T, tumor; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; qRT-PCR, quantitative reverse transcription polymerase chain reaction. *** $P < 0.001$, compared to the corresponding group.

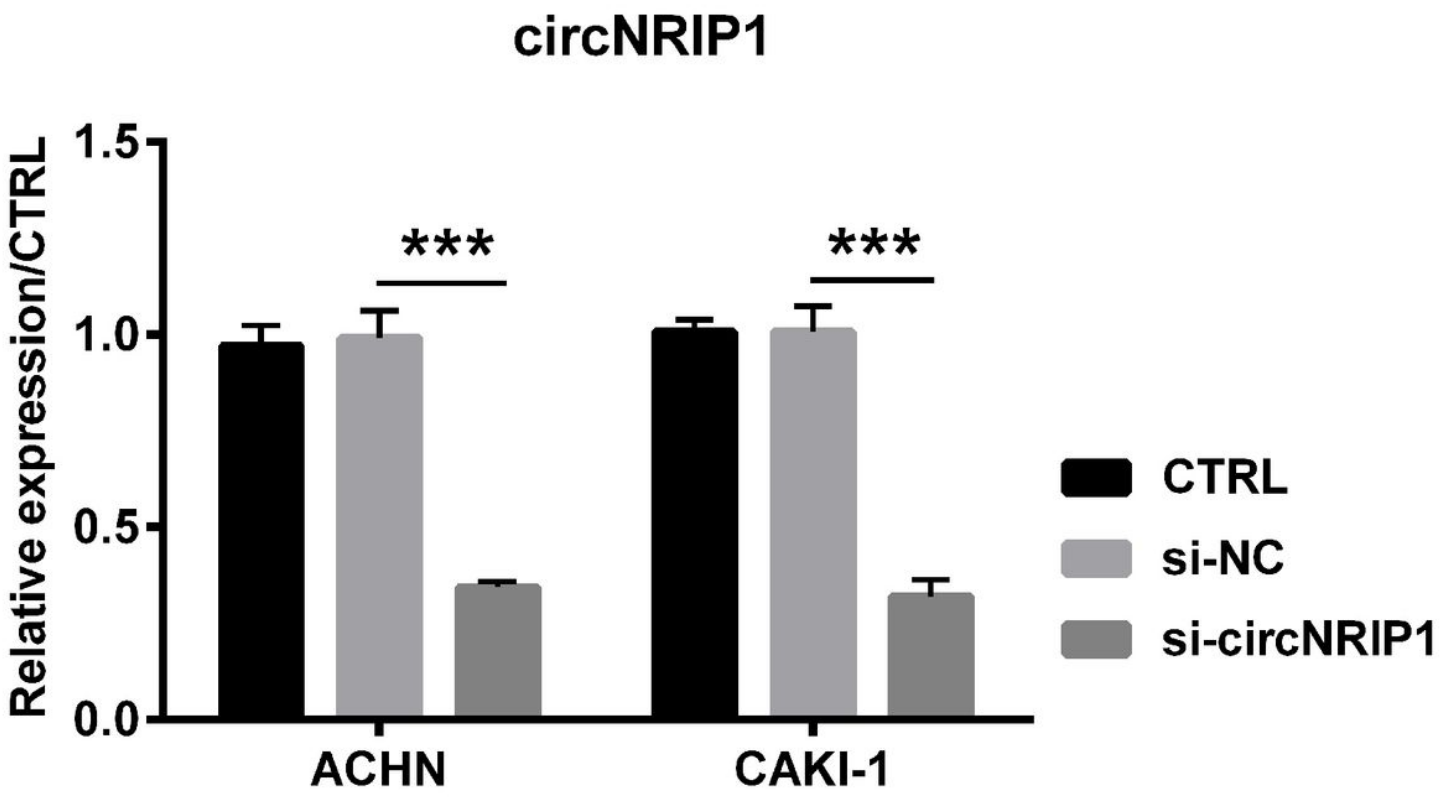

Figure 2

si-circNRIP1 inhibited the generation of circNRIP1. After the transfections of si-circNRIP1 and si-NC, the expression level of circNRIP1 was examined by qRT-PCR. CTRL, control; NC, negative control; si-NC, small-interfering RNA-NC; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; si-circNRIP1; small-interfering RNA-NRIP1; qRT-PCR, quantitative reverse transcription polymerase chain reaction. *** $P < 0.001$, compared to the corresponding group.

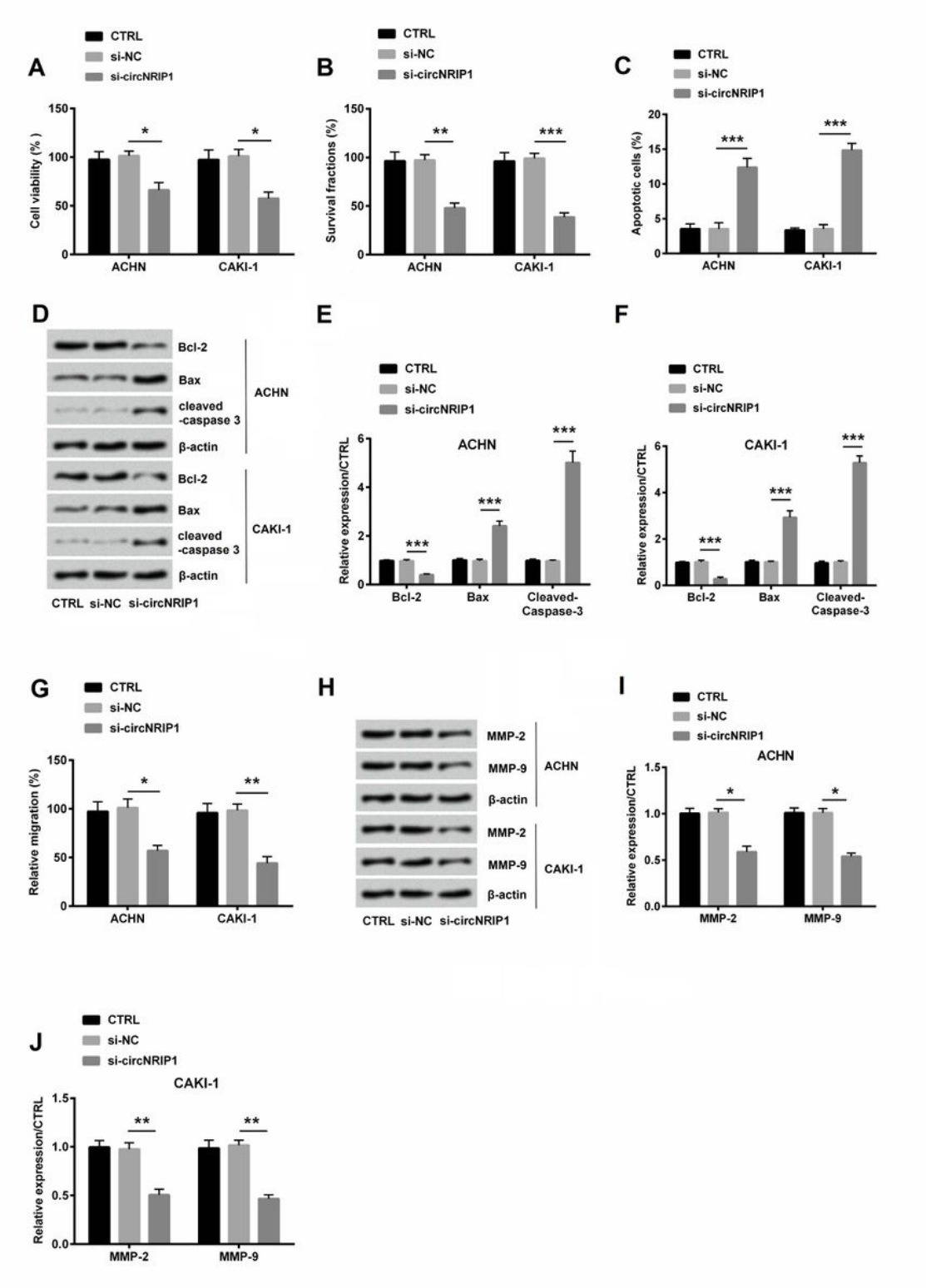

Figure 3

Knockdown of circNRIP1 prevented cell viability, colony formation and migration but promoted apoptosis. After the transfections of si-circNRIP1 and si-NC, (A) cell viability was explored by CCK-8; (B) the colony formation was examined by the colony formation assay; (C) the apoptotic rate was tested by annexin V-FITC/PI with flow cytometer; (D-F) the apoptosis-related proteins and (H-J) the migration-related proteins levels were identified by western blot; (G) the migration rate was detected with modified two-chamber

migration assay. CTRL, control NC, negative control; si-NC, small-interfering RNA-NC; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; si-circNRIP1; small-interfering RNA-NRIP1; MMP-2, matrix metalloprotein-2; MMP-9, matrix metalloprotein-9; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the corresponding group.

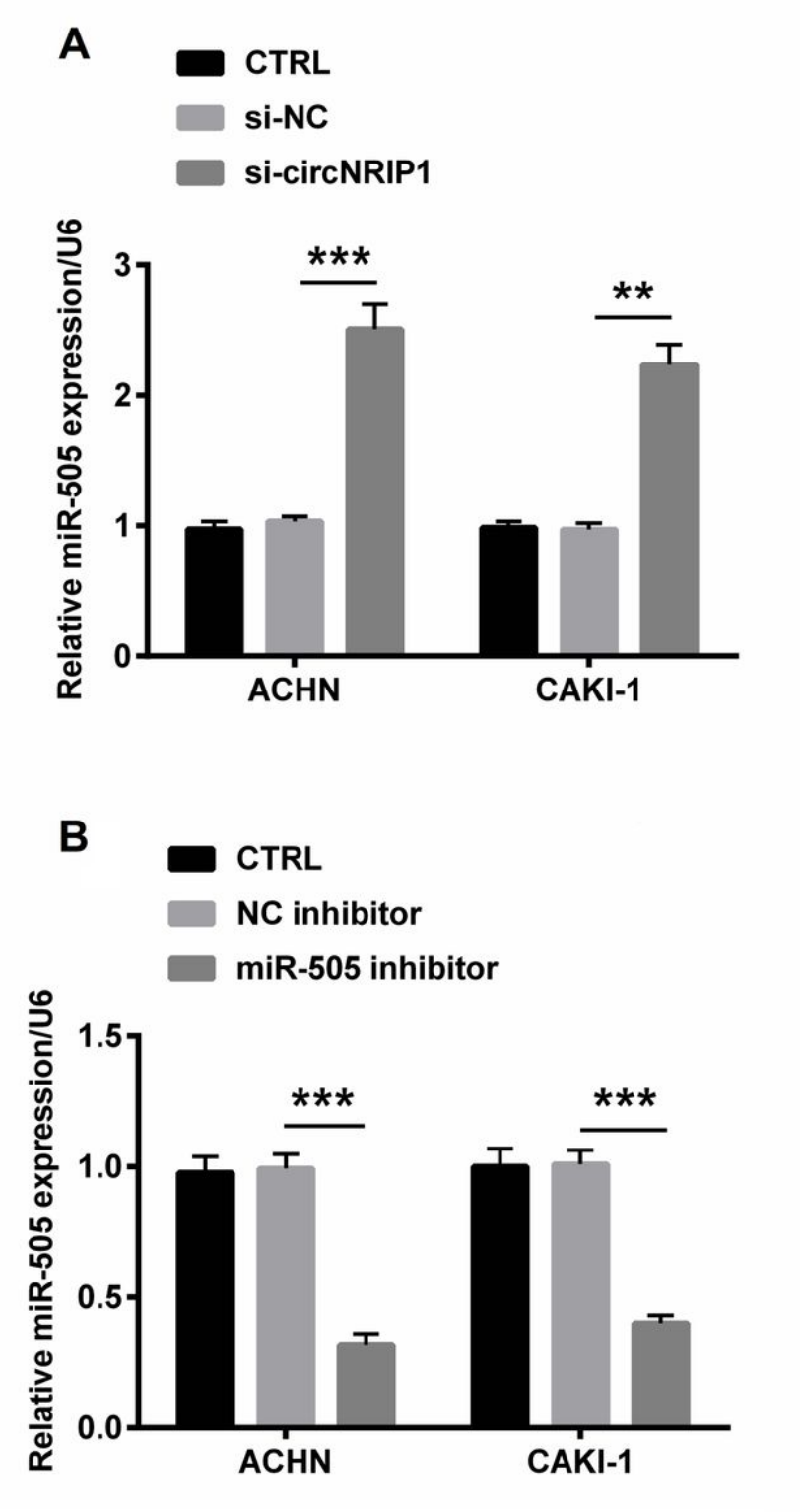

Figure 4

circNRIP1 silence promoted the production of miR-505. After the transfections of si-circNRIP1 and si-NC, (A) the expression level of circNRIP1, and after the transfections of miR-505 inhibitor and NC inhibitor (B) the expression level of miR-505 were tested by qRT-PCR. CTRL, control NC, negative control; si-NC, small-interfering RNA-NC; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; si-circNRIP1; small-interfering RNA-NRIP1; miR-505, mircoRNA-505; qRT-PCR, quantitative reverse transcription polymerase chain reaction. ** P < 0.01, *** P < 0.001, compared to the corresponding group.

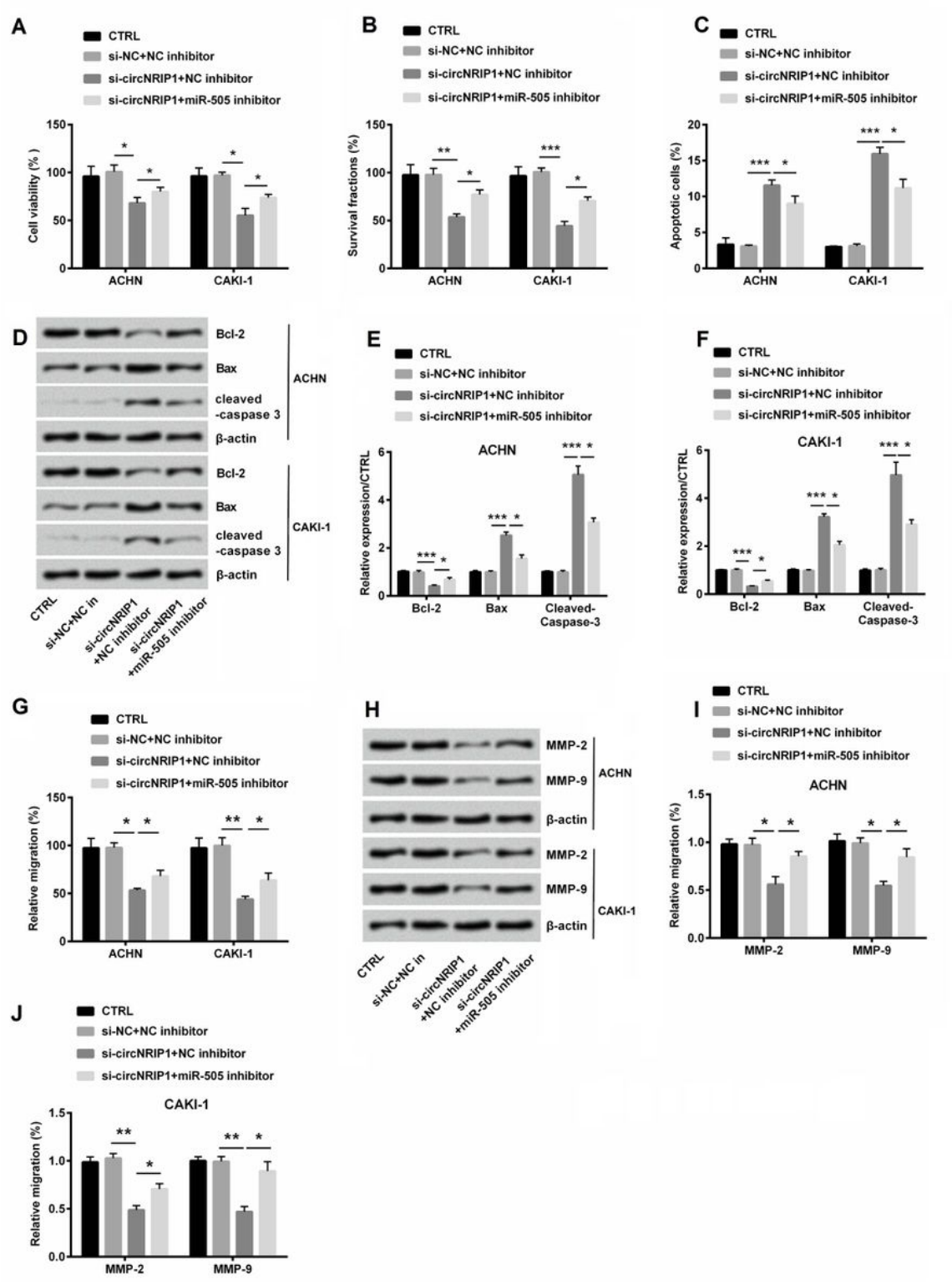

Figure 5

circNRIP1 silence constrained cell viability, colony formation and cell migration but accelerates apoptosis via up-regulating miR-505. After the transfections of si-circNRIP1 and the miR-505 as well as corresponding controls, (A) cell viability was explored by CCK-8; (B) the colony formation was examined by the colony formation assay; (C) the apoptotic rate was tested by annexin V-FITC/PI with flow cytometer; (D-F) the apoptosis-related proteins and (H-J) the migration-related proteins levels were identified by western blot; (G) the migration rate was detected with modified two-chamber migration assay. CTRL, control NC, negative control; si-NC, small-interfering RNA-NC; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; si-circNRIP1; small-interfering RNA-NRIP1; miR-505, mircoRNA-505; MMP-2, matrix metalloprotein-2; MMP-9, matrix metalloprotein-9; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the corresponding group.

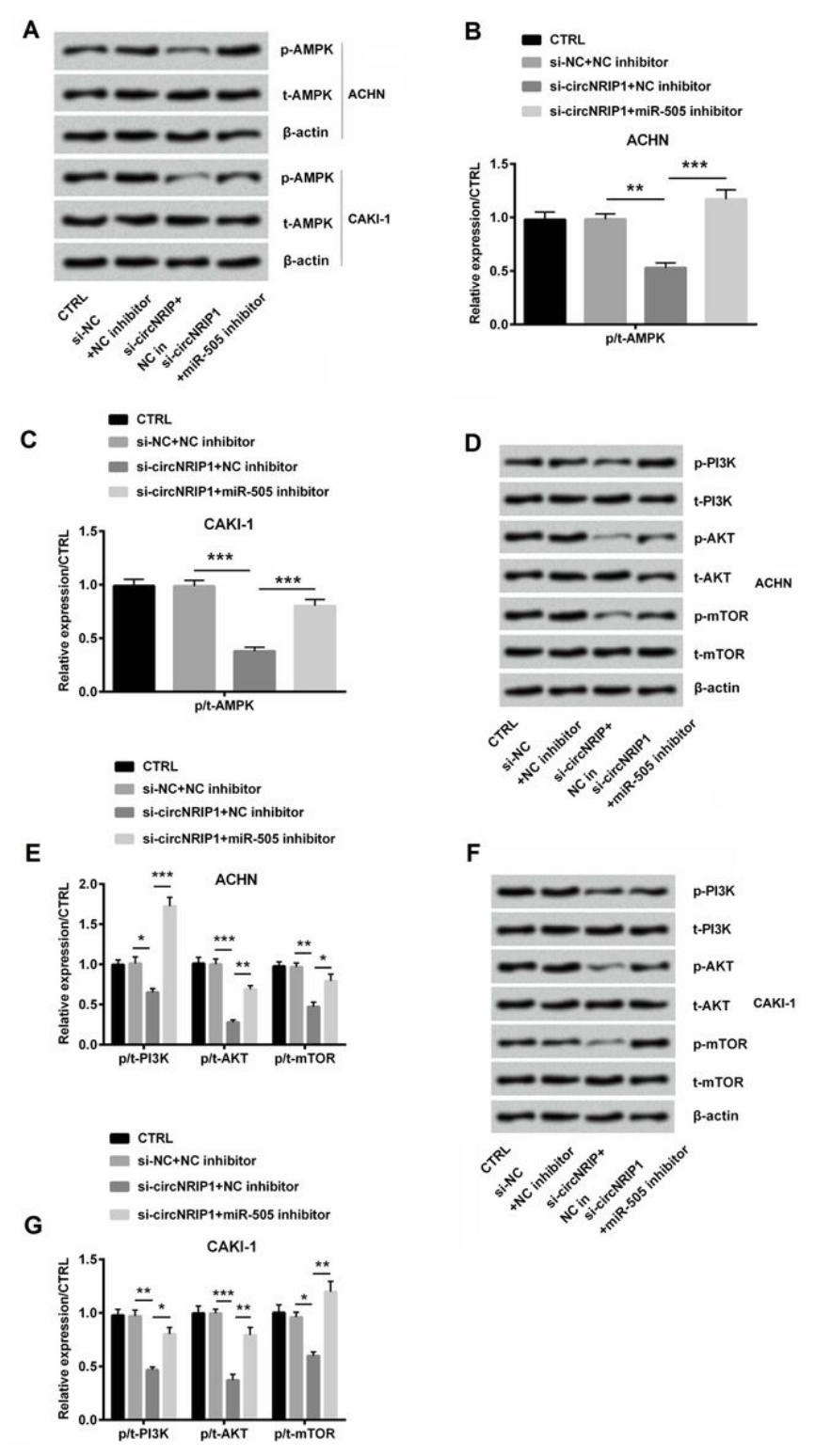

Figure 6

silencing circNRIP1 blocked AMPK and PI3K/AKT/mTOR pathways thorough stimulating miR-505. After the transfections of si-circNRIP1 and the miR-505 as well as corresponding controls, (A-C) the productions of AMPK proteins and (D-G) the productions of PI3K/AKT/mTOR proteinsm were assessed with western blot. CTRL, control NC, negative control; si-NC, small-interfering RNA-NC; NRIP1, nuclear receptor-interacting protein 1; circNRIP1, circular RNA-NRIP1; si-circNRIP1; small-interfering RNA-NRIP1;

miR-505, mircoRNA-505; AMPK, adenosine 5‘-monophosphate (AMP)-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the corresponding group.