Circular BANP knockdown inhibits the malignant progression of residual hepatocellular carcinoma after insufficient radiofrequency ablation

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Abstract

Background: Circular RNAs (circRNAs) are endogenous non-coding RNAs, some of which have pathological roles. The current study aimed to explore the role of circRNA BTG3-associated nuclear protein (circ-BANP) binding with let-7f-5p and its regulation of the toll-like receptor 4 (TLR4)/signal transducer and activator of transcription 3 (STAT3) signaling pathway in residual hepatocellular carcinoma (HCC) after insufficient radiofrequency ablation (RFA).

Methods: Circ-BANP, let-7f-5p, and TLR4 expressions in HCC samples were assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blotting. Bioinformatics prediction, RNA pull-down assay, and dual luciferase reporter gene assay were used to analyze the relationships among circ-BANP, let-7f-5p, and TLR4. Huh7 cells were used to generate an *in vitro* model of residual HCC, defined as Huh7-H cells, which were transfected with either a plasmid or the sequence of circ-BANP, let-7f-5p, or TLR4. Expression of circ-BANP, let-7f-5p, and TLR4 mRNA was determined by RT-qPCR. TLR4, STAT3, p-STAT3, vascular endothelial growth factor A, vascular endothelial growth factor receptor-2, and epithelial-mesenchymal transformation (EMT)-related factors proteins were determined by Western blotting. Cell proliferation was determined by cell counting kit-8 and 5-Ethynyl-2'-deoxyuridine (EdU) assay and cell migration and invasion by Transwell assay. Animal studies were performed by inducing xenograft tumors in nude mice.

Results: Circ-BANP and TLR4 mRNAs were upregulated in HCC tissues (the fold change for circ-BANP was 1.958 and that for TLR4 was 1.736 relative to para-tumors) and expression further increased following insufficient RFA (fold change for circ-BANP was 2.407 and that of TLR4 was 2.224 relative to para-tumors). Expression of let-7f-5p showed an opposite tendency (fold change for let-7f-5p in HCC tissues was 0.491 and that in tumors after insufficient RFA was 0.300 relative to para-tumors). Competitive binding of circ-BANP to let-7f-5p was demonstrated and TLR4 was identified as a target of let-7f-5p ($P < 0.01$). Knockdown of circ-BANP or elevation of let-7f-5p expression inhibited the TLR4/STAT3 signaling pathway, proliferation, invasion, migration, angiogenesis, and EMT in Huh7 and Huh7-H cells ($P < 0.01$). The effects induced by circ-BANP knockdown were reversed by let-7f-5p inhibition. Overexpression of TLR4 reversed the impact of let-7f-5p upregulation on the cells ($P < 0.01$). Silencing of circ-BANP inhibited the *in vivo* growth of residual HCC cells after insufficient RFA ($P < 0.01$).

Conclusions: Knockdown of circ-BANP upregulated let-7f-5p to inhibit proliferation, migration, and EMT formation in residual HCC remaining after insufficient RFA. Effects occur via regulation of the TLR4/STAT3 signaling pathway.

Keywords: circ-BANP; EMT; Hepatocellular carcinoma; Radiofrequency ablation; TLR4/STAT3

Introduction

Cancer is a leading cause of death globally.[1] Liver cancer is the 6th commonest cancer with ~841,000 new diagnoses and 782,000 deaths each year. Hepatocellular carcinoma (HCC) is the main pathological type, accounting for 80% of primary liver cancer cases.[2] Thus, HCC is a significant cause of cancer-related death throughout the world.[3] The therapeutic options of HCC are limited, leading to poor prognoses,[4] and the survival rate of HCC patients at 5 years is < 18%.[5] Radiofrequency ablation (RFA) is an alternative to surgery for HCC patients and is widely utilized due to its

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safety, repeatability, simplicity, and minimal invasiveness. Despite this, cases of residual HCC after insufficient RFA are on the increase. The HCC which recurs subsequent to RFA shows greater rates of cell proliferation, angiogenesis, and epithelial-mesenchymal transformation (EMT) formation accompanied by lower rates of apoptosis.[6] These observations indicate the urgent need to identify potential biomarkers for residual HCC after insufficient RFA.

Circular RNAs (CircRNAs) are a recently identified class of non-coding RNAs that are single-stranded and circular, lacking free 5'-end caps or 3'-end poly (A) tails.[7] Roles in the development of HCC have been proposed for circRNAs. Some examples include circ-ZEB1.33, which has been shown to promote the proliferation of human HCC,[8] and insulin-like growth factor-1 receptor (IGF1R), for which anti-apoptotic and pro-proliferative effects on HCC have been demonstrated.[9] Another member of the class of circRNAs, circRNA BTG3-associated nuclear protein (circ-BANP), has been revealed to facilitate the proliferation of colorectal cancer (CRC) cells[10] and to contribute to lung cancer progression.[11] However, the role of circ-BANP in HCC, especially residual HCC after insufficient RFA, remains unknown. MicroRNAs (miRNAs) are small non-coding RNAs with the capacity to suppress gene expression through binding 3'-untranslated regions (UTRs) and influencing mRNA stability or protein translation.[12] Let-7f-5p miRNA belongs to the let-7 family, which has been implicated in the biological processes of cancer cells.[13] Previous studies have shown that let-7f-5p influences the progression of bladder cancer[14] and CRC.[15] However, its role in HCC has been seldom explored. Moreover, any relationship between circ-BANP and let-7f-5p has been little scrutinized. Toll-like receptors are vital for immune system function and contribute to providing an advantageous microenvironment for cancer cells. Toll-like receptor 4 (TLR4) is a cancer stem cell marker for HCC[16] and it has been demonstrated that TLR4 expression is related to a poor prognosis for HCC patients.[17] It may be that TLR4 contributes to tumor angiogenesis since stimulation of TLR4 by lipopolysaccharide enhanced angiogenesis in a co-culture system consisting of primary human osteoblasts and outgrowth endothelial cells.[18] Although TLR4 has been confirmed as a target of let-7i under ischemic conditions,[19] the relationship between let-7f-5p and TLR4 warrants further investigation to clarify functional interactions. Furthermore, indications are that TLR4 interacts with signal transducer and activator of transcription 3 (STAT3)[20,21] and activation of the TLR4/STAT3 signaling pathway has been reported to promote HCC cell growth.[22]

The current study explored the role of circ-BANP and its interactions with let-7f-5p in the HCC which remains after insufficient RFA. The involvement of a TLR4-dependent signaling pathway was investigated. We explored the hypothesis that circ-BANP regulates let-7f-5p and affects residual HCC cell growth after insufficient RFA via the TLR4/STAT3 pathway.

Methods

Ethical approval

The protocol of this study was confirmed by the Ethics Committee of Beijing ChaoYang Hospital Affiliated to Capital Medical University (No. 2020-Ke-95) and based on the ethical principles for medical research involving human subjects of the *Helsinki Declaration*. Written informed consents were acquired from all patients before this study. Animal experiments were strictly in accordance with the Guide to the Management and Use of Laboratory Animals issued by the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of Beijing ChaoYang Hospital Affiliated to Capital Medical University.

Study subjects

Eighty-three HCC samples and para-tissues were harvested from patients who had undergone resection in Beijing ChaoYang Hospital Affiliated to Capital Medical University. Thirty-nine of the 83 were residual tumors obtained from HCC patients who had received insufficient RFA treatment. Tissues were stored at –80°C.

Cell culture

HCC cell line Huh7 (ATCC, Manassas, VA, USA) was cultured with Dulbecco's modified eagle medium containing 10% fetal bovine serum. Strongly proliferating cells were used for our experiments. Construction of the in *vitro* cancerous cell model after insufficient RFA was conducted according to a previously published method[23] and named Huh7-H.

Cell transfection

Cells were cultured for 24 h until 70% to 90% confluence was reached, after which transfection with small interfering RNA-negative control (si-NC), si-circ-BANP#1, si-circ-BANP#2, mimic-NC, let-7f-5p mimic, si-circ-BANP#1 + inhibitor NC, si-circ-BANP#1 + let-7f-5p inhibitor, let-7f-5p mimic + pcDNA NC, or let-7f-5p mimic + pcDNA TLR4 (GenePharma Co., Ltd., Shanghai, China) was achieved using Lipo 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). The medium was changed 6-h post-transfection and cells were collected after 48-h culture.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol and a TaKaRa reverse transcription kit was used to synthesize cDNA. PCR amplification was performed, and U6 and glyceraldehyde-3-phosphate dehydrogenase were included as internal references. Primer sequences are shown in Table 1. Changes in transcription were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequence.

Gene	Primer sequence (*5'-3'*)
let-7f-5p	Forward: 5'-GCCGCTGAGGTAGTAGATTGT-3' Reverse: *5'-GTCGTATCCAGTGCAGGGTCCGA* GGTATTCGCACTGGATACGACAACTAT-3'
U6	Forward: 5'-GCAGGAGGTCTTCACAGAGT-3' Reverse: 5'-TCTAGAGGAGAAGCTGGGGT-3'
circ-BANP	Forward: 5'-CAGGACGGTCAGCGTCGT-3' Reverse: 5'-GGCACAGCGTTGCTAATGAC-3'
TLR4	Forward: 5'-AGAAACTGCTCGGTCAGACG-3' Reverse: 5'-AATGGAATCGGGGTGAAGGG-3'
GAPDH	Forward: 5'-AGAAGGCTGGGGCTCATTTG-3' Reverse: 5'-AGGGGCCATCCACAGTCTTC-3'

circ-BANP: Circular RNA BTG3-associated nuclear protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TLR4: Toll-like receptor 4.

Western blotting analysis

Total protein was extracted, separated with 10% polyacrylamide gel electrophoresis, and transferred to membranes. Membranes were blocked with 5% skimmed milk and incubated overnight with primary antibodies against STAT3 (1: 500), p-STAT3 (1: 500) (Cell Signaling Technology, Beverly, MA, USA), E-cadherin (1: 5000), N-cadherin (1: 5000), Vimentin (1: 1000), TLR4 (1: 400), vascular endothelial growth factor A (VEGFA) (1: 1000), vascular endothelial growth factor receptor (VEGFR)-2 (1: 1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) (Abcam, Cambridge, UK) followed by incubation with secondary antibody for 2 h. Enhanced chemiluminescent (ECL) was used for development.

Dual luciferase reporter gene assay

Target sequences were predicted by bioinformatic website, starbase (http://starbase.sysu.edu.cn), and were amplified with let-7f-5p-bound circ-BANP 3'-UTR or TLR4 3"-UTR. Mutated sequences of circ-BANP 3'-UTR or TLR4 3'-UTR were introduced using site mutation kits. In addition, target sequences predicted by starbase were amplified with let-7 family (let-7a-5p, let-7b-5p, let-7d-5p, and let-7i-5p)-bound circ-BANP 3'-UTR and site mutation kits (NBsbio Co., Ltd., Beijing, China) used to introduce the circ-BANP 3'-UTR mutant sequence. The amplified circ-BANP 3'-UTR or TLR4 3'-UTR sequences and the corresponding mutant sequences were inserted into psi-CHECK2 plasmids to generate wild type (WT) and mutant type (MUT) circ-BANP 3'-UTR or TLR4 3'-UTR plasmids. The mimic and NC of let-7f-5p or the let-7 family (let-7a-5p, let-7b-5p, let-7d-5p and let-7i-5p) were co-transfected into cells with WT and MUT circ-BANP (or TLR4) 3'-UTR plasmid using Lipo 3000 transfection reagent. The activity of luciferase from the reporter gene was determined after 48 h.

RNA pull-down assay

Biotinylated let-7f-5p WT and MUT plasmids (50 nmol/L each) were used for the RNA pull-down assay, performed as previously described.[24]

Cell counting kit-8 (CCK-8) assay

Cells were cultured with 10 μL CCK-8 reagent added at 24, 48, and 72 h for continuous 2-h culture. The optical density at 450 nm was analyzed using a microplate reader.

EdU incorporation assay

Cells were seeded into 96-well plates and incubated with an EdU incorporation assay kit (Ribobio) according to the manufacturer's instructions. After adding 4',6-diamidino-2-phenylindole solution, fluorescence was determined using a fluorescent microscope (Leica).

Transwell assay

Cell migration and invasion were measured using a Transwell assay, according to a previously published method,[25] and a light microscope was employed for photographing and cell counting. Nine fields were examined.

Subcutaneous tumorigenesis in nude mice

Male BALB/c nu/nu mice aged 4 to 6 weeks (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were kept in sterile polycarbonate cages with free access to food and water. Huh7 and Huh7-H cells (1×10^7) that had been transfected with short hairpin (sh)-circ-BANP or sh-NC were injected subcutaneously into the left sides of the mice. The tumor volume was measured every 4 days and the mice were euthanized 28 days later and xenografts isolated and weighed.[23,26]

Statistical analysis

All data analyses were conducted using SPSS 22.0 software. Measurement data conforming to the normal distribution were expressed as mean ± standard deviation. Unpaired *t* test was performed for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test for pairwise comparisons. P value < 0.05 was indicative of a statistically significant difference.

Results

Let-7f-5p is downregulated while circ-BANP and TLR4 are upregulated in HCC

Expression of circ-BANP and TLR4 was upregulated in HCC tissues compared with non-cancerous controls and a further increase was found in tumors after insufficient RFA. By contrast, the expression of let-7f-5p had an opposite tendency [Figure 1A–D]. Expression of circ-BANP and TLR4 was upregulated in Huh7-H in comparison with Huh7 cells. Expression of let-7f-5p was downregulated in Huh7-H compare with Huh7 cells [Figure 1E and 1F].

Circ-BANP binds to let-7f-5p and let-7f-5p targets TLR4

Bioinformatic analysis predicted that circ-BANP could bind to let-7f-5p [Figure 2A]. Using a dual luciferase

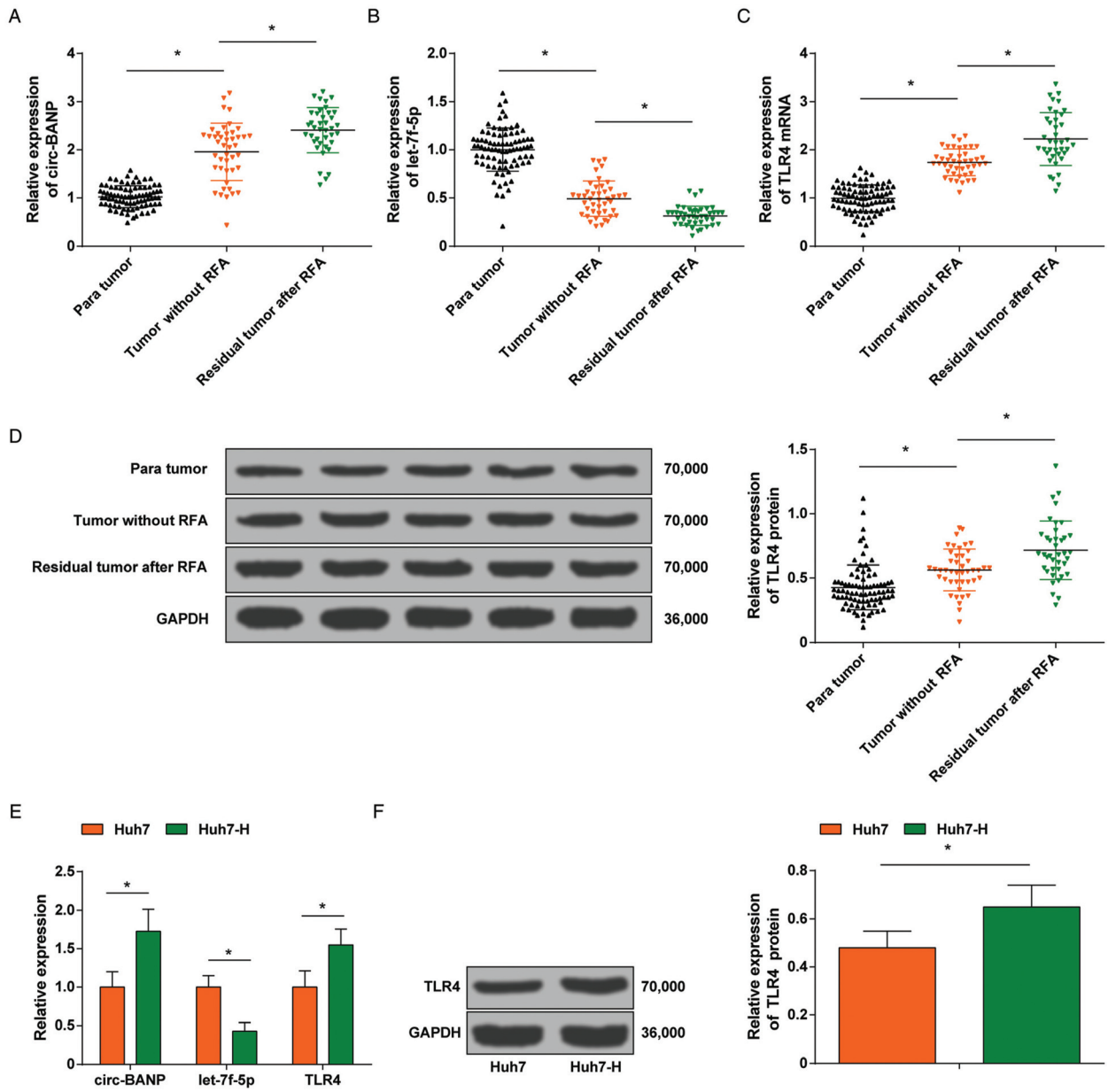

Figure 1: Let-7f-5p is downregulated while circ-BANPand TLR4 are upregulated in HCC, especially in tumors after insufficient RFA. (A–C) circ-BANP, let-7f-5p and TLR4 mRNA expression in HCC and adjacent normal tissues detected using RT-qPCR; (D) protein expression of TLR4 in HCC and adjacent normal tissues detected using Western blotting analysis; (E) circ-BANP, let-7f-5p and TLR4 mRNA expression in Huh7 and Huh7-H cells assessed by RT-qPCR; (F) protein expression of TLR4 in Huh7 and Huh7-H assessed by Western blotting analysis; $P < 0.01$; para-tumor: $n = 83$, tumor without RFA: $n = 44$; residual tumor after RFA: $n = 39$; repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, unpaired t test was performed for comparisons between two groups, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; circ-BANP: Circular RNABTG3-associated nuclear protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; TLR4: Toll-like receptor 4.

reporter gene assay, we were able to confirm that binding occurs between these two species [Figure 2B]. The results of an RNA pull-down assay indicated that the circ-BANP enrichment level in the Bio-let-7f-5p-WT group was significantly higher than in the Bio-let-7f-5p-MUT and Bio-probe NC groups. These findings suggest that circ-BANP may function as a ceRNA to sponge bind and sequester let-7f-5p [Figure 2C].

The results of the dual luciferase reporter gene assay indicated that, among the let-7 family members (let-7a-5p, let-7b-5p, let-7d-5p, let-7f-5p and let-7i-5p), let-7f-5p bound most tightly to circ-BANP [Supplementary Figure 1, http://links.lww.com/CM9/A803].

Co-transfection with TLR4-WT and let-7f-5p mimic in the dual luciferase reporter gene assay suppressed luciferase activity while co-transfection with TLR4-MUT and let-7f-5p mimic did not affect luciferase activity. These findings demonstrate a targeting relationship between let-7f-5p and TLR4 [Figure 2D and 2E].

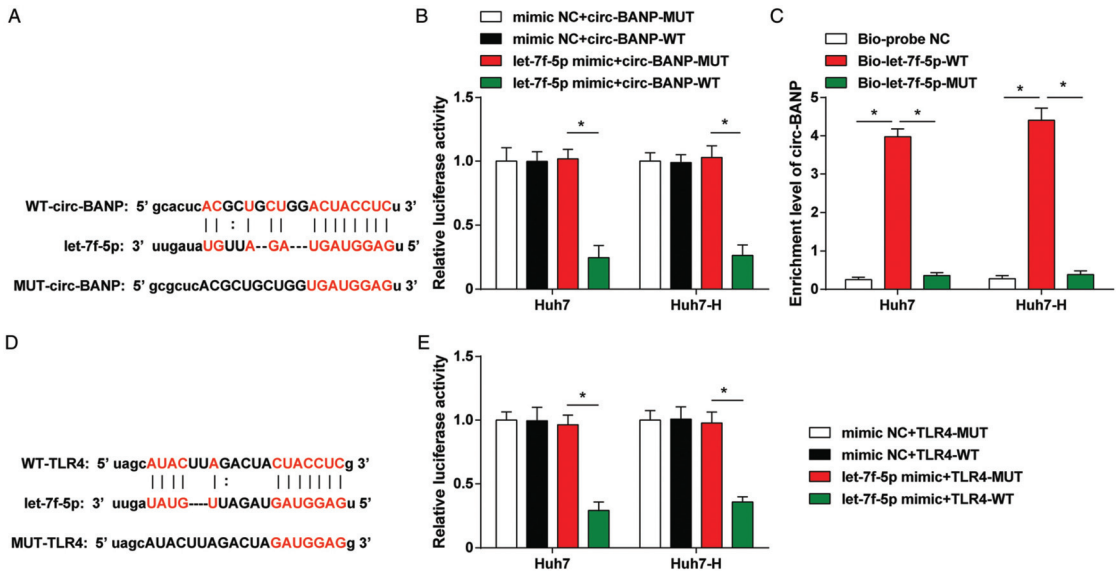

Figure 2: Circ-BANP binds to let-7f-5p and let-7f-5p targets TLR4. (A) Binding sites between circ-BANP and let-7f-5p predicted at starbase; (B) the binding relationship between circ-BANP and let-7f-5p confirmed using dual luciferase reporter gene assay; (C) the binding relationship between circ-BANP and let-7f-5p detected by RNA pull-down assay; (D) binding sites between let-7f-5p and TLR4 predicted at starbase; (E) targeting relationship between let-7f-5p and TLR4 confirmed using dual luciferase reporter gene assay; $^{*}P < 0.01$; repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; circ-BANP: Circular RNA BTG3-associated nuclear protein; MUT: Mutant type; TLR4: Toll-like receptor 4; WT: Wild type.

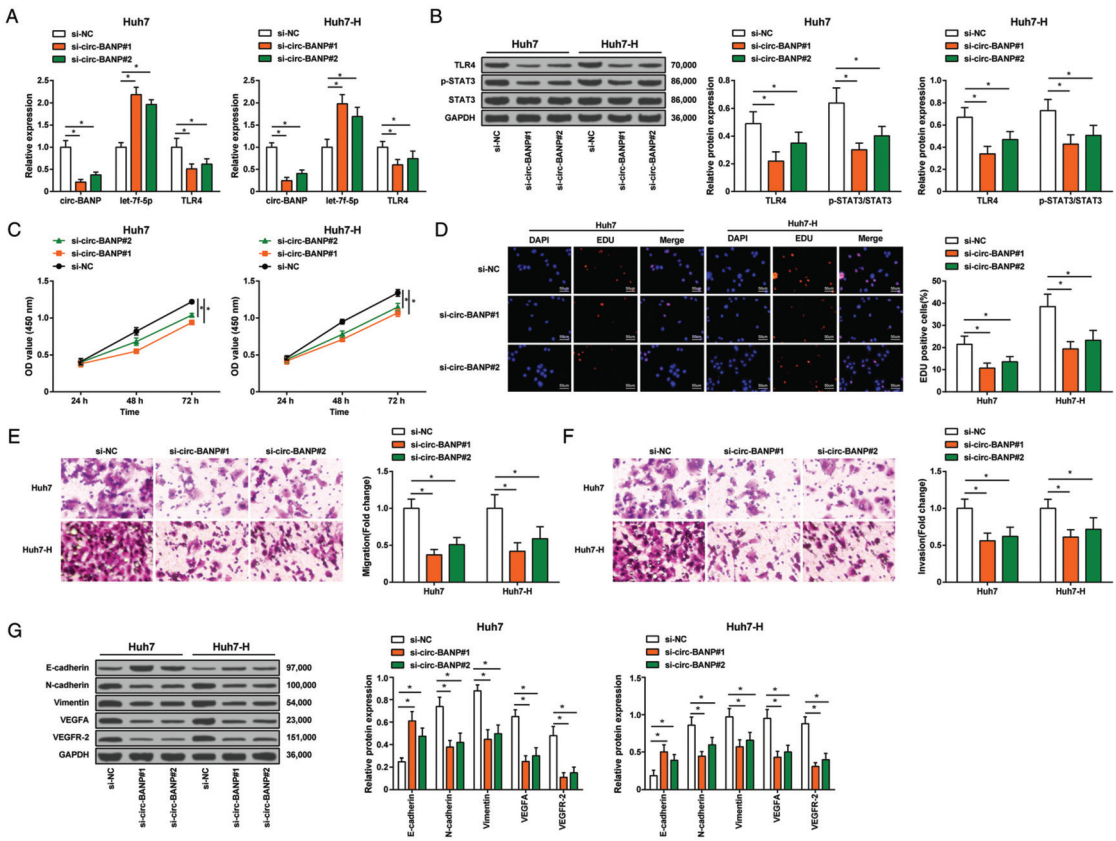

Figure 3: Circ-BANP inhibition represses proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA. (A) circ-BANP, let-7f-5p and TLR4 mRNA expression in cells after transfection assessed by RT-qPCR; (B) protein expression of TLR4, STAT3, and p-STAT3 in cells after transfection assessed by Western blotting analysis; (C) CCK-8 assay was used to evaluate cell proliferation after transfection; (D) EdU incorporation assay was employed to measure the proliferation rate of cells after transfection; (E) cell migration assessed by Transwell assay; (F) cell invasion assessed by Transwell assay; (G) protein expression of E-cadherin, N-cadherin, Vimentin, VEGFA, and VEGFR-2 in cells after transfection was determined using Western blotting analysis; $P < 0.05$, repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; CCK-8: Cell counting kit-8; circ-BANP: Circular RNA BTG3-associated nuclear protein; EMT: Epithelial-mesenchymal transformation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; STAT3: Signal transducer and activator of transcription 3; TLR4: Toll-like receptor 4.

Circ-BANP inhibition represses proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA

The circ-BANP expression in Huh7 and Huh7-H cells was knocked down using siRNA. RT-qPCR results indicated that circ-BANP expression was successfully downregulated by both si-circ-BANP#1 and si-circ-BANP#2, with si-circ-BANP#1 having the more potent interference effect. Knockdown of circ-BANP upregulated let-7f-5p, downregulated TLR4, and inactivated the TLR4/STAT3 signaling pathway [Figure 3A and 3B]. These results allow an approach to elucidating the molecular mechanism of circ-BANP activity. Results from CCK-8, EdU, and Transwell assays demonstrated that silencing of circ-BANP inhibited viability, invasion, and migration of Huh7 and Huh7-H cells [Figure 3C–F]. Expression of EMT- and angiogenesis-related proteins was evaluated using Western blotting analysis. Inhibition of circ-BANP increased expression of E-cadherin protein and decreased expression of N-cadherin, Vimentin, VEGFA, and VEGFR-2 proteins [Figure 3G].

Circ-BANP knockdown inhibits proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA via mediating let-7f-5p

To further investigate the binding relationship between circ-BANP and let-7f-5p, we performed experiments designed to rescue the circ-BANP knockdown phenotype by downregulating let-7f-5p expression. Results of RT-qPCR and Western blotting analyses demonstrated that downregulation of let-7f-5p reversed the inhibitory effect of circ-BANP knockdown on the TLR4/STAT3 signaling pathway [Figure 4A and 4B]. Downregulation of let-7f-5p reversed the impact of circ-BANP silencing on proliferation, migration, EMT formation, and angiogenesis of Huh7 and Huh7-H cells [Figure 4D-G].

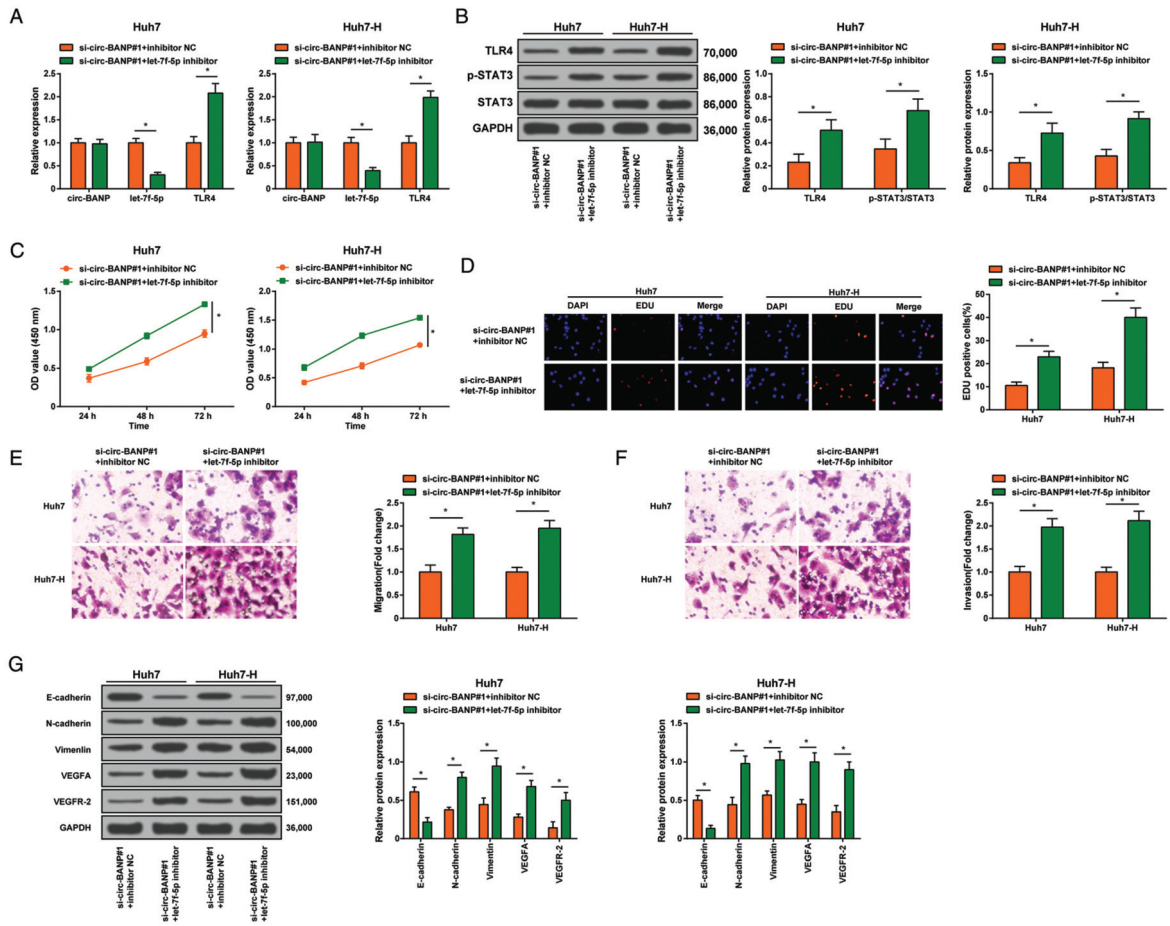

Figure 4: Circ-BANP knockdown inhibits proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA via mediating let-7f-5p. (A) circ-BANP, let-7f-5p and TLR4 mRNA expression in cells after transfection assessed by RT-qPCR; (B) protein expression of TLR4, STAT3, and p-STAT3 in cells after transfection assessed by Western blotting analysis; (C) CCK-8 assay was used to evaluate cell proliferation after transfection; (D) EdU incorporation assay was employed to measure the proliferation rate of cells after transfection; (E) cell migration assessed by Transwell assay; (F) cell invasion assessed by Transwell assay; (G) protein expression of E-cadherin, N-cadherin, Vimentin, VEGFA, and VEGFR-2 in cells after transfection was determined using Western blotting analysis; $^{*}P < 0.05$, repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; CCK-8: Cell counting kit-8; circ-BANP: Circular RNA BTG3-associated nuclear protein; EMT: Epithelial-mesenchymal transformation; DAPI: 4',6-diamidino-2-phenylindole; EDU: 5-ethynyl- 2'-deoxyuridine; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; STAT3: Signal transducer and activator of transcription 3; TLR4: Toll-like receptor 4; VEGFA: Vascular endothelial growth factor A; VEGFR-2: Vascular endothelial growth factor receptor 2.

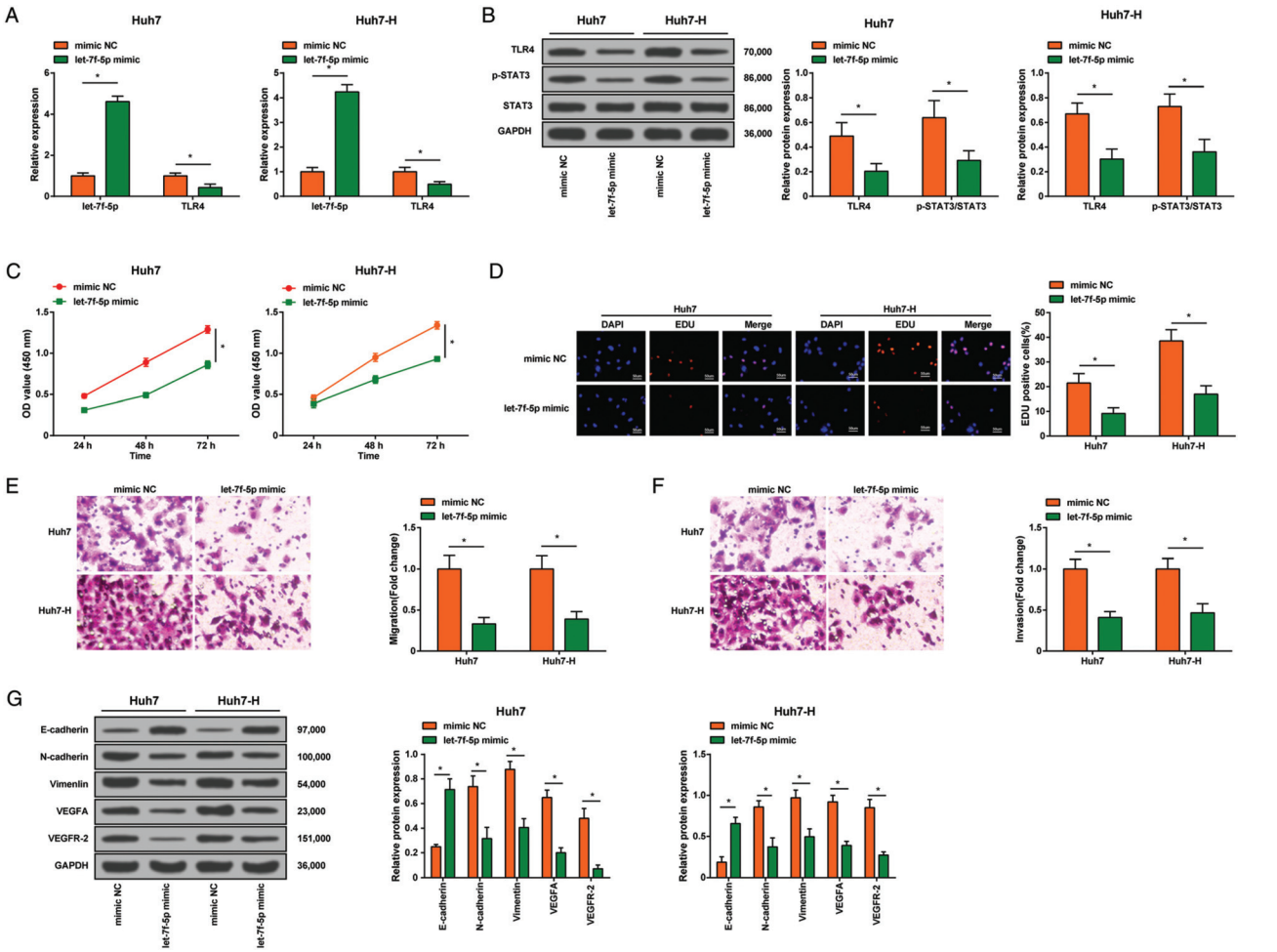

Figure 5: Elevated let-7f-5p reduces proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA. (A) circ-BANP, let-7f-5p and TLR4 mRNA expression in cells after transfection assessed by RT-qPCR; (B) protein expression of TLR4, STAT3, and p-STAT3 in cells after transfection assessed by Western blotting analysis; (C) CCK-8 assay was used to evaluate cell proliferation after transfection; (D) EdU incorporation assay was employed to measure the proliferation rate of cells after transfection; (E) cell migration assessed by Transwell assay; (F) cell invasion assessed by Transwell assay; (G) protein expression of E-cadherin, N-cadherin, Vimentin, VEGFA, and VEGFR-2 in cells after transfection was determined using Western blotting analysis; $^{*}P < 0.01$; repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; CCK-8: Cell counting kit-8; circ-BANP: Circular RNA BTG3-associated nuclear protein; EMT: Epithelial-mesenchymal transformation; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; STAT3: Signal transducer and activator of transcription 3; TLR4: Toll-like receptor 4.

Elevated let-7f-5p reduces proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA

Huh7 and Huh7-H cells were transfected with a let-7f-5p mimic. It was found that let-7f-5p mimic successfully upregulated let-7f-5p and inhibited activation of the TLR4/ STAT3 signaling pathway [Figure 5A and 5B]. Elevation of let-7f-5p also suppressed proliferation, migration, angiogenesis, and EMT formation in Huh7 and Huh7-H cells as measured by CCK-8, EdU, and Transwell assays [Figure 5C-G].

Let-7f-5p elevation targets TLR4 to suppress proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA

HCC cells were simultaneously transfected with the let-7f-5p mimic and pcDNA TLR4. When pcDNA TLR4 was present, the inhibitory effect of the let-7f-5p mimic on the activation of the TLR4/STAT3 signaling pathway was abolished [Figure 6A and 6B]. Furthermore, our experiments revealed that the role of let-7f-5p upregulation in inhibiting proliferation, migration, angiogenesis, and EMT formation of Huh7 and Huh7-H cells was abrogated by overexpression of TLR4 [Figure 6C–G].

Silencing circ-BANP inhibits in vivo growth of residual HCC cells after insufficient RFA

Experiments in nude mice were conducted to confirm the *in vivo* effects of circ-BANP on HCC. Huh7 and Huh7-H cells were transfected with sh-circ-BANP or sh-NC [Figure 7A] followed by subcutaneous injection into nude mice. Both the volume and the weight of xenografts were reduced after circ-BANP inhibition [Figure 7B–D].

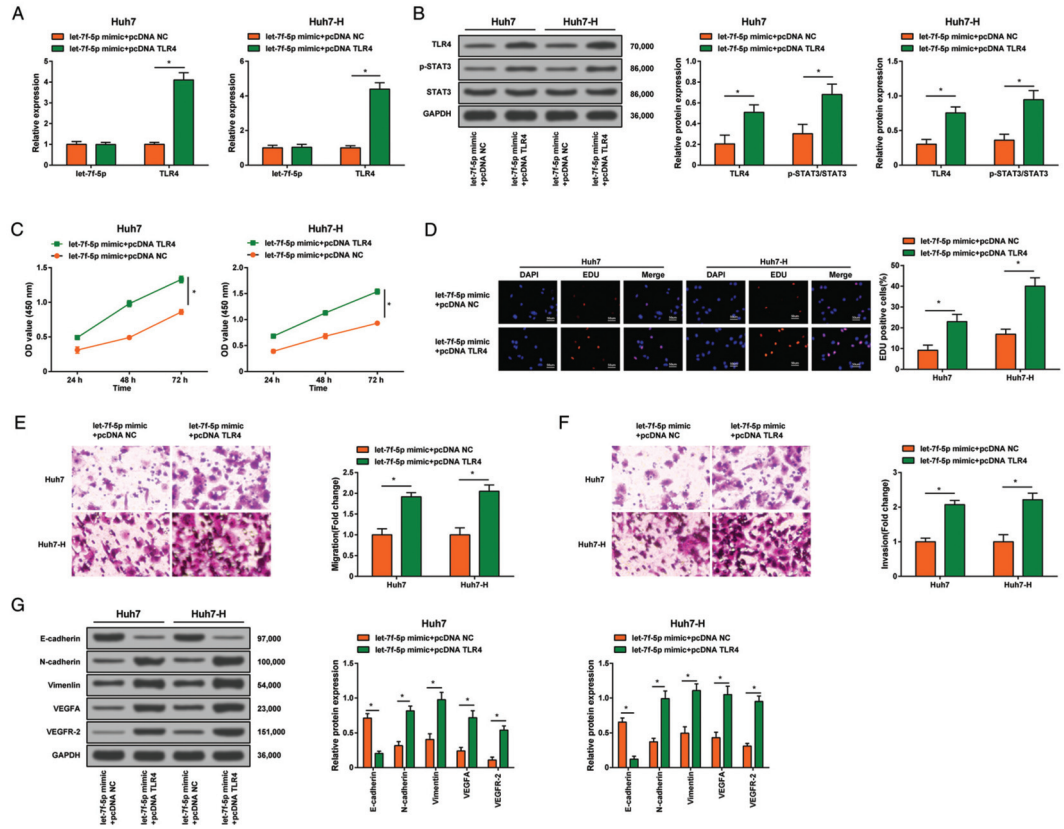

Figure 6: Let-7f-5p elevation targets TLR4 to restrain proliferation, migration, and EMT formation of residual HCC cells after insufficient RFA. (A) circ-BANP, let-7f-5p and TLR4 mRNA expression in cells after transfection assessed by RT-qPCR; (B) protein expression of TLR4, STAT3, and p-STAT3 in cells after transfection assessed by Western blotting analysis; (C) CCK-8 assay was used to evaluate cell proliferation after transfection; (D) EdU incorporation assay was employed to measure the proliferation rate of cells after transfection; (E) cell migration assessed by Transwell assay; (F) cell invasion assessed by Transwell assay; (G) protein expression of E-cadherin, N-cadherin, Vimentin, VEGFA, and VEGFR-2 in cells after transfection was determined using Western blotting analysis; $^{*}P < 0.01$; repetition = 3; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, unpaired *t* test was performed for comparisons between two groups, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; CCK-8: Cell counting kit-8; circ-BANP: Circular RNA BTG3-associated nuclear protein; EMT: Epithelial-mesenchymal transformation; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; STAT3: Signal transducer and activator of transcription 3; STAT3: Signal transducer and activator of transcription 3; TLR4: Toll-like receptor 4.

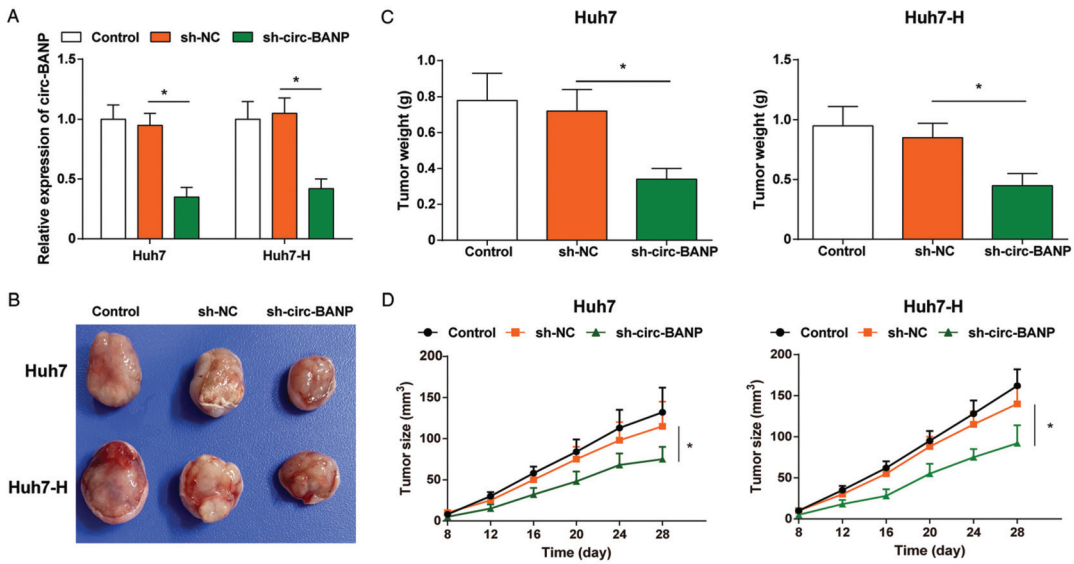

Figure 7: Silencing circ-BANP inhibits *in vivo* growth of residual HCC cells after insufficient RFA. (A) circ-BANP expression in Huh7 and Huh7-H cells after transfection of sh-circ-BANP or shNC was determined using RT-qPCR; (B) Huh7 and Huh7-H cells that had been transfected with sh-circ-BANP or sh-NC were subcutaneously injected into nude mice and 4 weeks later, the mice were euthanized. Images of tumor volume were exhibited; (C, D) tumor size was measured with a caliper rule every 4 days and tumor weight was measured at the end of the experiment; $^{*}P < 0.01$; n = 5; the measurement data conforming to the normal distribution were expressed as mean ± standard deviation, one-way ANOVA was used for comparisons among multiple groups and Tukey's multiple comparisons test was used for pairwise comparisons after one-way ANOVA. ANOVA: Analysis of variance; circ-BANP: Circular RNA BTG3-associated nuclear protein; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction.

Discussion

HCC accounts for a large portion of cancer-related deaths, making it a major public health challenge worldwide.[27] The incidence and severity of HCC are both increasing globally, while HCC prognoses remain disappointing because of the high rates of recurrence and metastasis. Thus, there is an urgent requirement for improved treatments for HCC.[28] The current study was designed to investigate the impact of circ-BANP adherence to let-7f-5p on the severity of HCC after insufficient RFA. We found that circ-BANP knockdown resulted in let-7f-5p upregulation, which had an inhibitory effect on proliferation, migration, angiogenesis, and EMT formation in residual HCC after insufficient RFA. Effects were achieved via inhibition of the TLR4/STAT3 signaling pathway.

Baseline values for expression of circ-BANP, let-7f-5p, and TLR4 were established and our findings showed that circ- BANP and TLR4 were upregulated in HCC tissues and further increased in tumors after insufficient RFA. By contrast, let-7f-5p expression evinced the opposite tendency. In previous work, Han *et al*[11] have verified overexpression of circ-BANP in lung cancer tissues and, more recently, markedly increased circ-BANP expression has been revealed in CRC tissues[10] Furthermore, decreased let-7f-5p expression has been reported in osteosarcoma cell lines,[13] as well as in carcinoid tumors of the lung.[29] Aberrant expression of TLR4 has also been reported in the cancerous state and it is known to be overexpressed in liver tumor tissues from patients with primary HCC.[30] In addition, circRNAs are known to function as ceRNAs for miRNAs and to have an impact on the stability of targeted genes during cancer development.[31]During the current study, we used the approaches of bioinformatic prediction, luciferase reporter assay and RNA pull-down assay to demonstrate that circ-BANP served to bind let-7f-5p which would otherwise target TLR4. Consistent with the results of the current study, previous work has indicated that circ-BANP sponges miR-503 in lung cancer.[11] The targeting relationship between TLR4 and let-7i under ischemic conditions has been previously confirmed.[19]

Acknowledging the abnormal expression of circ-BANP, let-7f-5p, and TLR4 in HCC, especially residual HCC tissues and cells after insufficient RFA treatment, the current study altered the expression of these species in Huh7 and Huh7-H cells to approach their roles. Our findings confirm that circ-BANP knockdown or let-7f-5p elevation inhibited proliferation, migration, angiogenesis, and EMT formation of residual HCC cells after insufficient RFA. Consistent with our findings, Zhu *et al*[10] have demonstrated potent suppression of CRC cell proliferation on circ-BANP downregulation, and inhibition of circ-BANP has also been shown to repress malignant episodes of lung cancer cells.[11] Our findings are further validated by the results of a recent study, which indicated that increased let-7f-5p expression results in the suppression of viability and migration of bladder cancer cells.[14] In addition, the current study used the approach of the rescue experiment to elucidate mechanisms of the circ-BANP/let-7f-5p/TLR4 axis in HCC. Our findings demonstrated that let-7f-5p downregulation reversed the role of circ-BANP silencing in HCC cells and the effect of let-7f-5p upregulation was abrogated by TLR4 overexpression. Recent studies have indicated the involvement of the TLR4/STAT3 signaling pathway in the regulation of migration and EMT of HCC cells[16] and Zhao et al[32] have reported that TLR4 activation promotes the angiogenesis of placental microvascular endothelial cells during severe preeclampsia pathogenesis. Furthermore, STAT3 showed a high level of expression in HCC and has been shown to be a significant marker for differentiating HCC from cirrhosis.[33] The results of these previous studies have been helpful in directing the current exploration of residual HCC after insufficient RFA treatment. We recognize that, due to limitations in funding and time, there remain some deficiencies in our work, such as the absence of an *in vivo* assay. We hope to address these deficiencies during our future studies.

In conclusion, our findings reveal that circ-BANP knockdown upregulates let-7f-5p, resulting in inhibition of proliferation, migration, and EMT formation in residual HCC after insufficient RFA. Effects are achieved via inhibition of the TLR4/STAT3 signaling pathway. The findings of the current study contribute to exploring therapeutic strategies of residual HCC after insufficient RFA. Further investigations are required to elucidate detailed mechanisms.

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Conflicts of interest

None.

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