Original Paper

Knockdown of Mir-135b Sensitizes Colorectal Cancer Cells to Oxaliplatin-Induced Apoptosis Through Increase of FOXO1

Yan Qin^a Longhai Li^a Fang Wang^a Xinyi Zhou^a Yankui Liu^a
Yuan Yin^b Xiaowei Qi^a

^aDepartment of Pathology, the Affiliated Hospital of Jiangnan University, Wuxi 4th People's Hospital, Wuxi, ^bWuxi Oncology Institute, the Affiliated Hospital of Jiangnan University, Wuxi 4th People's Hospital, Wuxi, China

Key Words

Crc • MiR-135b • FOXO1 • Bim • Noxa • Oxaliplatin

Abstract

Background/Aims: Aberrant expression of microRNAs (miRNAs) is found to be responsible for tumorigenesis, cancer development and chemoresistance. Although oxaliplatin is an effective chemotherapeutic drug for treatment of colorectal cancer (CRC), CRC cells can develop some mechanisms to evade oxaliplatin-induced cell death. It is urgent to explore the novel strategies to increase the chemosensitivity of CRC cells. **Methods:** QRT-PCR analysis was performed to detect the expression of miR-135b in CRC patients' serum and CRC cell lines. MTT assays were used to evaluate the effect of anti-miR-135b on oxaliplatin-induced cell death in CRC cell lines. Western blot, flow cytometry and luciferase reporter assays were performed to evaluate the potential mechanism and pathway of anti-miR-135b-promoted apoptosis in oxaliplatin-treated CRC cells. **Results:** Significant upregulation of miR-135b was observed in CRC cell lines and CRC patients' serum. Knockdown of miR-135b was found to sensitize colorectal cancer cells to oxaliplatin-induced cytotoxicity. Mechanically, knockdown of miR-135b increased the expression level of FOXO1 in CRC. As the downstream, the increased FOXO1 induced by anti-miR-135b promoted the expression of Bim and Noxa. Since Bim and Noxa act as key pro-apoptotic proteins in mitochondrial apoptosis, anti-miR-135b was able to enhance the oxaliplatin-induced apoptosis dependent on the anti-miR-135b/FOXO1 axis. **Conclusions:** Anti-miR-135b enhanced the anti-tumor effect of oxaliplatin on CRC. Combination with miR-135b antisense nucleotides may represent a novel strategy to sensitize CRC to oxaliplatin-based treatment.

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Y. Qin and L. Li contributed equally to this work.

Yuan Yin
and Xiaowei Qi

Wuxi Oncology Institute, the Affiliated Hospital of Jiangnan University (Wuxi 4th People's Hospital)
No. 200, Huihe Road, Wuxi 214000, Jiangsu Province (China)
E-Mail wuxiyuanyin@yeah.net; wuxixiaoweiqi@yeah.net

Introduction

Due to the change of eating habits and lifestyle, incidence of colorectal cancer (CRC) is increasing rapidly in recent years. Nowadays, CRC has become the third common malignancies around the world. What is worse, as CRC is aggressive with high potential of metastasis, CRC is the second leading cause of cancer-associated mortality [1, 2]. Despite the great advancement of cancer therapy in recent years, surgery and chemotherapy are still the main approaches for treatment of CRC [3, 4]. However, cancer cells usually develop mechanisms to evade cell death induced by chemotherapy [5, 6]. To solve this major problem, it is urgent to explore the potential mechanisms of chemoresistance and take strategies to increase the chemosensitivity of CRC cells.

Oxaliplatin is one of the most effective chemotherapeutic drugs used for treatment of CRC. As a platinum-based anti-cancer drug, oxaliplatin exhibits high activity to bind with DNA nucleobases. Due to the interaction with oxaliplatin and DNA in CRC cells, apoptosis pathway is triggered followed by damage of DNA [7, 8]. However, cancer cells in numerous CRC patients usually show low sensitivity to oxaliplatin treatment [9]. CRC cells can develop some mechanisms to evade oxaliplatin-induced cell death. For example, cancer cells may upregulate drug efflux transporters to pump out the platinum-based chemotherapeutic drugs [10, 11], and cancer cells with acquired resistance to oxaliplatin expresses higher levels of anti-apoptotic proteins such as survivin and BIRC6 compared to the parental cells [12, 13]. Therefore, novel chemotherapeutic adjuvants are required to increase the chemosensitivity to oxaliplatin in CRC treatment.

MicroRNAs (miRNAs) are a group of endogenous and non-coding RNAs. Cellular miRNAs modulates expression of numerous genes by binding to the 3'-untranslated region (3'-UTR) of targeted mRNA [14, 15]. Therefore, miRNAs have strong biological activities to regulate tumorigenesis, cancer proliferation, development and apoptosis [16, 17]. Recently, studies demonstrate that many cases of chemotherapy failure are induced by aberrant expression of miRNAs in various cancers [18-21]. Therefore, correcting the disorder of miRNAs has been considered as a novel approach to sensitize cancer cells to chemotherapy. The aim of this study is to explore the potential role of miR-135b in regulating chemosensitivity to oxaliplatin in CRC.

Materials and Methods

CRC cell lines and patients' specimens

Human CRC cell lines HT29, SW620 and SW480 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum. Cells were cultured in humidified atmosphere containing 5% CO₂ at 37 °C. For detection of miR-135b *in vivo*, serum derived from 25 primary CRC patients and 25 healthy controls was collected at Affiliated Hospital of Jiangnan University (Wuxi 4th People's Hospital, Wuxi, China) between April 2015 and December 2016. The median age of the patients was 58 years (range, 34-81 years), and the patients were chosen randomly. We obtained informed consent from all of them. The experimental protocols were approved by the ethics committee of the Affiliated Hospital of Jiangnan University (Wuxi 4th People's Hospital).

Detection of miR-135b expression

Relative expression of miR-135b was detected according to the quantitative real-time polymerase chain reaction (qRT-PCR) assays. Briefly, total RNA from cell lines and patients' serum was extracted by TRIzol reagent (Invitrogen) and reversely transcribed by One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, China) according to the manufacturer's instructions. Mature miR-135b levels were measured SYBR Premix Ex Taq (TaKaRa) on the Applied Biosystems 7500 Sequence Detection system (Applied Biosystems, USA). MiR-135b primer sequence is as follows: 5'-TATGGCTTTTCATTCTATGTGA-3'. Relative expression of miR-135b was normalized to U6 snRNA according to the 2^{-ΔΔCT} method [22].

Cell transfection

Mature human miR-135b mimics (5'-UAUGGCUUUUCAUCCUAUGUGA-3'), miR-135b antisense-oligonucleotide (anti-miR-135b, 5'-UCACAUAGGAAUGAAAAGCCAU-3') and negative control oligonucleotides (NCO, 5'-UUAUUCGUGUUAUUAGCGUCCU-3') were purchased from GenePharma Co. Ltd. (Shanghai, China). For Knockdown of FOXO1, its specific siRNA was purchased from Santa Cruz Biotechnology (sc-35382). For overexpression of FOXO1, open reading frame of FOXO1 gene was amplified and inserted into the pcDNA3.1 eukaryotic expression plasmid (Invitrogen). Cells were transfected with miR-135b mimics (50 pmol/ml), anti-miR-135b (50 pmol/ml), NCO (50 pmol/ml), FOXO1 siRNA (50 pmol/ml) and FOXO1 plasmid (2 µg/ml) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Cell viability assay

Transfected cells were seeded into 96-well plates at a density of 5×10^3 cell/well. After cellular adhesion, culture medium containing oxaliplatin was added to each well. 48 h after incubation, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) reagent (100 µL medium + 20 µL MTT solution) was added to each well for 4 h incubation. After the cells were suspended in 150 µL of dimethyl sulfoxide (DMSO), the absorbance of each well at 570 nm was read on a spectrophotometer.

Luciferase reporter assay

FOXO1 3' UTR containing predicted miR-135b binding sites and corresponding mutant sites was cloned into the pMIR-firefly luciferase reporters (Invitrogen) and named as pMIR-WT FOXO1. The mutant FOXO1 reporter was created by mutating the seed regions of the miR-135b binding sites (AAAGCCAUA) by using the site-directed mutagenesis kit (Takara, Japan) and named as pMIR-MT FOXO1. pMIR-firefly luciferase reporters, pRL-TK renilla plasmids (Promega, USA) and miR-135b mimics (or anti-miR-135b or NCO) were co-transfected into cells by using Lipofectamine 2000 (Invitrogen). 48 h later, luciferase activities were measured by using Dual Luciferase Assay System (Promega) according to the manufacturer's instructions.

Western blot analysis

Cells were lysed in RIPA buffer (Cell Signaling Technology, USA) to extract the total proteins. After measurement of protein concentration, equal amounts of protein from each sample were separated by 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. Next, the membranes were blocked in 5% skimmed milk and probed with primary antibodies of FOXO1 (#2880, Cell Signaling Technology), Bim (sc-374358, Santa Cruz Biotechnology, USA), Noxa (sc-56169, Santa Cruz Biotechnology), Bax (sc-7480, Santa Cruz Biotechnology), MCL-1 (#94296, Cell Signaling Technology), cytochrome c (#11940, Cell Signaling Technology), caspase-9 (#9502, Cell Signaling Technology), caspase-3 (#9662, Cell Signaling Technology), Apaf-1 (#8969, Cell Signaling Technology), GAPDH (#5174, Cell Signaling Technology), β-actin (#3700, Cell Signaling Technology) and Cox IV (#4850, Cell Signaling Technology). Subsequently, proteins on the membranes were probed with HRP-conjugated secondary antibodies, and then visualized by using enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc, USA).

Separation of mitochondria fraction and cytoplasm fraction

Cellular mitochondria was extracted by using Mitochondria/Cytosol Fraction Kit (BioVision, USA) according to the manufacturer's instructions. They were considered as the mitochondria fraction. The remaining cell lysate was used as the cytoplasm fraction. Expression of Bax and cytochrome c in cellular mitochondria fraction and cytoplasm fraction was detected by using western blot assays

Immunoprecipitation

Cells were lysed in RIPA buffer (Cell Signaling Technology) followed by incubation with primary antibodies overnight at 4°C. Protein A agarose beads were then supplemented into the cell lysate and maintained for 2 h. After centrifugation, the resulting beads were mixed with the SDS loading buffer and boiled at 95 °C for 5 mins for preparation of following western blot analysis.

Cell apoptosis

Cells were collected and stained with Annexin-V and Propidium iodide (PI) according to the manufacturer's instructions (Sigma-Aldrich, USA). Cell apoptosis was determined by flow cytometry.

In vivo tumorigenesis assays

Anti-miR-135b-SW620 was conducted to stably express miR-135b antisense nucleotide sequence by using a lentiviral-based system (Genechem Co., Ltd., Shanghai, China) for tumorigenesis assays. Xenograft tumors were generated via the subcutaneous injection of anti-miR-135b-SW620 or control SW620 into the 4-5-week-old BALB/c nude mice (5×10^6 cells per mouse). After xenografts reached 0.5 cm in diameter, animals were treated with oxaliplatin i.p. twice a week (2 mg/kg) until the mice were killed 28 days post-injection. The animal care and experimental protocols were approved by the Animal Care Committee of the Affiliated Hospital of Jiangnan University (Wuxi 4th People's Hospital).

Statistical analysis

Data were obtained from at least three independent experiments and presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by using SPSS 16.0 software. For comparison analysis, two-tailed Student's t-test was used to determine the statistical differences between two groups. One-way analysis of variance (ANOVA) and Bonferroni's post hoc test were used to determine the differences between three or more groups. Differences were considered significant when the values of $P < 0.05$.

Results

Expression of miR-135b is overexpressed in CRC

First, we performed qRT-PCR analysis to investigate the expression profile of miR-135b in CRC. In cell lines, we found that expression level of miR-135b in SW480, SW620 and HT29 CRC cells was significantly higher than that in FHC which is a normal fetal colon cell line [23] (Fig. 1A). In patients' serum, we observed significant increase of miR-135b level in CRC patients compared to the healthy controls (Fig. 1B). These data suggested that miR-135b may function as a tumor promoter in CRC.

Knockdown of miR-135b sensitizes CRC cells to oxaliplatin-induced cytotoxicity

As the miR-135b was overexpressed in CRC cells, we corrected this disorder of miR-

Fig. 1. Expression of miR-135b was overexpressed in CRC. A: QRT-PCR assays were performed to analyze the expression profile of miR-135b in CRC cell lines SW480, SW620 and HT29 and normal colon cell line FHC. * $P < 0.05$ vs. FHC cell line. B: QRT-PCR assays were performed to detect the miR-135b level in CRC patients' and healthy controls' serum. * $P < 0.05$ vs. healthy controls.

Fig. 2. Knockdown of miR-135b sensitizes CRC cells to oxaliplatin-induced cytotoxicity. A: Effect of anti-miR-135b on changing cellular level of miR-135b in SW480, SW620 and HT29 CRC cells. * $P < 0.05$. B: Sensitization of anti-miR-135b on oxaliplatin-induced cytotoxicity (0-30 μ M) to SW480, SW620 and HT29 CRC cells. * $P < 0.05$.

135b by transfection with miR-135b antisense-oligonucleotide (anti-miR-135b) in SW480, SW620 and HT29 cells. We then showed the strong effect of anti-miR-135b on decreasing the cellular level of miR-135b in these CRC cell lines (Fig. 2A). Interestingly, we found that combination with anti-miR-135b sensitized CRC cells to oxaliplatin-induced cytotoxicity (Fig. 2B). We therefore demonstrated that anti-miR-135b can be used as a sensitizer to enhance the anti-tumor effect of oxaliplatin on CRC *in vitro*.

Knockdown of miR-135b increases FOXO1 expression in CRC

To explore the mechanism by which anti-miR-135b sensitized oxaliplatin-induced cytotoxicity to CRC, the online bioinformatics databases of TargetScan, miRanda, and PicTar were used to predict the potential target of miR-135b. FOXO1, a reported tumor suppressor in multiple cancers [24-26], was found to contain putative binding site paired with miR-135b at the 3' UTR of FOXO1 mRNA (Fig. 3A). To confirm the miR-135b/FOXO1 axis in CRC, we inserted the wild-type or mutant 3' UTR of FOXO1 into the pMIR-reporter followed by performance of dual-luciferase reporter assays. We found that co-transfection with miR-135b mimic significantly decreased the luciferase activities of pMIR-reporter contained wild type FOXO1 3'-UTR but not the mutant pMIR-reporter or the empty one. On the contrary, anti-miR-135b was found to increase the luciferase activities of pMIR-reporter carrying wild type FOXO1 3'-UTR (Fig. 3B). Furthermore, results of western blot analysis showed that protein level of FOXO1 was negatively regulated by miR-135b in CRC cells (Fig. 3C). We demonstrated that FOXO1 was the target of miR-135b, and treatment with anti-miR-135b was able to increase the FOXO1 expression in CRC.

Anti-miR-135b sensitizes CRC cells to oxaliplatin-induced cytotoxicity through the increase of FOXO1

To investigate whether the sensitization of anti-miR-135b on oxaliplatin-induced cytotoxicity to CRC was mediated by FOXO1 pathway, we transfected the HT29, SW620 and

Fig. 3. Anti-miR-135b increases FOXO1 expression in CRC. A: Putative binding site paired with miR-135b at the 3' UTR of FOXO1 mRNA. B: Effect of miR-135b mimics or inhibitors on changing luciferase activities of the pMIR-reporter carrying FOXO1 3'-UTR in HT29, SW620 and SW480 CRC cells. * $P < 0.05$ vs. NCO group. C: Effect of miR-135b mimics or inhibitors on changing protein level of FOXO1 in HT29, SW620 and SW480 CRC cells.

Fig. 4. Anti-miR-135b sensitizes CRC cells to oxaliplatin-induced cytotoxicity through the FOXO1 pathway. A: FOXO1 siRNA antagonized the effect of anti-miR-135b on sensitizing HT29, SW620 and SW480 CRC cells to oxaliplatin-induced cytotoxicity (2 μ M). * $P < 0.05$ vs. NCO + oxaliplatin group. # $P < 0.05$ vs. anti-miR-135b + oxaliplatin group. B: Effect of FOXO1 plasmid on enhancing oxaliplatin-induced cytotoxicity (2 μ M) to HT29, SW620 and SW480 CRC cells. * $P < 0.05$ vs. NCO + oxaliplatin group.

SW480 cells with FOXO1 small interfere RNA (FOXO1 siRNA) to antagonize the effect of anti-miR-135b on FOXO1 upregulation in CRC. We observed that the sensitization of anti-miR-135b on oxaliplatin-induced cytotoxicity to CRC was abolished due to the FOXO1 siRNA co-transfection (Fig. 4A). On the other hand, we simulated the effect of anti-miR-135b on FOXO1 by using recombinant FOXO1 plasmid. The results showed that transfection with FOXO1 plasmid sensitized HT29, SW620 and SW480 CRC cells to oxaliplatin-induced cytotoxicity, similarly with anti-miR-135b (Fig. 4B). These data indicated that FOXO1 expression profile regulated chemosensitivity to oxaliplatin in CRC, and anti-miR-135b was found to sensitize CRC cells to oxaliplatin-induced cytotoxicity through the increase of FOXO1.

Anti-miR-135b/FOXO1 axis promotes Bim and Noxa expression in oxaliplatin-treated CRC cells

Bim and Noxa expression is directly transactivated by FOXO1 [27, 28]. Furthermore, previous studies have demonstrated that expression profile of Bim and Noxa in cancer cells, which is regulated by FOXO1 pathway, determines the efficiency of chemotherapy. Overexpression of Bim and Noxa usually reduces the chemoresistance in cancer treatment [29-32]. Thus, we focused on these two proteins downstream of anti-miR-135b/FOXO1 axis in oxaliplatin-treated CRC cells. In addition, as oxaliplatin-induced cell death in SW620 can be augmented due to the anti-miR-135b co-treatment, we chose the CRC cell line of SW620 for the following mechanical research. Results of western blot analysis showed that anti-miR-135b and oxaliplatin treatment increased the protein level of FOXO1 in nucleus. Furthermore, we found that expression of both Bim and Noxa can be promoted by anti-miR-135b treatment. However, co-transfection with FOXO1 siRNA decreased the Bim and Noxa expression in oxaliplatin-treated SW620 cells (Fig. 5A). It indicated that anti-miR-135b/FOXO1 axis promotes Bim and Noxa expression in oxaliplatin-treated CRC cells. We next investigated the function of Bim and Noxa in CRC cells co-treated with anti-miR-135b and oxaliplatin. The results showed that anti-miR-135b promoted interaction with Bim and Bax (Fig. 5B), and thus inducing translocation of Bax from cytoplasm into mitochondria in oxaliplatin-treated SW620 cells (Fig. 5C). Besides, interaction with Noxa and MCL-1 also can be enhanced due to the anti-miR-135b transfection in oxaliplatin-treated SW620 cells (Fig. 5D). However, co-transfection with FOXO1 siRNA was found to inhibit the activities of Bim and Noxa in anti-miR-135b and oxaliplatin co-treated SW620 cells. We thus demonstrated the key role of anti-miR-135b/FOXO1 axis in enhancing Bim and Noxa function in oxaliplatin-treated CRC cells.

Fig. 5. Anti-miR-135b/FOXO1 axis promotes Bim and Noxa expression in oxaliplatin-treated CRC cells. A: Expression of FOXO1, Bim and Noxa in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). B: Interaction with Bim and Bax in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). C: Translocation of Bax from cytoplasm into mitochondria in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). D: Interaction with Noxa and MCL-1 in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M).

Anti-miR-135b/FOXO1 axis promotes mitochondrial apoptosis in oxaliplatin-treated CRC cells

Bim and Noxa functions as pro-apoptotic proteins dependent on mitochondrial pathway [33, 34]. Since the activities of Bim and Noxa were regulated by anti-miR-135b/FOXO1 axis, we next explored the effect of anti-miR-135b on mitochondrial apoptosis in oxaliplatin-treated CRC cells. As shown in Fig. 6A, combination with anti-miR-135b and oxaliplatin induced more significant release of cytochrome c from mitochondria into cytoplasm in SW620 CRC cells compared to the oxaliplatin monotherapy group. In the presence of cytochrome c in cytoplasm, induced by combination with anti-miR-135b and oxaliplatin, amount of Apaf-1/caspase-9 complex was significantly increased (Fig. 6B). Due to the interaction with Apaf-1 and caspase-9 in anti-miR-135b and oxaliplatin co-treated CRC cells, caspase-9 and its downstream of caspase-3 was cleaved (Fig. 6C) to turn to the active form. Subsequently, caspases-dependent apoptosis was induced in SW620 cells under the combination treatment with anti-miR-135b and oxaliplatin (Fig. 6D). However, co-transfection with FOXO1 siRNA was found to suppress the mitochondrial apoptosis pathway in anti-miR-135b and oxaliplatin co-treated SW620 cells. These data demonstrated anti-miR-135b/FOXO1 axis promotes mitochondrial apoptosis in oxaliplatin-treated CRC cells.

Fig. 6. Anti-miR-135b/FOXO1 axis promotes mitochondrial apoptosis in oxaliplatin-treated CRC cells. A: Release of cytochrome c from mitochondria into cytoplasm in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). B: Interaction with Apaf-1 and caspase-9 in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). C: cleavage of caspase-9 and caspase-3 in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). D: Cell apoptosis in SW620 CRC cells treated with NCO, anti-miR-135b, FOXO1 siRNA and oxaliplatin (2 μ M). *P<0.05 vs. oxaliplatin + NCO group. #P<0.05 vs. oxaliplatin + anti-miR-135b group.

Fig. 7. Anti-miR-135b enhances anti-tumor effect of oxaliplatin on CRC in vivo. A: SW620 cells transduced with empty lentiviral vector or lentiviral vector carrying anti-miR-135b sequence were subcutaneously injected into the of nude mice (n=28, 7 mice for each group). After xenografts reached 0.5 cm in diameter, animals were treated with oxaliplatin i.p. twice a week (2 mg/kg) until the mice were killed 28 days post-injection. B: Expression level of miR-135b in each group of xenografts. *P<0.05 vs. control SW620 group. #P<0.05 vs. control SW620 group + oxaliplatin group. C: Expression level of FOXO1, Bim and Noxa in each group of xenografts.

Fig. 8. Effect of anti-miR-135b/FOXO1 axis on changing the chemosensitivity of CRC cells to cisplatin and 5-fluorouracil. MTT assays were performed to evaluate the viability of HT29, SW620 and SW480 cells after treatment with NCO, anti-miR-135b, FOXO1 siRNA, cisplatin (2 μ M) and 5-fluorouracil (1 μ M). * P <0.05 vs. NCO + cisplatin group. # P <0.05 vs. anti-miR-135b + cisplatin group. & P <0.05 vs. NCO + 5-fluorouracil group. ^ P <0.05 vs. anti-miR-135b + 5-fluorouracil group.

Anti-miR-135b enhances anti-tumor effect of oxaliplatin on CRC in vivo

To verify that anti-miR-135b enhances anti-tumor effect of oxaliplatin on CRC *in vivo*, a xenograft model of SW620 cells (transduced with empty lentiviral vector or lentiviral vector carrying anti-miR-135b sequence) in nude mice was constructed. Under the equal dose of oxaliplatin treatment, we observed that tumor sizes were significantly smaller in the anti-miR-135b-SW620 group compared to the control SW620 group (Fig. 7A). Subsequently, we purified the tumor tissues resected from mice. Expression of anti-135b was found to be significantly decreased in the anti-miR-135b-SW620 cells compared to the control SW620 cells (Fig. 7B). On the other hand, protein level of FOXO1 was increased in the anti-miR-135b-SW620 cells, and knockdown of miR-135b was found to promote the expression of Bim and Noxa in the oxaliplatin-treated SW620 cells in mice (Fig. 7C). Together, we demonstrated that combination with anti-miR-135b was able to enhance the anti-tumor effect of oxaliplatin on CRC *in vivo*.

Anti-miR-135b upregulates FOXO1 expression to sensitize CRC cells to some other chemotherapeutic drugs

To investigate whether the sensitization of anti-miR-135b on chemotherapy was broad-spectrum, we co-treated the HT29, SW620 and SW480 CRC cells with anti-miR-135b and some other chemotherapeutic drugs such as cisplatin and 5-fluorouracil. We found that combination with anti-miR-135b was able to increase the cytotoxicity of cisplatin and 5-fluorouracil (Fig. 8) to CRC cells. However, knockdown of FOXO1 was found to abolish the sensitization of anti-miR-135b on cisplatin and 5-fluorouracil-induced cytotoxicity in these CRC cells. We thus demonstrated that knockdown of miR-135b sensitized CRC cells to chemotherapy through increase of FOXO1 expression.

Discussion

MiRNAs have been validated to regulate tumorigenesis, cancer proliferation, apoptosis and chemosensitivity. Among these miRNAs, miR-135b expression is reported to be elevated in many cancers due to the gene mutations (such as gene of *Adenomatous Polyposis Coli*, *APC*) [35, 36]. Overexpression of miR-135b is found to be involved for cancer progression, metastasis and invasion [37-39]. In CRC, studies also demonstrate that miR-135b overexpression is correlated with tumor stage and poor clinical outcome [36]. It is clear that miR-135b is an important oncogenic effector in cancers. Moreover, some studies suggest that miR-135b acts as a contributor to anti-apoptosis and chemoresistance in CRC [40, 41]. However, the potential mechanisms are required to be explored.

In the present study, we demonstrated significant upregulation of miR-135b in CRC cell lines and patients' serum. It suggested that miR-135b may be a novel therapeutic target for CRC. However, we observed that miR-135b antisense-oligonucleotide (anti-miR-135b) single treatment can not induce remarkable cell death in the mentioned CRC cell lines. We therefore using anti-miR-135b for adjuvant therapy on CRC. Oxaliplatin is an effective chemotherapeutic drug for treatment of CRC. However, cancer cells in numerous CRC patients usually show low sensitivity to oxaliplatin treatment [9]. Meaningfully, we found that combination with anti-miR-135b was able to enhance the oxaliplatin-induced cytotoxicity against CRC cell lines. *In vivo* tumorigenesis assays, we also found that the anti-miR-135b-stably expressed CRC tumors exhibited more sensitivity to oxaliplatin treatment. Taken together, our work showed that miR-135b can be a potential therapeutic target for oxaliplatin-based chemotherapy in CRC.

Forkhead box O 1 (FOXO1) is an important transcription factor that participates in a variety of biological processes, including DNA repair, cell cycle transition, oxidative, apoptosis and stress response [42, 43]. FOXO1 acts as a tumor suppressor, and decrease of which has been reported to promote cell proliferation and tumorigenesis in many cancers [44, 45]. As the downstream, FOXO1 promotes transcriptional activities of Bim and Noxa, which functions as pro-apoptotic proteins dependent on mitochondrial pathway [27, 28, 33, 34]. Therefore, FOXO1 is a key regulator of apoptosis pathway in cancer cells.

In the present study, we found that FOXO1 was the target of miR-135b in CRC. Overexpression of miR-135b induced downregulation of FOXO1, which was found to be responsible for low sensitivity of CRC cells to oxaliplatin treatment. As a result of anti-miR-135b adjuvant therapy, cellular FOXO1 was significantly increased. As a feedback loop mechanism, upregulated FOXO1 may further induce suppression of miR-135b [36]. As the downstream of anti-miR-135b/FOXO1 pathway, expression of Bim and Noxa was promoted. Bim in cancer cells induces translocation of Bax from cytoplasm to mitochondria to form the stable protein-permeable pore on mitochondria [46, 47]. Noxa in cancer cells interact with MCL-1 which is a key anti-apoptotic protein [48] to inhibit its function. We therefore explored that anti-135b/FOXO1 axis promoted mitochondrial apoptosis in oxaliplatin-treated CRC cells. It may be the potential mechanism by which anti-miR-135b exerted its effect on sensitizing CRC cells to oxaliplatin-based chemotherapy.

In conclusion, we demonstrate here that knockdown of miR-135b enhances efficacy of oxaliplatin-based treatment in CRC by targeting FOXO1. However, further efforts are required to test the effect of anti-miR-135b adjuvant treatment on clinical use of oxaliplatin for cancer patients.

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Disclosure Statement

The authors declare to have no conflicts of interest

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