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

DAN LIAO1,2*, TONG LI1*, CAIGUO YE1*, LIUYAN ZENG3, HUAHUI LI1, XINGXIANG PU4, CONGCONG DING1, ZHIWEI HE1 and GUO-LIANG HUANG1

1China-American Cancer Research Institute, Dongguan Scientific Research Center, Key Laboratory for Epigenetics of Dongguan City, Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, Guangdong Medical University, Dongguan, Guangdong 523808; 2Department of Gynaecology and Obstetrics, Dongguan Third People’s Hospital, Affiliated Dongguan Shilong People’s Hospital of Southern Medical University, Dongguan, Guangdong 523326; 3Department of Health Management Center, The Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524001; 4Department II of Chest Internal Medicine, Hunan Cancer Hospital The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, Hunan 410013, P.R. China

Received June 29, 2017; Accepted November 15, 2017

DOI: 10.3892/etm.2017.5522

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 bioinformative 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.
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 miRNAsSeq 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 median 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 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., Waltham, MA, USA) and maintained at 37°C in a humidified cell incubator of 5% CO₂.

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 Reproter 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
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 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.

**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
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.

Acknowledgements

This study was supported by the Science and Technology Planning Project of Guangdong Province (grant no. 2013B021800070), the Training Plan for Outstanding Young Teachers in Higher Education Institutions of Guangdong Province (grant no. YQ201403/YQ2014086), the Hunan Provincial Natural Science Foundation of China (grant no. 2015jj6066), and the Project of Hunan Provincial Department of Health (grant no. B2012-095).

References


