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RETRACTION

Retraction: Hsa_circ_0003645 shows an oncogenic role by sponging microRNA-1299 in hepatocellular carcinoma cells

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RESEARCH ARTICLE

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Hsa_circ_0003645 shows an oncogenic role by sponging microRNA-1299 in hepatocellular carcinoma cells

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Abstract

Background: Studies found circular RNAs (circRNAs) and microRNAs (miRNAs) are abnormally expressed in hepatocellular carcinoma. Cancer process might be modulated by circRNA-miRNA node. Here, we focused on hsa_circ_0003645 and miR-1299.

Methods: RT-qPCR was used to examine hsa_circ_0003645 and miR-1299 in hepatocellular carcinoma tissues and cells. Hsa_circ_0003645 and miR-1299 were regulated by transfection. The phenotypes of hepatocellular carcinoma cells were evaluated through cell viability, colony, apoptosis, migration and invasion, and proteins associated with apoptosis and motility. Targeting relationship between hsa_circ_0003645 and miR-1299 was confirmed by dual-luciferase reporter assay. Changes in signaling transduction were monitored by Western blot.

Results: Hsa_circ_0003645 was notably enhanced in patients with hepatocellular carcinoma tissues and (Huh7 and SK-HEP-1) cells. Silencing hsa_circ_0003645 blocked the growth of hepatocellular carcinoma cells by inhibiting colony formation, inducing apoptosis, impeding migration and invasion, promoting the expression of apoptosis-associated proteins, and decreasing the expression of motility-relevant proteins. miR-1299 was targeted by hsa_circ_0003645 and upregulated by silencing hsa_circ_0003645. miR-1299 deficiency reversed the role of hsa_circ_0003645 silence in growth of hepatocellular carcinoma cells and signaling transduction of PI3K/mTOR pathway.

Conclusion: Silencing hsa_circ_0003645 buffered the growth of hepatocellular carcinoma cells. miR-1299 was responsible for the role of hsa_circ_0003645 silence.

KEYWORDS

apoptosis, hepatocellular carcinoma, Hsa_circ_0003645, microRNA-1299, migration and invasion

1 | INTRODUCTION

There were 841 000 incident cases and 782 000 deaths of liver cancer worldwide in 2018, mainly hepatocellular carcinoma.[1] The primary risk factors are hepatitis B and C infection as well as alcohol consumption, and overweight and metabolic syndromes are considered as the relevant risk factors.[2-4] Resection, transplantation, and ablation are considered for treatment according to the profiles of tumor burden and liver function.[5,6] However, it is no longer amenable to curative therapies when it is diagnosed at the advanced stage. Considering that the liver is lightly infected by non-viral or viral genes as well as small-molecule delivery systems, gene therapy exhibits the potency by delivering adeno-associated virus or small-molecular delivery systems.[7]

Increasing reports showed non-protein coding RNAs are abnormally expressed in hepatoma, including long non-coding RNAs,[8] circular RNA (circRNAs),[9] and microRNAs (miRNAs).[10] Further evidence confirmed that dysregulated non-protein coding RNAs participate in the pathological process of human hepatoma disease.[8-10] For example, circPOK back-spliced from *Zbtb7a* gene functions as a non-coding proto-oncogenic RNA by inducing pro-proliferative and pro-angiogenic factors in the scenario of mesenchymal tumor progression, while its linear counterpart is a tumor suppressor by coding transcription factor Pokemon.[11] More than function in tumorigenesis, circRNAs are also accepted as diagnostic biomarkers.[12] For instance, circRNA-0068669 has been identified as a biomarker for metastasis happening in hepatocellular carcinoma.[13] Besides, after analyzing the clinicopathological characteristics of hepatocellular carcinoma, a study considered that the abnormal expression of circRNAs shows the clinical significance.[14]

CircRNAs from back-spliced exons are characterized by loop structures which are covalently closed and are mainly enriched in the cytoplasm.[15] miRNAs are consisted of 20-22 nucleotides (nt) and function as small non-coding RNAs in almost all biological pathways, including proliferation, differentiation, and apoptosis in human cancers.[16,17] Intriguingly, the modulatory association between circRNAs and miRNAs has been proposed as a pathological mechanism of hepatoma disease. Several reports have investigated and confirmed the process. For example, circHIPK3 derived from *HIPK3* gene is able to sponge 9 miRNAs by interacting with 18 potential binding sites and continually induces human cell growth.[18] Moreover, the modulatory node consisting of circRNA-miRNA-mRNA has been extensively proposed.[19] The modulatory role of circRNAs in signaling transduction has been identified as another functional mechanism.[20]

Here, we explored expression profile of hsa_circ_0003645 in hepatocellular carcinoma cells. Further, we attempted to reveal the role of hsa_circ_0003645 by silencing it. Inhibition of hsa_circ_0003645 modulated phenotypes by affecting the growth, motility, and apoptosis. Further studies demonstrated that these effects were related to miR-1299, and PI3K/mTOR pathway might participate in this process.

2 | MATERIALS AND METHODS

2.1 | Collection of clinical specimens

We enrolled 30 patients (20 males and 10 females) aged 38.9-72.3 years, with histologically confirmed hepatocellular carcinoma, in Hwa Mei Hospital, University of Chinese Academy of Science. All patients had not received any treatment before the identification. All specimens were frozen at −80°C immediately after surgical resection. The paired adjacent liver tissues were obtained during operation. Each patient provided the written informed consent. This study was approved by the ethics committee of Hwa Mei Hospital, University of Chinese Academy of Science. The tissues were collected for analyzing hsa_circ_0003645 and miR-1299 by qRT-PCR method.

2.2 | Cell lines and transfection

Transformed human liver epithelial-3 (THLE-3) cells (CRL-11233 from ATCC, Rockville, MD, USA), Huh7 cells (RCB1366 from RIKEN BRC, Koyadai), and SK-HEP-1 cells (HTB-52 from ATCC) were cultured according to standard protocols provided by the manufacturers. Transfection was performed using Lipofectamine RNAiMAX Reagent (Invitrogen). siRNA for hsa_circ_0003645 was designed and generated by GENESEED, and siRNA-negative control (si-NC) was provided meanwhile. Anti-miR™ miRNA, an inhibitor for miR-1299 (anti-miR-199), and its corresponding negative control (anti-NC) were provided by Thermo Fisher Scientific. RT-qPCR was carried out for detecting the interference efficiency as described below.

2.3 | Viability assay

Cell viability was measured using cell counting kit (Abmole Bioscience). Shortly, the cells were grown in a 96-well plate in a concentration of 3×10^3 cells each well. At the indicated times after transfection, 10 μL CCK reagent was incubated with the cells in each well for 4 hours at 37°C. The absorbance at 450 nm was detected using a microplate reader (BioTek).

2.4 | Colony formation

Appropriately 1×10^4 cells were seeded onto 10-cm dishes. The cells were subjected to PBS wash and 0.5% crystal violet staining 2 weeks after culture. The staining continued for 20 minutes, and the colony was counted using inverted microscope (Olympus).

2.5 | Apoptosis assay

Apoptotic cells were confirmed by flow cytometry after staining using Annexin V and PI (BD Biosciences). Appropriately 1×10^6 cells

were collected, washed using PBS, and suspended in binding buffer. Then, the cells were stained in Annexin V-FITC and PI for 15 minutes at room temperature in darkness. Flow cytometric analysis was implemented using FACS CantoII flow cytometer (Becton Dickinson). The cells stained by Annexin V were counted as apoptotic cells.

2.6 | Migration and invasion

To evaluate motile phenotypes, Boyden chamber inserted by the polycarbonate membrane with 8-μm pores (Corning Costar Co.) was utilized here. In terms of invasion assay, the inserts were conditioned with Matrigel (BD Biosciences). In short, lower chamber was filled with complete media, and 2×10^4 cells in 500 μL serum-free medium were placed into upper chamber. The chamber was maintained at 37°C. The cells migrating or invading to the bottom surface of the membrane were fixed using methanol and stained using crystal violet. Continually, microscopic inspection was carried out using inverted microscope.

2.7 | Dual-luciferase reporter assay

The hsa_circ_0003645 sequence bearing target sites predicted between miR-1299 and hsa_circ_0003645 was constructed into psiCheck vector (Promega) (circ-WT). Mutant hsa_circ_0003645 was generated using In-Fusion cloning kit (Clontech) (circ-MUT). HEK 293T/17 cells (CRL-11268 from ATCC) were maintained in DEME (Gibco) supplemented with 10% FBS. Co-transfection with miR-1299 mimic or miR-NC and circ-WT or circ-MUT was implemented in the presence of Lipofectamine RNAiMAX Reagent. Cellular lysates were collected for luciferase assaying 48 hours after transfection. Assay for luciferase was done using Dual-Luciferase Reporter Assay System (Promega) on Turner Biosystems 20/20 luminometer (Promega). The fluorescence was corrected using pRL-TK (Promega).

2.8 | RNA isolation and RT-qPCR for hsa_circ_0003645 and miR-1299

Whole RNA from hepatocellular carcinoma tissues, the paired adjacent liver tissues, and cells was isolated and purified using QIAzol Lysis Reagent provided in the miRNeasy Mini Kit (QIAGEN), and RiboLock RNase Inhibitor (Thermo Fisher Scientific) was utilized in this procedure. For hsa_circ_0003645 detection, RNase R (Epicenter) was used to eliminate liner RNA. Then, RNA was reversely transcribed into cDNA using ReverTra Ace qPCR Kit (TOYOBO) and random primers. Real-time PCR for miR-1299 and hsa_circ_0003645 was carried out using SYBR Green Reaction Mix (Applied Biosystems) on ABI 7300 SYSTEM (Applied Biosystems). Divergent primers for hsa_circ_0003645 were designed using method exploited by Zhong et al[21] Specific primers were used for miR-1299 reported by Wang et al[22] Hsa_circ_0003645 or miR-1299 expression was digitalized using $2^{-\triangle\triangle Ct}$ method and normalized to GAPDH or U6, respectively.

2.9 | Western blotting

Cellular lysates were prepared using RIPA lysis buffer (Beyotime). Bicinchoninic Acid Protein Assay Kit (Pierce) was used for investigating protein content. Proteins in lysis were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were delivered onto nitrocellulose membranes (Amersham Biosciences), followed by sealing in 5% non-fat milk. Primary antibodies, containing antibodies against Bax (AHP2716 from Bio-Rad; 1:1000), cleaved caspase 3 (9661 from CST; 1:1000), cytochrome c (orb127957 from Biorbyt; 1:1000), β-actin (4967 from CST; 1:1000), N-cadherin (orb225524 from Biobyt; 1:500), vimentin (ab137321 from Abcam; 1:2000), β-catenin (AP32836PU-T from OriGene; 2 μg/mL), PI3K (3811 from CST; 1:1000), phospho-PI3K (Ser249) (13857 from CST, 1:1000), mTOR (2972 from CST; 1:1000), and phospho-mTOR (2971 from CST; 1:1000), were used to probe the indicated proteins. Then, primary antibodies were recognized by secondary antibody linked by horseradish peroxidase (ab6721 from Abcam; 1:5000). Enhanced Chemiluminescence Substrate Kit (Amersham Bioscience) was incubation with the membranes, and then, the protein bands were imaged on Western blotting System (Bio-Rad).

2.10 | Statistical analysis

The experimental data were collected from at least three independent examinations. Statistical analysis was implemented using GraphPad Prism 6 software (GraphPad). Statistical significance was evaluated through Student's *t* test, and the calculated *P*-values <.05 were accepted.

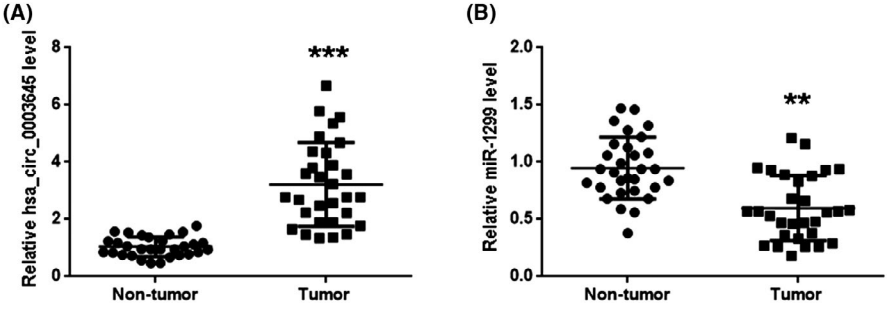

FIGURE 1 Expression profile of hsa_circ_0003645 and miR-1299 in hepatocellular carcinoma tissues. A total of 30 patients aged 38.9-72.3 years were enrolled, including 20 males and 10 females. RT-qPCR was applied to measure the expression of hsa_circ_0003645 and miR-1299. One-way ANOVA followed by Tukey's test, $^{**}P < .01$, $^{***}P < .001$

3 | RESULTS

3.1 | Hsa_circ_0003645 was accumulated, and miR-1299 was downregulated in hepatocellular carcinoma tissues

To investigate the expression profile of hsa_circ_0003645 and miR-1299 in hepatocellular carcinoma, we enrolled 30 patients with histologically confirmed hepatocellular carcinoma. After analysis by RT-qPCR method, we observed the accumulation of hsa_circ_0003645 in hepatocellular carcinoma tissues (***P < .001) (Figure 1A). However, miR-1299 was significantly downregulated in hepatocellular carcinoma (**P < .01) (Figure 1B).

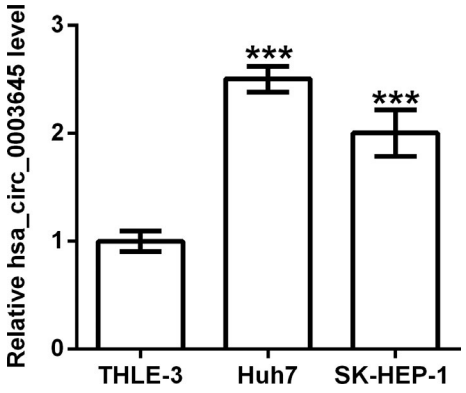

FIGURE 2 Expression profile of hsa_circ_0003645 in hepatocellular carcinoma cells. Following cell lysis, RT-qPCR was carried out. Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, ***P < .001

3.2 | Hsa_circ_0003645 was upregulated in hepatocellular carcinoma cells

Hsa_circ_0003645 is located on Chr16:19656207-19663412, with 356 nt spliced sequence length. Hsa_circ_0003645 consists of exon23, exon24, exon25, and exon26 from the host gene encoding VPS35L. To investigate the role of hsa_circ_0003645 in hepatocellular carcinoma cells, RT-qPCR analysis proceeded in Huh7 and SK-HEP-1 cells with THLE-3 cells as a control. As shown in Figure 2, compared with THLE-3 cells, human liver adenocarcinoma Huh7 (***P < .001) and SK-HEP-1 (***P < .001) cells suggested an accumulation of hsa_circ_0003645.

3.3 | Silencing hsa_circ_0003645 prohibited the phenotypes of hepatocellular carcinoma cells

We further confirmed the modulatory role of hsa_circ_0003645 in cellular viability, colony formation, apoptosis, and migration and invasion. First, we generated siRNA for hsa_circ_0003645 and transfected the siRNA into THLE-3, Huh7, and SK-HEP-1 cells. Second, RT-qPCR analysis of hsa_circ_0003645 was performed at 72 hours after transfection. Hsa_circ_0003645 was decreased in siRNA-treated THLE-3 (***P < .001), Huh7 (***P < .001), and SK-HEP-1 (***P < .001) cells (Figure 3A). Third, the viability was evaluated at 0, 12, 24, 48, and 72 hours after transfection. We noticed the viability of THLE-3 cells was not impinged by hsa_circ_0003645 silence (P > .05) (Figure 3B). By contrast, the viability of Huh7 (Figure 3C) and SK-HEP-1 (Figure 3D) was repressed at 48 hours (both **P < .01) and 72 hours (both ***P < .001) after transfection.

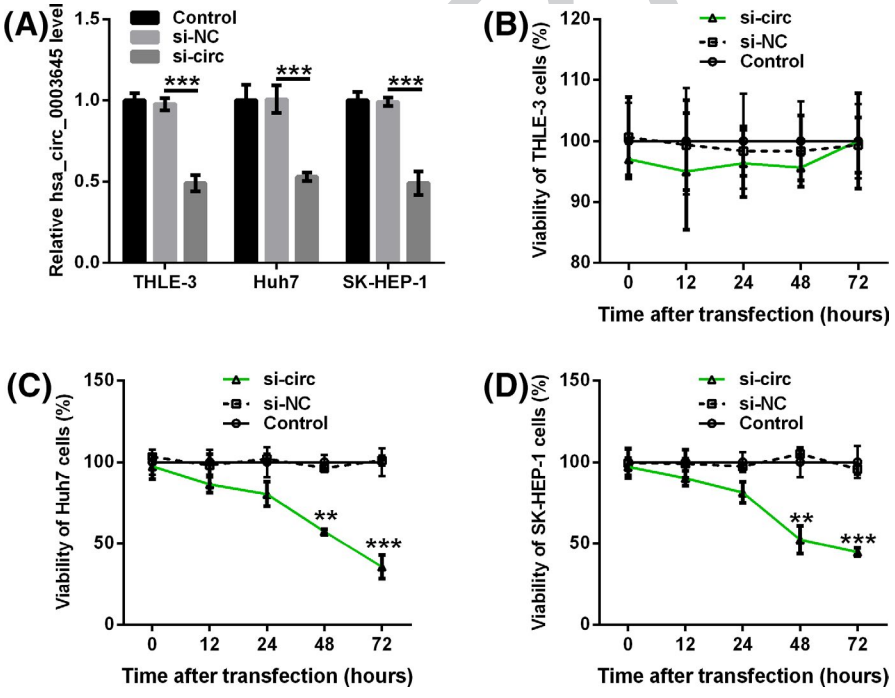

FIGURE 3 Silencing hsa_circ_0003645 decreased cellular viability of hepatocellular carcinoma cells. siRNA targeting hsa_circ_0003645 was transfected into THLE-3, Huh7, and SK-HEP-1 cells. (A) The transfected cells were lysed, followed by RT-qPCR analysis 72 h after transfection. The viability of (B) THLE-3, (C) Huh7, and (D) SK-HEP-1 cells was investigated at 0, 12, 24, 48, and 72 h after transfection. Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, **P < .01, ***P < .001

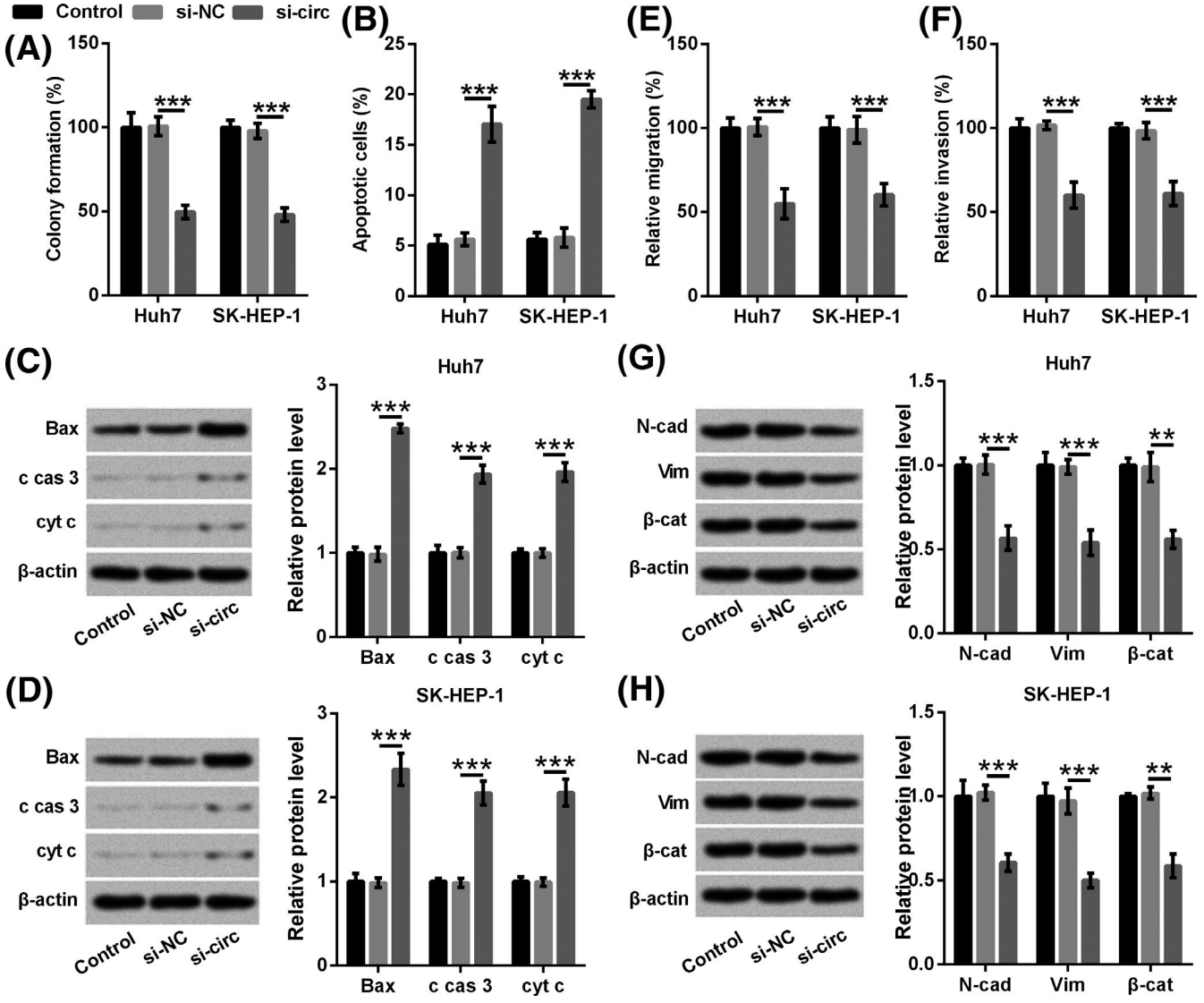

FIGURE 4 Silencing hsa_circ_0003645 controlled cancer phenotypes of hepatocellular carcinoma cells. At 72 h after transfection with siRNA targeting hsa_circ_0003645, (A) the ability of the transfected cells to form colony was examined by observation using microscope, (B) the number of apoptotic cells by flow cytometer, (C and D) Bax (21 kDa), cleaved caspase 3 (c cas 3) (17 kDa), and cytochrome c (cyt c) (15 kDa) by Western blot, (E and F) migration and invasion by microscope, and (G-H) N-cadherin (N-cad) (140 kDa), vimentin (Vim) (54 kDa), and β-catenin (β-cat) (92 kDa) by Western blot. Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, $^{**}P < .01$, $^{***}P < .001$

Subsequently, we analyzed the activity of Huh7 and SK-HEP-1 cells at 72 hours after transfection in colony genesis, apoptosis, and migration and invasion. We observed that the formation of colony by Huh7 ($^{***}P < .001$) or SK-HEP-1 ($^{***}P < .001$) cells was inhibited (Figure 4A). Apoptosis of Huh7 ($^{***}P < .001$) and SK-HEP-1 ($^{***}P < .001$) cells was strikingly intensified after transfection with si-circ (Figure 4B). Interestingly, protein expression of apoptotic markers Bax (both $^{***}P < .001$), cleaved caspase 3 (both $^{***}P < .001$) and cytochrome c (both $^{***}P < .001$) was potentiated in siRNA-treated Huh7 (Figure 4C) and SK-HEP-1 (Figure 4D) cells. Additionally, silencing hsa_circ_0003645 suppressed the capacity of Huh7 (both $^{***}P < .001$) and SK-HEP-1 (both $^{***}P < .001$) cells to migrate (Figure 4E) and invade (Figure 4F). We further ascertained the results by Western blot method, and we observed that siRNA for hsa_circ_0003645 reduced the abundance of N-cadherin (both $^{***}P < .001$), vimentin (both $^{***}P < .001$), and β-catenin (both $^{**}P < .01$) in Huh7 (Figure 4G) and SK-HEP-1 (Figure 4H) cells. Together, the results supported the notion that silencing hsa_circ_0003645 relived the cancer phenotypes of hepatocellular carcinoma cells.

3.4 | A complementary relationship between hsa_circ_0003645 and miR-1299

As predicted using circRNA Interactome Database, hsa_circ_0003645 shared the complementary sequences with miR-1179, miR-1299, miR-136, miR-139-3p, miR-335, miR-377, miR-521-5p, miR-516b, miR-518a-5p, miR-527, miR-578, miR-605, miR-619, miR-626, miR-657,

and miR-885-5p. Considering miR-1299 exhibits an inhibitory role in the proliferation of hepatocellular carcinoma cells, we associated the tumor-suppressive role of hsa_circ_0003645 silence with miR-1299. Then, we identified the modulatory role of hsa_circ_0003645 in the expression of miR-1299 by RT-qPCR analysis and confirmed whether hsa_circ_0003645 associates with miR-1299 using dual-luciferase reporter assay. The cells treated by siRNA for hsa_circ_0003645 showed an upregulation of miR-1299 (both ***P < .001) (Figure 5A). As expected, miR-1299 mimic targeting hsa_circ_0003645 decreased the luciferase activity (***P < .001) (Figure 5B).

Given that silencing hsa_circ_0003645 was capable of inducing miR-1299 expression, we postulated that hsa_circ_0003645 might impact the phenotypes of hepatocellular carcinoma cells. To confirm the possibility, we transfected anti-miR-1299 into the cells poorly expressing hsa_circ_0003645. The downregulation of miR-1299 (both ***P < .001) was noticed (Figure 6A). Anti-miR-1299 altered the ability of hsa_circ_0003645 silence to repress the viability (both **P < .01) (Figure 6B) and colony formation (*P < .05 or **P < .01) (Figure 6C). Additionally, the number of apoptotic cells was declined (*P < .05) (Figure 6D), and the accumulation of Bax (both ***P < .001), cleaved caspase 3 (*P < .05, ***P < .001), and cytochrome c (**P < .01, ***P < .001) was decreased (Figure 6E,F) in the cells lowly expressing hsa_circ_0003645 and miR-1299. As for the alteration in motile capacity, we found miR-1299 silence elicited the restoration of migration (**P < .01) and invasion (**P < .01, *P < .05) in part (Figure 6G,H). What's more, silencing miR-1299 elevated the expression of N-cadherin (*P < .05, **P < .01), vimentin (*P < .05, **P < .01), and β-catenin (both **P < .01) in Huh7 (Figure 6I) and SK-HEP-1 (Figure 6J) cells. Collectively, hsa_circ_0003645 silence-mediated alteration of miR-1299 might be a functional mechanism repressing the phenotypes of hepatocellular carcinoma cells.

3.5 | Bluntness of PI3K/mTOR was triggered by hsa_circ_0003645 silence-caused restoration of miR-1299

Then, we investigated whether there is a modulatory effect of hsa_circ_0003645 on signaling transduction of PI3K/mTOR cascade. Results from Western blot assay suggested hsa_circ_0003645 led to the decline in phosphorylation of PI3K (both ***P < .001) and mTOR (both ***P < .001) in Huh7 (Figure 7A) and SK-HEP-1 cells (Figure 7B). However, the phosphorylation of PI3K (both *P < .05) and mTOR (both *P < .05) was restored in the cells (Figure 7A,B) transfected with si-circ and anti-miR-1299. Together, the results revealed hsa_circ_0003645-mediated downregulation of miR-1299 was involved in the modulation of signaling transduction in PI3K/mTOR cascade.

4 | DISCUSSION

Recent studies have validated that circRNAs, as wells as their corresponding linear RNAs, are dysregulated in tumor tissues relative to

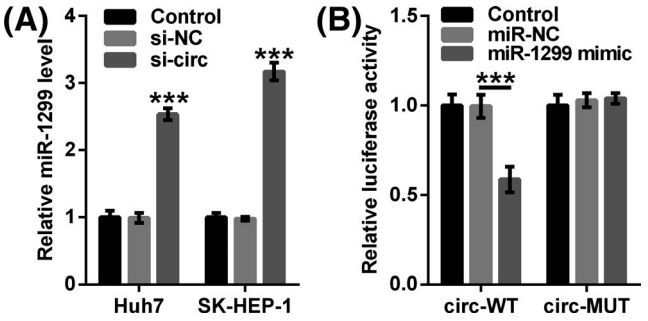

FIGURE 5 Hsa_circ_0003645 was an upstream regulator of miR-1299. (A) At 72 h after transfection with siRNA targeting hsa_circ_0003645, the cellular lysate was collected to measure miR-1299 by RT-qPCR. (B) The validation of hsa_circ_0003645 targeted by miR-1299 was done in HEK 293T/17 cells using luciferase reporter assay. psiCheck carrying gene encoding Firefly luciferase served as an expressive vector, and pRL-TK carrying gene encoding Renilla functioned as a control vector. Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, ***P < .001

the normal tissues.[21] CircRNAs regulate the pathological processes of malignancies.[23,24] There is mounting evidence demonstrating that circRNAs are involved in cancer pathogenesis through sponging miRNAs,[18,25] and an analysis implied that circRNA-miRNA code shows a potency for cancer diagnosis and treatment.[26] Here, we verified the existence of hsa_circ_0003645 and its relevance to miR-1299 in hepatocellular carcinoma cells.

Results from circRNA microarray have proved circRNAs are abnormally expressed in hepatocellular carcinoma.[27,28] According to the transcriptome analysis of circRNAs, hsa_circ_0003645 is located on chr16:19656207-19663412 and is spliced from C16orf62 mRNA.[29] CircRNA microarray analyses found hsa_circ_0003645 was differently expressed in hepatocellular carcinoma.[30,31] To delineate the expression profile of hsa_circ_0003645 in hepatocellular carcinoma cells, we performed RT-qPCR for THLE-2, Huh7, and SK-HEP-1 cells. We identified hsa_circ_0003645 was enriched in hepatocellular carcinoma Huh7 and SK-HEP-1 cells. Its upregulation has been reported in patients with non-small cell lung cancer.[32] However, experimental validation is required to illuminate whether hsa_circ_0003645 is universally upregulated in human cancer.

In terms of the biological role of hsa_circ_0003645, An et al reported that hsa_circ_0003645 shows an oncogenic role.[32] Consequently, we hypothesized that inhibiting the expression of hsa_circ_0003645 might prohibit the phenotypes of hepatocellular carcinoma cells. As expected, silencing hsa_circ_0003645 suppressed the viability, clonogenic activity, and motility while induced apoptosis. We further analyzed its biological function from a molecular perspective. Results showed hsa_circ_0003645 altered the expression of hallmarks of apoptosis as well as migration and invasion. The possible mechanisms remain unknown. Studies showed a subset of circRNAs regulates protein expression through sponging miRNA[33] or affecting transcription rate of host gene,[34] suggesting

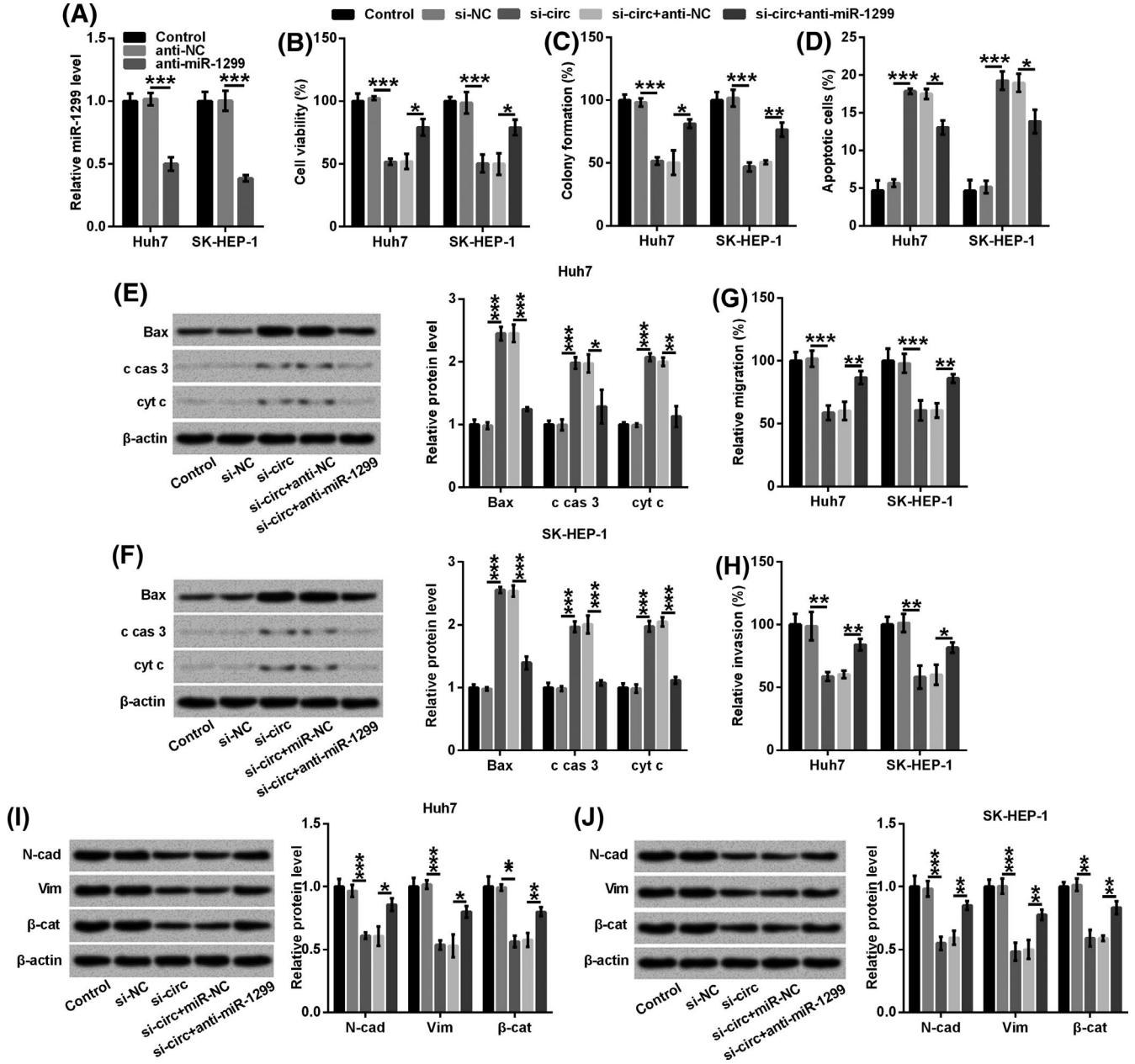

FIGURE 6 Suppressive role of hsa_circ_0003645 in cancer phenotypes of hepatocellular carcinoma cells was mediated by miR-1299 upregulation. At 72 h after transfecting anti-miR-1299 into the cells poorly expressing hsa_circ_0003645, (A) RT-qPCR was carried out to confirm miR-1299 was downregulated in the cells, (B) the viability by microplate reader, (C) colony by microscope, (D) apoptotic cells by flow cytometer, (E-F) Bax (21 kDa), cleaved caspase 3 (c cas 3) (17 kDa), and cytochrome c (cyt c) (15 kDa) by Western blot, (G-H) migration and invasion by microscope, and (I-J) N-cadherin (N-cad) (140 kDa), vimentin (Vim) (54 kDa), and β-catenin (β-cat) (92 kDa) by Western blot. Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

the diversity of their functions from physiological activities to pathological events.

Hsa_circ_0003645 was predicted to interact with miR-1299 by analytical tool including miRDB and circRNA Interactome.[30] This database allows mapping of circRNA-miRNA contacts at single-circRNA resolution. Here, we validated the negatively regulatory capacity of hsa_circ_0003645 in miR-1299 expression. Further, we ascertained the target relationship between miR-1299 and hsa_circ_0003645 at the mRNA dimension. Notably, results showed hsa_circ_0003645 silence inhibited the growth while induced apoptosis dependent on miR-1299 upregulation triggered by silencing hsa_circ_0003645. Recent evidence showed miR-1299 is a tumor suppressor as miR-1299 buffered proliferation by targeting 3′-untranslated region (3′-UTR) of cyclin-dependent kinase 6 (CDK6).[35] Further, it proved occupying 3′-UTR by miRNAs results in downregulation of CDK6 at the protein level.[36] Further studies are required to prove whether

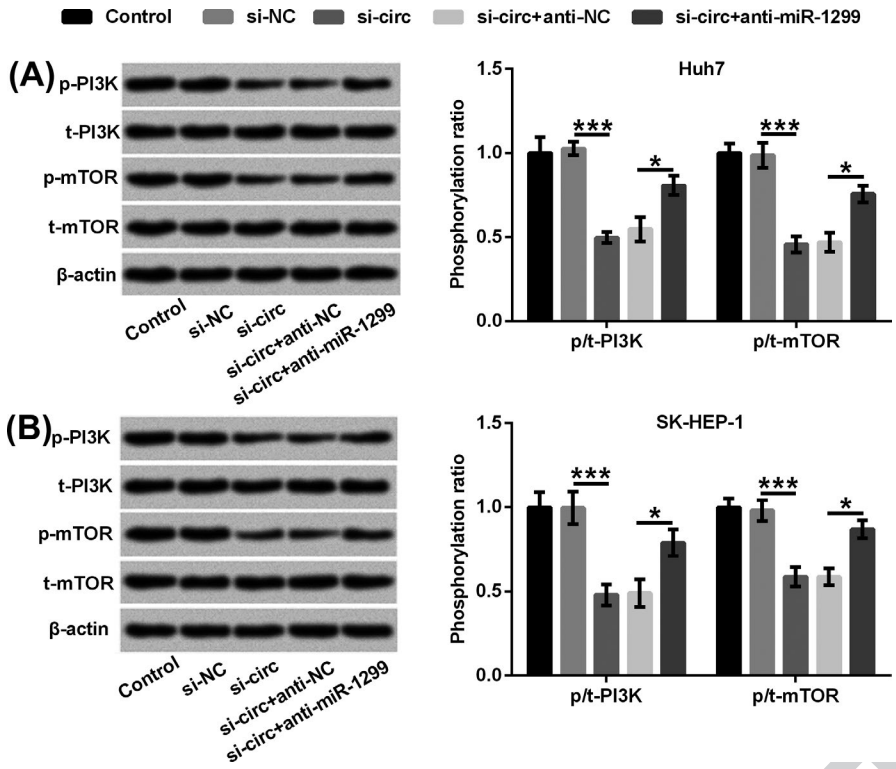

FIGURE 7 Reactivation of PI3K/mTOR pathway in hsa_circ_0003645-silenced cells required the downregulation of miR-1299. At 72 h after transfecting anti-miR-1299 into (A) Huh7 and (B) SK-HEP-1 cells low expressing hsa_circ_0003645, Western blot assay was done for detecting PI3K (100 kDa), phospho-PI3K (Ser249) (100 kDa), mTOR (289 kDa), and phospho-mTOR (Ser2448) (289 kDa). Data were the means ± standard deviation from three independent experiments. One-way ANOVA followed by Tukey's test, $^{*}P < .05$, $^{***}P < .001$

hsa_circ_0003645 might modulate the transcription of proteins involved in apoptosis as well as migration and invasion by binding miR-1299.

It has been established the abnormal activation of PI3K/mTOR is included in the pathogenesis of human cancer diseases.[37] Bluntness of signaling transduction in PI3K/mTOR pathway sensitizes hepatocellular carcinoma cells to cisplatin-induced apoptosis.[38] Ensuing alteration in the downstream targets of PI3K/mTOR signaling pathway contributes to the sensitivity to apoptosis and inhibits extensive proliferation.[39] The observation that the phosphorylated PI3K and mTOR were downregulated in the cells deficient in hsa_circ_0003645 implied that activation of the transducers was intervened. However, this pathway was blocked when the cells were deficient in hsa_circ_0003645 and miR-1299 at the same time. These findings implied that PI3K/mTOR pathway was blocked by silencing hsa_circ_0003645, and miR-1299 was involved in this process.

Our data expressly demonstrated that the abundance of hsa_circ_0003645 in hepatocellular carcinoma cells was a high oncogenic event since we observed that silencing hsa_circ_0003645 blocked the over-proliferation and induced apoptosis. miR-1299 was responsible for the role of hsa_circ_0003645 silence and involvement of PI3K/mTOR pathway. Further experiments should be carried out to better understand the role of miR-1299 in the translation of its target mRNA.

AUTHORS' CONTRIBUTIONS

Ming Shu conceived and designed the experiments. Qiuyun Yu and Jinhua Dai performed the experiments and analyzed the data. Qiuyun Yu and Ming Shu manuscript writing and revision. All authors read and approved the final manuscript.

ETHICAL APPROVAL

All procedures performed in the 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. This study was approved by the ethics committee of Hwa Mei Hospital, University of Chinese Academy of Science. Written informed consent was obtained from the patient.

CONSENT FOR PUBLICATION

All patients were informed and agree to publish.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.
2. Chuang SC, Lee YC, Wu GJ, Straif K, Hashibe M. Alcohol consumption and liver cancer risk: a meta-analysis. *Cancer Causes Control*. 2015;26(9):1205-1231.
3. Maucort-Boulch D, de Martel C, Franceschi S, Plummer M. Fraction and incidence of liver cancer attributable to hepatitis B and C viruses worldwide. *Int J Cancer*. 2018;142(12):2471-2477.
4. Marengo A, Rosso C, Bugianesi E. Liver cancer: connections with obesity, fatty liver, and cirrhosis. *Annu Rev Med*. 2016;67:103-117.

5. Mokdad AA, Singal AG, Yopp AC. Advances in local and systemic therapies for hepatocellular cancer. *Curr Oncol Rep*. 2016;18(2):9.
6. Cooper A, Downs S, Skill N, Mangus R, Kubal C, Maluccio M. Liver transplantation or resection for treatment of hepatocellular carcinoma in patients with well-compensated cirrhosis: a decision analysis model. *OBM Transplant*. 2019;3(2):21.
7. Mahato M, Jayandharan GR, Vemula PK. Viral-and Non-viral-Based Hybrid Vectors for Gene Therapy. In: Jayandharan GR, ed.*Gene and Cell Therapy: Biology and Applications*. Singapore: Springer; 2018:111-130.
8. Huang JL, Zheng L, Hu YW, Wang Q. Characteristics of long non-coding RNA and its relation to hepatocellular carcinoma. *Carcinogenesis*. 2014;35(3):507-514.
9. Fu L, Jiang Z, Li T, Hu Y, Guo J. Circular RNAs in hepatocellular carcinoma: functions and implications. *Cancer Med*. 2018;7(7):3101-3109.
10. Liu X, Wang T, Wakita T, Yang W. Systematic identification of microRNA and messenger RNA profiles in hepatitis C virus-infected human hepatoma cells. *Virology*. 2010;398(1):57-67.
11. Guarnerio J, Zhang Y, Cheloni G, et al. Intragenic antagonistic roles of protein and circRNA in tumorigenesis. *Cell Res*. 2019;29(8):628-640.
12. Zhang H, Shen Y, Li Z, et al. The biogenesis and biological functions of circular RNAs and their molecular diagnostic values in cancers. *J Clin Lab Anal*. 2019;34(1):e23049.
13. Yao T, Chen Q, Shao Z, Song Z, Fu L, Xiao B. Circular RNA 0068669 as a new biomarker for hepatocellular carcinoma metastasis. *J Clin Lab Anal*. 2018;32(8):e22572.
14. Fu L, Wu S, Yao T, et al. Decreased expression of hsa_circ_0003570 in hepatocellular carcinoma and its clinical significance. *J Clin Lab Anal*. 2018;32(2):e22239.
15. Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res*. 2015;25(8):981-984.
16. Bruce JP, Hui AB, Shi W, et al. Identification of a microRNA signature associated with risk of distant metastasis in nasopharyngeal carcinoma. *Oncotarget*. 2015;6(6):4537-4550.
17. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6(4):259-269.
18. Zheng Q, Bao C, Guo W, et al. Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat Commun*. 2016;7:11215.
19. Li Z, Ruan Y, Zhang H, Shen Y, Li T, Xiao B. Tumor-suppressive circular RNAs: Mechanisms underlying their suppression of tumor occurrence and use as therapeutic targets. *Cancer Sci*. 2019;110(12):3630-3638.
20. Fu HW, Lin X, Zhu YX, et al. Circ-IGF1R has pro-proliferative and anti-apoptotic effects in HCC by activating the PI3K/AKT pathway. *Gene*. 2019;716:144031.
21. Bachmayr-Heyda A, Reiner AT, Auer K, et al. Correlation of circular RNA abundance with proliferation–exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues. *Sci Rep*. 2015;5:8057.
22. Wang Y, Lu Z, Wang N, Zhang M, Zeng X, Zhao W. MicroRNA-1299 is a negative regulator of STAT3 in colon cancer. *Oncol Rep*. 2017;37(6):3227-3234.
23. Hsiao KY, Lin YC, Gupta SK, et al. Noncoding effects of circular RNA CCDC66 promote colon cancer growth and metastasis. *Can Res*. 2017;77(9):2339-2350.
24. Guarnerio J, Bezzi M, Jeong JC, et al. Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. *Cell*. 2016;166(4):1055-1056.
25. Hansen TB, Kjems J, Damgaard CK. Circular RNA and miR-7 in cancer. *Can Res*. 2013;73(18):5609-5612.
26. Verduci L, Strano S, Yarden Y, Blandino G. The circRNA-microRNA code: emerging implications for cancer diagnosis and treatment. *Molecular oncology*. 2019;13(4):669-680.
27. Fu L, Yao T, Chen Q, Mo X, Hu Y, Guo J. Screening differential circular RNA expression profiles reveals hsa_circ_0004018 is associated with hepatocellular carcinoma. *Oncotarget*. 2017;8(35):58405-58416.
28. Huang XY, Huang ZL, Xu YH, et al. Comprehensive circular RNA profiling reveals the regulatory role of the circRNA-100338/miR-141-3p pathway in hepatitis B-related hepatocellular carcinoma. *Sci Rep*. 2017;7(1):5428.
29. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA (New York, NY)*. 2013;19(2):141-157.
30. Qiu L, Wang T, Ge Q, et al. Circular RNA signature in hepatocellular carcinoma. *J Cancer*. 2019;10(15):3361-3372.
31. Barrett T, Suzek TO, Troup DB, et al. NCBI GEO: mining millions of expression profiles–database and tools. *Nucleic Acids Res*. 2005;33(Database issue):D562-D566.
32. An J, Shi H, Zhang N, Song S. Elevation of circular RNA circ_0003645 forecasts unfavorable prognosis and facilitates cell progression via miR-1179/TMEM14A pathway in non-small cell lung cancer. *Biochem Biophys Res Comm*. 2019;511(4):921-925.
33. Zhao Y, Alexandrov PN, Jaber V, Lukiw W. Deficiency in the Ubiquitin Conjugating Enzyme UBE2A in Alzheimer's disease (AD) is linked to deficits in a natural circular miRNA-7 Sponge (circRNA; ciRS-7). *Genes*. 2016;7(12):E116.
34. Ashwal-Fluss R, Meyer M, Pamudurti NR, et al. circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell*. 2014;56(1):55-66.
35. Zhu H, Wang G, Zhou X, et al. miR-1299 suppresses cell proliferation of hepatocellular carcinoma (HCC) by targeting CDK6. *Biomed Pharmacother*. 2016;83:792-797.
36. Lena AM, Mancini M, Rivetti di Val Cervo P, et al. MicroRNA-191 triggers keratinocytes senescence by SATB1 and CDK6 downregulation. *Biochem Biophys Res Commun*. 2012;423(3):509-514.
37. De Luca A, Maiello MR, D'Alessio A, Pergameno M, Normanno N. The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opin Ther Targets*. 2012;16(Suppl 2):S17-S27.
38. Sheng J, Shen L, Sun L, Zhang X, Cui R, Wang L. Inhibition of PI3K/mTOR increased the sensitivity of hepatocellular carcinoma cells to cisplatin via interference with mitochondrial-lysosomal crosstalk. *Cell Prolif*. 2019;52(3):e12609.
39. Zhang X, Jin B, Huang C. The PI3K/Akt pathway and its downstream transcriptional factors as targets for chemoprevention. *Curr Cancer Drug Targets*. 2007;7(4):305-316.

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