

miR-221 inhibits autophagy and targets TP53INP1 in colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-associated mortalities worldwide. MicroRNAs (miRNAs/miRs) serve important roles in tumor development, progression and metastasis. miR-221 has been reported to modulate proliferation, apoptosis, cell cycle distribution and cell migration in a variety of cancers. However, the function of miR-221 in the autophagy of cancer is unclear. In the present study, the role of miR-221 in the autophagy of CRC cells was investigated and its associated target was identified. Survival analysis using The Cancer Genome Atlas data suggested that a higher expression of miR-221 was associated with poor survival in patients with CRC. A Cell Counting kit-8 assay revealed that miR-221 promoted CRC cell proliferation. Autophagy flux analyzed by microtubule-associated protein 1 light chain 3 (LC3) turnover indicated that miR-221 reduced autophagy

in CRC cells using different protease inhibitors (E64d and pepstatin A; Bafilomycin A1) in nutrient-rich medium or under starvation conditions. Tumor protein 53-induced nuclear protein 1 (TP53INP1) was identified as a potential novel target of miR-221 by bioinformatic prediction. The protein expression of TP53INP1 was inversely regulated by miR-221 in CRC cells. Furthermore, luciferase activity assays were performed and indicated that miR-221 may regulate the luciferase activity of wild-type TP53INP1 without interfering with the activity of mutant TP53INP1. These data suggested that miR-221 may promote the cell proliferation of CRC via the inhibition of autophagy and targeted TP53INP1.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-associated death worldwide (1). The morbidity rates of CRC are increasing substantially in a number of countries within Eastern Asia and Eastern Europe which were previously at low risk (2). The multifactorial etiology of CRC involves lifestyle and dietary factors, such as smoking, red and processed meat consumption, and excessive alcohol consumption (3). Autophagy is a vital transformational switch among mechanisms that are involved in the pathogenesis of CRC (4). Autophagy may act as a suppressor during early stages and as a promoter during advanced stages of CRC (4,5). It is important to determine the regulative mechanisms of autophagy in CRC.

Recent studies suggests that the post-transcription and translation regulation mediated by microRNAs (miRNAs/miRs) contribute significantly to autophagy in cancer (6). It is found that miR-23b-3p inhibits autophagy in gastric cancer cells (7) and miR-26 suppresses autophagy in hepatocellular carcinoma cells (8). Whereas miR-193b is suggested to induce autophagy in oesophageal cancer cells (9). It is interesting that different miRNAs play diverse roles in the regulation of autophagy through various targets.

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Abbreviations: CRC, colorectal cancer; miRNAs, microRNAs; TP53INP1, tumor protein 53-induced nuclear protein 1; TCGA, The Cancer Genome Atlas; LC3, microtubule-associated protein 1 light chain 3

Key words: autophagy, colorectal cancer, miR-221, tumor protein 53-induced nuclear protein 1

Recently, miR-221 is indicated to inhibit autophagy activity by modulating the p27/CDK2/mTOR axis in heart failure (10). Upregulation of miR-221 is also found to inhibit hypoxia/re-oxygenation induced autophagy (11). Inhibition of autophagy by miR-221 in human umbilical vein endothelial cells is further reported (12). Although miR-221 is found to regulate autophagic gene in cancer (13), no direct evidence suggests a role of miR-221 in cancer related autophagy (14). In this study, we demonstrated that miR-221 inhibited autophagy activity in CRC cells and targeted tumor protein 53-induced nuclear protein 1 (TP53INP1), a regulator of autophagy (15).

Materials and methods

Expression of miR-221 in CRC using The Cancer Genome Atlas (TCGA) data. TCGA, launched by the National Institute of Health (NIH), is a public funded project that create a comprehensive 'atlas' of cancer genomic profiles (16). miRNA data were extracted from TCGA (<http://tcga-data.nci.nih.gov/>) for CRC tumors (accessed May, 2015). Level 3 Illumina miRNASeq patients with complete follow-up information were used for miRNA expression analysis. The reads per million miRNAs mapped data unit was evaluated for miR-221. The expression of miR-221 was divided into two groups according to the mean expression level.

Cell culture. Human colon cancer cell line CACO2, HT29, HCT116 and SW620 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37°C in a humidified cell incubator of 5% CO₂.

RNA extraction and quantitative reverse transcription polymerase chain reactions. Total RNA was isolated from cultured cells using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand cDNA were synthesized from 1 µg of total RNA using MMLV reverse transcription kit according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA) with specific reverse transcription primer. Real-time quantitative PCR analysis was performed using FS Universal SYBR-Green Master reagents (Roche Diagnostics, Indianapolis, IN, USA) in an Applied Biosystems PRISM 7500 instruments (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for miR-221 were as follows: Reverse transcription, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGAAACC-3'; forward, 5'-CGAGCTACATTGTCTGCTGGGT-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'. U6 RNA was used as an internal control for miRNA normalization. Primer set for U6 was purchased from RiboBio Co., Ltd. All samples were run in triplicate and the 2^{-ΔΔCT} method was applied to quantify the relative expression of miR-221.

Plasmids, cell transfection and Cell Counting kit-8 (CCK-8) assay. Overexpression and down-expression plasmids of

miR-221 plus negative control plasmids were purchased from GenePharma Co., Ltd. (Shanghai, China) Transfection of cell lines was carried out using jetPEI (Polyplus-transfection), according to the manufacturer's instructions. Cells with specific transfection were seeded in 96-well plates. CCK-8 solution was added at particular periods of time and incubated for 1 h at 37°C. Absorbance was detected at 450 nm using Synergy² Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Western blot and autophagy flux analysis. Cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was assessed by the BCA assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amount of proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with the rabbit anti-LC3 II monoclonal antibody (1:1000, #2775; Cell Signaling Technology, Inc., Danvers, MA, USA) or the rabbit anti-TP53INP1 polyclonal antibody (1:500, sc-68919; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C and then with goat anti-rabbit IgG infrared-dye-conjugated secondary antibodies (1:10,000, 142801A; Abbkine Scientific Co., Ltd., Wuhan, China) for 1 h at room temperature. β-actin (1:1,000, sc-376421; Santa Cruz Biotechnology, Inc.) was used as a loading control. Protein bands were detected by Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Autophagy flux was measured with LC3II turnover using protease inhibitors, cells were cultured in nutrient-rich medium (DMEM containing 10% FBS) or starvation conditions (DMEM without FBS) in the absence or presence of E64d (E8640, 10 µg/ml, 4 h; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and pepstatin A (P5318, 10 µg/ml, 4 h; Sigma-Aldrich; Merck KGaA), or Bafilomycin A1 (ab120497, 0.1 µM, 2 h; Abcam, Cambridge, UK). Expression of LC3II was quantified and the ratio of LC3II between cells treated without/with protease inhibitors was applied to represent autophagy flux (17).

miR-221 target gene prediction and luciferase reporter assay. TargetScan 6.2 (18), miRanda-rel 2010 (19), and RNA22 v2.0 (20) were applied for bioinformatic analysis. The binding sites were further predicted. The human 3'UTR of TP53INP1 and a mutant of the putative binding site to the seed sequence of miR-221 were cloned into the pGL3-control vector (Promega Corporation) downstream of the firefly luciferase gene to generate the vector pGL3-TP53INP1-WT and pGL3-TP53INP1-MUT by GenePharma Co., Ltd. For the luciferase reporter assay, cells were co-transfected with pGL3-TP53INP1-WT or pGL3-TP53INP1-MUT, and the pRL-TK vector (as calibration) using jetPEI (Polyplus-transfection). Luciferase activity was determined with the Dual Luciferase Reporter Assay system (Promega Corporation).

Statistical analysis. Quantitative data was presented as mean ± standard deviation (SD). Student's t-test was applied to compare the mean value of each group. Overall survival was estimated with Kaplan-Meier method. Results were

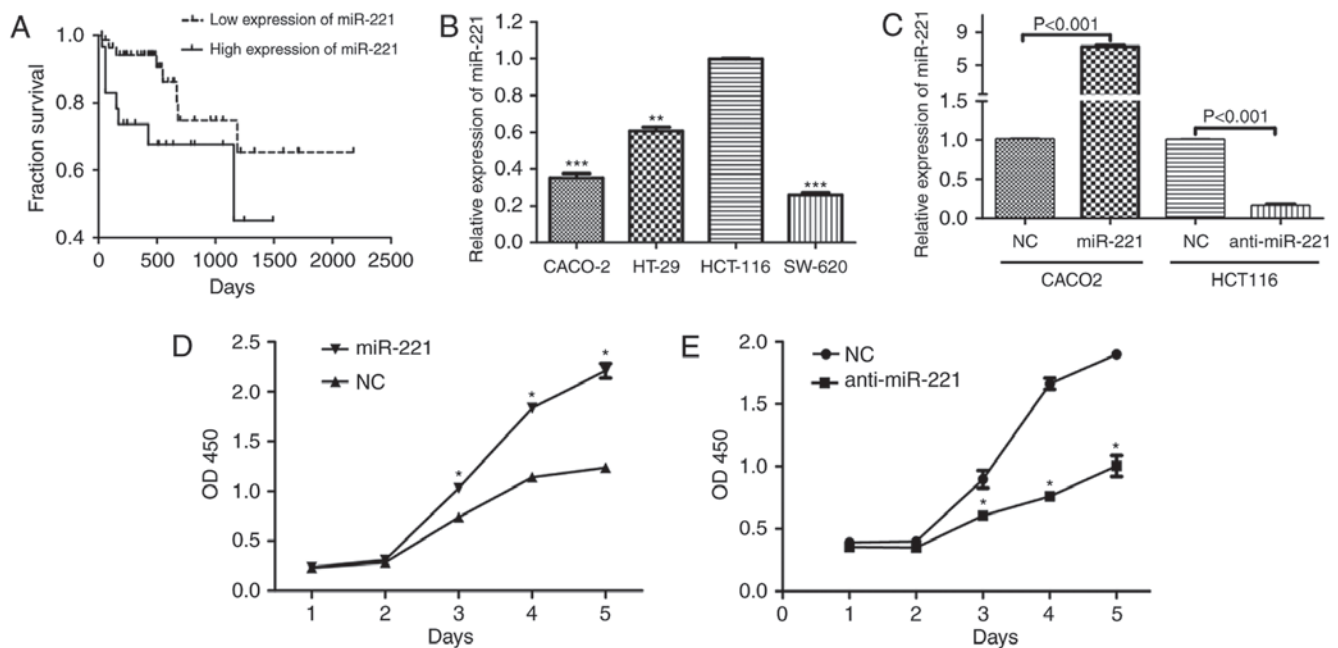


Figure 1. Expression of miR-221 and CRC survival. (A) Association between miR-221 expression and CRC survival was estimated by performing Kaplan-Meier curve analysis using The Cancer Genome Atlas data. Log-rank test, $P=0.059$; Breslow test, $P=0.027$; Tarone Ware test, $P=0.031$. (B) Relative mRNA expression of miR-221 in CRC cells. ** $P<0.01$ and *** $P<0.001$ vs. HCT116 cells. (C) The mRNA expression of miR-221 in transfected CRC cells. $P<0.001$ as indicated. (D) Overexpression of miR-221 promoted cell growth in CACO2 cells. * $P<0.05$ vs. NC. (E) Downregulation of miR-221 suppressed cell growth in HCT116 cells. * $P<0.05$ vs. NC. miR, microRNA; CRC, colorectal cancer; NC, negative control.

considered statistically significant at $P<0.05$. All analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Results

miR-221 regulates survival of CRC. miRNA data for CRC patients from TCGA were used to analyze miR-221 affecting CRC patient survival. The result of Kaplan-Meier method indicated that higher expression of miR-221 was associated with worse patient survival (log-rank test, $P=0.059$; Breslow test, $P=0.027$; Tarone Ware test, $P=0.031$; Fig. 1A). The relative expression of miR-221 was detected in CRC cells. The data showed that the expression of miR-221 was high in HCT116 cells and low in CACO2 and SW620 cells (Fig. 1B). Based on the expression level of miR-221, overexpression of miR-221 in CACO2 and anti-expression in HCT116 cells was implemented respectively in this study. Transfection with miR-221 significantly increased the expression of miR-221 in CACO2 cells, while transfection with anti-miR-221 reduced miR-221 expression in HCT116 cells (Fig. 1C). CCK-8 assay was applied to assess miR-221 affecting CRC cells growth. The data suggested that overexpression of miR-221 promoted cell growth in CACO2 cells whereas downregulation of miR-221 inhibited cell growth in HCT116 cells (Fig. 1D and E).

miR-221 impairs autophagy in CRC cells. Autophagy flux analyzed by LC3 turnover was performed to determine autophagy of CRC cells modulated by miR-221. The ratio of LC3II quantified by western blot analysis between cells treated without/with protease inhibitors was used to reflect autophagy flux. The results suggested that LC3II ratio was inversely regulated by miR-221 in both nutrient-rich medium

and starvation condition using different protease inhibitors. LC3II ratio was decreased in CACO2 cells with overexpression of miR-221 whereas LC3II ratio was increased in HCT116 cells with downregulation of miR-221 in E64d and pepstatin A (Fig. 2A and B). Similar result was obtained in Bafilomycin A1 treatment, which suggested that LC3II ratio was significantly downregulated in CACO2 cells with overexpression of miR-221 whereas LC3II ratio was increased in HCT116 cells with down-expression of miR-221 (Fig. 2C and D). These data indicated that miR-221 reduced autophagy in CRC cells (Fig. 2).

TP53INP1 is a target of miR-221. Bioinformatic analysis identified TP53INP1 as a potential target of miR-221. Among the predicted targets of hsa-miR-221 by the three programs, 446 miRNA-regulated target genes were identified by Targetscan, 5,670 transcripts were identified by miRanda, and 10,184 transcripts by RNA 22. We focused our attention on TP53INP1, which is a regulator of autophagy (15). Western blot analysis showed that the protein expression of TP53INP1 was low in HCT116 cells and high in CACO2 and SW620 cells (Fig. 3A). The expression pattern of TP53INP1 protein was inversely correlated with the expression of miR-221. Overexpression of miR-221 in CACO2 cells repressed TP53INP1 expression whereas downregulation of miR-221 in HCT116 cells upregulated TP53INP1 expression (Fig. 3B and C).

The binding sites of TP53INP1 and miR-221 were predicted and luciferase plasmids were constructed and designated as pGL3-TP53INP1-WT and pGL3-TP53INP1-MUT (Fig. 3D). The luciferase activity assay indicated that overexpression of miR-221 suppressed luciferase activity of wild-type TP53INP1 in CACO2 cells whereas downregulation of

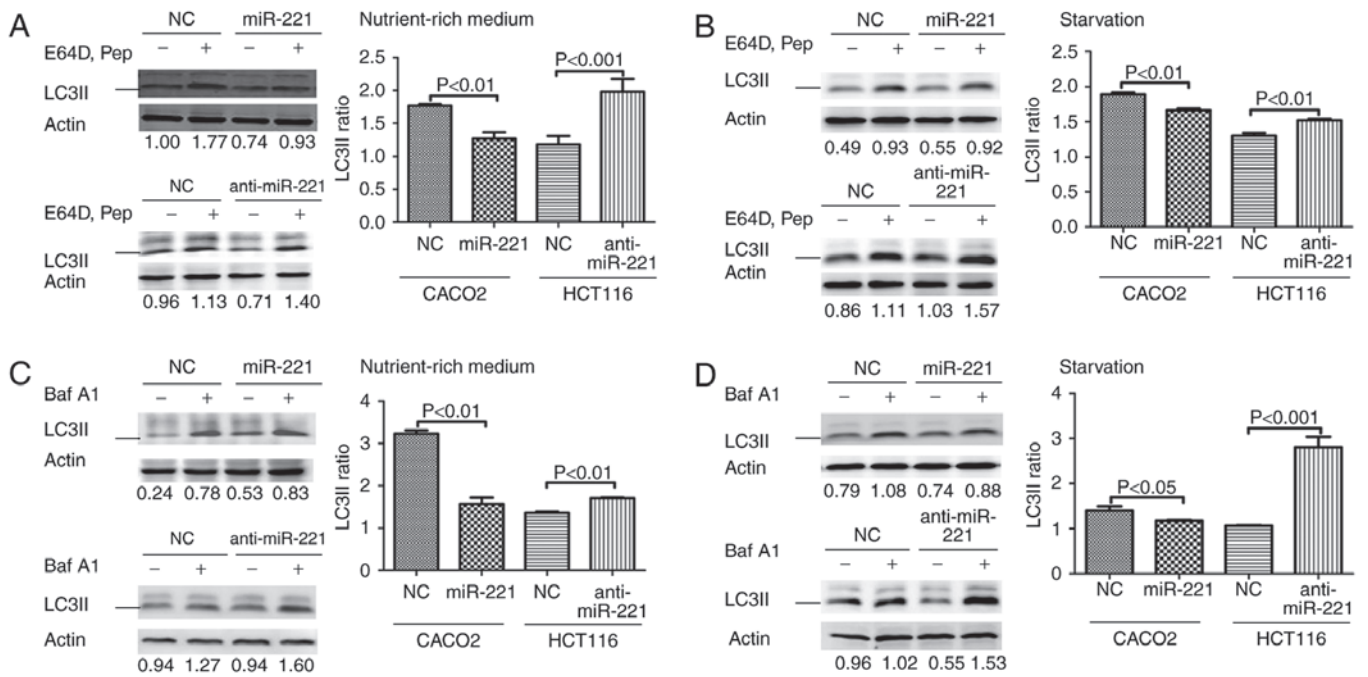


Figure 2. miR-221 inhibits autophagy in CRC cells. Autophagy flux indicated by LC3II turnover was modulated by miR-221 overexpression in CACO2 cells and downexpression in HCT116 cells in different cultivated conditions and drug treatments. (A) Cells were incubated in nutrient-rich medium in the absence (-) or presence (+) of E64d and pepstatin A. (B) Cells were incubated under starvation conditions in the absence (-) or presence (+) of E64d and pepstatin A. (C) Cells were incubated in nutrient-rich medium in the absence (-) or presence (+) of Bafilomycin A1. (D) Cells were incubated under starvation conditions in the absence (-) or presence (+) of Bafilomycin A1. $P < 0.05$, $P < 0.01$ and $P < 0.001$, as indicated. miR, microRNA; CRC, colorectal cancer; NC, negative control; Baf A1, Bafilomycin A1; Pep, pepstatin A.

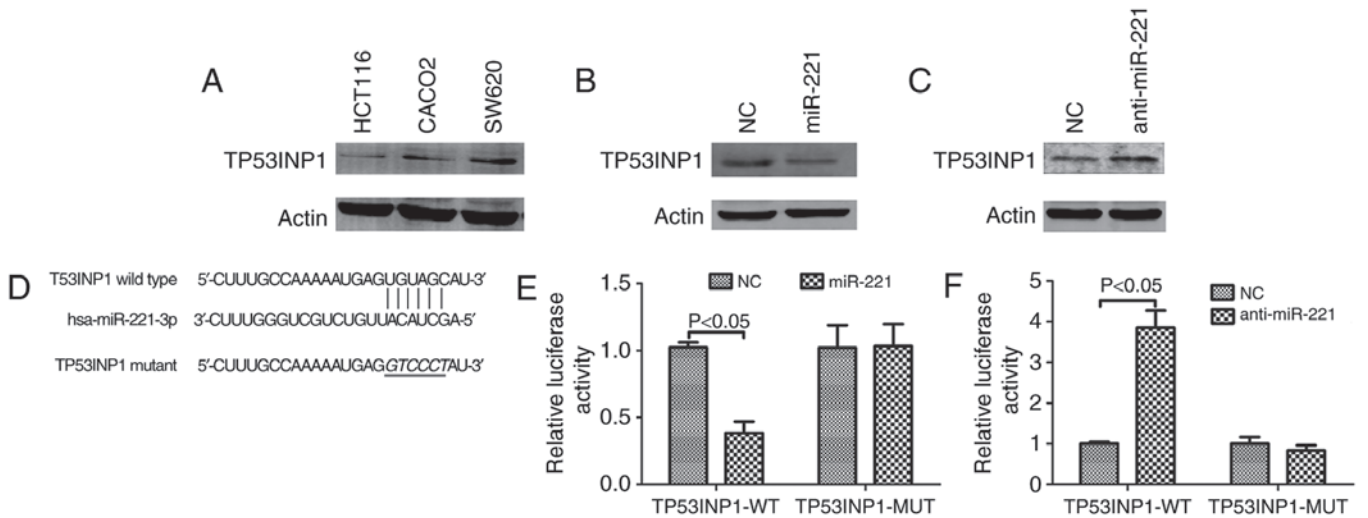


Figure 3. TP53INP1 is a target of miR-221. (A) TP53INP1 protein expression in CRC cells. (B) Overexpression of miR-221 downregulated TP53INP1 protein expression in CACO2 cells. (C) Downregulation of miR-221 upregulated TP53INP1 protein expression in HCT116 cells. (D) WT and MUT of putative miR-221 targeting sequences in TP53INP1 mRNA. (E) Overexpression of miR-221 suppressed luciferase activity of WT TP53INP1 in CACO2 cells. (F) Downregulation of miR-221 increased luciferase activity of WT TP53INP1 in HCT116 cells. $P < 0.05$, as indicated. TP53INP1, tumor protein 53-induced nuclear protein 1; miR, microRNA; CRC, colorectal cancer; NC, negative control; WT, wild-type; MUT, mutant.

miR-221 increased luciferase activity of wild-type TP53INP1 in HCT116 cells without interfering the activity of mutant TP53INP1 (Fig. 3E and F).

Discussion

The role of miR-221 is considered to act as an oncogene or a tumor suppressor in tumor progression depending on tumor

background (21). Overexpression of miR-221 is found in the majority of epithelial tumors (22). miR-221 is a potential biomarker for cancer diagnosis and prognosis (23-25). miR-221 modulates the proliferation, apoptosis, cell cycle distribution and cell migration in a variety of cancers (21). However, the function of miR-221 in autophagy of cancer is not clarified. In this study, we demonstrated that miR-221 inhibited autophagy activity and thereby promoted cell survival in CRC.

Autophagy is a cellular degradation process by eliminating damaged or superfluous proteins, and unnecessary or dysfunctional cellular components (26,27). Autophagy is a dual function player in cancer progression. On one hand autophagy plays oncosuppressive functions by the clearance of potentially harmful components; on the other hand it facilitates to overcome cellular stress during cancer progression (26). The core pathway of autophagy involves at least five molecular components regulated by a panel of proteins such as Atg1, Atg8, Atg9, Atg12, Beclin 1 and LC3 (28).

Recently, TP53INP1 is found to be a regulator of autophagy (15). TP53INP1 is reported to interact with ATG8-family proteins and LC3 to induce autophagy-dependent cell death (29). Two miRNAs, miR-30a and miR-205, are suggested to suppress autophagy by targeting TP53INP1 (30,31). Our data with bioinformative prediction, expression analysis and luciferase assay indicated that TP53INP1 was a target of miR-221 in CRC cells, which might elucidate the inhibitory effect of autophagy by miR-221. In cardiac remodeling, the inactivation of mTOR abolishes the inhibitory effect of miR-221 on autophagy (10). mTOR as a miR-221 target is also reported in hepatocellular carcinoma cells (32). Overexpression of p27 is found to rescue the impairment of miR-221 on autophagic flux in cardiac remodeling (10). In human umbilical vein endothelial cells, miR-221 is found to inhibit autophagy by modulating a PTEN/Akt signaling pathway (12). miR-221 targeting PTEN/Akt is also reported in several cancer cells during cancer development (33-35). Our results implicated a potential role of TP53INP1 on the effect of miR-221 on autophagy of CRC.

In conclusion, our findings explore a new role of miR-221 in autophagy regulation of cancer. The tumor promotion function of miR-221 in CRC could be the result of autophagy inhibition. TP53INP1 is a new target of miR-221 in CRC cells. Although further experiments should be performed, our data suggested that the effect of miR-221 on autophagy might be mediated by TP53INP1.

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