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Original Article

Long non-coding RNA UPAT promotes cell proliferation via regulating the miR-133a/IGF1R axis in colorectal cancer

Xin Liu[1], Wenxiao Wang[1], Lei Zhang[2], Chenggang Yang[1]

[1]Department of Gastrointestinal Surgery, Liaocheng People's Hospital, Liaocheng 252000, Shandong Province, China; [2]Department of General Surgery, Liaocheng Dongchangfu People's Hospital, Liaocheng, China

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Abstract: Many long noncoding RNAs (lncRNAs) are dysregulated in human cancers and play critical roles in tumor development and progression. It has been reported that long noncoding RNA UPAT promotes colon tumorigenesis by inhibiting degradation of UHRF1. However, the function and molecular mechanism of UPAT in colorectal cancer (CRC) needs to be further studied. In this study, we found that UPAT expression was inversely correlated to miR-133a expression in colorectal cancer tissues and cells. In addition, knockdown of UPAT inhibited cell proliferation and cell cycle progression of colorectal cancer. Further mechanistic studies revealed that UPAT could sponge endogenous miR-133a and inhibit its activity. Moreover, both UPAT knockdown and miR-133a overexpression in CRC cell lines led to cell proliferation and cell cycle progression was inhibited. We also found that insulin-like growth factor 1 receptor (IGF1R), a known target of miR-133a, was inhibited by both UPAT knockdown and miR-133a overexpression. Furthermore, tumor growth suppression was retarded with miR-133a down-regulated in UPAT knockdown of colorectal cancer cells xenografts. Taken together, our work provides the first evidence of a UPAT-miR-133a-IGF1R regulatory network in CRC and reveals that UPAT is a potential new oncogene for CRC.

Keywords: lncRNA UPAT, proliferation, miR-133a, IGF1R, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer type and the third leading cause of cancer-related deaths worldwide [1]. The occurrence and progression of CRC is a multi-step process involving deregulation of multiple oncogenes and tumor suppressors [2]. Although great efforts have been made to understand the complicated pathogenesis of CRC and to improve its treatment, CRC remains a severe disease. Therefore, it is important to elucidate the molecular mechanisms underlying CRC proliferation.

Long noncoding RNAs (lncRNAs, >200 nucleotides in length) have limited or no protein-coding capacity [3, 4]. Previous studies have demonstrated that lncRNAs play important roles in diverse biological processes, including embryonic development, cell growth and tumorigenesis, by regulating gene expression at the chromatin organization, transcriptional, and post-transcriptional levels [5]. For example, UCA1 is up-regulated in CRC tissues and predicted poor prognosis in two independent CRC cohorts [6]. The lncRNA H19 promotes cell proliferation by competitively binding to miR-200a and derepressing β-Catenin expression in CRC [7]. However, the expression and function of lncRNA UPAT in CRC remain unclear.

In the present study, we found that the expression levels of the lncRNA UPAT were inversely correlated with miR-133a in CRC tissues and cells. Moreover, mechanistic analysis revealed that UPAT functioned as a ceRNA to regulate expression of IGF1R through competition for miR-133a. Further studies showed that both UPAT knockdown and miR-133a overexpression led to CRC cell proliferation and CRC cells xenograft tumor growth inhibition. Taken together, our study demonstrates that the long noncoding RNA UPAT promotes cell proliferation via

regulating the miR-133a/IGF1R axis in colorectal cancer.

Material and methods

Clinical ethics

The patients underwent surgery for excision of a primary tumor in the Nanjing Medical University Affiliated Cancer Hospital (Nanjing, China). Written informed consent was obtained from the patients before samples collection. All experiments were approved by the Ethical Committee of the Nanjing Medical University Affiliated Cancer Hospital.

Tissue samples and cell lines

We collected 25 paired CRC tissues and the corresponding adjacent normal tissues. All tumor and paired adjacent normal tissues were confirmed by experienced pathologists. Informed written consent was obtained from all patients included in this study. The CRC cell lines CW-2, SW1116, and SW480 cells were purchased from Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM Medium (Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA) in a humidified 5% CO_2 atmosphere at 37°C.

Cell transfection

The sequences of miR-133a mimic, inhibitor, and UPAT siRNA were all synthesized by GenePharma Company (Shanghai, China). Oligonucleotide and plasmid transfection were conducted by using the Lipofectamine™ 2000 transfection reagent (Invitrogen, USA), followed by the protocol recommended by the manufacturer. After 48 h transfection, the SW480 cells were collected and used for further investigations.

Real-time PCR

Total RNA was isolated by TRIZOL Reagent (Invitrogen, USA) following the manufacturer's instructions. After RNA extraction, RNA samples were reversely transcribed by High Capacity cDNA Reverse Transcription Kit (TAKARA, Japan). RT-PCR was performed using a SYBR premix Ex Taq kit (TAKARA, Japan) on the 7300 Real-time PCR system (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The miR-133a and U6 primer sequences used were obtained from Shanghai GenePharma. (Shanghai, China). The expression levels of U6 and GAPDH were used as controls, respectively. The relative fold changes of candidate genes were analyzed by using $2^{-\Delta\Delta CT}$ method.

Western blotting

Cell lysates were lysed by RIPA buffer (Sigma-Aldrich, USA) with Complete Protease Inhibitor Cocktail (Roche, USA). Protein concentration was measured with the Bio-Rad protein assay kit. 50 μg protein extractions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to 0.22 μm nitrocellulose membranes (Sigma-Aldrich. USA). The membrane was blocked in 5% nonfat milk and incubated with diluted antibodies against IGF1R (Cell Signaling, USA) and GAPDH (Cell Signaling, USA), followed by incubation with a HRP-conjugated secondary antibody (Santa Cruz, USA). ECL chromogenic substrate was used to visualize the bands and the intensity of the bands was quantified by densitometry (Quantity One software; Bio-Rad). GAPDH was used as control.

Cell Counting Kit-8 assay

Cell proliferation was monitored by the Cell Counting Kit-8 (CCK8) assay (Promega Corporation, USA) every 24 h following the manufacturer's protocol. In brief, the transfected cells were plated in 96-well plates (3×10^4 cells/well) and then 10 μl of CCK8 solution was added and incubated for 2 h. Each solution was measured spectrophotometrically at 450 nm.

Cell cycle analysis

First, cells were seeded in six-well plates at 2×10^5 cells/well. Forty-eight hours after transfection, cells were fixed in 70% ethanol and stained with 20 μg/mL propidium iodide (PI). Next, cell cycle distribution was analyzed on a flow cytometer (FACSCalibur, BD Biosciences). The experiment was repeated at least three times.

Luciferase assay

The fragment from UPAT containing the predicted hsa-miR-133a binding site was chemically synthesized and cloned into the pmiRGLO vec-

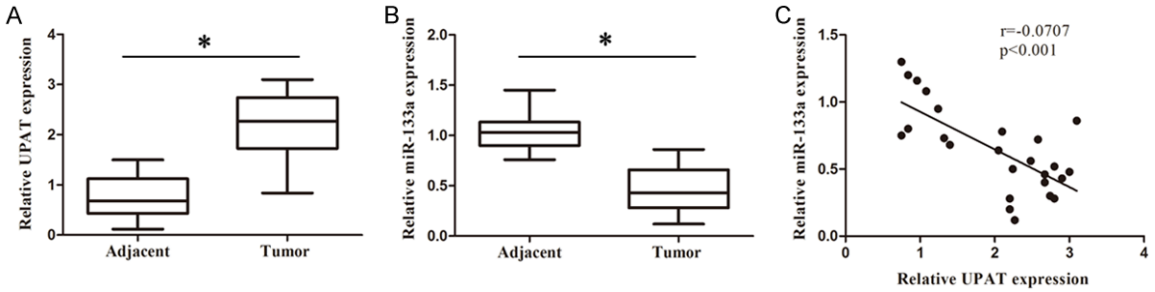

Figure 1. UPAT and miR-133a are inversely expressed in clinical CRC tissues. A and B. Relative expression of UPAT and miR-133a in gastric cancer tissues was analyzed by real-time PCR and was normalized to normal tissues. C. A statistically significant inverse correlation between UPAT and miR-133a in CRC specimens (Spearman's correlation analysis, r = -0.0707; P < 0.001). The expression levels of U6 and GAPDH were used as controls, respectively. Results are represented as the mean ± SD based on three independent experiments, *P < 0.05.

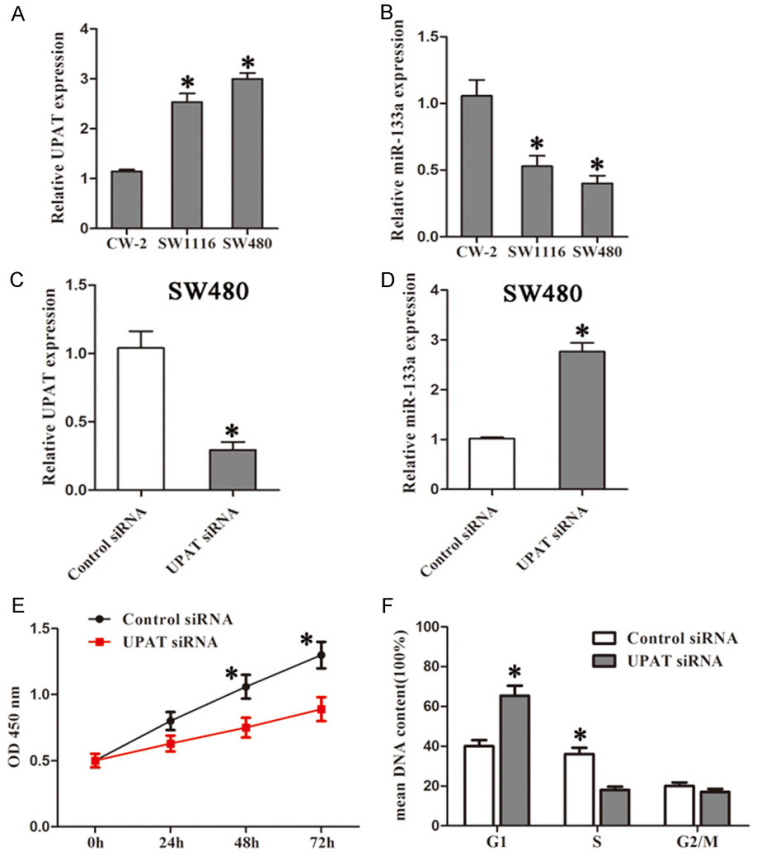

Figure 2. Knockdown of UPAT inhibits cell proliferation and cell cycle progression of CRC cells. A and B. Relative expression of UPAT and miR-133a in CRC cells was analyzed by real-time PCR and was normalized to immortalized CW-2 cells. The expression levels of U6 and GAPDH were used as controls, respectively. C and D. Relative UPAT and miR-133a expression was analyzed by real-time PCR after SW480 cells transient transfection with UPAT siRNA. E. Cell proliferation was measured by CCK-8 assay at the indicated times after SW480 cells transient transfection with UPAT siRNA. F. Cell cycle analysis with flow cytometry. Cells were stained with propidium iodide and analyzed. Results are represented as the mean ± SD based on three independent experiments, *P < 0.05.

tor (Genewiz, USA). The resulting vector was called the reporter vector pmiRGLO-UPAT-wild type (pmiRGLO-UPAT-wt). The corresponding mutants were created by mutating the hsa-miR-133a seed region binding site, which was called the reporter vector pmiRGLO-UPAT-mutated-type (pmiRGLO-UPAT-mut). miR-133a mimic or control was co-transfected with the reporter vectors containing either the targeting sequences or the corresponding mutants using Transfection reagent (Invitrogen, USA) according to the protocol. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

Tumor formation in nude mice

CRC cells transfected with si-UPAT, co-transfected with siUPAT, and miR-133a inhibitor, or control were subcutaneously injected into male nude mice at 6 weeks of age (Vital River Laboratories, Beijing, China). The mice were sacrificed after 4 to 6 weeks and examined for the growth of subcutaneous tumors. Measurements were taken weekly and tumor volumes were calculated using the formula V = length x width

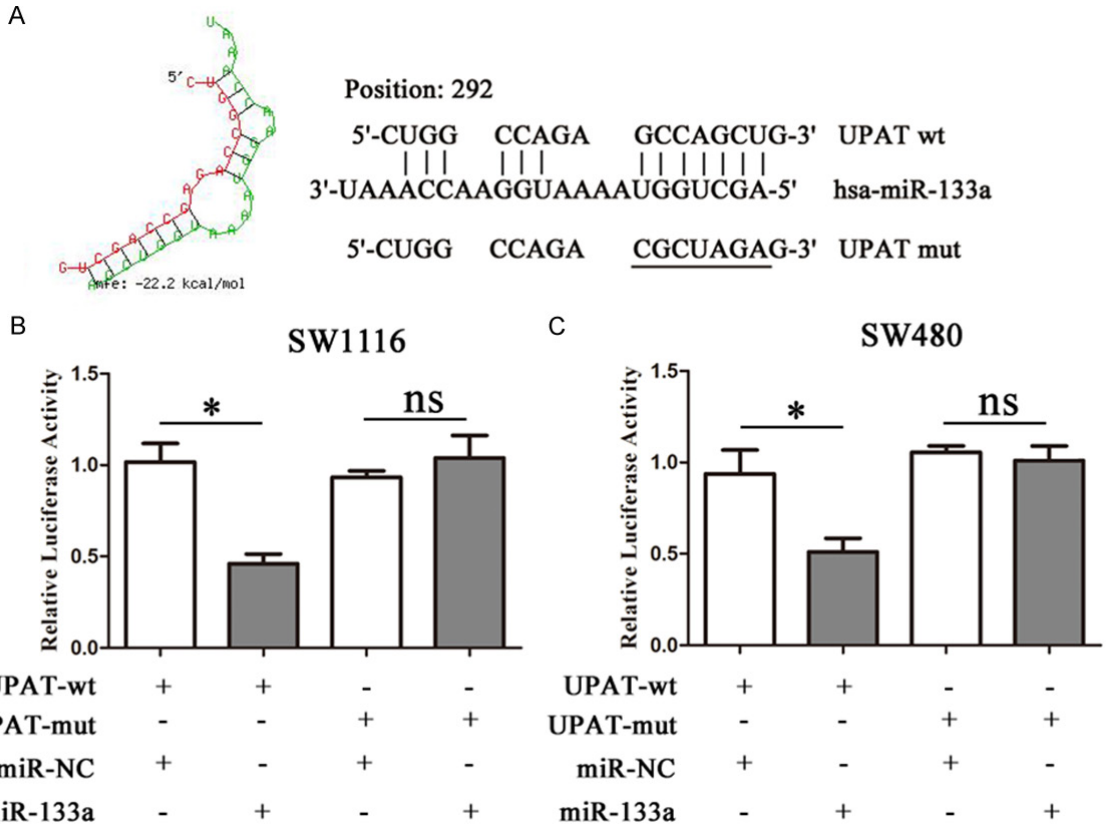

Figure 3. UPAT acts as a molecular sponge of miR-133a in CRC cells. A. miR-133a binding sequence in UPAT-wt and sequence of UPAT-mut. B and C. Luciferase activity of the indicated group in SW1116 and SW480 cells by transfected miR-133a mimic + pmiRGLO vector, miR-133a mimic + pmiRGLO-UPAT-WT or miR-133a mimic + pmiRGLO-UPAT-MUT. Results are represented as the mean ± SD based on three independent experiments, *$P < 0.05$.

^ 2/2. The animals were sacrificed 4 weeks after injection.

Statistical analysis

Data were expressed as the mean ± SD of at least three independent experiments. Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, USA). Student's t test was performed to analyze the data. Values of $P < 0.05$ were considered significant.

Results

Inverse expression of UPAT and miR-133a in clinical GC tissues and cells

Real-time PCR revealed that compared with adjacent tissues, tumors exhibited a significantly higher UPAT expression (**Figure 1A**) and a significantly lower miR-133a expression (**Figure 1B**). Pearson expression analysis suggested an inverse relationship between UPAT and miR-133a expression in tumors (**Figure 1C**, r = -0.0707, $P < 0.001$). *In vitro*, UPAT was significantly higher expressed in SW1116 and SW480 compared with CW-2 cells (**Figure 2A**), while miR-133a was expressed significantly lower (**Figure 2B**).

Knockdown of UPAT inhibits cell proliferation and cell cycle progression in vitro

To further explore the roles of UPAT on proliferation and cell cycle progression of CRC cells, SW480 cells were transfected with UPAT siRNA or control siRNA. Transfection of UPAT siRNA decreased UPAT levels in CRC cells (**Figure 2C**). At the same time, knockdown of UPAT substantially upregulated miR-133a levels in CRC cells (**Figure 2D**). CCK8 assays showed that cell proliferation was inhibited in CRC cells transiently transfected with UPAT siRNA compared with controls (**Figure 2E**). To further investigate wh-

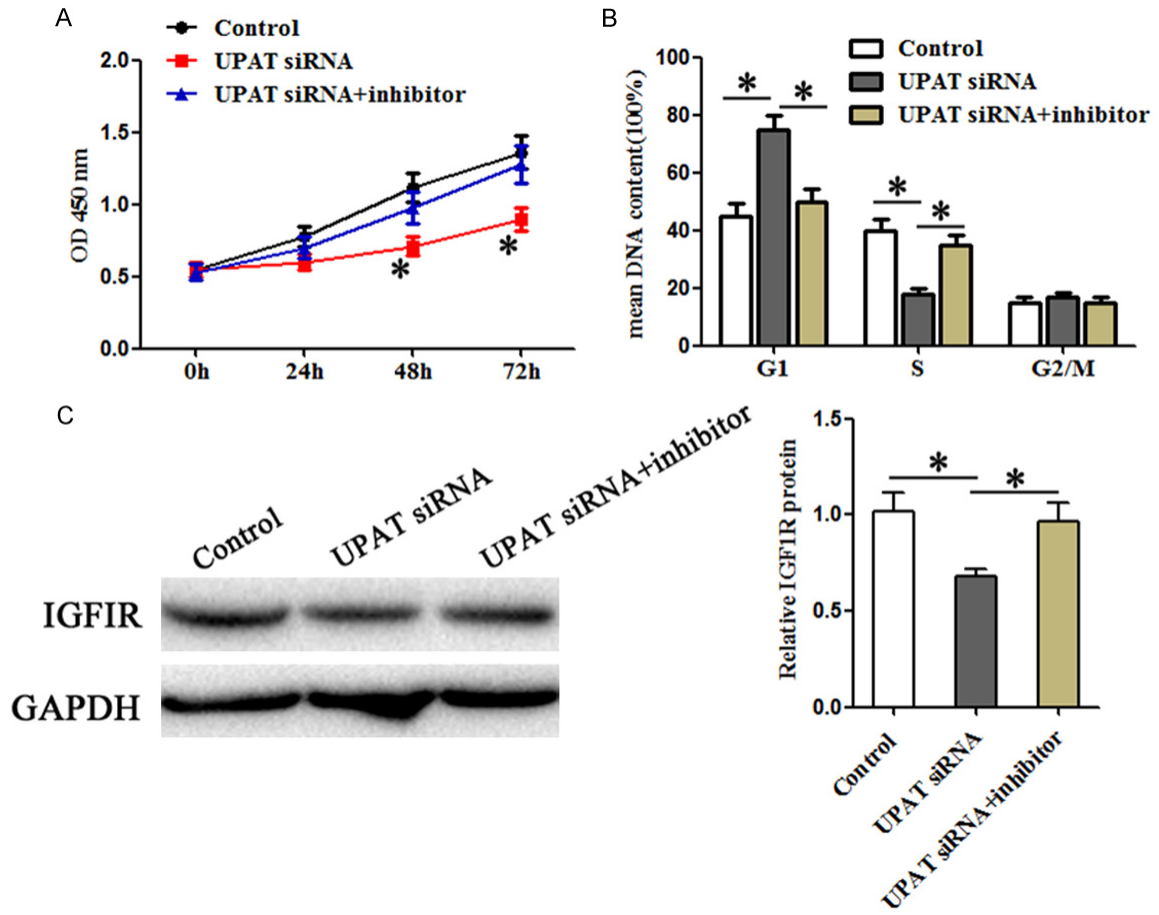

Figure 4. UPAT knockdown and miR-133a overexpression inhibit CRC cell proliferation. A and B. CCK-8 cell proliferation assays and flow cytometry assays were performed in SW480 cells after transfection with UPAT siRNA or control, or co-transfection with UPAT siRNA + miR-133a inhibitor or control. C. Relative protein expression levels of IGF1R were analyzed by Western blot analysis after transfecting UPAT siRNA or control, or co-transfection with UPAT siRNA + miR-133a inhibitor or control. Results are represented as the mean ± SD based on three independent experiments, $*P < 0.05$.

ether the effect of knockdown UPAT on proliferation of CRC cells reflected cell-cycle arrest, cell-cycle progression was analyzed by flow-cytometry. The results revealed that SW480 cells transfected with UPAT siRNA had an obvious cell-cycle arrest at the G1-G0 phase and had a decreased G2-S phase (**Figure 2F**). These results indicate that UPAT strongly promotes cell proliferation.

LncRNA UPAT acts as a molecular sponge of miR-133a

We performed a search for miRNAs that have complementary base pairing with GAPLINC, using online software program RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). The results demonstrated that miR-133a formed complementary base pairing with UPAT (**Figure 3A**). To further investigate whether miR-133a is a functional target of UPAT, a dual-luciferase reporter assay was performed in both SW1116 and SW480 cells. We found that co-transfection of pmiRGLO-UPAT-wt and miR-133a mimic strongly decreased the luciferase activity while co-transfection of pmiRGLO-UPAT-mut and miR-133a mimic did not change luciferase activity (**Figure 3B** and **3C**). Thus, these results demonstrate that miR-133a is a UPAT-targeting miRNA.

miR-133a reverses the promoting effects of UPAT in CRC cells

Although our experiments had confirmed that miR-133a was a target of UPAT, the function of

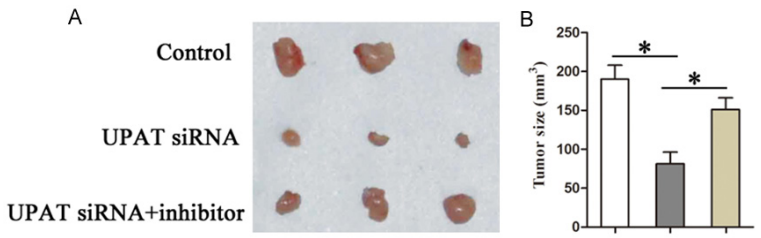

Figure 5. miR-133a reverses the promoting effects of UPAT on tumor growth in xenograft models of CRC. A. SW480 cells were transfected with control, UPAT siRNA, and UPAT siRNA + miR-133a inhibitor. The cells were injected subcutaneously into 9 nude mice per flank. Surgical resections of SW480 xenograft tumors on week 4 for animals were shown. B. Measurements of tumor volumes were shown. Statistically significant differences are indicated: *$P < 0.05$; Student's t test.

miR-133a in UPAT-induced promotion in CRC cells remained unclear. In order to confirm whether UPAT could promote CRC cells through the UPAT-miR-133a-IGF1R axis, we co-transfected UPAT siRNA and miR-133a inhibitor or control in SW480 cells. The CCK-8 assays (**Figure 4A**) and flow cytometry assays (**Figure 4B**) showed that miR-133a could largely reverse the promoting effect of UPAT on CRC cell proliferation and cell cycle progression. Western blot also revealed that the promotion of IGF1R protein expression by UPAT could be largely reversed by miR-133a (**Figure 4C**). These results indicate that miR-133a can reverse the promoting effects of UPAT in CRC cells and UPAT can promote CRC cells through the UPAT-miR-133a-IGF1R axis.

miR-133a reverses the promoting effects of UPAT on tumor growth in xenograft models of CRC

Furthermore, to determine whether UPAT could promote tumor growth in xenograft models of CRC through the UPAT-miR-133a pathway, we assessed tumor growth of xenografts derived from SW480 cells that were co-transfected with UPAT siRNA and miR-133a inhibitor or control prior to subcutaneous injection into nude mice. Our results show that both UPAT knockdown and miR-133a overexpression inhibited tumor growth of the SW480 xenograft (**Figure 5A** and **5B**). Collectively, these results support a role for miR-133a in reversal of the promoting effects of UPAT on tumor growth in xenograft models of CRC.

Discussion

Colorectal cancer is one of the leading causes of cancer mortality and the third most common malignant neoplasm all over the world [8]. Mainstream tumorigenic processes involved in CRC are characterized by phenotypic multistep progression cascades [9]. Reliable identification of CRC progression-specific targets has huge implications for its prevention and treatment [10, 11]. Thus, exploration of novel diagnostic and therapeutic molecular targets for CRC is particularly crucial.

LncRNAs play critical regulatory roles in diverse cellular processes such as chromatin remodeling, transcription, post-transcriptional processing, and intracellular trafficking [12]. Recent studies have shown that lncRNAs can act as oncogenes or tumor suppressor genes to affect tumorigenesis [13]. UPAT, whose alias is AOC4P, was originally detected in hepatocellular carcinoma and acted as a tumor suppressor by enhancing Vimentin degradation and suppressing the EMT [14]. Additionally, UPAT has been reported to promote colon tumorigenesis by inhibiting degradation of UHRF1 [15]. However, the function and molecular mechanism of UPAT in CRC are still unclear.

In the present study, we found that UPAT expression was inversely correlated to miR-133a expression in colorectal cancer tissues and cells. In addition, knockdown of UPAT inhibited cell proliferation and cell cycle progression of colorectal cancer. Further mechanistic studies revealed that UPAT could sponge endogenous miR-133a and inhibit its activity. These findings suggest that UPAT may promote CRC cell proliferation by acting as a molecular sponge of miR-133a.

miR-133a was reported to be downregulated in a variety of cancers, including esophageal squamous cell carcinoma, bladder cancer, prostate cancer, and colorectal cancer [16-19]. Therefore, we examined the role of UPAT siRNA on miR-133a expression level and miR-133a

targets. We focused on the target genes IGF1R as it was previously reported to be repressed by miR-133a [20]. The results showed that miR-133a inhibitor reversed the suppression effects of UPAT siRNA on cell growth and IGF1R expression in CRC cells. We also examined the role of GAPLINC on miR-378 in xenograft models of GC. The results were similar, showing that miR-133a reversed the suppression effects of UPAT siRNA on tumor growth in xenograft models of CRC.

In summary, the present study provides novel evidence of a UPAT-miR-133a-IGF1R regulatory network in CRC and reveals that UPAT is a potential new oncogene for CRC.

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Disclosure of conflict of interest

None.

Address correspondence to: Chenggang Yang, Department of Gastrointestinal Surgery, Liaocheng People's Hospital, 67 Dongchang West Road, Dongchangfu District, Liaocheng 252000, Shandong Province, China. Tel: +86 635-8276110; Fax: +86 635-8966225; E-mail: bakerham123@163.com

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