MicroRNA-155 increases colon cancer chemoresistance to cisplatin by targeting forkhead box O3

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Abstract. To investigate the effect of microRNA (miR)-155 on colon cancer chemoresistance to cisplatin and its mechanism. Reverse transcription quantitative polymerase chain reaction was used to measure the levels of miR-155 and forkhead box O3 (FOXO3) in colon cancer specimens and cell lines. Overexpression of miR-155 and miR-155 inhibitor were transfected into colon cancer cell lines to investigate its role of chemoresistance to cisplatin in colon cancer. MTS assays were used to analyse cell viability in vitro. In vivo tumor formation assays were performed in C57BL/6 wild type and miR-155 knockout mice (miR-155-/-). A luciferase reporter assay was used to measure the translation of FOXO3. Additionally, the expression of FOXO3 was detected by western blot analysis. It was identified that miR-155 was markedly upregulated in colon cancer tissue and cell lines. Overexpression of miR-155 enhanced colon cancer cell chemoresistance to cisplatin in vitro and tumorigenesis in vivo. In addition, overexpression of miR-155 was associated with decreased levels of FOXO3, primarily through inhibiting the expression of FOXO3 to increase colon cancer resistance to cisplatin. The present study demonstrated that miR-155 increased colon cancer drug resistance and decreased FOXO3 expression in vivo and in vitro. This may provide a novel method for the treatment of drug-resistant colon cancer.

Introduction

Colorectal cancer (CRC) was the third leading cause of cancer-associated mortality worldwide in 2015, despite the advancements in the diagnosis and treatment (1). A total of ~50% of patients with colon cancer will develop liver metastasis and the 5-year survival rate for metastatic colon cancer is only 10-15% in the United States of America (2,3). Metastasis is a major cause of mortality in patients with colon cancer and is considered incurable due to a lack of effective therapy (4). To identify the novel molecules serving key roles in colon cancer, a number of studies focused on determining the microRNA (miRNA/miR) associated with this type of cancer (5-7).

Forkhead box O3 (FOXO3) is one of the most comprehensively characterized members of the FOXO family of transcription factors. It is a tumor suppressor, and is a potent transcriptional activator which triggers the expression of a program of genes involved in cell cycle arrest, DNA repair, hypoxia and apoptosis (8,9). It has been identified that the FOXO3 transcription factor serves as a target of anti-tumor drugs in different types of cancer, including breast cancer, chronic myeloid leukemia and colon cancer (10,11). It also affects the sensitivity of colon cancer cells to cisplatin (11). FOXO3 activity may be regulated by post-translational modifications, including phosphorylation (12), but the molecular mechanisms regulating the expression of the FOXO3 expression remain unclear.

miRs are naturally-occurring endogenous single-stranded RNA usually measuring 18-24 nucleotides in length (13). A total of >700 miRs have been identified in the human genome. A function of microRNA is the downregulation of target proteins expression by mRNA cleavage and decay or translational repression (14). miRs have been implicated in the regulation of gene expression essential for organ development, cellular differentiation, homeostasis and those involved in tumor occurrence (15-17). miRNA expression is often dysregulated in cancer tissues and they may function either as tumor suppressors or oncogenes (oncomiRs) (18). miR-155 has been demonstrated to be an oncogenic miRNA in certain tumors, including liposarcoma and leukaemia (19-21). miR-155 transgenic mice have also been demonstrated to develop acute lymphocytic lymphoma or leukaemia (22). miR-155 is upregulated in gastric cancer and serves as a prognosis biomarker in these patients (23). Increased miR-155 expression is involved in poor prognosis in pancreatic and lung cancers (24,25), while in breast cancer miR-155 contributes to the progression of invasion (26,27). There are numerous target genes of miR-155, including inositol polyphosphate-5-phosphatase D and CCAAT/enhancer binding protein β, suppressor of
cytokine signaling 1 and FOXO3 (28). However, the function of miR-155 in colon cancer is not understood, although it has been identified that miR-155 is upregulated in colon cancer (29,30). It remains unknown if miR-155 targets FOXO3 to effect colon cancer cell viability.

In the present study, clinical tissues from patients with colon cancer were collected and miR-155 and FOXO3 expression levels were detected. It was identified that the expression levels of miR-155 and FOXO3 were increased and decreased, respectively, in colon cancer tissues and human cell lines. miR-155 was transfected into colon carcinoma HT29 and SW620 cell lines, and it was determined that FOXO3 expression was decreased at the protein level in a dose-dependent manner, suggesting that they are negatively associated. The sensitivity of colon carcinoma cells to chemotherapy drugs cisplatin and paclitaxel was detected, and it was demonstrated that miR-155 increased chemoresistance. The present study suggests a novel pathway and target for colon cancer therapy.

Materials and methods

Cell lines and reagents. Human colon carcinoma HT29 and SW620 cell lines were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co. Ltd, China), 100 units/ml penicillin and 100 g/ml streptomycin at 37°C in a humidified chamber with 5% CO₂. Samples from patients with colon cancer and para-carcinoma tissues were collected by radical colon resection from the People's Hospital of Rizhao City (Rizhao, China). The present study was approved by the Ethical Committee of the People's Hospital of Rizhao City (Rizhao, China). The present study was approved by the Ethical Committee of the People's Hospital of Rizhao City (Rizhao, China). All the patients were informed and written consent was obtained. Cisplatin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). miR-155-5p mimic (C-3006 47-05-0005) and scrambled control oligonucleotide (CN-001000-01-05; sequence, 5'-CCCUAUCACGAUUAGCAUUAAUU-3') were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit was purchased from Promega Corporation (Madison, WI, USA). All the PCR primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using TRIzol® (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For mRNA detection, cDNA for PCR was obtained by RT-PCR following the manufacturer's protocol of a Toyobo SYBR-Green RT-PCR Master Mix kit (Toyobo Life Science, Osaka, Japan). cDNA was subjected to qPCR using the SYBR-Green PCR reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI StepOnePlus (ABI) instrument (Thermo Fisher Scientific, Inc.). For miR-155 detection, polyAs were added into RNA using Esherichia coli polyA polymerase (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). A total of 2 µg tailed total RNA was reverse transcribed with miR reverse transcriptase primer (1 µM; Shanghai GenePharma Co., Ltd., Shanghai, China). qPCR was performed with miR-155 forward (F) and reverse (R) primers, and the thermocycler conditions were as follows: 95°C for 10 min; then 41 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. All primers used for qPCR analysis were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), as follows: FoxO3 F, AGTGGATGTTGC GCTGTGT; FoxO3 R, CTGTCGACCAGGCGGATCAGCA GTTTTTTTTTTTTTTTTTTTTTTTTTTTTT; miR-155 F, TTA ATGCTAATCTGTAAGG; miR-155 R, GCTGTCAAC GATAACGCTAAGCAG; and U6 R, AACGCTTACGATTTGCGT. The relative amount of target mRNA was determined using the comparative threshold (Cq) method by normalizing target mRNA Cq values to those of GAPDH or U6 (32).

Plasmid construction and stable/transient transfection of miR-155. To construct the human FOXO3 recombinant plasmid, the FOXO3 gene (NCBI Reference Sequence: NM_001455.3) was cloned into pCMV-tag2a vector (Agilent Technologies; Thermo Fisher Scientific, Inc.). A human genomic fragment of 65 bp containing the miR-155 precursor DNA sequence (NCBI Reference Sequence: NR_030784.1) was cloned into the pcDNA3.1(-)-myc-his vector (Invitrogen; Thermo Fisher Scientific, Inc.). The recombinant plasmid was pcDNA3.1-miR-155. The primers for construction were as follows; underlined nucleotides represent BamH I and HindIII sites: miR-155 P1, GATCC TGTATAGCTAACTGTGATAGGGTTTTGCCCTCA ACTGACTCTCATATATTAGCATTACAAGCA; miR-155 P2, AGCTCTTGTTAAGCTGCTATATATGGTAGCTAGT GGGGAGAAAACCCCTATCAGATTAGCATTACAGG; FOXO3-P1, ATTAGGATCTACGAGGCGACGCCGCG TTC; and FOXO3-P2, GCAGAAGTTTTCTGTCGCC AGCTCTAG. To generate a cell line stably expressing miR-155, HT29 and SW620 cells were transfected with 200 ng pcDNA3.1-miR-155 using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h transfection, following 800 mg/ml G418 selection, the single clone that over-expressed miR-155 was identified. For miR155 transient transfection, miR-155 mimics (Invitrogen; Thermo Fisher Scientific, Inc.) were used to transfect the 2 cell lines.

 Luciferase assays. The online database search TargetScanHuman 6.2 (http://www.targetscan.org/), microRNA (http://www.microrna.org) and miR TarBase (http://mirtarbase.mbc.nctu.edu.tw/php/search.php) were used to predict a potential target of miR-155 (Date of access, January 2016). The wild type (WT) or mutant 3'-UTR of FOXO3 containing the putative miR-155 binding sites was synthesized and inserted into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) to generate the recombinant constructs, pmirGLO-FOXO3-3'UTR-WT and pmirGLO-FOXO3-3'UTR-M, respectively. miR-155 mimics or miR-inhibitor using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 48 h. The primers for 3'UTR WT and mutant were as follow;
FOXO3-3'UTR-WT P1, AAACTTTTGCAAAAAAAGCA TTAGGCATAT; FOXO3-3'UTR-WT P2, CTAGATATGCCT AATTGGTTTATGC AAGAGT TT; FOXO3-3'UTR-M P1, AAACTCTTTGCATAAAAACGATAAGGCATAT; and FOXO3-3'UTR-M P2, CTAGATATGCCTUAUGGTTTTTATGC AAGAGTTT. Underlined nucleotides represent PmeI and XbaI sites, respectively. Italicized nucleotides represent the miR-155 target sequence. Bold italicized nucleotides represent the sites of mutation. Cells were harvested 48 h following transfection for the measurement of luciferase activity with a Dual-Luciferase Reporter Assay system (Promega Corporation) according to manufacturer's protocol. Renilla luciferase activity was applied as the method of normalization for firefly luciferase activity.

Cell proliferation assays. The effect of miR-155-5p on cisplatin chemotherapy sensitivity of colon cancer cell lines was detected using Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega Corporation) according to manufacturer's protocol. Cells grown in regular media were plated on 96-well plates (5,000 cells/well) and treated with cisplatin (0, 10, 50, 100 and 200 µM) at 37°C in a 5% CO₂ humidified atmosphere for 48 h. Subsequently, 20 µl combined MTS/phenozenethioseolate was pipetted into each well and incubated for 4 h at 37°C. A total of 25 µl 10% SDS (Boster Biological Thecnology, Pleasanton, CA, USA) was added to each well to stop the reaction. Absorbance was detected at 490 nm using a 96-well plate reader.

Western blot analysis. Tissues from the CT-26 tumors (2x10⁵ cells/mouse; purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were collected and lysed cells by lysis buffer (Tris 20 mM, NaCl 150 mM, 1% Triton X-100 and 1% cocktail protease inhibitors). All of the protein extraction process was carried out according to the product specification. The BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was applied to detect the protein concentration. Then total protein (30 µg) were separated by electrophoresis on a 12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred onto nitrocellulose membranes (Merck KGaA). The membranes were blocked in 5% non-fat milk solution for 2-4 h at 4°C, and then washed twice with TBST solution (0.1% Tween-20). Membranes were incubated overnight at 4°C with the following antibodies. FOXO3 polyclonal antibodies (1:1,000; Abcam, Cambridge, UK; cat. no. ab23683) or β-actin (1:3,000, cat. no. BM0626, Specificity: monoclonal mouse; Boster Biological Technology, USA) monoclonal antibodies were used as primary antibodies, followed by goat anti-rabbit (cat. no. A21020; dilution: 1:1,000; AmeyJet Scientific, Wuhan, China) or goat anti-mouse secondary antibodies (cat. no. A21010; dilution: 1:1,000) conjugated with horseradish peroxidase (ProteinTech Groups, Inc., Chicago, IL, USA) incubated for 2 h at 4°C. The protein analysis was performed using Millipore ECL Western Blotting Substrate on a UVP ChemDoc-it imaging system (Chemidoc-IT 510; Visionworks LS, UVP, LLC, USA).

Caspase 3 activity detection. Colon cancer cells treated with or without cisplatin were seeded at a density of 2x10⁵ cells/well in a 6-well plate with 2 ml culture medium. Following 24 h incubation, cells were treated with 200 µM cisplatin to induce apoptosis. Caspase-3 activity was measured using a Caspase 3 Fluorometric activity assay kit (cat no. C1115; Beyotime Institute of Biotechnology) following the manufacturer's protocol.

Flow cytometry. Colon cancer cells treated with or without cisplatin were harvested by 500 µl 0.25% trypsin and then centrifugation 5 min at room temperature (241.5 x g). A total of 500 µl binding buffer (including precooled 70% ethanol and 0.5 mmol/l EDTA) was added to each tube and incubated overnight at 4°C, then intracellularly stained with phycoerythrin-anti-human Ki67 mAb (cat no. 350503; dilution, 1:20; BioLegend, Inc., San Diego, CA, USA) in staining buffer (PBS containing 0.5% BSA) for 30 min at room temperature in the dark. Samples were washed twice with PBS, and analyzed by flow cytometry using a BD C6 auri flow cytometry and Expo 32-ADC v. 1.2B software (both BD Biosciences, Franklin Lakes, NJ, USA).

Animal model. Pathogen-free 10 weeks old male WT C57BL/6 mice (mean weight:24.26±3.04 g, n=7) and pathogen-free 10-week-old male miR-155 knockout mice (miR-155-/-) (mean weight, 24.75±2.93 g; n=6) were obtained from Jackson Laboratory (Ben Harbour, ME, USA). The animals were housed under standard conditions (18-29°C, 0.03%) in a 12 h light/dark cycle with access to water and food (ad libitum). The mouse colon cancer CT26 cell line (2x10⁵ cells per mouse) was inoculated into the backs of the mice by hypodermic inoculation (matrix glue was adminis- tered to the mice in order to prevent the spread of the tumor, and multiple tumors were not observed). A total of 2 days following tumor cell inoculation, mice were treated with cisplatin (300 µM) every 2 days for 10 days by intraperito- neal injection. The tumor sizes were measured every 3 days using calipers from 1st day of drug treatment to the 30th day, and tumor volume was calculated as: Tumor volume=[length x width (b^2)]/2 (33). The data are presented as the mean volume ± standard deviation (SD). The Ethical Committee of the People's Hospital of Rizhao approved all animal experimental procedures.

Statistical analysis. SPSS software (version 16; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All data were calculated as the mean ± standard deviation. A one-way analysis of variance and Student Newman-Keuls post-hoc test were used to analyze the results between treated and control groups, and an unpaired two-tailed Student's t-test was used to compare two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-155 is negatively associated with FOXO3 in colon cancer tissues. To investigate miR-155 expression in colon cancer and para-carcinoma tissues, samples (colon cancer, n=25; para-carcinoma, n=20) from Rizhao People's Hospital were collected. Total RNA of tissues was extracted, and qPCR was performed. The results indicated that miR-155 expression was
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Increased in colon cancer tissues compared with para-carcinoma tissues, while FOXO3 expression was decreased in colon cancer tissues compared with para-carcinoma tissues (Fig. 1A and B). The plasmid pcDNA3.1-miR-155 was transfected into colon cancer HT29 and SW620 cell lines, and the mRNA expression of FOXO3 was also detected. FOXO3 mRNA levels decreased following miR-155 enhancement compared with the control group (Fig. 1C-E).

FOXO3 is a direct target of miR-155 in colon cancer cells. Based on an online database search for targets of miRNAs that were identified in previous studies (34,35), it was suggested that FOXO3 is a potential target of miR-155. Then, whether miR-155 mediated FOXO3 was determined. pmirGLO-UTR WT or M plasmids were constructed and transfected into HT-29 cells. It was demonstrated that reduced firefly luciferase expression indicated the binding of miR-155 to the cloned miRNA target sequence, and the mutant UTR group was not altered (Fig. 2A). This suggested that miR-155 may bind to position 1497-1503 of FOXO3 3’UTR and FOXO3 be directly targeted by miR-155 in human colon cancer cells (Fig. 2B).

miR-155 decreases chemosensitivity to cisplatin in colon cancer cells. Different concentrations of cisplatin (0, 10, 50, 100 and 200 µM) were used to treat HT29 and SW620 cells for 48 h. The viability of cells was detected using...
a Non-Radioactive Cell Proliferation Assay kit. HT29 cells demonstrated increased sensitivity compared with HT29-miR-155 cells, which stably expressed high levels of miR-155 (Fig. 3A). The same result was exhibited in SW620 cells (Fig. 3B). Antigen Ki-67 is a nuclear protein that is associated with cellular proliferation and is a cellular marker for proliferation (36). Ki67 was detected in HT29 and HT29-miR-155 cells treated with cisplatin by flow cytometry. (D) Ki67 was detected in SW620- and SW620-miR-155 cells treated with cisplatin by flow cytometry. (E) Ki67 percentages in colon cancer cells. miR, microRNA; PE, phycoerythrin; Iso, control group.

Figure 3. Effect of miR-155 on colon cancer cell sensitivity to the chemotherapy drug cisplatin. (A) miR-155 increased the viability of HT-29 cells following cisplatin treatment. (B) miR-155 increased the viability of SW620 cells following cisplatin treatment. (C) Ki67 was detected in HT-29 and HT-29-miR-155 cells treated with cisplatin by flow cytometry. (D) Ki67 was detected in SW620- and SW620-miR-155 cells treated with cisplatin by flow cytometry. (E) Ki67 percentages in colon cancer cells. miR, microRNA; PE, phycoerythrin; Iso, control group.

miR-155 blocks the caspase 3 activity induced by cisplatin. Cisplatin may induce apoptosis in HT-29 and SW620 cells (37-39). miR-155 mimics were transfected into these two cell lines to obtain HT-29-miR-155 and SW620-miR-155 cells. Colon cancer cells were treated with cisplatin for 48 h and harvested for caspase 3 activity detection. High miR-155 expression decreased the caspase 3 activity induced by cisplatin in HT-29 and SW620 cells (Fig. 4A and B).

FOXO transcription factors have been indicated to regulate apoptosis and cell cycle-associated genes (40-42). When FOXO3 and miR-155 were co-transfected into colon cancer cells, it was demonstrated that cisplatin efficiently induced caspase 3 activity (Fig. 4C and D). These data suggested that...
miR-155 decreased colon cancer sensitivity to cisplatin by targeting FOXO3.

**miR-155 promotes colon cancer growth in mice.** C57BL/6 (WT) and mir-155 knockout mice (miR-155-/-) were divided into 4 groups at random. CT26 cells were inoculated into the backs of the mice. The tumor-bearing mice were treated with 300 µM cisplatin for 10 days. Tumor length (a) and width (b) were measured using vernier calipers. Tumor volume was calculated using the formula: V (mm$^3$) = (a x b$^2$)/2.

The results showed that: the tumor weight was (0.7184±0.1547) g and (0.6728±0.1415) g in the control group of C57 WT mouse and miR-155-/- mouse, respectively, and the tumor weight was (0.5649±0.1252) g and (0.4937±0.1096) g in the cisplatin group of C57 WT mouse and miR-155-/- mouse, respectively. It was observed that tumor growth was significantly reduced in the miR-155-/- mice compared with the WT mice (Fig. 5A and B). It was also identified that FOXO3 expression in tumor tissues of miR-155-/- was increased compared with that of the WT mice (Fig. 5C). The data additionally suggested that miR-155 enhanced tumor growth and chemoresistance by targeting the FOXO3 gene.

**Discussion**

Although the mechanism by miR-155 regulation is not well understood, it has been suggested that the oncogenic microRNA miR-155 is enhanced in numerous types of cancer (43-46). However, miR-155 expression in colon cancer has not been identified. In the present study, to the best of our knowledge, the effect of miR-155 targeting FOXO3 gene in colon cancer cells was demonstrated for the first time. Tissues from patients with colon cancer were collected for miR-155 detection. The results indicated that miR-155 was increased in the colon cancer tissues, but not in para-carcinoma tissues. This result was in accordance with similar previous studies that have detected miR-155 expression in colon cancer (29,30).

As a prominent oncomiR, miR-155 may target the mismatch repair genes MutL Homolog 1, MutS Homolog (MSH) 2 and MSH6 and contribute to the microsatellite instability (MSI) phenotype in colon cancer (47). It downregulates B-cell lymphoma (Bcl)-6 and causes V-Myc avian myelocytomatosis viral oncogene homolog and Cyclin D1 upregulation, which promotes cell proliferation (48). In renal cancer cells, miR-155 may function as an oncogene by targeting BTB domain and CNC homolog (49). In the present study, miR-155 targets were predicted using TargetScanHuman 6.2, and it was identified that FOXO3 3'UTR was able to bind with miR-155. This prediction was associated with the results revealed by Zhang et al (50).

In the present study, FOXO3 expression in colon cancer was negatively associated with miR-155 expression. To additionally demonstrate the interaction between FOXO3 and miR-155, a recombinant plasmid pmirGLO-FOXO3-3'UTR-WT expressing the FOXO3 3'UTR wild type and a pmirGLO-FOXO3-3'UTR-M plasmid with the FOXO3 3'UTR with a small number of mutant bases, were constructed. Then, miR-155 mimics and inhibitors were co-transfected into HT-29 cells and luciferase reporter assays demonstrated that miR-155 directly bound to the FOXO3 3'UTR.

The functions of miR-155 in various types of cancer are different: It has been suggested that miR-155 is associated with the development of liver, leukemia, breast, lung and stomach tumors (51-53). Conversely, miR-155 may serve to prevent cancer in transgenic mice by promoting proper immune function. To clarify the function of miR-155 in colon cancer,
the effect of miR-155 on chemoresistance was evaluated in the present study. Cisplatin was used to treat HT-29, SW620, HT-29-miR-155 and SW620-miR-155 cells. miR-155 increased the resistance of colon cancer cells to cisplatin. This result is consistent with results from Yu et al (54). However, the novel observation of the present study was that in colon cancer cells, miR-155 increased chemoresistance by targeting FOXO3. In the miR-155-/- mice, CT26 cell growth was inhibited, and these cells demonstrated an increased sensitivity to cisplatin compared with the WT mice.

To conclude, the present study identified that miR-155 may promote colon cancer growth and increase colon cancer cells chemoresistance to cisplatin by directly targeting FOXO3. This suggests a novel pathway for the treatment and cure of colon cancer in the future.

Competing interests

The authors declare that they have no competing interests.

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