European Review for Medical and Pharmacological Sciences 2019; 23: 2786-2793

HCP5 promotes colon cancer development by activating AP1G1 via PI3K/AKT pathway

W.-K. YUN[1], Y.-M. HU[1], C.-B. ZHAO[1], D.-Y. YU[1], J.-B. TANG[2]

[1]Department of Radiotherapy, Harbin Medical University Cancer Hospital, Harbin, China.
[2]Department of Oncology, Harbin Medical University Cancer Hospital, Harbin, China.

Abstract. – OBJECTIVE: To explore whether HCP5 participates in the pathogenic progression of colon cancer (CC) and its underlying mechanism.

PATIENTS AND METHODS: HCP5 expression in CC tissues and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between the HCP5 expression and tumor stage of CC patients was then analyzed. After CC cells were transfected with HCP5-siRNA, the proliferation and migration capacities were detected by cell counting kit-8 (CCK-8), colony formation and transwell assay, respectively. Cell cycle was examined by flow cytometry. Western blot was conducted to detect protein expressions of HCP5, AP1G1 and relative molecules in the PI3K/AKT pathway. Rescue experiments were performed by co-transfection of HCP5-siRNA and AP1G1-siRNA into CC cells, followed by cell function detection.

RESULTS: HCP5 was highly expressed, whereas AP1G1 was lowly expressed in CC tissues and cell lines. Besides, CC patients with stage III-IV presented higher expression of HCP5 than those with stage I-II. The knockdown of HCP5 in CC cells down-regulated proliferation and migration capacities, and arrested cell cycle in the G0/G1 phase, which was reversed by the AP1G1 knockdown. In addition, HCP5 knockdown up-regulated AP1G1 expression, whereas down-regulated the expression of relative proteins in the PI3K/AKT pathway.

CONCLUSIONS: HCP5 was significantly increased in CC and enhanced the proliferation and migration of CC cells by inhibiting the AP1G1 expression. HCP5 promoted CC development by activating the PI3K/AKT pathway.

Key Words:
CC, HCP5, AP1G1, PI3K/AKT pathway.

Introduction

Colon cancer (CC) is a common malignancy in the digestive tract and frequently occurs at the junction of the rectum and sigmoid colon[1]. In recent years, the incidence of CC has been on the rise. Although current treatments for CC have been advanced, including surgical resection, radiotherapy and chemotherapy, the clinical outcomes are still not satisfactory. It is reported that 50% of CC patients have a poor prognosis and the five-year survival rate of less than 5%[2,3].

With the in-depth study of tumor pathogenesis, the signaling pathways related to tumor cell proliferation and differentiation have been well recognized[4]. The PI3K/AKT pathway is thought to be a crucial pathway in regulating a variety of cell activity[5]. It is reported that the PI3K/AKT pathway is closely related to the occurrence and development of malignant tumors, such as lung cancer, breast cancer and kidney cancer. The exploration of the significance of key genes involved in the PI3K/AKT pathway in cancer cells will contribute to further revealing the occurrence and development of malignancies[6].

Abnormal expression of lncRNAs exerts important roles in the occurrence and progression of malignant tumors and provides a new promising strategy for tumor treatment[7-9]. Depending on the transcript size, non-coding RNAs can be divided into long non-coding RNAs (lncRNAs) and small non-coding RNAs (small ncRNAs)[10]. LncRNAs contain more than two hundred nucleotides and cannot be translated into proteins[8]. LncRNA HCP5 is mainly expressed in the immune system and often considered to be associated with herpes zoster and other serious skin reactions[11]. Recent studies have reported differential expression of HCP5 in multiple cancers. For example, HCP5 was significantly downregulated in patients with ovarian cancer[12]. In addition, the HCP5 gene was also considered to be a susceptibility locus for HCV-associated hepatocellular carcinoma[13]. However, the role of HCP5 in CC remains unknown.

Corresponding Author: Jiebing Tang, MD; email: jess_tong@163.com

This study aimed to explore the role of HCP5 in the occurrence and progression of CC. Demonstration of the regulatory effect of HCP5 on the PI3K/AKT pathway might provide novel therapeutic targets for CC treatment.

Patients and Methods

Sample Collection

CC tissues and paracancerous tissues were harvested from patients undergoing surgical resection in our hospital from June 2015 to March 2018. Enrolled patients had complete clinical data and signed an informed consent before the study. All experimental procedures were approved by the Ethics Committee of Harbin Medical University Cancer Hospital. CC tissues were immediately preserved in liquid nitrogen. Based on the tumor stage, 22 CC cases were classified as stage I-II and 16 were stage III-IV.

Cell Culture and Transfection

Colonic epithelial cell line (NCM460) and CC cell lines (LoVo, CaCo2, SW620, SW480 and HCT116) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HCT116 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and the others were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS. One day prior to cell transfection, cells were seeded into the 6-well plates at a density of 4×10^4 cells per well. Cells were transfected with corresponding plasmids when the confluence was up to 50% following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The culture medium was replaced 6 hours later.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA in cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. Primers used in the study were as follows: GAPDH forward: 5′-ATCACTGCCACCCAGAAGAC-3′, GAPDH reverse: 5′-ATGAGGTCCACCACCCTGTT-3′; HCP5 forward: 5′-GACTCTCCTACTGGTGCTTGGT-3', HCP5 reverse: 5′-CACTGCCTGGTGAGCCTGTT-3′; AP1G1 forward: 5′-GAGTTTTGCCCTGGTGAATG-3′, AP1G1 forward: 5′-GCAAGGAAGATTCCAGATGC-3′.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, the immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Cell Counting Kit-8 (CCK-8) Assay

SW480 and HCT116 cells were seeded into 96-well plates with 2×10^3 cells per well. Totally 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well. About 4 hours later, the medium was replaced and cells were incubated with the CCK8 solution for 1 h. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells were digested for preparing cell suspension at a density of 2×10^5/mL. A total of 200 cells were seeded in each well of the 6-well plates. Colonies visible to the naked eye were fixed with hematoxylin for 30 min, observed and captured using a microplate.

Transwell Assay

SW480 or HCT116 cells were resuspended in serum-free medium at a density of 2×10^5/mL. Totally 100 μL of cells suspension was added in the upper transwell chamber of the 24-well plates for 24 hours. The penetrating cells in the lower chamber were fixed and stained with 20% Giemsa. In each well, 5 randomly selected fields were captured for cell counting.

Cell Cycle Detection

Cells were washed with phosphate-buffered saline (PBS) three times, digested with trypsin

and then centrifuged. Subsequently, cells were fixed with 75% ethanol at -20°C for 8 h or longer. Before cell cycle detection, fixed cells were centrifuged and washed with PBS, followed by incubation with 0.5 mg/mL ribotide for 10 min. Finally, cells were incubated with 10 μL of propidium iodide (PI) (1 mg/mL) in the dark for 30 min, and cell cycle was detected using flow cytometry.

Statistical Analysis

GraphPad Prism 5 (La Jolla, CA, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The difference of the measurement data was analyzed using the *t*-test. $p<0.05$ was considered statistically significant.

Results

HCP5 was Highly Expressed in CC

We first detected the expression level of HCP5 in CC and paracancerous tissues by qRT-PCR. It is indicated that HCP5 was significantly higher in CC tissues than that in paracancerous tissues (Figure 1A). Subsequently, we divided CC tissues into high-level group (stage III-IV) and low-level group (stage I-II) according to the tumor stage. Higher expression of HCP5 was observed in CC patients with stage III-IV compared with those with stage I-II (Figure 1B). Similarly, we detected HCP5 expression in colonic epithelial cell line and CC cell lines. HCP5 showed an increased expression in LoVo, CaCo2, SW620, SW480 and HCT116 cells compared to the NCM460 cells (Figure 1C). SW480 and HCT116 cells were then selected for the following experiments. To further explore the effect of HCP5 on the pathogenesis of CC, two lines of HCP5-siRNA were constructed and tested for their transfection efficacies in SW480 and HCT116 cells. Both two lines of HCP5-siRNA reduced the HCP5 level in CC cells, while the HCP5-siRNA-1 showed a better knockdown efficiency (Figure 1D). HCP5-siRNA-1 was then utilized for the following exploration.

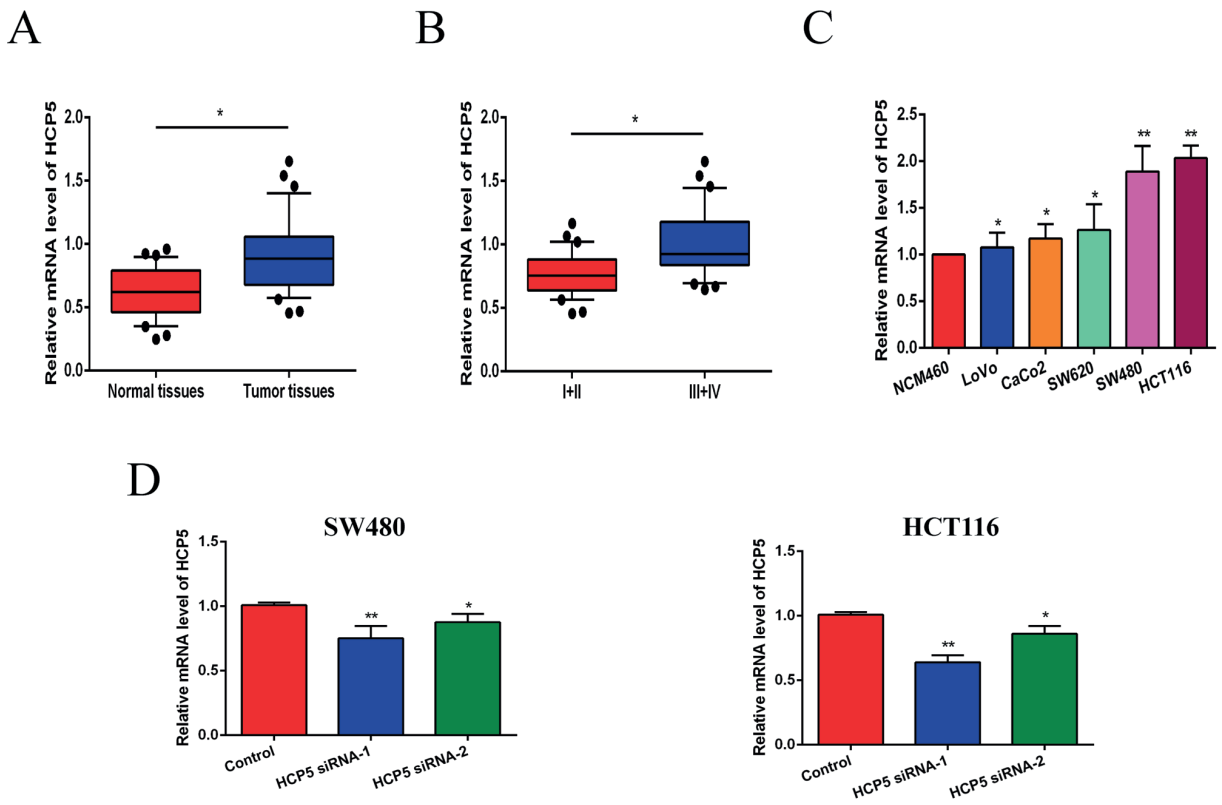

Figure 1. HCP5 was highly expressed in colon cancer. ***A,*** HCP5 expression in colon cancer tissues and paracancerous tissues detected by qRT-PCR. ***B,*** HCP5 expression in colon cancer tissues with stage I-II or stage III-IV detected by qRT-PCR. ***C,*** HCP5 expression in NCM460, LoVo, CaCo2, SW620, SW480 and HCT116 cells. ***D,*** HCP5 expression in SW480 and HCT116 cells after transfection of HCP5-siRNA.

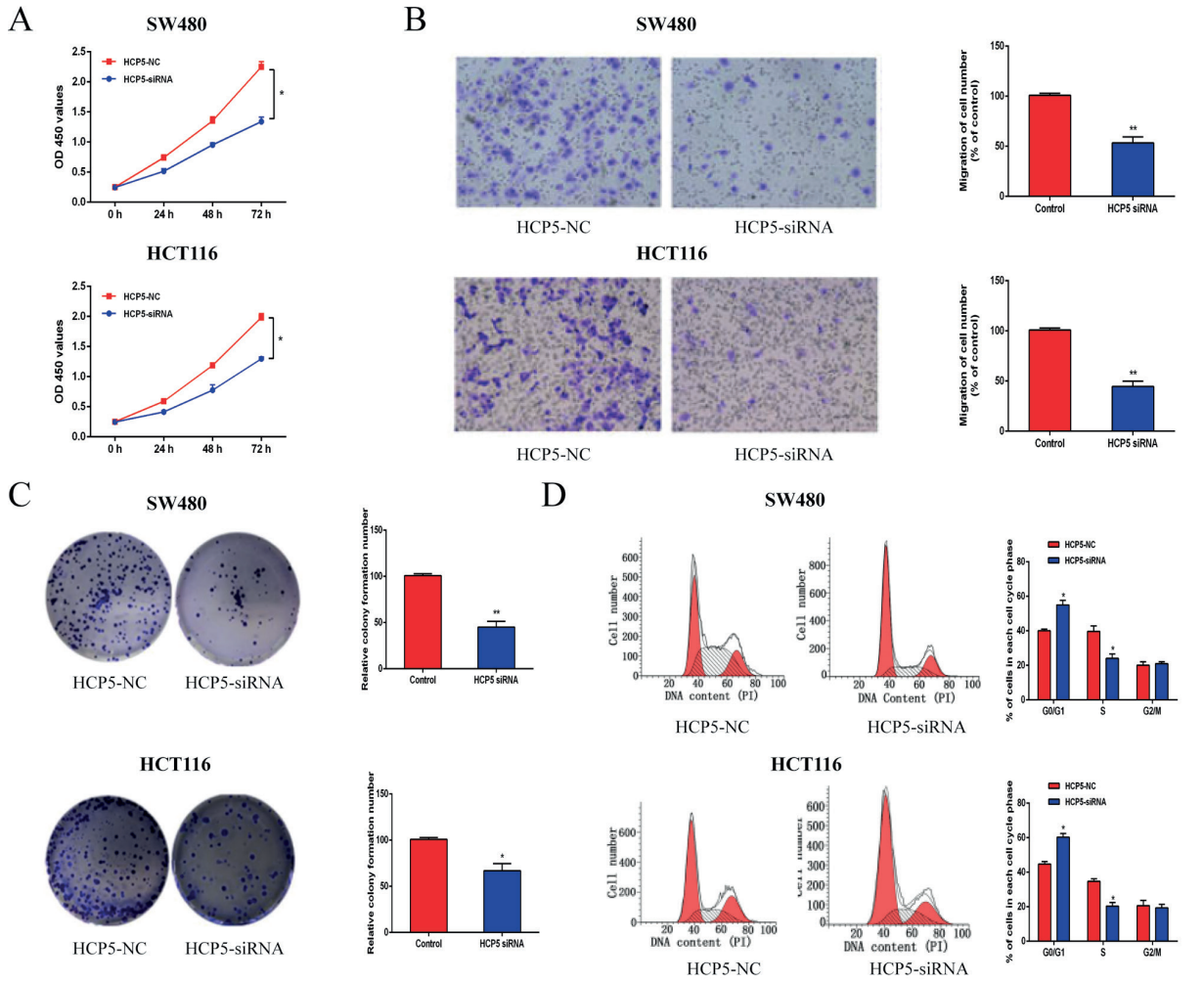

Figure 2. HCP5 knockdown inhibited proliferative and migratory capacities of colon cancer cells. ***A,*** Cell proliferation was measured by CCK-8 assay at 0, 24 h, 48 h and 72 h after transfection of HCP5-siRNA in SW480 and HCT116 cells, respectively. ***B,*** Cell migration after transfection of HCP5-siRNA in SW480 and HCT116 cells. ***C,*** Colony formation after transfection of HCP5-siRNA in SW480 and HCT116 cells. ***D,*** Cell cycle after transfection of HCP5-siRNA in SW480 and HCT116 cells.

HCP5 Knockdown Inhibited Proliferative and Migratory Capacities of CC Cells

We transfected HCP5-siRNA-1 into SW480 and HCT116 cells to knock down HCP5 expression, followed by detection of cell proliferation and migration. Cell proliferation was measured by CCK-8 assay at 0, 24 h, 48 h and 72 h after transfection of HCP5-siRNA, respectively. It was found that after HCP5 knockdown, the proliferation capacity of CC cells was significantly lower than that of the normal group (Figure 2A). The transfection of HCP5-siRNA in SW480 and HCT116 cells also decreased the number of migratory cells (Figure 2B). The colony formation assay further demonstrated an inhibited cell proliferation by knockdown of HCP5 (Figure 2C). Subsequently, we analyzed the proportion of cells in G0/G1 phase, S phase and G2/M phase by flow cytometry. The results showed that the proportion of cells in the G0/G1 phase was markedly increased while decreased in S phase after transfection of HCP5-siRNA (Figure 2D). Above data suggested that the HCP5 knockdown inhibited both proliferation and migration capacities of SW480 and HCT116 cells.

HCP5 Regulated the PI3K/AKT Pathway by Inhibiting AP1G1

We further examined the AP1G1 expression in CC and paracancerous tissues by qRT-PCR, and found that AP1G1 was significantly decreased in CC tissues (Figure 3A). Next, the AP1G1 expres-

sion in CC cells transfected with HCP5-siRNA was also detected. The results indicated that the AP1G1 expression was significantly increased accompanied by the decreased HCP5 expression (Figure 3B). Similar results were also obtained at the protein level (Figure 3C). AP1G1-siRNA was then constructed and tested for its transfection efficacy. Both mRNA and protein levels of AP1G1 decreased by AP1G1-siRNA transfection in SW480 and HCT116 cells (Figure 3D, 3E). To further elucidate the interaction among HCP5, AP1G1, and the PI3K/AKT pathway, CC cells were divided into the negative control group, the HCP5-siRNA group and the AP1G1-siRNA+HCP5-siRNA group. HCP5-siRNA transfection up-regulated the AP1G1 expression in CC cells and down-regulated the expressions of p-PI3K, p-AKT, and VEGF. However, protein expressions of p-PI3K, p-AKT, and VEGF were enhanced in AP1G1-siRNA+HCP5-siRNA group, indicating that HCP5 activated the PI3K/AKT pathway by inhibiting AP1G1 in CC (Figure 3F).

AP1G1 Reversed the Role of HCP5 in Proliferative and Migratory Capacities of CC Cells

To further discuss the interaction between HCP5 and AP1G1, rescue experiments were then conducted. We found that the proliferation capacity in the AP1G1-siRNA+HCP5-siRNA group was remarkably elevated compare to that of the AP1G1-siRNA group (Figure 4A). Besides, the migration capacity was also increased by co-transfection of AP1G1-siRNA and HCP5-siRNA (Figure 4B). Our data indicated that HCP5 might exert its function in regulating the proliferation and migration of CC cells by inhibiting the AP1G1 expression.

Discussion

As a common malignant tumor of the digestive tract, CC occurs mostly in the middle-aged and elderly population. The etiology and pathogene-

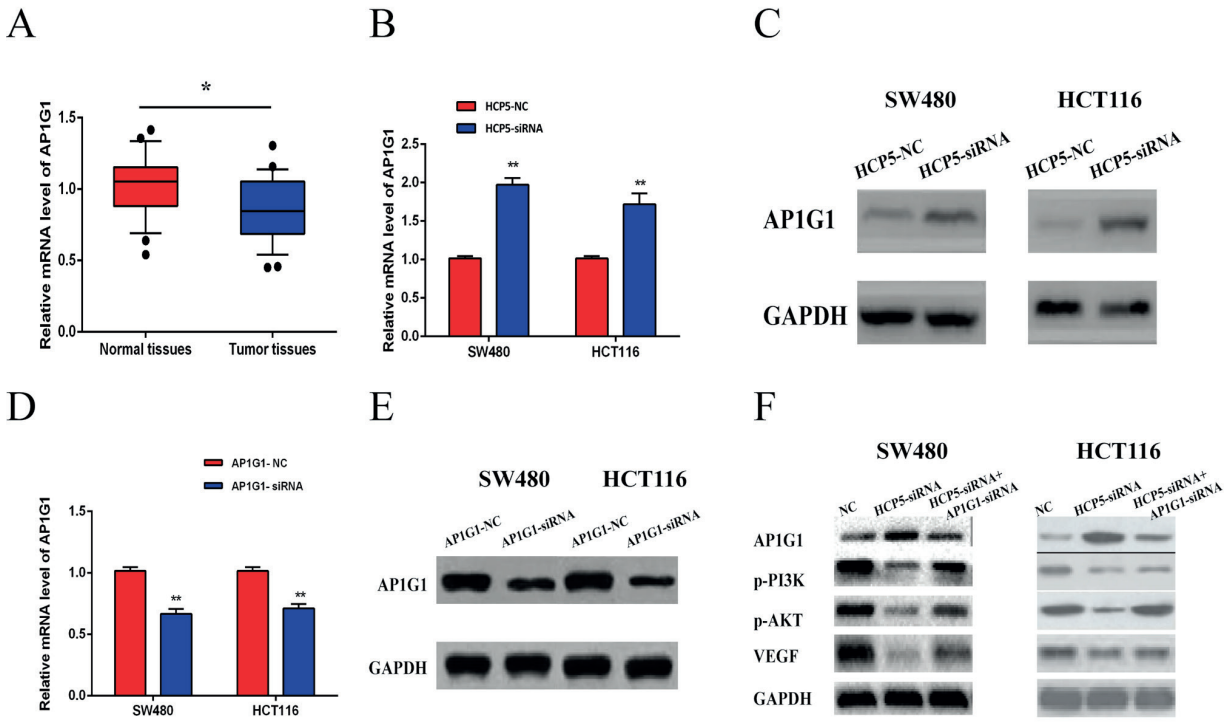

Figure 3. HCP5 regulated PI3K/AKT pathway by inhibiting AP1G1. ***A,*** AP1G1 expression in colon cancer tissues and paracancerous tissues detected by qRT-PCR. ***B,*** The mRNA level of AP1G1 after transfection of HCP5-siRNA in SW480 and HCT116 cells. ***C,*** Protein level of AP1G1 after transfection of HCP5-siRNA in SW480 and HCT116 cells. ***D,*** The mRNA level of AP1G1 after transfection of AP1G1-siRNA in SW480 and HCT116 cells. ***E,*** Protein level of AP1G1 after transfection of AP1G1-siRNA in SW480 and HCT116 cells. ***F,*** Protein levels of key genes in the PI3K/AKT pathway after co-transfection of HCP5-siRNA and AP1G1-siRNA in SW480 and HCT116 cells.

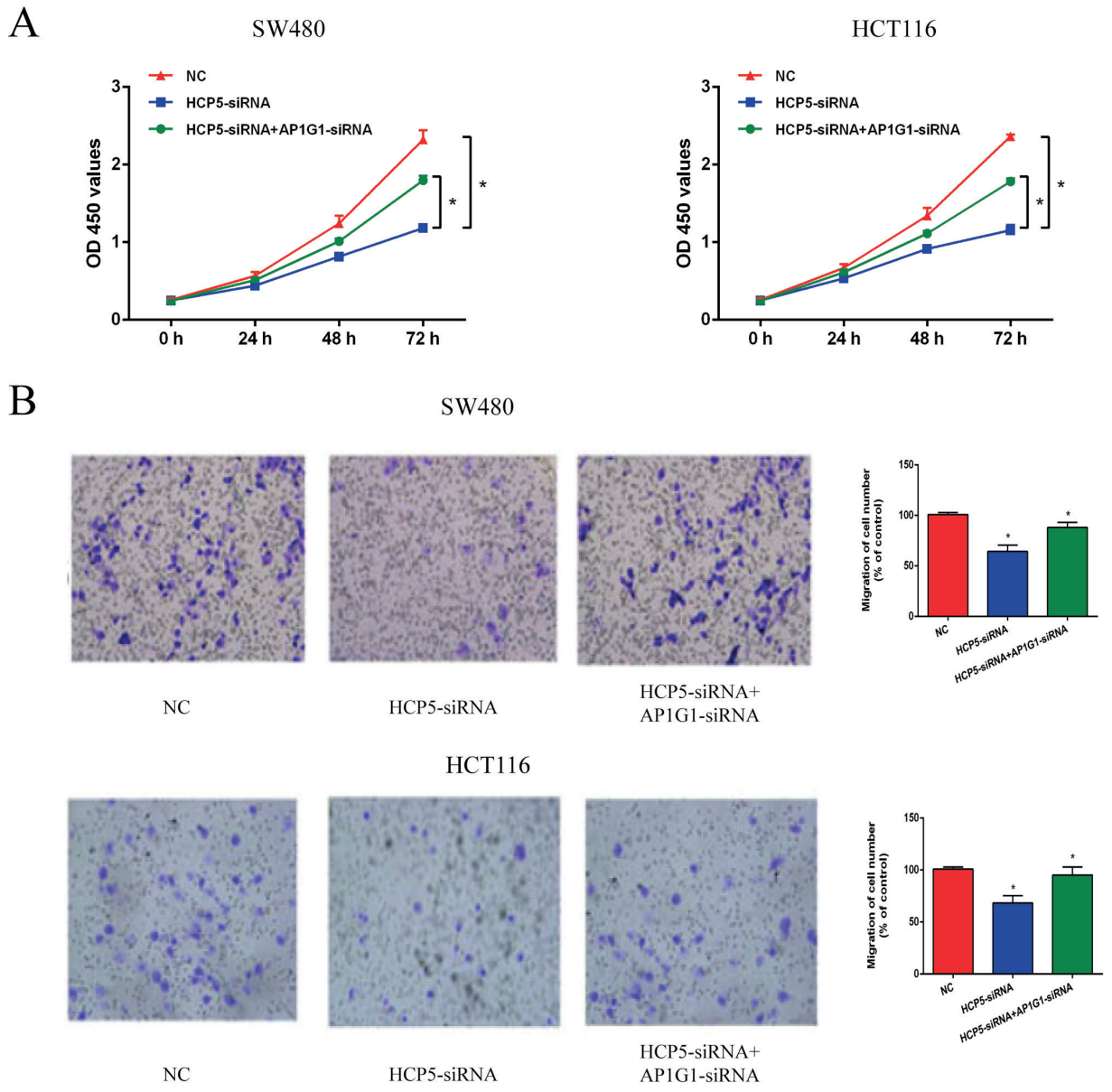

Figure 4. AP1G1 reversed the role of HCP5 in proliferative and migratory capacities of colon cancer cells. ***A,*** Proliferation after co-transfection of HCP5-siRNA and AP1G1-siRNA in SW480 and HCT116 cells. ***B,*** Migration after co-transfection of HCP5-siRNA and AP1G1-siRNA in SW480 and HCT116 cells.

sis of CC have not been fully elucidated yet. At present, CC development is believed as a dynamic process, which involves environmental factors and genetic factors[14]. The occurrence and progression of the tumor are related to disordered cell metabolism, cell cycle and signaling pathways. The oncogene activation and tumor-suppressor gene inactivation are fundamental for tumor pathogenesis.

With the advanced development of tumor biology, abnormally activated PI3K/AKT pathway is found in multiple tumors, including breast cancer, ovarian cancer, endometrial cancer, nasopharyngeal cancer and CC[15-19].

The occurrence of CC is also accompanied by abnormal expressions of various oncogenes and tumor-suppressor genes. The role of the PI3K/AKT pathway in CC has also been well studied. It is reported that the PI3K/AKT pathway promoted the invasion and metastasis of malignancies by reducing the intercellular adhesion, increasing the tumor cell motility, regu-

lating the growth factor receptors and affecting the expression of the extracellular matrix. Activated PI3K disturbs the normal function of the PI3K/AKT pathway, thus affecting cell proliferation, differentiation, apoptosis and transport of intracellular substances. PI3K further stimulates the AKT activity to improve tumor cell proliferation and survival.

The crucial roles of lncRNAs in the occurrence and development of tumors have been reported recently[20,21]. LncRNAs function by affecting apoptosis, metastasis and infiltration of tumor cells, which provide new ideas for tumor therapy. Researches[22] have shown that the proportion of tumor-associated lncRNAs (18%) is twice than that of tumor-associated proteins (9%). HCP5 is located on chromosome 6q21.3 and has been previously reported to be expressed in immune system-associated cells. The specific role of HCP5 in malignancies, however, has not been fully elucidated[23].

In the present work, HCP5 was highly expressed in CC tissues and cell lines. HCP5 knockdown reduced the proliferation and invasion capacities of CC cells through the PI3K/AKT pathway. In addition, we found that AP1G1 was lowly expressed in CC and might be inhibited by HCP5. Rescue experiments further confirmed that the regulatory effect of HCP5 on CC cells were partially reversed by the AP1G1 knockdown.

Conclusions

We showed that HCP5 was highly expressed in CC and enhanced the proliferation and migration of CC cells by inhibiting the AP1G1 expression. Besides, HCP5 promoted CC development by activating the PI3K/AKT pathway. Our study provided a new therapeutic target for early diagnosis and treatment of CC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
2) Vizin T, Christensen IJ, Nielsen HJ, Kos J. Cathepsin X in serum from patients with colorectal cancer: relation to prognosis. Radiol Oncol 2012; 46: 207-212.
3) Van Cutsem E, Oliveira J. Primary colon cancer: ESMO clinical recommendations for diagnosis, adjuvant treatment and follow-up. Ann Oncol 2009; 20 Suppl 4: 49-50.
4) McCubrey JA, Abrams SL, Fitzgerald TL, Cocco L, Martelli AM, Montalto G, Cervello M, Scalisi A, Candido S, Libra M, Steelman LS. Roles of signaling pathways in drug resistance, cancer initiating cells and cancer progression and metastasis. Adv Biol Regul 2015; 57: 75-101.
5) McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, Salisbury JL, Mazzarino MC, Stivala F, Libra M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 2006; 46: 249-279.
6) McNamara CR, Degterev A. Small-molecule inhibitors of the PI3K signaling network. Future Med Chem 2011; 3: 549-565.
7) Deng G, Sui G. Noncoding RNA in oncogenesis: a new era of identifying key players. Int J Mol Sci 2013; 14: 18319-18349.
8) Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
9) Yang BF, Cai W, Chen B. LncRNA SNHG12 regulated the proliferation of gastric carcinoma cell BGC-823 by targeting microRNA-199a/b-5p. Eur Rev Med Pharmacol Sci 2018; 22: 1297-1306.
10) Deng G, Sui G. Noncoding RNA in oncogenesis: a new era of identifying key players. Int J Mol Sci 2013; 14: 18319-18349.
11) Liu Y, Helms C, Liao W, Zaba LC, Duan S, Gardner J, Wise C, Miner A, Malloy MJ, Pullinger CR, Kane JP, Saccone S, Worthington J, Bruce I, Kwok PY, Menter A, Krueger J, Barton A, Saccone NL, Bowcock AM. A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci. PLoS Genet 2008; 4: e1000041.
12) Liu N, Zhang R, Zhao X, Su J, Bian X, Ni J, Yue Y, Cai Y, Jin J. A potential diagnostic marker for ovarian cancer: involvement of the histone acetyltransferase, human males absent on the first. Oncol Lett 2013; 6: 393-400.
13) Lange CM, Bibert S, Dufour JF, Cellerai C, Cerny A, Heim MH, Kaiser L, Malinverni R, Mullhaupt B, Negro F, Semela D, Moradpour D, Kutalik Z, Bochud PY. Comparative genetic analyses point to HCP5 as susceptibility locus for HCV-associated hepatocellular carcinoma. J Hepatol 2013; 59: 504-509.
14) Ranzani M, Annunziato S, Adams DJ, Montini E. Cancer gene discovery: exploiting insertional mutagenesis. Mol Cancer Res 2013; 11: 1141-1158.
15) Tokunaga E, Kimura Y, Mashino K, Oki E, Kataoka A, Ohno S, Morita M, Kakeji Y, Baba H, Maehara Y. Activation of PI3K/Akt signaling and hormone resistance in breast cancer. Breast Cancer 2006; 13: 137-144.
16) Wang S, Lei Y, Cai Z, Ye X, Li L, Luo X, Yu C. Girdin regulates the proliferation and apoptosis of pan-

creatic cancer cells via the PI3K/Akt signalling pathway. Oncol Rep 2018; 40: 599-608.

17) Paramee S, Sookkhee S, Sakonwasun C, Na Takuathung M, Mungkornasawakul P, Nimlamool W, Potikanond S. Anti-cancer effects of Kaempferia parviflora on ovarian cancer SKOV3 cells. BMC Complement Altern Med 2018; 18: 178.

18) Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res 2012; 18: 5856-5864.

19) Ni J, Peng Y, Yang FL, Xi X, Huang XW, He C. Overexpression of CLEC3A promotes tumor progression and poor prognosis in breast invasive ductal cancer. Onco Targets Ther 2018; 11: 3303-3312.

20) Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.

21) Giannakakis A, Coukos G, Hatzigeorgiou A, Sandaltzopoulos R, Zhang L. miRNA genetic alterations in human cancers. Expert Opin Biol Ther 2007; 7: 1375-1386.

22) Khachane AN, Harrison PM. Mining mammalian transcript data for functional long non-coding RNAs. PLoS One 2010; 5: e10316.

23) Teng H, Wang P, Xue Y, Liu X, Ma J, Cai H, Xi Z, Li Z, Liu Y. Role of HCP5-miR-139-RUNX1 feedback loop in regulating malignant behavior of glioma cells. Mol Ther 2016; 24: 1806-1822.