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miRNA-217 inhibits proliferation of hepatocellular carcinoma cells by regulating KLF5

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Abstract. – OBJECTIVE: The aim of this study was to figure out the effect of microRNA-217 on the proliferation of hepatocellular carcinoma (HCC) cells, and to explore its influence on KLF5 expression and the underlying regulatory mechanisms.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of microRNA-217 in tumor tissues and paracancerous tissues of 60 patients with HCC. Meanwhile, the relationship between microRNA-217 expression and HCC pathological parameters was analyzed. In HCC cell lines including HepG2 and Bel-7402, negative control group (NC) and microRNA-217 overexpression group were set up, and qRT-PCR was performed to further verify their transfection efficiency. In addition, Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) assay were performed to analyze the effect of microRNA-217 on the biological function of HCC cells. Finally, the potential mechanism of KLF5, the downstream gene of microRNA-217, was explored using bioinformatics analysis and cell recovery experiments.

RESULTS: QRT-PCR results showed that microRNA-217 level in tumor tissues of HCC patients was conspicuously lower than that in adjacent tissues, and the difference was statistically significant. Compared with patients with high expression of microRNA-217, the pathological stage was higher and the overall survival rate was lower in patients with low expression. Compared with the NC group, the cell proliferation ability of the microRNA-217 mimics group was conspicuously decreased. Subsequently, in the HCC cell line and tissue verification, the expression of KLF5 was found remarkably increased, and microRNA-217 exhibited a negative correlation with KLF5 level. In addition, the overexpression of microRNA-217 conspicuously reduced the protein expression of CD31, Ki-67, c-Myc, MMP-2, and MMP-9. In cell recovery experiment, it was found that the overexpression of KLF5 could counteract the effect of microRNA-217 mimics on the cell proliferation of HCC, thereby inhibiting the malignant progression of this disease.

CONCLUSIONS: The above studies demonstrated that microRNA-217 was markedly associated with the pathological stage and poor prognosis of HCC, and could inhibit the malignant progression of this disease. In addition, our investigation has showed that microRNA-217 might be capable of inhibiting cell proliferation of HCC *via* regulating KLF5.

Key Words:
MicroRNA-217, KLF5, Hepatocellular carcinoma, Proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and malignant tumors in the world, with its incidence ranks fifth in malignant tumors and its mortality ranks third[1-3]. The comprehensive treatment of liver cancer includes surgical resection, transcatheter arterial chemoembolization, liver transplantation and radiofrequency ablation. Surgical treatment is the preferred method for liver cancer treatment. However, although liver transplantation can obtain satisfactory results, the development of this surgery is greatly limited due to the lack of donor liver[4-6]. Hepatic arterial chemoembolization and radiofrequency ablation have relatively good curative effects. However, due to the insidious onset of liver cancer, most patients are diagnosed at an advanced stage, and only 30% to 40% of patients can receive radical treatment, for most of the advanced liver cancer patients have to undergo palliative treatment[6,7]. Due to the limitations of various treatments, the recurrence and metastasis of the disease itself, the severity of the combined liver disease and liver failure during treatment, the therapeutic effects of various treatments are still limited. Therefore, it

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is urgent to do the research on the pathogenesis of liver cancer, and further develop new treatment methods, to improve the prognosis of patients with liver cancer and the quality of their life[8,9].

MicroRNA is a 22-24 nucleotide-sized molecule that has been found to regulate gene expression in recent years. It binds mainly to the 3'-Untranslated Regions (3'-UTR) of the target gene and regulates gene expression at the post-transcriptional level[10-12]. If the microRNA is fully complementary to the 3'-UTR of the target gene, the expression of the target gene can be reduced by the microRNA by degrading mRNA of the target gene. But if the microRNA is not completely complementary to the 3'-UTR of the target gene, the expression of target genes is reduced *via* blocking protein translation[12-14]. In addition to the special mechanism of regulating gene expression, microRNA expression is also tissue-specific, which means that the expression level of the same microRNA molecule in different tissues may be different, and the expression levels of different microRNA molecules in the same tissue are also not the same[14]. Besides, the same microRNA molecule can have different kinds of target genes, and different microRNA molecules can also regulate the expression of the same gene, which constitutes a complex regulatory network in the body[15]. MicroRNA-217, a kind of microRNA molecule, has recently been extensively studied in tumors[16-18]. It has recently been discovered that miRNAs may be a novel therapeutic target for human tumors and have been found to regulate KLF5 signaling in a variety of tumor cells. Therefore, targeting KLF5 in hepatoma cells may be a potential target therapy[19,20]. Subsequently, we speculated through bioinformatics analysis that microRNA-217 may target KLF5 in tumor cells to inhibit cell proliferation of hepatocellular carcinoma, which can become a new direction of tumor-targeted therapy.

This study separately described the possible role and potential mechanism of microRNA-217 and KLF5 in the development of hepatocellular carcinoma. This may bring new ideas for the diagnosis and treatment of this disease.

Patients and Methods

Patients and HCC Samples

Tumor tissues and adjacent tissues were collected of 60 patients aged 42-89 years with hepatocellular carcinoma who underwent radical collection. All subjects underwent no radiotherapy or chemotherapy before surgery. The pathological type and staging criteria of hepatocellular carcinoma were performed according to the international association of cancer (UICC) hepatocellular carcinoma staging criteria. The informed consent has been signed by patients and/or their families. Our research was approved by the Ethics Oversight Committee.

Cell Lines and Reagents

Six human HCC cells (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one hu-

Table I. Association of miR-217 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	MiR-217 expression		*p*
		High (%)	Low (%)	
Age (years)				0.792
<60	25	13	10	
≥60	35	21	14	
Gender				0.399
Male	29	19	10	
Female	31	17	14	
T stage				0.008
T1-T2	35	26	9	
T3-T4	25	10	15	
Lymph node metastasis				0.129
No	37	25	12	
Yes	23	11	12	
Distance metastasis				0.107
No	42	28	14	
Yes	18	8	10	

man normal liver cell line (LO2) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an incubator with 5% CO_2 at 37°C. Cells were passaged with 1 x trypsin + EDTA (Ethylene Diamine Tetraacetic Acid) when grown to 80% - 90% of confluence.

Transfection

Negative control (NC) and microRNA-217 overexpression sequence (microRNA-217 mimics) were purchased from Shanghai Jima Company (Shanghai, China). Cells were seeded in 6-well plates and grown to a cell density of 70%. Afterward, transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), and cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell functional experiment after 48 hours.

Cell Proliferation Assay

After 48 h of transfection the cells were collected and seeded into 96-well plates with 2000 cells per well. After culturing for 24 h, 48 h, 72 h and 96 h respectively, the cells were treated with Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

To demonstrate the role of microRNA-217 in cell proliferation, EDU assay (RiboBio, Guangzhou, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μm EDU for 2 h, stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI), and the number of EDU-positive cells was detected by fluorescence microscopy. The display rate of EDU positive was shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

Quantitative Real Time-Polymerase Chain Reaction

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, and Real Time-PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real Time-PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: microRNA-217: forward: 5'-TTAGCTCAGGATCATCATCATTTACATAGATAGGG-3'; reverse: 5'-AACACTCGAGTGAGAGAAGAGAGTGCCTAGA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'- AACGCTTCACGAATTTGCGT-3'; KLF5: forward: 5'-CTTCCACAACAGGCCACTTACTT-3', reverse: 5'-AGAAGCAATTGTAGCAGCATAGGA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Each sample was subjected to a three-well repeated experiment. A Bio-Rad PCR instrument was used to analyze and process the data (Bio-Rad, Hercules, CA, USA). The β-actin and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 minutes at 4°C. The total protein concentration was calculated by the HCCA Protein Assay Kit (Pierce, Waltham, MA, USA). Anti-KLF5, CD31, Ki-67, c-Myc, MMP-2, MMP-9 rabbit anti-human monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from GenScript (Piscataway, NJ, USA), with internal reference control using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Tissue protein concentration was determined by the Bradford method. Then, the protein samples experienced sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and membrane transfer. The membrane was blocked overnight, and the primary and secondary antibodies were added for electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) coloration. The image was

Table II. Univariate and multivariate analysis of prognostic factors in for overall survival.

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age (years)	1.687 (0.682-2.318)	0.244	-	-
Gender	1.446 (0.782-1.994)	0.129	-	-
Tumor size	1.285 (0.895-1.827)	0.114	-	-
Histological grade	1.192 (0.572-2.442)	0.092	-	-
Lymph nodes metastasis	3.261 (1.337-5.018)	0.002	2.885 (1.124-4.572)	0.006
Clinical stage	3.032 (1.248-4.869)	0.007	2.857 (1.123-4.362)	0.013
LEF1-AS1 expression	3.162 (1.225-4.654)	0.008	2.942 (1.169-4.156)	0.014

semi-quantitatively analyzed by alpha SP image analysis software.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Univariate analysis was performed using the χ^2 test and the exact probability Fisher test. Multivariate analysis was performed using COX regression analysis. Patient survival was analyzed using the Kaplan-Meier method and intergroup curves were compared using the Log-rank test. $p<0.05$ was considered statistically significant.

Results

Downregulated MicroRNA-217 in Primary Lung Cancer Tissues, and Reduced Expression of MicroRNA-217 Connected With Advanced Clinical Procedure and Poor Patient Prognosis

The hepatocellular carcinoma tissues and cell lines were verified by qRT-PCR. The results showed that the expression level of microRNA-217 in tumor tissues was conspicuously lower than that in the adjacent tissues (Figure 1A and 1B). In addition, compared with LO2, microRNA-217 was conspicuously down-regulated in HCC cells, especially in HepG2 and Bel-7402 cell lines, with the difference most significant, so we chose these two cells for follow-up experiment (Figure 1C). According to the expression of microRNA-217 in 60 pairs of tumor tissues and paracancerous tissues, we divided these tissues into high expression group and low expression group to explore the relationship between microRNA-217 expression and prognosis of HCC patients. The Kaplan-Meier survival curves showed that low expression of microRNA-217 was conspicuously associated with poor prognosis of HCC ($p<0.05$; Figure 1D).

Subsequently, we further analyzed the relationship between microRNA-217 expression and age, gender, clinical stage, lymph node metastasis and distant metastasis of HCC patients. As shown in Table 1, low expression of microRNA-217 was positively correlated with the clinical stage of HCC, but not with age, gender, lymph node metastasis, and distant metastasis. Therefore, the above results suggested that microRNA-217 might be a new biological indicator for predicting the malignant progression of HCC.

Upregulation of MicroRNA-217 Inhibited Cell Proliferation

To explore the effects of microRNA-217 on HCC cell function, we first successfully constructed the microRNA-217 overexpression model and verified it by qRT-PCR (Figure 2A). Then, we performed cell proliferation experiment in HepG2 and Bel-7402 cell lines, respectively. The result of CCK8 assay showed that the proliferation rate of cells in the microRNA-217 mimics group was conspicuously decreased compared with the NC group (Figure 2B). Subsequently, using cell cloning and EdU assay, it was confirmed that tumor cell proliferation was markedly reduced in the microRNA-217 mimics group compared with the NC group, suggesting that the cell proliferative capacity was inhibited (Figure 2C, 2D).

KLF5 Was Highly Expressed in HCC Tissues and Cell Lines

The qRT-PCR assay was performed to verify the level of KLF5 in hepatocellular carcinoma tissues and cell lines, and the results showed that the expression of KLF5 was remarkably increased in HCC tumor tissues compared to adjacent tissues (Figure 3A). In addition, KLF5 was notably higher

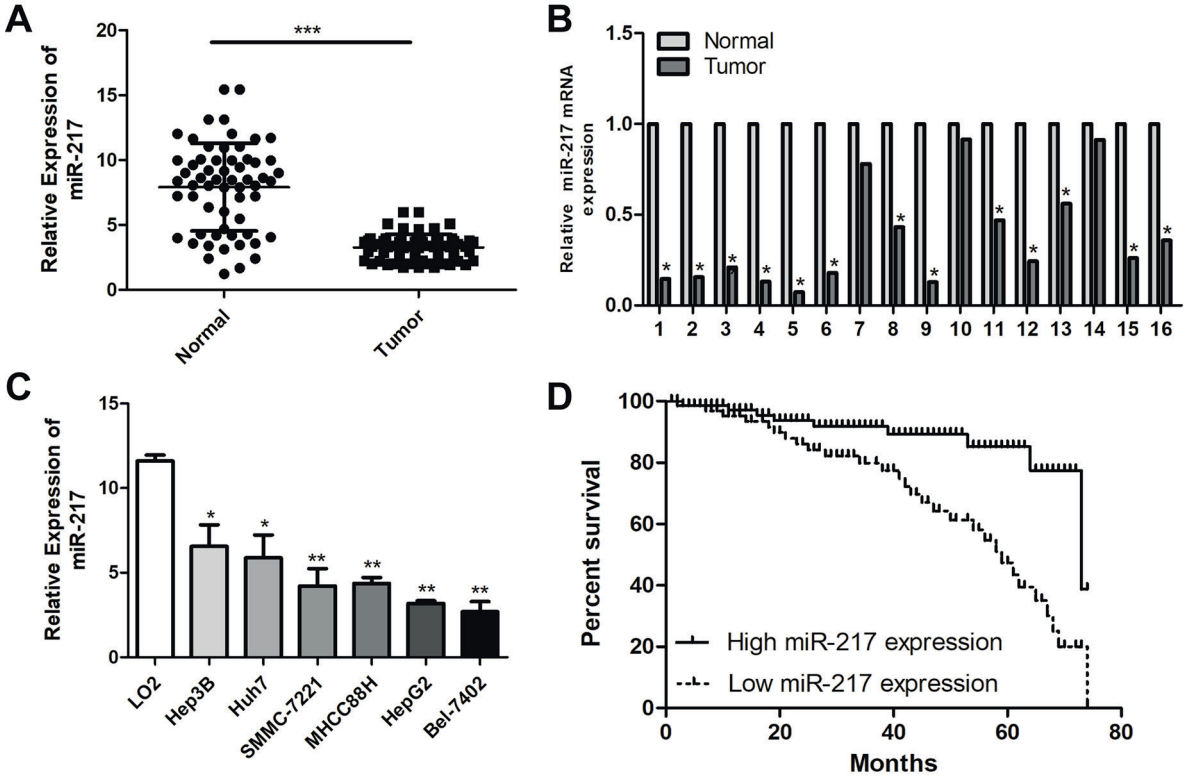

Figure 1. MicroRNA-217 is lowly expressed in hepatocellular carcinoma tissues and cell lines. **A, B,** qRT-PCR detection of differential expression of microRNA-217 in hepatocellular carcinoma tumor tissues and paracancerous tissues. **C,** qRT-PCR detection of microRNA-217 expression levels in hepatocellular carcinoma cell lines. **D,** Kaplan-Meier survival curve of hepatocellular carcinoma patients based on microRNA-217 expression. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

in HCC cells than in LO2 (Figure 3B). Therefore, we detected the levels of microRNA-217 and KLF5 using qRT-PCR in hepatocellular carcinoma tissues. The results showed that microRNA-217 and KLF5 were negatively correlated in tumor tissues of HCC patients (Figure 3C). Similarly, the expression of KLF5 was significantly reduced in the HepG2 and Bel-7402 cell lines after overexpression of microRNA-217 (Figure 3D).

Upregulated MicroRNA-217 Decreased the Expression of KLF5 Related Signaling Pathway

To further explore the way in which microRNA-217 promotes malignant progression of hepatocellular carcinoma, we examined the expression of key proteins in the KLF5-related pathway after overexpression of microRNA-217. Western blot result revealed that the up-regulation of microRNA-217 markedly reduced the expression of key proteins including KLF5, CD31, Ki-67, c-Myc, MMP-2, and MMP-9 in the pathway, thereby inhibiting the progression of HCC (Figure 3E).

MicroRNA-217 Exactly Inhibited KLF5 Gene Expression

To further explore the way in which microRNA-217 inhibits the malignant progression of hepatocellular carcinoma, we overexpressed KLF5 in the HCC cells which had been transfected with NC and microRNA-217 mimics, and the transfection efficiency was examined by PCR and Western blot experiments (Figure 4A and 4B). Subsequently, we demonstrated by cell cloning and EdU experiments that the overexpression of KLF5 counteracted the effect of microRNA-217 mimics on HCC cell proliferation (Figure 4C and 4D).

Discussion

Liver cancer is one of the most common malignant tumors of the digestive tract in China. An important feature of liver cancer is that once the patient is diagnosed, it is often in the middle and late stages, which is extremely unfavorable for

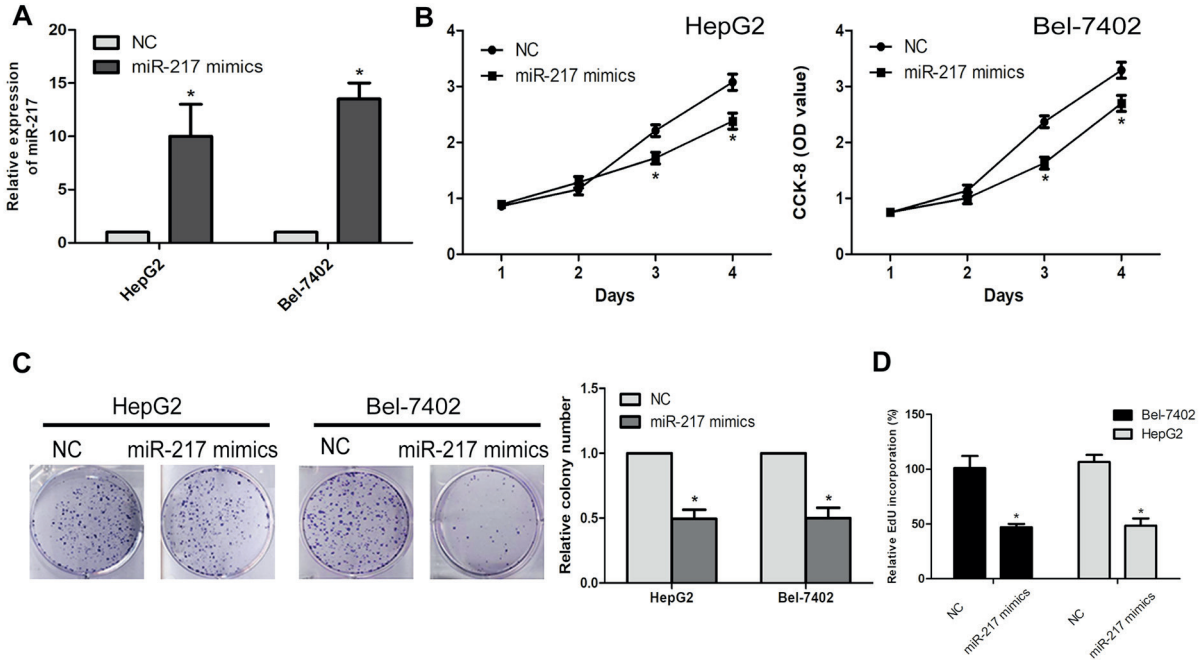

Figure 2. MicroRNA-217 mimics inhibits the proliferation of hepatocellular carcinoma cells. **A,** qRT-PCR validates the transfection efficiency of microRNA-217 after transfection of NC and microRNA-217 mimics in HepG2 and Bel-7402 cell lines. **B,** CCK-8 assay was performed to detect the proliferation of hepatocyte cancer cells in HepG2 and Bel-7402 cell lines. **C,** Cell cloning assay to detect the proliferation of hepatocyte cancer cells in HepG2 and Bel-7402 cell lines (magnification: 10×). **D,** EdU assay to detect the proliferation of hepatocyte cancer cells in HepG2 and Bel-7402 cell lines. Data are mean ± SD, *$p<0.05$.

the treatment of the disease. Therefore, the early diagnosis of liver cancer is very meaningful for patients[1-3]. However, the pathogenic factors of liver cancer are very complicated, and the recognized HBV is an important pathogenic factor. But there are still many cases in which HBV carriers do not develop liver cancer, so individual differences also exist in the pathogenic process of the disease[21,22]. Many studies[22-23] have been carried out on the pathogenesis of liver cancer, trying to find high-specific and critical diagnostic markers, laying the foundation for early diagnosis. In recent years, the role of epigenetic regulation in the pathogenesis of diseases, especially malignant tumors, has received increasing attention[7-9]. Therefore, it is crucial to explore an early non-invasive diagnostic technique for hepatocellular carcinoma and specific treatment based on the gene level.

The regulation of genes by microRNA molecules at the post-transcriptional level is one of the hotspots of current research[10-12]. MicroRNAs can be involved in the regulation of many cellular life processes such as proliferation, senescence, apoptosis, and autophagy by regulation of target genes[10,13]. If the microRNA molecule inhibits the expression of the oncogene, it will function as a tumor suppressor gene, but if the microRNA inhibits the expression of the tumor suppressor gene, it conversely becomes a carcinogenic microRNA molecule[13-16]. MicroRNA-217, one of the miRNA molecules, has been discovered for a long time, but its biological function has just begun to be studied. It has been verified[16-18] to play a role in inhibiting tumors and participate in many physiological and pathological processes such as osteosarcoma, cervical cancer, etc. To explore the role of microRNA-217 in hepatocellular carcinoma, the expression of microRNA-217 in 60 cases of hepatocellular carcinoma tissues and paracancerous tissues was detected by RT-PCR. As a result, microRNA-217 level in hepatocellular carcinoma tissues was found down-regulated and conspicuously lower than that in the adjacent tissues. In addition, its expression level was found notably correlated with clinical stage. Therefore, we believed that microRNA-217 might play a role in tumor suppression in HCC. The expression of KLF5 was then examined by RT-PCR, and the results

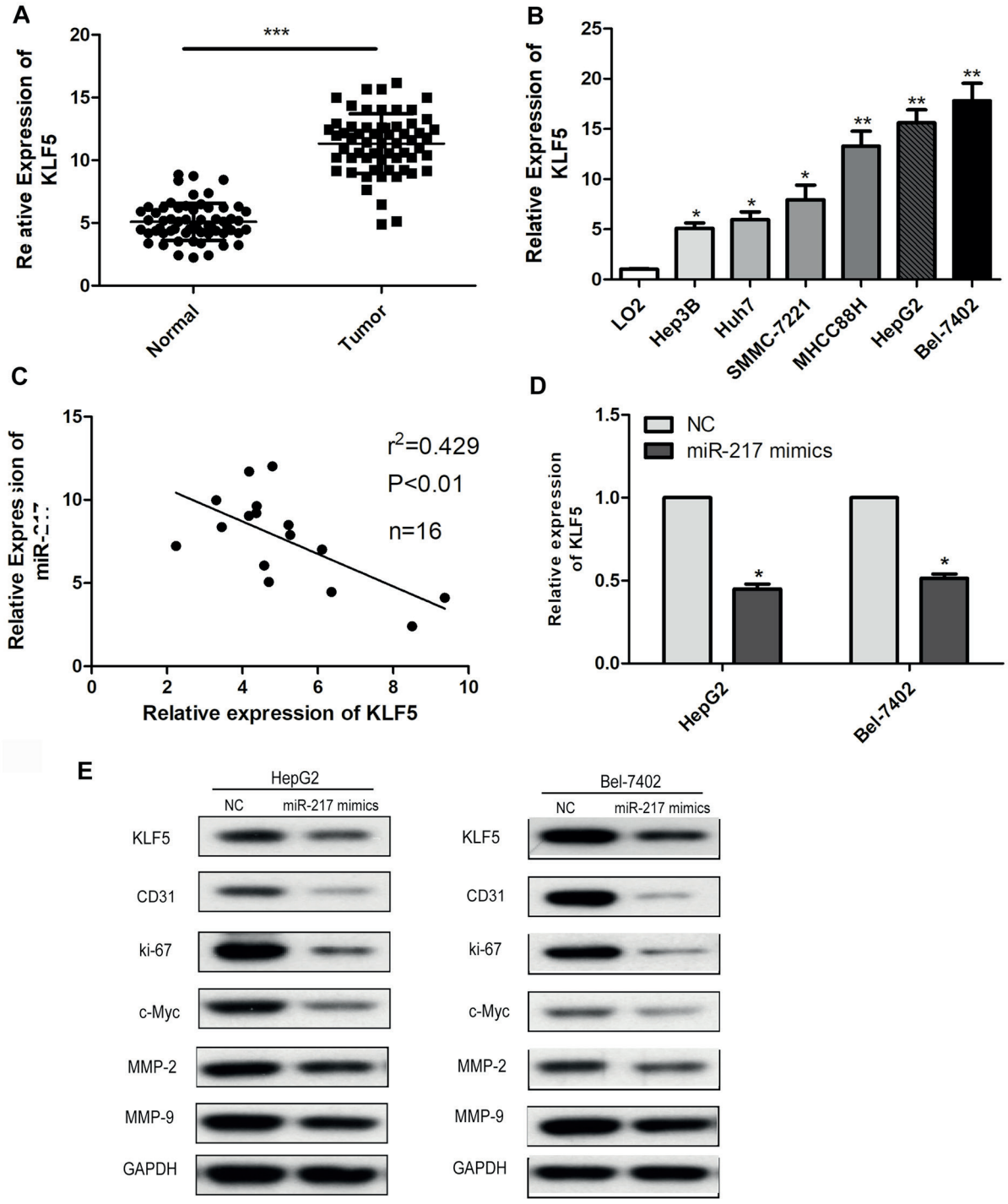

Figure 3. KLF5 is highly expressed in hepatocellular carcinoma tissues and cell lines. **A,** qRT-PCR detection of differential expression of KLF5 in hepatocellular carcinoma tumor tissues and adjacent tissues. **B,** qRT-PCR detection of KLF5 expression levels in hepatocellular carcinoma cell lines. **C,** miR-217 in hepatocellular carcinoma tissues was markedly negatively correlated with the expression level of KLF5. **D,** qRT-PCR verified the expression level of KLF5 after transfection of microRNA-217 mimics in HepG2 and Bel-7402 cell lines. **E,** Western blotting revealed the protein expression levels of KLF5, CD31, Ki-67, c-Myc, MMP-2 and MMP-9 in HepG2 and Bel-7402 cell lines after microRNA-217 was overexpressed. Data are mean ± SD, *p< 0.05, **p<0.01, ***p<0.001.

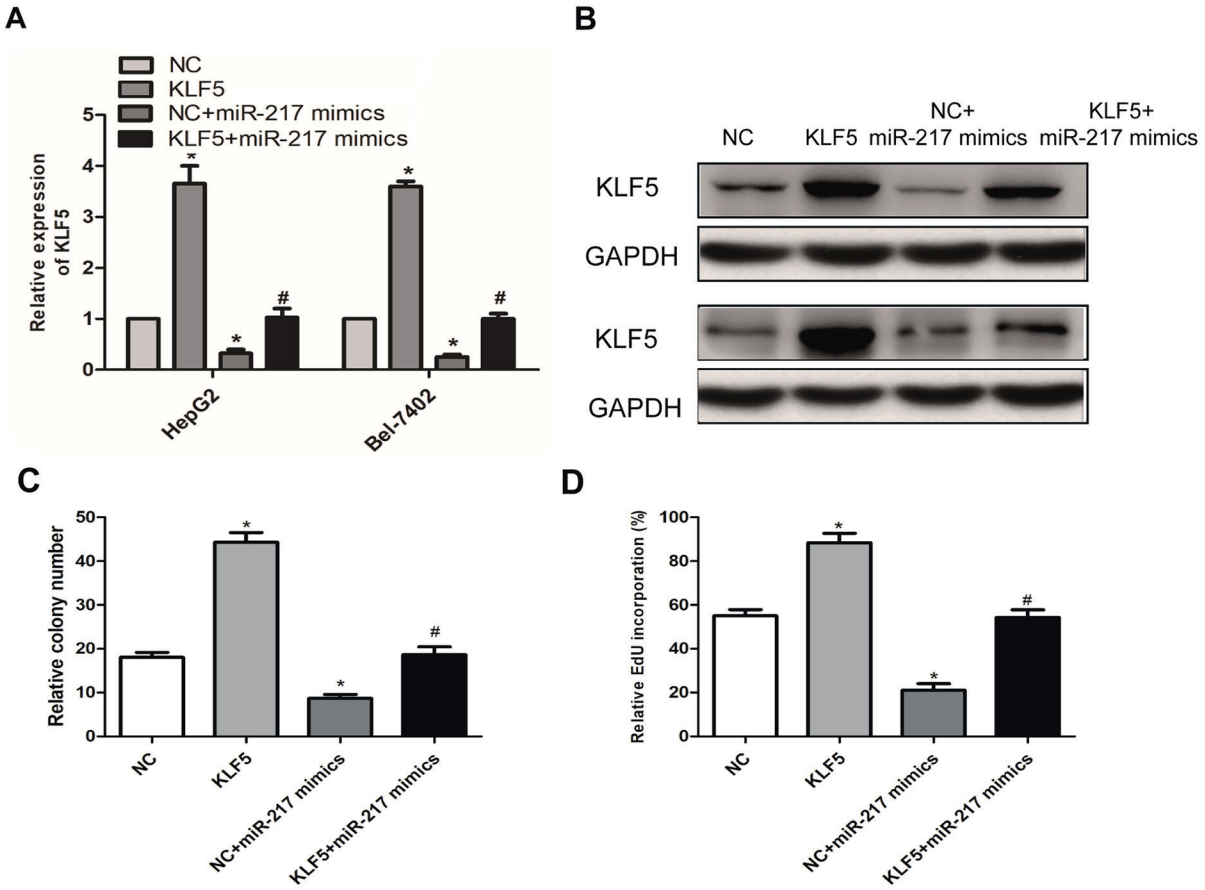

Figure 4. MicroRNA-217 regulates the expression of KLF5 in hepatocellular carcinoma tissues and cell lines. **A,** detection of KLF5 expression levels in microRNA-217 and KLF5 co-transfected cell lines by qRT-PCR. **B,** Detection of KLF5 expression levels in microRNA-217 and KLF5 co-transfected cell lines by Western blot. **C,** Cell cloning assay detects the proliferation of hepatocellular carcinoma cells after co-transfection of microRNA-217 and KLF5. **D,** EdU assay detects the role of microRNA-217 and KLF5 in co-transfection of hepatocellular carcinoma positive cells. Data are average ± SD, *#$p<0.05$.

showed that its level in tumor tissues was conspicuously up-regulated compared with matched adjacent tissues. The expression of microRNA-217 was different in various tissues and different hepatocellular carcinoma cell lines. The levels of microRNA-217 were found the lowest in HepG2 and Bel-7402 cell lines, which were then chosen for follow-up experiments.

To further explore the effect of microRNA-217 on the biological function of HCC, we constructed a microRNA-217 overexpression model using miRNA-217 mimics. The results of CCK8, cell cloning and EdU experiments indicated that microRNA-217 could inhibit the proliferation of HCC. In addition, the expression levels of KLF5, CD31, Ki-67, c-Myc, MMP-2, and MMP-9 were significantly decreased after microRNA-217 was up-regulated, and the development of HCC was thereby inhibited. In the current work, microRNA-217 was found markedly down-regulated in hepatocellular carcinoma, suggesting that this microRNA might have a potential role in hepatocellular carcinoma. However, its molecular mechanism remains unclear.

Research evidence[19-21] has shown that there is a mutually regulated relationship between microRNA molecules and their target molecules. In this issue, bioinformatics analysis showed that microRNA-217 inhibited cell proliferation by acting on KLF5. It was found that KLF5 could express in hepatocellular carcinoma cells and promote the malignant progression of tumor cells. To explore the effect and interaction of microRNA-217 and KLF5 on the development and progression of hepatocellular carcinoma, we further verified that microRNA-217 mimics remarkably down-regulated the expression of KLF5 mRNA and protein. Subsequently, we used a cell recovery experiment

to verify that the overexpression of KLF5 counteracted the ability of microRNA-217 mimics to promote HCC cell proliferation. The above findings suggested that there might exist a feedback regulation loop, which was, KLF5 could reverse the biological effects of microRNA-217 on HCC cells, thereby jointly affecting the malignant progression of hepatocellular carcinoma.

Conclusions

MicroRNA-217 was conspicuously associated with pathological stage and poor prognosis of HCC patient; besides, it could inhibit the malignant progression of HCC by regulating KLF5.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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