Research Article

Rui Xiang[#], Min Feng[#], Xin Zhou, Lihong Ma, Ningfei Dong*

circAPLP2 promotes colorectal cancer progression by upregulating HELLS by targeting miR-335-5p

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Abstract

Background – Colorectal cancer (CRC) is one of the deadliest cancers in the world. Increasing evidence suggests that circular RNAs (circRNAs) are implicated in CRC pathogenesis. This study aimed to determine the role of circAPLP2 and explore a potential mechanism of circAPLP2 action in CRC.

Methods – The expression of circAPLP2, miR-335-5p and helicase lymphoid-specific (HELLS) mRNA in CRC tissues and cells was measured by quantitative real-time polymerase chain reaction (qPCR). The functional effects of circAPLP2 on cell cycle progression/cell apoptosis, colony formation, cell migration, invasion and glycolysis metabolism were investigated by flow cytometry assay, colony formation assay, wound healing assay, transwell assay and glycolysis stress test. Glycolysis metabolism was also assessed by the levels of glucose uptake and lactate production. The protein levels of HELLS and HK2 were detected by western blot. The interaction between circAPLP2 and miR-335-5p, or miR-335-5p and HELLS was verified by dual-luciferase reporter assay. The role of circAPLP2 on solid tumor growth in nude mice was investigated.

Results – circAPLP2 and HELLS were overexpressed, but miR-335-5p was downregulated in CRC tissues and cells. Functional analyses showed that circAPLP2 knock-down suppressed CRC cell cycle progression, colony

formation, migration, invasion and glycolysis metabolism, induced cell apoptosis and blocked solid tumor growth in nude mice. Moreover, miR-335-5p was a target of circAPLP2, and miR-335-5p could also bind to HELLS. Rescue experiments presented that miR-335-5p inhibition reversed the effects of circAPLP2 knockdown, and HELLS overexpression abolished the role of miR-335-5p restoration. Importantly, circAPLP2 could positively regulate HELLS expression by mediating miR-335-5p.

Conclusion – circAPLP2 triggered CRC malignant development by increasing HELLS expression via targeting miR-335-5p, which might be a novel strategy to understand and treat CRC.

Keywords: circAPLP2, miR-335-5p, HELLS, colorectal cancer

1 Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related death, second only to lung cancer, liver cancer and stomach cancer [1]. In gender, CRC is the second common cancer in females and the third most common cancer in males [2]. Most CRC cases are found in Western countries (55%), but due to the rapid development of other countries in the past, this trend is changing, making CRC a problem of globalization [3,4]. Mutations in specific genes contribute to CRC, just like other types of cancer. These mutations can appear in oncogenes, tumor suppressor genes and genes related to DNA repair mechanisms [1]. Recently, the dysregulation of circular RNAs (circRNAs) in cancers has aroused much concern [5]. The exploration in the function of CRC-specific circRNAs will help to further understand the pathogenesis of CRC.

circRNAs are a cluster of non-coding RNAs (ncRNAs) that harbor covalently closed-loop structures. Numerous circRNAs originate from the exon region of protein-coding genes, in the process of “back-splicing” [6,7]. Compared with linear RNAs, circRNAs are more stable because of

[#] These authors contributed equally.

* **Corresponding author: Ningfei Dong**, Department of Gastroenterology, West Hospital of Zibo Central Hospital, No. 10 Shanghai Road, Zhangdian District, Zibo, Shandong, 255000, China, e-mail: dd7282dd@163.com, tel: +86-186-7818-7282

Rui Xiang, Min Feng, Xin Zhou, Lihong Ma: Department of Gastroenterology, West Hospital of Zibo Central Hospital, No. 10 Shanghai Road, Zhangdian District, Zibo, Shandong, 255000, China

the lack of 5' caps and 3' ends, and circRNAs are gradually acknowledged to be novel biomarkers in cancer [5]. circRNAs are shown to be differently expressed in a tissue-specific manner in tumors. For example, certain circRNAs were identified to be significantly upregulated or downregulated in CRC tissues compared with adjacent normal tissues by RNA-sequencing technology [8,9]. Increasing studies manifested that circRNAs participated in CRC development, functioning as oncogenes or tumor suppressors, such as circ_0079993 and circ_0026344 [10,11]. As for circAPLP2, RNA-sequencing data suggested that it was highly expressed in CRC tissues [12]. However, its function is not fully disclosed.

circRNAs regulate gene expression in various ways, acting as microRNA (miRNA) sponges, RNA-binding protein sponges or regulators of transcription and translation [13–15]. miRNAs are well-known to be crucial for the initiation, progression and dissemination of human cancers, and are defined as treasures for cancer diagnosis and therapy [16]. The interaction between circRNA and miRNA has been identified in several studies. For example, miR-335-5p was a target of circZMYM2, and circZMYM2 targeted miR-335-5p to promote pancreatic cancer development [17]. It is still unknown whether miR-335-5p can be targeted by circTLK1 and whether miR-335-5p is involved in the circTLK1 regulatory pathway in CRC.

Helicase lymphoid-specific (HELLS) is shown to be upregulated in CRC tissues in Gene Expression Profiling Interactive Analysis (GEPIA) database, hinting that HELLS may play functions in CRC development. Given that miRNAs regulate gene expression by binding to their 3' untranslated region (3'UTR) [18], bioinformatics analysis presents that miR-335-5p can bind to HELLS 3'UTR. It is worth exploring whether miR-335-5p is involved in CRC progression through the suppression of HELLS.

In this study, we not only investigated the expression of circAPLP2 in CRC tissues and cells, but also explored the functions of circAPLP2 on the development of CRC both *in vitro* and *in vivo*. Besides, we provided a potential mechanism of circAPLP2 action in CRC associated with miR-335-5p and HELLS, aiming to further understand the progression of CRC from the perspective of circAPLP2.

2 Materials and methods

2.1 Clinical samples

CRC tissues ($n = 33$) and matched normal tissues ($n = 33$) were collected from CRC patients recruited from West

Hospital of Zibo Central Hospital. The use of these tissues was approved by these subjects with the written informed consent. All samples frozen by liquid nitrogen were stored at -80°C freezer. This study was conducted with the approval of the Ethics Committee of West Hospital of Zibo Central Hospital.

2.2 Cell lines and cell culture

CRC cell lines, including SW480, HCT116, SW620, LOVO and HT29, and normal intestinal epithelial cells (HIEC6) were purchased from Bena Culture Collection (Beijing, China). According to the guideline, SW480, SW620 and HIEC6 cells were cultured in 90% DMEM (Bena Culture Collection) containing 10% FBS, and HCT116 cells were cultured in 90% RPMI-1640 (Bena Culture Collection) containing 10% FBS. LOVO cells were cultured in 90% F-12K medium (Bena Culture Collection) containing 10% FBS, and HT29 cells were cultured in 90% McCoy's 5a medium (Bena Culture Collection) containing 10% FBS. These cells were placed in a 37°C incubator supplemented with 5% CO_2 for cell culture.

2.3 Quantitative real-time polymerase chain reaction (qPCR)

First, total RNA was isolated using the Total RNA Extraction Kit (Novland BioPharma; Shanghai, China). The reverse transcription of circRNA and mRNA was performed using One Step Reverse Transcription PCR Kit (Novland BioPharma), and the reverse transcription of miRNA was performed using One Step Stemaim-it miR Reverse Transcription Quantitation Kit (Novland BioPharma). QPCR reactions were carried out using the SYBR Green Mix (Novland BioPharma) under a CFX 96-touch Real-Time PCR System (Bio-Rad; Hercules, CA, USA). GAPDH or U6 was used as the normalization control, and the relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The sequences of used primers were listed as follows:

circAPLP2, F: 5'-CTTTCAAGTGTCTCGGCTCT-3' and R: 5'-TCTTTTGTTCACAAAGCAGCTCT-3'; APLP2, F: 5'-GCAACCGAATGGACAGGGTA-3' and R: 5'-TCAGAGTCTGCCTCTCTGCT-3'; HELLS, F: 5'-GCCATGAGAGACCGAAATGC-3' and R: 5'-ACTCCCTGATTAGACGGCAC-3'; GAPDH, F: 5'-CAATGACCCCTTCATTGACC-3' and R: 5'-GACAAGCTTCGGTTCTCAG-3'; miR-335-5p, F: 5'-TCAAGAGCAATAACGAAAAATGT-3' and R: 5'-GCTGTCAACGATACGTACGT-3'; U6, F: 5'-GGTCGGGCAGGAAAGAGGGC-3' and R: 5'-GCTAATCTTCTCTGTATCGTTCC-3'.

2.4 RNase R treatment

Total RNA isolated from SW480 and HCT116 cells were exposed to RNase R (1 U/ μ g; Epicentre; Madison, WI, USA) at 37°C for 30 min. Then, the expression of circAPLP2 and linear APLP2 was detected by qPCR as mentioned above.

2.5 Cell transfection

We used small interference RNA (siRNA) to mediate circAPLP2 knockdown, and siRNA targeting circAPLP2 (si-circAPLP2) and siRNA negative control (si-NC) were assembled by Genescript (Shanghai, China). Besides, miR-335-5p mimic, miR-335-5p inhibitor and their matched negative control (miR-NC mimic or miR-NC inhibitor) were purchased from Ribobio (Guangzhou, China). The pCD-ciR vector harboring circAPLP2 sequence was used for circAPLP2 overexpression (pCD-ciR-circAPLP2; Genescript; Guangzhou, China), with pCD-ciR vector as the control. The pcDNA vector harboring HELLS sequence was used for HELLS overexpression (pcDNA-HELLS; Sangon; Shanghai, China), with pcDNA-NC as the negative control. Cells were transiently transfected with these oligonucleotides or plasmids using Lipofectamine 3000 (Invitrogen; Carlsbad, CA, USA).

2.6 Flow cytometry assay

For cell cycle distribution analysis, cells were harvested, digested with trypsin and centrifuged for 15 min. Cell pellets were resuspended with 70% ethanol for fixing at -20°C overnight. Afterward, cells were washed with PBS twice and incubated with RNase A (180 μ g/mL; Invitrogen) for 15 min at 37°C. Then, cells were stained with propidium iodide (PI; 50 μ g/mL; Invitrogen) for 30 min at room temperature in the dark. Cells were analyzed by flow cytometry system (BD Biosciences, San Jose, CA, USA).

The apoptotic cells were detected using the Bioscience™ Annexin V-FITC Apoptosis Detection Kit (Invitrogen). Cells were harvested and centrifuged for 10 min. The cells were then washed with PBS twice and resuspended in binding buffer, and then stained with Annexin V-FITC and PI in the dark at room temperature for 15 min. The stained cells for apoptosis detection were analyzed by a flow cytometry system (BD Biosciences).

2.7 Colony formation assay

SW480 and HCT116 cells were seeded in 6-well plates at a density of 200 cells per well and cultured for 12 days with replacement with fresh medium every 3 days in a 37°C incubator containing 5% CO₂. Subsequently, colonies were washed with PBS, fixed with methanol and stained with 0.1% crystal violet (Sangon). The images of colonies were captured, and the number of colonies was counted under a microscope (Olympus; Tokyo, Japan).

2.8 Wound healing assay

SW480 and HCT116 cells were seeded into a 6-well plate with a density of 2.0×10^5 cells/well. Then, the monolayer was gently scratched using a 10 μ L pipette tip, ensuring that the tip is perpendicular to the bottom of the well. Next, the monolayer with a wound was gently washed twice with PBS to remove the detached cells. After culturing for 24 h, the representative images of migration distance were recorded under a microscope (40 $\times$; Olympus), and the data were analyzed using the Image J software (NIH, Bethesda, MA, USA).

2.9 Transwell assay

For migration assay, cells (5×10^4) were resuspended in serum-free medium and plated on the top of uncoated transwell chambers (Corning Incorporated; Corning, NY, USA). For invasion assay, cells (5×10^4) were transferred on the top of transwell chambers pre-coated with Matrigel matrix (Corning Incorporated). Meanwhile, the bottom of chambers in these two assays was filled with culture medium containing 10% FBS. After incubation for 24 h, the remaining cells attached to the inserts were fixed with methanol and stained with 0.1% crystal violet (Sangon). Cells were then photographed and counted under a microscope (100 $\times$; Olympus).

2.10 Glycolysis stress test

Extracellular acidification rate (ECAR) was measured to monitor glycolysis metabolism using a Seahorse XF96 Analyzer Glycolysis (Seahorse Bioscience, Santa Clara, CA, USA) in line with the guidelines. Briefly, SW480 and HCT116 cells in culture medium containing 10% FBS were seeded in XF 96-well plates. For glycolysis

stress test, D-glucose (10 mM), oligomycin (1 μ M) and 2-deoxyglucose (2-DG; 100 mM) were serially added into the wells at the indicated time points, and the value of corresponding ECAR (mPH/min) was assessed.

2.11 Glucose uptake and lactate production

To monitor glycolysis metabolism, the levels of glucose uptake and lactate production were detected using the Glucose Uptake Assay Kit (Abcam; Cambridge, MA, USA) and Lactate Assay Kit (Abcam) according to the manuscript's protocols.

2.12 Western blot

Total proteins were extracted using RIPA Lysis Buffer (Sangon) and quantified by BCA Protein Assay Kit (Sangon). The equal amount of protein was isolated by 12% SDS-PAGE and then transferred on PVDF membranes (Bio-Rad). The protein-included membranes were blocked by 5% skim milk and then incubated with the primary antibodies, including anti-hexokinase 2 (anti-HK2; ab227198; Abcam), anti-HELLS (ab3851; Abcam) and anti-GAPDH (ab9485; Abcam) overnight at 4°C. Then, the membranes were incubated with the secondary antibody (ab205718; Abcam) for 1.5 h at room temperature. The target proteins were detected by enhanced chemiluminescence (ECL; Sangon) reagents and quantified using Image J software.

2.13 Dual-luciferase reporter assay

The target relationship between miR-335-5p and circAPLP2 or HELLS was predicted by the bioinformatics tool starbase v3.0 (<http://starbase.sysu.edu.cn/>). To verify the predicted relationship, dual-luciferase reporter assay was performed. In brief, the fragment of wild-type circAPLP2, including the miR-335-5p binding site, was amplified and cloned into PGL4 reporter plasmid, naming as WT-circAPLP2. The fragment of mutant-type circAPLP2 containing mutants on the miR-335-5p binding site was amplified and cloned into PGL4 reporter plasmid, named as MUT-circAPLP2. Similarly, WT-HELLS and MUT-HELLS were also constructed. SW480 and HCT116 cells were cotransfected with miR-335-5p mimic or miR-NC mimic and WT-circAPLP2, MUT-circAPLP2, WT-HELLS or MUT-HELLS and incubated for 48 h. The luciferase activity in SW480 and HCT116 cells with different transfection was measured using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI, USA).

2.14 Animal experiments

For stable circAPLP2 knockdown, short hairpin RNA targeting circAPLP2 (sh-circAPLP2; Genepharma) was packaged into lentiviral vector by Genepharma, with sh-NC as the negative control. Lentiviral vector harboring sh-circAPLP2 or sh-NC was transfected into 293FT cells. SW480 cells were infected with the viral supernatants, and sh-circAPLP2 or sh-NC was infected into SW480 cells. The experimental mice (Balb/c; Female; 6 weeks old) were purchased from SLAC Animal Lab. (Shanghai, China) and divided into two groups (sh-circAPLP2 group and sh-NC group; $n = 6$ per group). SW480 cells harboring sh-circAPLP2 or sh-NC were subcutaneously injected into nude mice to allow tumor growth. The status of tumor growth was observed every 3 days, and tumor volume ($\text{length} \times \text{width}^2 \times 0.5$) was recorded. After 4 weeks, all mice were sacrificed by cervical dislocation, and tumor tissues were excised for weighting and expression detection. All procedures were approved by the Animal Care and Use of Committee of West Hospital of Zibo Central Hospital.

2.15 Statistical analysis

All experiments were repeated at least three times. All data were presented as mean $\pm$ standard deviation (SD). $P < 0.05$ was set as statistically significant. The difference was analyzed using the unpaired Student's *t*-test for comparisons of two groups or ANOVA for multiple group comparisons. Pearson correlation coefficient was adopted to analyze the correlation between two parameters. All statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA).

3 Results

3.1 circAPLP2 abundantly existed in CRC tissues and cells and was resistant to RNase R

circAPLP2 (hsa_circ_0000372) was derived from the exon2-exon3 of APLP2 mRNA, and the schematic diagram of circAPLP2 formation is shown in Figure 1a. The expression pattern of circAPLP2 in CRC was unclear, and our result

Figure 1: circAPLP2 was upregulated in CRC tissues and cells, and resistant to RNase R. (a) The schematic diagram of circAPLP2 formation. (b) The expression of circAPLP2 in CRC tissues and paired normal tissues detected by qPCR. (c) The expression of circAPLP2 in cancer cells (SW480, HCT116, SW620, LOVO and HT29) and non-cancer cells (HIEC6) detected by qPCR. (d) The effects of RNase R or Mock treatment on the expression of circAPLP2 and linear APLP2. * $P < 0.05$.

showed that the expression of circAPLP2 was remarkably increased in CRC tissues compared with normal tissues (Figure 1b). Besides, circAPLP2 expression was also shown to be elevated in CRC cell lines, including SW480, HCT116, SW620, LOVO and HT29 cells, compared with that in HIEC6 cells (non-cancer cells) (Figure 1c). Next, we used RNase R to examine the stability of circAPLP2, and the result presented that RNase R significantly reduced the expression of linear APLP2 mRNA but unaffected the expression of circAPLP2 compared with Mock treatment (Figure 1d). In short, circAPLP2 was upregulated in CRC, and circAPLP2 was stable and resistant to RNase R.

3.2 circAPLP2 knockdown induced cell cycle arrest and cell apoptosis but suppressed colony formation, migration, invasion and glycolysis metabolism

To explore the role of circAPLP2 in CRC, we used siRNA-mediated circAPLP2 knockdown to investigate the effects of circAPLP2 in CRC cells. The data showed that circAPLP2 expression level was strikingly decreased after

si-circAPLP2 transfection (Figure 2a). In function, circAPLP2 knockdown noticeably induced cell cycle arrest at the G0/G1 stage in SW480 and HCT116 cells (Figure 2b). The ability of colony formation was notably suppressed by circAPLP2 knockdown (Figure 2c). Compared to si-NC transfection, si-circAPLP2 transfection significantly promoted cell apoptosis (Figure 2d). From wound healing and transwell assays, we monitored that the capacity of cell migration was strikingly inhibited in SW480 and HCT116 cells transfected with si-circAPLP2 compared with si-NC (Figure 2e and f), and the capacity of cell invasion was also inhibited by si-circAPLP2 transfection in SW480 and HCT116 cells by transwell assay (Figure 2g). Moreover, glycolysis stress test showed that the ECAR value was decreased in SW480 and HCT116 cells transfected with si-circAPLP2 compared with si-NC with sequential addition of Glucose, Oligomycin and 2-DG (Figure 2h), and the levels of glucose uptake and lactate production were notably decreased after circAPLP2 knockdown (Figure 2i and j). In addition, the expression of HK2 (a marker of glycolysis) was also significantly reduced in cells with circAPLP2 knockdown (Figure 2k). In short, circAPLP2 knockdown blocked a series of CRC cell malignant behaviors, including colony

Figure 2: circAPLP2 knockdown induced cell cycle arrest and apoptosis but suppressed colony formation, migration, invasion and glycolysis metabolism. (a) The efficiency of circAPLP2 knockdown was examined using qPCR. Then, the effects of circAPLP2 knockdown on (b) cell cycle progression, (c) colony formation, (d) cell apoptosis, (e and f) cell migration, (g) cell invasion and (h) glycolysis metabolism in SW480 and HCT116 cells were detected by flow cytometry assay, colony formation assay, flow cytometry assay, wound healing assay, transwell assay and glycolysis stress test. (i and j) Glucose uptake and lactate production were examined using detection kits. (k) The expression of a glycolysis-related protein, HK2, was measured by western blot. * $P < 0.05$.

formation, cell cycle progression, migration, invasion and glycolysis metabolism.

3.3 circAPLP2 targeted miR-335-5p and suppressed its expression

We attempted to identify the target miRNAs of circAPLP2, thus providing a mechanism to elucidate the role of circAPLP2 in CRC. Bioinformatics analysis (starbase v3.0) showed that circAPLP2 harbored targeting site (miR-335-5p response element) with miR-335-5p (Figure 3a). For further validation, WT-circAPLP2 and MUT-circAPLP2 reporter plasmids were constructed, and the data showed that the luciferase activity was markedly impaired in

SW480 and HCT116 cells with cotransfection of miR-335-5p mimic and WT-circAPLP2 but not MUT-circAPLP2 (Figure 3b and c), verifying the interaction between circAPLP2 and miR-335-5p. In addition, circAPLP2 expression was remarkably increased in SW480 and HCT116 cells transfected with pcDNA-circAPLP2 compared with pcDNA-NC (Figure 3d). Then, in pcDNA-circAPLP2-transfected cells, the expression of miR-335-5p was decreased, while the expression of miR-335-5p was remarkably increased in si-circAPLP2-transfected cells (Figure 3e). Furthermore, the expression of miR-335-5p was lower in CRC tissues and cell lines (SW480, HCT116 and SW620) than those in normal tissues and non-cancer cells (HIEC6), respectively (Figure 3f and g). Pearson correlation analysis showed that miR-335-5p expression was negatively

Figure 3: circAPLP2 targeted miR-335-5p to suppress miR-335-5p expression. (a) The binding site between circAPLP2 and miR-335-5p was analyzed by starbase v3.0. (b and c) The relationship between circAPLP2 and miR-335-5p was verified by dual-luciferase reporter assay. (d) The efficiency of circAPLP2 overexpression was examined using qPCR. (e) The effects of circAPLP2 overexpression and knockdown on the expression of miR-335-5p were examined using qPCR. (f) The expression of miR-335-5p in CRC and normal tissues detected by qPCR. (g) The expression of miR-335-5p in cancer cells (SW480, HCT116 and SW620) and non-cancer cells (HIEC6) detected by qPCR. (h) The association between miR-335-5p expression and circAPLP2 expression in CRC tissues was analyzed by Pearson correlation coefficient. * $P < 0.05$.

associated with circAPLP2 expression in CRC tissues (Figure 3h). In short, circAPLP2 functioned as a molecular sponge of miR-335-5p and suppressed miR-335-5p expression.

3.4 miR-335-5p deficiency abolished the effects of circAPLP2 knockdown in CRC cells

We performed rescue experiments to verify whether circAPLP2 played functions in CRC by targeting miR-335-5p. The expression of miR-335-5p was visibly lessened in cells transfected with miR-335-5p inhibitor compared with miR-NC inhibitor (Figure 4a). In function, circAPLP2 knockdown-induced cell cycle arrest was alleviated by the reintroduction of miR-335-5p inhibitor (Figure 4b). The ability of colony formation was suppressed by circAPLP2 knockdown in SW480 and HCT116 cells but restored by miR-335-5p inhibitor (Figure 4c). circAPLP2

knockdown-induced cell apoptosis was partly blocked by the inhibition of miR-335-5p (Figure 4d). Besides, the migration distance in wound healing assay and the number of migrated cells in transwell assay were blocked in cells after si-circAPLP2 transfection but renovated in cells after si-circAPLP2 + miR-335-5p inhibitor cotransfection (Figure 4e and f). Also, the number of invaded cells was elevated in cells after si-circAPLP2 + miR-335-5p inhibitor cotransfection compared with si-circAPLP2 transfection (Figure 4g). Glycolysis stress test showed that the value of ECAR was weakened in cells transfected with si-circAPLP2 but recovered in cells transfected with si-circAPLP2 + miR-335-5p inhibitor (Figure 4h). The levels of glucose uptake and lactate production were also recovered by the reintroduction of miR-335-5p inhibitor (Figure 4i and j). The expression of HK2 impaired by circAPLP2 knockdown was enhanced by miR-335-5p inhibition (Figure 4k). In short, circAPLP2 knockdown blocked a series of CRC cell malignant behaviors by increasing miR-335-5p expression.

Figure 4: miR-335-5p inhibition reversed the effects of circAPLP2 knockdown. (a) The efficiency of miR-335-5p inhibitor was examined using qPCR. (b) Cell cycle progression, (c) the ability of colony formation, (d) cell apoptosis, (e and f) cell migration, (g) cell invasion, (h) glycolysis metabolism, (i and j) the levels of glucose uptake and lactate production, and (k) the expression of HK2 in SW480 and HCT116 cells transfected with si-circAPLP2, si-NC, si-circAPLP2 + miR-335-5p inhibitor or si-circAPLP2 + miR-NC inhibitor were examined using flow cytometry assay, colony formation assay, flow cytometry assay, wound healing assay, transwell assay, glycolysis stress test, detection kits and western blot. * $P < 0.05$.

3.5 HELLS was a target of miR-335-5p

Additionally, we analyzed the potential target mRNAs of miR-335-5p. Bioinformatics analysis predicted that miR-335-5p bound to HELLS 3'UTR through a special binding site (Figure 5a), indicating that HELLS might be a target of miR-335-5p, which was further verified by dual-luciferase reporter assay (Figure 5b). In the following assay, we detected that the expression of miR-335-5p was pronouncedly promoted in cells transfected with miR-335-5p mimic compared with miR-NC mimic (Figure 5c). Besides, miR-335-5p negatively regulated HELLS expression because

HELLS expression was notably increased in SW480 and HCT116 cells transfected with miR-335-5p inhibitor but decreased in cells transfected with miR-335-5p mimic (Figure 5d). We next examined the expression pattern of HELLS in CRC. The result showed that HELLS was upregulated in CRC tissues and cell lines (SW480, HCT116 and SW620) compared with that in normal tissues and non-cancer cells (HIEC6), respectively (Figure 5e–g). Moreover, Pearson correlation analysis introduced that HELLS expression was negatively associated with miR-335-5p expression in CRC tissues (Figure 5h). In short, miR-335-5p bound to HELLS and suppressed its expression.

Figure 5: miR-335-5p bound to HELLS 3'UTR. (a) The binding site between miR-335-5p and HELLS 3'UTR was analyzed by starbase v3.0. (b) The relationship between miR-335-5p and HELLS was verified by dual-luciferase reporter assay. (c) The efficiency of miR-335-5p mimic was examined using qPCR. (d) The effects of miR-335-5p enrichment and inhibition on the expression of HELLS were examined using western blot. (e and f) The expression of HELLS in CRC and normal tissues was detected by qPCR and western blot. (g) The expression of HELLS in HIEC6, SW480, HCT116 and SW620 cells was detected by western blot. (h) The association between HELLS expression and miR-335-5p expression in CRC tissues was analyzed by Pearson correlation coefficient. * $P < 0.05$.

3.6 miR-335-5p overexpression induced cell cycle arrest and cell apoptosis but suppressed cell colony formation, migration, invasion and glycolysis metabolism by repressing HELLS expression

We next performed rescue experiments to determine whether miR-335-5p exerted its role by degrading HELLS. The detection of HELLS overexpression efficiency showed that the expression of HELLS was remarkably enhanced in SW480 and HCT116 cells transfected with pcDNA-HELLS compared with pcDNA-NC (Figure 6a). In function, miR-335-5p mimic transfection notably induced cell cycle arrest and cell apoptotic rate, which was alleviated by the reintroduction of pcDNA-HELLS (Figure 6b and d). The ability of colony formation in SW480 and HCT116 cells was markedly blocked by miR-335-5p overexpression but partly enhanced by synchronous HELLS overexpression (Figure 6c). Besides, the capacities of migration and invasion were suppressed in

SW480 and HCT116 cells transfected with miR-335-5p mimic but recovered in cells transfected with miR-335-5p mimic + pcDNA-HELLS (Figure 6e–g). Moreover, the value of ECAR in glycolysis stress test and the levels of glucose uptake and lactate production were also weakened in miR-335-5p mimic-transfected cells but restored in miR-335-5p mimic + pcDNA-HELLS-transfected cells (Figure 6h–j). The expression of HK2 was decreased in SW480 and HCT116 cells transfected with miR-335-5p mimic but reinforced in cells transfected with miR-335-5p mimic + pcDNA-HELLS (Figure 6k). In short, miR-335-5p overexpression blocked CRC cell malignant behaviors by targeting HELLS.

3.7 circAPLP2 positively regulated HELLS expression by targeting miR-335-5p

Further dual-luciferase reporter assay discovered that the cotransfection of WT-HELLS 3'UTR and miR-335-5p remarkably reduced luciferase activity in SW480 and

Figure 6: miR-335-5p restoration suppressed CRC development *in vitro* by targeting HELLS. (a) The efficiency of HELLS overexpression was examined using western blot. (b) Cell cycle progression, (c) the ability of colony formation, (d) cell apoptosis, (e and f) cell migration, (g) cell invasion, (h) glycolysis metabolism, (i and j) the levels of glucose uptake and lactate production, and (k) the expression of HK2 in SW480 and HCT116 cells transfected with miR-335-5p mimic, miR-NC mimic, miR-335-5p mimic + pcDNA-HELLS or miR-335-5p mimic + pcDNA-NC were examined using flow cytometry assay, colony formation assay, flow cytometry assay, wound healing assay, transwell assay, glycolysis stress test, detection kits and western blot. * $P < 0.05$.

HCT116 cells, while further WT-circAPLP2 but not MUT-circAPLP2 reintroduction partly recovered luciferase activity (Figure 7a and b). Interestingly, we found that HELLS expression at the mRNA level in CRC tissues was positively associated with circAPLP2 expression (Figure 7c). Besides, the expression of HELLS was strikingly decreased in SW480 and HCT116 cells transfected with si-circAPLP2 compared with si-NC but partly recovered in cells transfected with si-circAPLP2 + miR-335-5p inhibitor compared with si-circAPLP2 + miR-NC inhibitor (Figure 7d). We deduced that circAPLP2 functioned as miR-335-5p sponge to increase the expression of HELLS.

3.8 circAPLP2 downregulation suppressed tumor growth *in vivo*

Animal experiments presented that tumor volume and tumor weight in the sh-circAPLP2 group were significantly lower than that in the sh-NC group (Figure 8a and b), suggesting that circAPLP2 downregulation inhibited tumor growth. Besides, the expression of circAPLP2 and HELLS was declined, while the expression of miR-335-5p was elevated in tumor tissues from the sh-circAPLP2 group relative to the sh-NC group (Figure 8c–e), indicating that circAPLP2 promoted tumor growth *in vivo* by mediating the miR-335-5p/HELLS axis.

Figure 7: circAPLP2 positively regulated HELLS expression by targeting miR-335-5p. (a and b) The interactions among circAPLP2, miR-335-5p and HELLS were further analyzed by dual-luciferase reporter assay. (c) The association between HELLS expression and circAPLP2 expression in CRC tissues was analyzed by Pearson correlation coefficient. (d) The expression of HELLS in SW480 and HCT116 cells transfected with si-circAPLP2, si-NC, si-circAPLP2 + miR-335-5p inhibitor or si-circAPLP2 + miR-NC inhibitor was detected by western blot. $*P < 0.05$.

4 Discussion

The use of molecular markers for risk stratification, early detection and prognostic markers of CRC shows promise in molecular medicine [19]. circRNA satisfies a variety of suitable characteristics as biomarkers, including non-digestible and degraded, high abundance and wide distribution [20]. For example, circRNA expression profile revealed that circDDX17 was notably downregulated in CRC tissues, and low expression of circDDX17 contributed to CRC cell proliferation, migration and invasion [12]. Similarly, circBANP was presented to be upregulated in CRC tissues by circRNA expression profile, and circBANP knockdown suppressed CRC cell proliferation and served as a prognostic and therapeutic marker [21]. A circRNA profile from a previous study also monitored that circAPLP2 expression was strikingly enhanced in CRC tissues [12], while its detailed functions were not expounded. In our study, the expression of circAPLP2 was remarkably higher in 33 CRC tissues compared with that in matched normal tissues. Functional assays presented that underexpression of circAPLP2 weakened colony formation ability, the capacities of migration and invasion, and glycolysis metabolism, but promoted CRC cell cycle arrest and apoptosis. Also, *in vivo* assay showed that circAPLP2 knockdown blocked solid tumor growth. Our study provided sufficient evidence that circAPLP2

downregulation blocked CRC malignant development both *in vitro* and *in vivo*, suggesting that circAPLP2 was an oncogene in CRC.

It is a canonical phenomenon that circRNAs act as potent molecular sponges of miRNAs, sequestering miRNA activity and thus affecting the expression of downstream target genes [14]. In this paper, miR-335-5p was regarded as a target of circRNA through the prediction of bioinformatics analysis and the validation of dual-luciferase reporter assay. Interestingly, the role of miR-335-5p had been partly defined. Previous studies documented that miR-335-5p expression was remarkably declined in the plasma of patients with colorectal adenoma by high-depth small RNA sequencing [22]. In addition, miR-335-5p was markedly downregulated in CRC tissues and cells, and forced expression of miR-335-5p impaired CRC cell proliferation, migration and invasion, as well as lung and liver metastasis *in vivo* by mediating its target genes [23,24]. These studies strongly corroborated our observation of a lower abundance of miR-335-5p in clinical CRC tissues and cell lines, and miR-335-5p restoration induced cell cycle arrest and apoptosis but inhibited colony formation ability, migration, invasion and glycolysis metabolism.

Further study illustrated that HELLS was a target gene of miR-335-5p. HELLS was stated to regulate chromatin remodeling and mediate epigenetic silencing of

Figure 8: circAPLP2 downregulation impaired tumor growth *in vivo*. (a) Nude mice were injected SW480 cells with sh-circAPLP2 or sh-NC, and tumor volume in each mouse was detected every 3 days. (b) Tumor weight was detected after 4 weeks when all mice were killed. (c and d) The expression of circAPLP2 and miR-335-5p in these tumor tissues was detected by qPCR. (e) The expression of HELLS in these tumor tissues was detected by western blot. * $P < 0.05$.

tumor suppressor genes, thus aggravating the progression of cancers [25,26]. HELLS was demonstrated to be a downstream target of Forkhead Box M1 (FOXM1), and HELLS was used to be a biomarker for early cancer detection and an indicator for malignant progression [27]. Importantly, a study exposed that HELLS was highly expressed in CRC tissues, and high HELLS expression was associated with high TNM stage and low overall survival [28]. Besides, HELLS knockdown suppressed CRC cell proliferation and promoted cell cycle arrest [28]. Consistent with these findings, we also defined HELLS as an oncogene because its expression was strikingly increased in CRC tissues and cells. Moreover, HELLS overexpression abolished the role of miR-335-5p restoration and recovered CRC cell malignant activities.

In conclusion, higher expression of circAPLP2 was detected in CRC tissues and cells. circAPLP2 knockdown was associated with inhibitory colony formation ability, migration/invasion capacity and glycolysis metabolism. Besides, circAPLP2 knockdown also blocked solid tumor growth *in vivo*. Further mechanism analysis demonstrated that circAPLP2 played pro-cancer effects in CRC by upregulating HELLS via targeting miR-335-5p. Our study enriched the role of circAPLP2 in CRC and broadened the insights into CRC pathogenesis from the perspective of circAPLP2.

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