

Replication Factor C4 in human hepatocellular carcinoma: A potent prognostic factor associated with cell proliferation

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SUMMARY Replication Factor c4 (RFC4) has been found to play important roles in many carcinomas and is correlated with poor prognosis. The present study was performed to investigate the specific role of RFC4 in hepatocellular carcinoma (HCC) and the underlying molecular mechanism. Public datasets including TCGA and GTEx were applied to explore the expression of RFC4 in HCC and its association with HCC prognosis. The results of bioinformatics analysis showed that RFC4 was overexpressed in HCC tissues compared with noncancerous tissues and significantly correlated with poor prognosis for HCC. Through immunohistochemistry, the association between RFC4 expression and clinical-pathological features of HCC patients was evaluated. Western blots were applied to investigate relative protein expression. Then *in vivo* and *in vitro* experiments were performed to explore the function of RFC4 in HCC tumor cells. The present results suggest that high level expression of RFC4 is associated with tumor size. In addition, RFC4 knockdown suppressed the cell proliferation and sphere formation of hepatoma cells *in vitro*. Moreover, silencing of RFC4 significantly decreased the growth of tumors in a xenograft tumor model. In conclusion, our study indicates that RFC4 is a potential prognostic predictor associated with poor outcomes for HCC patients. Furthermore, knocking down RFC4 could significantly inhibit tumor progression both *in vitro* and *in vivo*. Therefore, the present study can shed new light on the understanding of molecular mechanisms of HCC and may provide molecular targets and diagnostic biomarkers for the treatment of HCC.

Keywords RFC4, hepatocellular carcinoma, prognostic marker

1. Introduction

As the eukaryotic clamp loader, replication factor C (RFC) loads the DNA polymerase δ processivity factor proliferating cell nuclear antigen (PCNA) at primer-template junctions (1-4). RFC is a heteropentameric complex consisting of one large subunit (Rfc1) and four small subunits (Rfc2, -3, -4, and -5), all of which are essential for viability in yeast (5-7). Consistent with their role in DNA replication, mutant strains of the subunits display defects in the DNA replication checkpoint pathway (7-9). By interacting with RPA1, RFC4 is required for both DNA replication and DNA damage checkpoints in *Saccharomyces cerevisiae* (7). Therefore, the deregulation of the RFC4 can contribute to cell proliferation and tumorigenesis and its aberrant expression may indicate it is a promising prognostic marker in several malignancies (10-14). However, the role of RFC4 in cancer initiation and progression and its correlation with HCC remains unclear.

In this study, we systematically explored the roles of RFC4 in HCC. The correlation between RFC4 expression and HCC prognosis was investigated. Furthermore, the function of RFC4 in tumor proliferation was also explored. Consequently, our findings in the study may provide further understanding of HCC development and lead to an improved diagnosis.

2. Materials and methods

2.1. Bioinformatics analysis

Data from GTEx database and TCGA database were applied for differential genetic analysis. Differential gene expression and survival analysis were measured using the GEPIA website (<http://gepia.cancer-pku.cn>). One-way ANOVA was applied for gene expression analysis between cancer and non-cancerous liver tissues. The disease-free survival time and overall survival time were obtained from the TCGA public

database. The results are shown below.

2.2. Patients and samples

Approved by our institutional research ethics committee, 142 patients with hepatocellular carcinoma who have been proved by pathology were incorporated into the study. Fresh samples were collected just after surgery and fixed in 10% formalin before embedding in paraffin wax. Patients' clinical and pathological data including age, gender, number of tumor nodes, tumor sizes and AFP level were identified. The histopathology of each specimen was reviewed by board-certified pathologists in our institution. Written informed consent was obtained from patients before the study. We have also complied with the World Medical Association Declaration of Helsinki involving the ethical conduct of research involving human subjects.

2.3. Immunohistochemistry

The paraffin-embedded tissues were sliced into 4µm sections and baked at 75°C for 45 minutes. The sections were de-waxed in xylene and rehydrated in graded ethanol. Then, EDTA (PH = 8.0) and 3% H₂O₂ in methanol treated the slices for 10 minutes. The tissue sections were cultured with anti-RFC4 antibodies (rabbit, ab156780, Abcam) at a 1:250 dilution overnight at 4°C. Then second antibody was added and incubated at room temperature for 30 minutes. After DAB staining, the sections were counterstained using haematoxylin, dehydrated, and cleared with xylene. For the results analysis, a semi-quantitative H-score was computed for each sample by multiplying the staining intensities (0: negative, 1: weak staining, 2: moderate staining, 3: strong staining) and distribution areas (0-100%). All samples were classified as high expression and low expression group respectively according to the distribution of H-score.

2.4. Cell culture and transfection

Both human hepatocellular carcinoma cell lines (Hep3B and SNU-475) used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SNU-475 was cultured in RPMI-1640 medium while Hep3B was cultured in Dulbecco's modified Eagle's medium (DMEM), both contained 1% penicillin streptomycin and 10% fetal bovine serum. Cells were maintained at 37°C with 5% CO₂. Both cell lines were maintained in our institution. For *in vitro* study, both cell lines were transfected with shRNA to silence RFC4 according to the manufacture instructions. Short hairpin RNA (Target sequence: AAGGATCGAGGAGTAGCTGCCAG and GGACCACCTGGAAGTGGAAAAAC) was obtained from the National Core Facility for Manipulation of

Gene Function by RNAi, miRNA, miRNA sponges, and CRISPR/Genomic Research Center, Academia Sinica, Taipei, Taiwan. The stable cell lines were verified by RT-qPCR and Western blot before proceeding to the next experiment.

2.5. RT-PCR

All RNA extractions were performed from cells by using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific) according to protocols. After measuring content by ultraviolet analysis, RNA was used to synthesize cDNA for qPCR analysis. Quantitative PCR was performed on a Smart Cycler using SGExcel FastSYBR Mixture (With Low ROX) Plus (Sango Biotech, China). To further analyze the real-time PCR data, we applied a comparative threshold cycle (Ct) method, which compares differences in CT values between target RNA and common control (15). Forward and reverse primers are shown below (RFC4, F, 5'-GCGGAAACCTGAGGAACGAGCC-3' and R, 5'-TGGCAGCTACTCCTCGATCCTTG-3'; GAPDH, F, 5'-GAGTCAACGGATTTGGTCGT-3' and R, 5'-TTGATTTGGAGGGATCTCG-3').

2.6. Western blot

Cell samples were lysed in RIPA lysis buffer, and protein concentrations were determined using the bicinchoninic acid (BCA) method. Then, an equal amount of denatured protein sample was loaded per lane, separated in SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% dry milk for 1h at room temperature, primary antibodies including RFC4 (1:1,500 dilution, ab156780, Abcam), mouse anti-β-actin (1:1,000 dilution, ab8226, Abcam plc, Cambridge, UK) were used. After incubation and washing, membranes were further incubated with secondary antibodies (polyclonal goat anti-rabbit/mouse, 1:10,000 dilutions, Rockland Immunochemicals Inc, PA) for 30 min at 37°C and detected by chemiluminescence. Protein bands were visualized by applying the SuperSignal[™] West Femto Chemiluminescent Substrate (Pierce Biotechnology, USA).

2.7. Colony formation array

Hep3B and SNU-475 cells were seeded and cultured on 60 mm² plates at an initial density of 800/well, each group was measured in 3 parallel wells. After 2 weeks, cells were washed and then fixed with 10% formaldehyde for 15 min at room temperature. Cells were then stained with Giemsa for 15 min. Colony numbers were counted using an optical microscope.

2.8. MTT assay

Both Hep3B and SNU-475 cells were seeded and

cultured on 96-well plates at a density of 4,000 cells/well. The cell's proliferation capacity was measured using MTT (methyl thiazolyl tetrazolium) assay. 0.02 mL of 5 mg/mL MTT reagent were added into each well for 24 hours at 37°C. Then the medium was replaced by 0.15 mL of dimethyl sulfoxide (DMSO, Sigma) for 10 min incubation. Microplate spectrophotometer (Thermo Scientific, Franklin, MA) was applied to measure the optical density at 570 nm. All experiments were performed in triplicate.

2.9. Tumorigenicity assay

For *in vivo* xenograft studies, 5×10^6 Hep3B cells transfected with RFC4 shRNA and controls were subcutaneously injected into the left flank of 8-week-old BALB/c nude mice (Slac Laboratory Animal Co. Ltd, Shanghai, China). Mice were euthanized at 4 weeks post injection, and the tumors were excised and fixed for subsequent histopathological examination and analysis. Meanwhile, tumor volumes were measured twice a week after two weeks, tumor volume = $1/2$ (length \times width²). All animal experiments were approved by the Animal Care and Use Committee of our institution.

2.10. Flow cytometry

Cell cycle progression was analyzed by flow cytometry. HCC cells were trypsinized, counted, washed, fixed by dropwise addition of 70% ethanol and stored at 4°C until analysis. Then cells were washed with PBS, resuspended

in 50 ug/mL propidium iodide (PI) solution and analyzed by flow cytometry.

2.11. Statistical analyses

All data in our study were analyzed by SPSS.22.0. Data are presented as mean \pm SEM. Student's *t*-test were used for continuous variables, χ^2 tests were applied to analyze categorical variables. Survival of patients was plotted using Kaplan-Meier method. In this study, a *p* value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. RFC4 was overexpressed in hepatocellular carcinoma

To explore the potential roles of RFC4 in hepatocellular carcinoma, we first analyzed the mRNA expression of RFC4 in hepatocellular carcinoma and normal liver tissues by Bioinformatics analysis. Differential gene expression analysis was conducted from GTEx and TCGA database and 369 primary HCC samples and 160 normal liver tissues were analyzed. As Figure 1A shows, RFC4 was significantly overexpressed in HCC. Moreover, we further explored the expression of RFC4 in several HCC cell lines through public dataset (CCLE) and further verified *via* RT-PCR. As Figure 1B-C shows, RFC4 represented a significantly higher expression level in HCC cell lines compared with normal liver cell lines. Next, we investigated the expression of

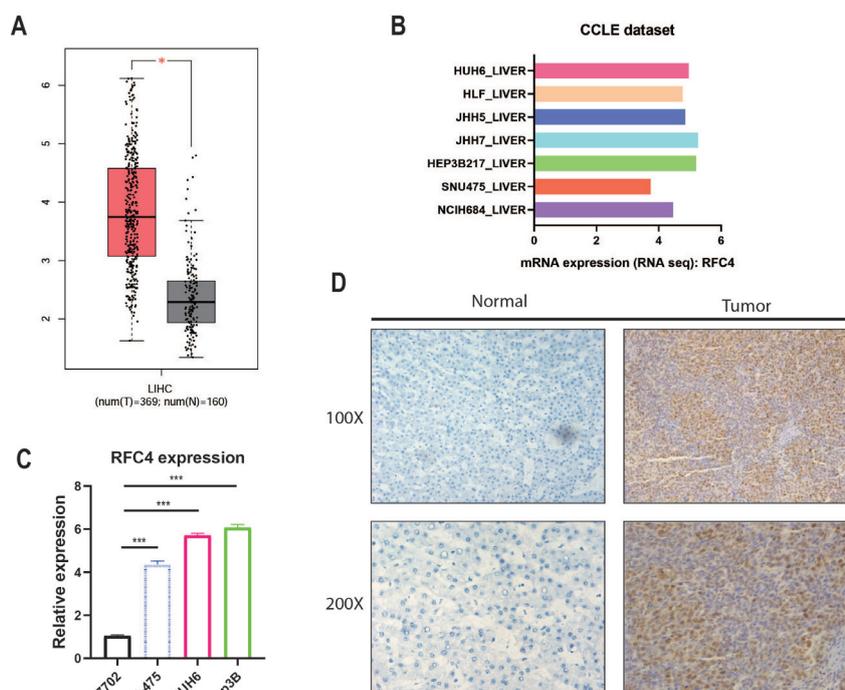


Figure 1. The expression of RFC4 in human HCC cell lines and tissues. (A) The expression of RFC4 mRNA level in HCC and normal liver tissues. **(B)** RFC4 mRNA expression in the CCLE dataset. **(C)** RFC4 mRNA expression in normal liver cell and HCC cell lines were explored by RT-PCR. **(D)** Immunostaining showed high and low expression of RFC4 in HCC and normal liver tissues.

RFC4 protein in HCC and normal liver tissues from 142 patients *via* IHC. Similar to mRNA expression, the expression of RFC4 protein was significantly overexpressed in HCC (Figure 1D). Moreover, the positive signal of RFC4 was mainly expressed in the nuclei of tumor cells and showed typical strong staining (Figure 1D). In contrast, normal liver tissues barely expressed RFC4 (Figure 1D).

3.2. High expression of RFC4 correlated with clinicopathological variables and prognosis of HCC

In order to investigate the correlation between RFC4 protein expression status and clinical-pathological characters of HCC patients, we divided HCC patients into two groups according to the expression of RFC4 protein. The associations between RFC4 protein expression and clinicopathological features are shown in Table 1. High expression of RFC4 protein was

significantly correlated with bigger tumor size ($p = 0.029$). As for other clinicopathological features including age, gender, number of tumor nodes and AFP level, no associations were found (all $p > 0.05$). On the other hand, we assessed the association between RFC4 expression and prognosis to identify the prognostic value of RFC4 for HCC. Overall survival (OS) and disease-free survival (DFS) information were obtained from TCGA database. A significant correlation was found between RFC4 expression and adverse clinical outcome including short OS and DFS of HCC patients (Figure 2A-B).

3.3. RFC4 promotes the proliferation of HCC cancer cells

To understand the potential roles of RFC4 in malignant behavior of HCC, we inhibited the expression of RFC4 in HCC cancer cells. RFC4 expression was knocked

Table 1. Relationships of RFC4 and clinicopathological characteristics in 142 patients with hepatocellular carcinoma

Feature	All, $n = 142$	RFC4 expression		χ^2	p
		Low, $n = 60$	High, $n = 82$		
Age (year)				3.169	0.075
< 60	80	39	41		
≥ 60	62	21	41		
Gender				0.021	0.886
Male	72	30	42		
Female	70	30	40		
Number of tumor nodes				3.388	0.066
Single	60	20	40		
Multiple ≥ 2	82	40	42		
Tumor size				4.760	0.029
< 5 cm	63	33	30		
≥ 5 cm	79	27	52		
AFP (ng/mL)				0.483	0.487
< 50	52	20	32		
≥ 50	90	40	50		

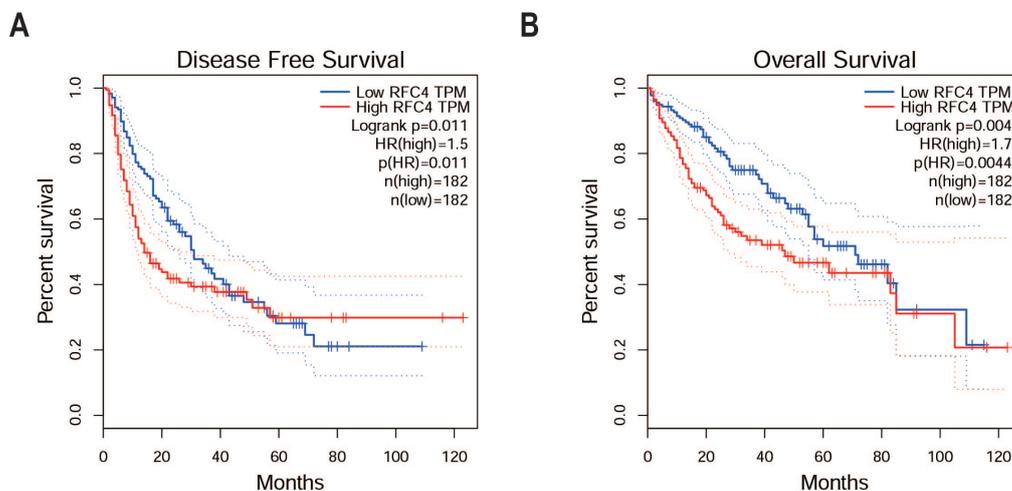


Figure 2. High expression of RFC4 as a prognostic factor of human HCC. (A) Kaplan-Meier survival analysis of disease-free survival for RFC4 expression in HCC. **(B)** Kaplan-Meier survival analysis of overall survival for RFC4 expression in HCC.

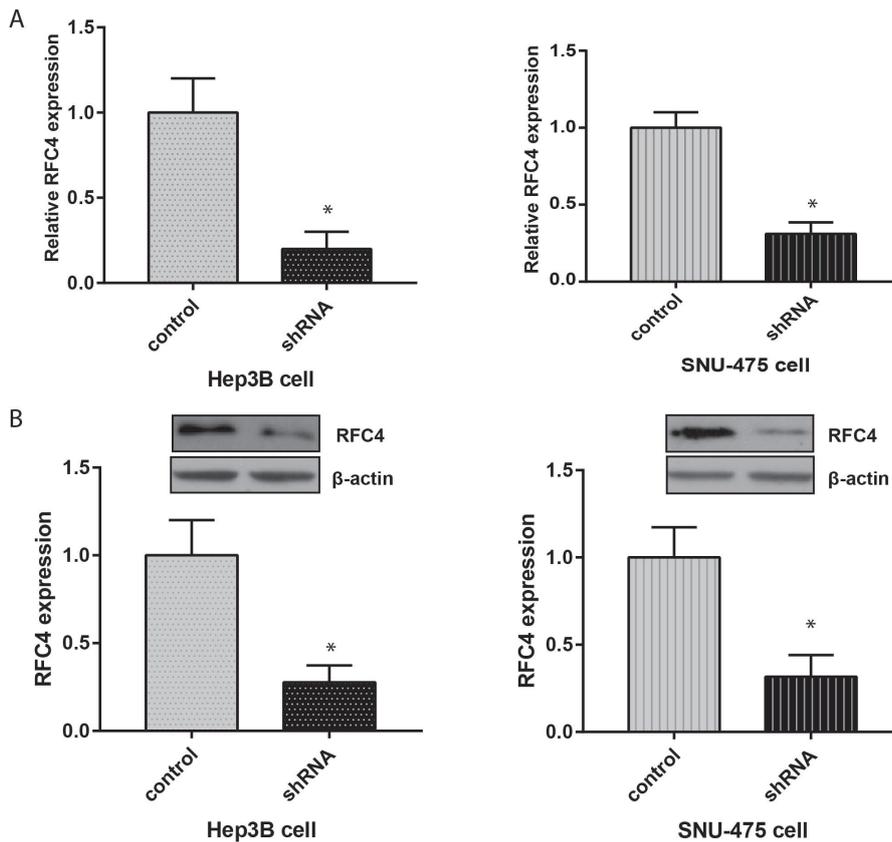


Figure 3. Stably knocking down of RFC4 by shRNA in both Hep3B and SNU-475 cells. (A, B) RT-PCR and Western blot showed lower expression level of RFC4 in shRNA group ($p < 0.05$), compared to the control group, just as expected.

down in both Hep3B and SNU-475 cells using shRNA. Both RT-PCR and Western blot results showed that shRNA worked as we expected, the expression of RFC4 was dramatically decreased in the shRNA group (Figure 3A-B).

Next, we tested whether loss-of-function of RFC4 is correlated with the proliferation of HCC cells by MTT assay and colony formation arrays. We found that down-regulation of the expression of RFC4 significantly inhibited the proliferation of Hep3B and SNU-475 cells compared to controls ($p < 0.05$), manifested as decreased cell proliferation rate and colony formation (Figure 4A-B). Flow cytometry further indicated that cell cycle arrest was induced after knocking down RFC4 (Figure 4C). Cells remaining in G2 phase significantly increased after RFC4 ablation.

3.4. Down-regulation of the expression of RFC4 suppressed the tumorigenicity of HCC cells *in vivo*

To further explore whether the role of RFC4 is associated with abnormal *in vitro* behavior and can be translated into abnormal tumorigenesis *in vivo*, cells from RFC4 shRNA group and control group were injected subcutaneously into athymic mice respectively. The tumor volumes were measured twice a week after two weeks. As the growth curve shows in Figure 5A, tumor growth of shRNA group was significantly slower than that of control group ($p < 0.05$). Meanwhile, we

also performed IHC to detect the expression of RFC4 in subcutaneous tumors. Consistently, RFC4 expression was dramatically decreased in shRNA group, which indicated that an effective and stable knocking-down of RFC4 was built into mice tumors (Figure 5B).

4. Discussion

In this study, we explored the potential role of RFC4 in HCC. We demonstrated that RFC4 was overexpressed in HCC tissues and high RFC4 expression represents a poor clinical outcome. Furthermore, we investigated the function of RFC4 in HCC aggression. Consistent with previous reports, RFC4 may be an effective marker and probably even a potential therapeutic target for HCC.

RFC family members play important roles in eukaryotic DNA replication and DNA repair activities following DNA damage. Among them, RFC4, which encodes the fourth largest subunit of the RFC complex is also involved in several other biological processes (7,16). Deregulation of RFC4 may contribute to cell proliferation and tumorigenesis. Moreover, it has been reported to be deregulated in diverse malignancies, including cervical cancer, colorectal cancer, and prostate cancer (10-13). In our study, we indicated that RFC4 plays an important role in tumor cell proliferation *via* MTT assay and colony formation. The knockdown of RFC4 expression in HCC lines by siRNA resulted in

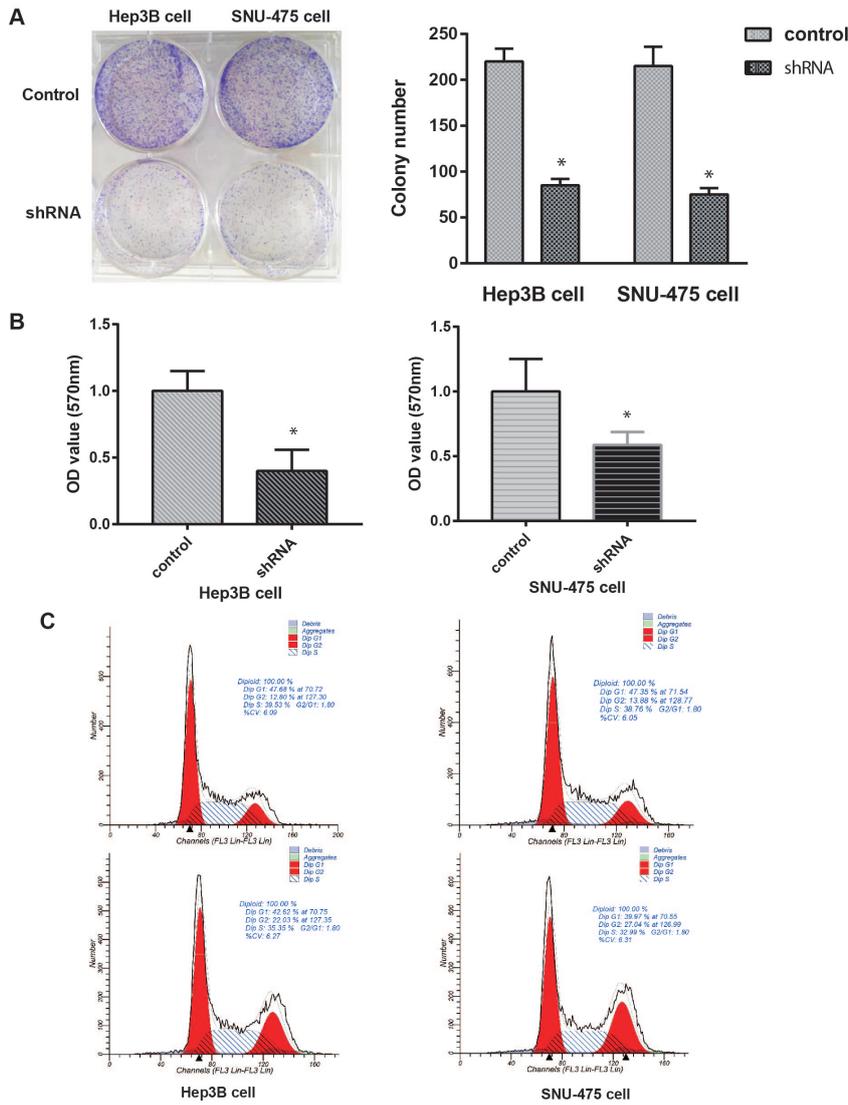


Figure 4. Knocking down RFC4 in HCC cancer cells inhibited tumor cell proliferation. (A) Typical images of colony-forming assay and its quantification demonstrated that colony rate of shRNA group was significantly lower than control group in both cancer cell lines ($p < 0.05$). (B) OD value of MTT assay suggested that cell proliferation rate of shRNA group was lower than control group in both cancer cell lines ($p < 0.05$). (C) Cell cycle arrest of HCC cells induced by RFC4 ablation. Cell cycle was analyzed by flow cytometry.

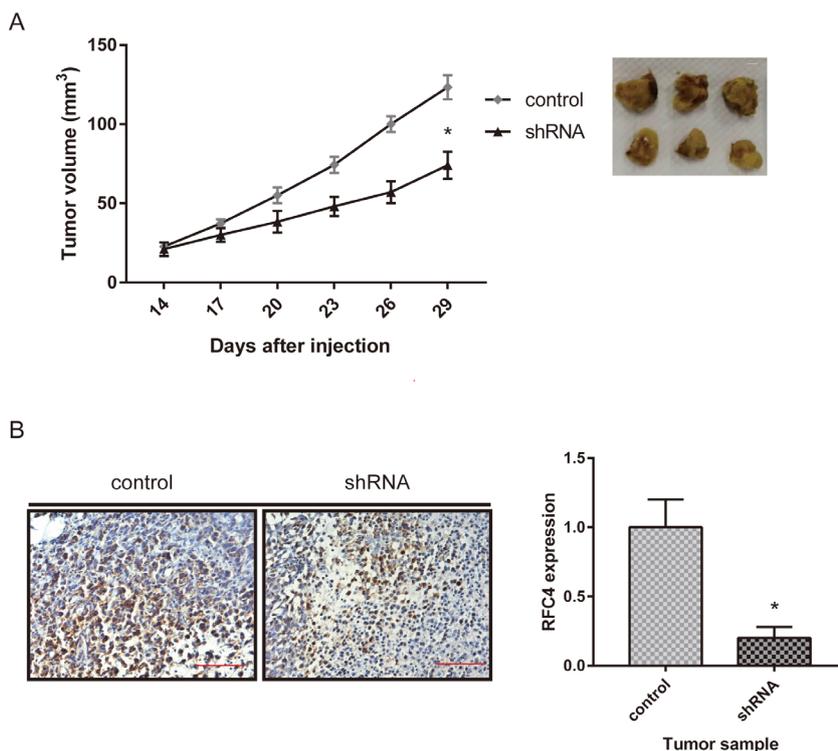


Figure 5. The influence of RFC4 on tumor growth of HCC in mice. (A) Representative images of tumors and tumor growth curves from both groups are shown. Tumor volumes of shRNA group were smaller than that of the control group ($p < 0.05$). Scale bar 2 mm. (B) Immunohistochemistry demonstrated that the expression of RFC4 was dramatically decreased in mice tumors ($p < 0.05$), which suggested a successful construction of RFC4 knocked down model in mice. Scale bar 100 μ m.

a significant decrease in cell proliferation. This result may suggest that RFC4 is involved in DNA replication in cancer cells. Consistent with our results, Arai *et al.* found that knock down of RFC4 in HepG2 cells arrested the growth of HepG2 cells in S phase and increased cell apoptosis.

The current status of HCC diagnosis and treatment urgently requires us to search for potent molecular targets for therapies directed against hepatocellular carcinoma. Several studies have reported that aberrant expressions of RFC4 can be a promising prognostic marker and even a therapeutic target in a number of malignancies (14,15,17). In our present analyses, data from public databases suggested that high expression of RFC4 was correlated with clinical-pathological characteristics and poor OS and DFS of HCC patients. Meanwhile, we also found that high expression of RFC4 protein was significantly correlated with larger tumor size ($p = 0.029$). All the above results suggested that RFC4 is a potential molecular target for modulating the growth of hepatocellular cancer cells. Similar to our findings, Wang *et al.* identified RFC4 as a radioresistant factor that promotes NHEJ-mediated DNA repair in colorectal cancer cells *via* genome-wide RNAi screen and the expression level of RFC4 predicted radiotherapy responsiveness (10). By using public databases, Kong *et al.* demonstrated that five core genes including RFC4 and relevant cell cycle-related pathways play significant roles in HCC progression and prognosis and could greatly improve knowledge about HCC progression (11). Niu *et al.* found that RFC4 was not only changed in high-grade squamous intraepithelial lesions, but also significantly changed in squamous cell carcinomas, indicating that its dysregulation may contribute to cervical cancer development (12). In addition, using immunohistochemistry analysis in a tissue microarray comprising 331 surgically resected colorectal cancer sections, Xiang *et al.* reported that RFC4 is frequently overexpressed in colorectal cancer, and is associated with tumor progression and a worse survival outcome (13).

In conclusion, we elaborately explored the roles of RFC4 in HCC. Though more research is still needed to explore and verify the exact underlying mechanisms of HCC development and progression, we speculate that RFC4 can be a prognostic marker and even a therapeutic target for HCC.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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