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MicroRNA-23a Antisense Enhances 5-Fluorouracil Chemosensitivity Through APAF-1/Caspase-9 Apoptotic Pathway in Colorectal Cancer Cells

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ABSTRACT

Current literature provided information that alteration in microRNA expression impacted sensitivity or resistance of certain tumor types to anticancer treatment, including the possible intracellular pathways. The microRNA-23a (miR-23a)-regulated apoptosis in response to the 5-fluorouracil (5-FU)-induced mitochondria-mediated apoptotic pathway was determined in this study. The miR-23a expression in 5-FU-treated and untreated colon cancer cells and tissues was assessed using real-time PCR analysis. To determine the function of miR-23a in the regulation of 5-FU-induced apoptosis, cell-proliferation, cytotoxicity, and apoptosis analyses were performed. Dual luciferase reporter assay was used to identify the apoptosis-related target gene for miR-23a. The activity of caspases-3, -7, and -9 were also assessed in miR-23a antisense and 5-FU treated tumor cells. A xenograft tumor model was established to evaluate the biological relevance of altered miR-23a expression to the 5-FU-based chemotherapy in vivo. We found that the expression of miR-23a was increased and the level of apoptosis-activating factor-1 (APAF-1) was decreased in 5-FU-treated colon cancer cells compared to untreated cells. The activation of the caspases-3 and 7 was increased in miR-23a antisense and 5-FU-treated colon cancer cells compared to negative control. APAF-1, as a target gene of miR-23a, was identified and miR-23a antisense-induced increase in the activation of caspase-9 was observed. The overexpression of miR-23a antisense up-regulated the 5-FU induced apoptosis in colon cancer cells. However, the miR-23a knockdown did not increase the antitumor effect of 5-FU in xenograft model of colon cancer. This study shows that miR-23a antisense enhanced 5-FU-induced apoptosis in colorectal cancer cells through the APAF-1/caspase-9 apoptotic pathway. J. Cell. Biochem. 115: 772–784, 2014.

KEY WORDS: 5-FLUOROURACIL (5-FU); COLORECTAL CANCER (CRC); microRNA-23a (miR-23a); Apoptosis-Activating Factor-1(APAF-1); APOPTOSIS

Based on the GLOBOCAN 2008 estimates, colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide [Jemal et al., 2011]. In addition to surgical procedures, adjuvant chemotherapy is often used to prolong the patient's survival time, especially during the advanced stages of the disease. 5-FU is the most commonly used chemotherapeutic agent for CRC. In fact, fewer than 25% of patients with advanced CRC have shown major responses after 5-FU-based chemotherapy, and, in many cases, patients who initially responded to 5-FU ultimately became resistant. Consequently, resistance to this drug is a major obstacle in CRC chemotherapy [Shin et al., 2009]. 5-FU is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen. It exerts its anticancer effects through the inhibition of thymidylate synthase and the incorporation of its active metabolites into RNA and DNA, so as to influence the uracil metabolism, which is reported as a potential target for antimetabolite chemotherapy [Longley et al., 2003; Karasawa et al., 2009]. Moreover, the futile cycles of misincorporation stimulate the cellular excision system, and subsequently cause the DNA strand to brake and eventually lead to apoptosis in the cancer cell [Grem, 2000].

MicroRNAs (miRNAs) comprise a novel class of noncoding regulatory RNA molecules. One of the main roles of miRNAs is to regulate the expression of target genes during various crucial cell processes such as apoptosis, differentiation and development by interacting with complementary sites in the 3′ untranslated region (3′-UTR) [Bartel, 2004]. On the basis of these findings, the expression patterns of miRNAs have been systematically examined in carcinomas [Lu et al., 2005; Ng et al., 2009]. Numerous studies indicate that

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miRNAs can be used as regulatory molecules in human cancer chemotherapy [Meng et al., 2006; Ranade et al., 2010]. Recently, an increasing amount of evidence suggests that altered expression of miRNA-21 (miR-21) in cancer cells could modulate the functional expression of critical genes involved in cell survival in response to chemotherapeutic stress [Meng et al., 2006; Rossi et al., 2007; Bourguignon et al., 2009]. Rossi et al. showed that miR-23a was up-regulated in 5-FU-treated C22.20 and HC.21 colon cancer cells. However, the mechanism of miR-23a involvement in the regulation of 5-FU chemosensitivity has not yet been determined [Rossi et al., 2007].

The cytosolic protein apoptosis-activating factor-1 (APAF-1), the human homolog of the *Caenorhabditis elegans* cell death protein CED-4, plays a central role in caspase-9-dependent activation of caspase-3 in the general mitochondrial apoptotic pathways in various cell types [Cecconi et al., 1998; Yoshida et al., 1998]. Previous studies have shown that apoptosis is one of the primary cytotoxic mechanisms of chemotherapeutic agents, including 5-FU [Kaufmann and Earnshaw, 2000]. Inhibition of the apoptotic pathway is one of the main factors responsible for the onset of drug resistance in cancer [Karasawa et al., 2009]. B-cell lymphoma 2 (Bcl-2) proteins have also arisen as regulators of apoptosis in 5-FU treatment of human colon cancer cell lines [Violette et al., 2002; Feng et al., 2006; Wang et al., 2007]. 5-FU, like most anticancer drugs, such as mitoxantrone, cyclophosphamide, cisplatin, and imatinib, induces mitochondrion-toxicity and disturbs mitochondrial functions [Finsterer and Ohnsorge, 2013]. Moreover, altered mitochondrial bioenergetic features are also a characteristic of 5-FU-resistant human colon cancer cells [Shin et al., 2005]. The cytochrome-*c*/APAF-1/caspase-9 pathway might play an important role in chemotherapy-induced apoptosis [Danial and Korsmeyer, 2004; Adams and Cory, 2007]. However, it is not clear whether 5-FU induces apoptosis in colorectal cancer by this APAF-1/caspase-9 apoptotic pathway.

Currently, 5-FU plays a central role in many chemotherapeutic regimens for CRC. For this reason, it is important to understand the mechanisms that underline 5-FU chemotherapeutic sensitivity and resistance. Recently, miRNA microarray technology has been used to identify novel genes regulating 5-FU resistance, and the potential biomarkers of 5-FU resistance, such as miR-196a, miR-31, miR-200, miR-143, and miR-145, have been proposed [Slaby et al., 2009; Schee et al., 2010; Nugent et al., 2011]. In the present study, we examined the expression of miR-23a in response to 5-FU treatment in colon cancer cells and showed, for the first time, that miR-23a antisense may enhance 5-FU-induced apoptosis in vitro.

MATERIALS AND METHODS

CELL CULTURE AND MATERIALS

Human HCT116 and HT29 colon carcinoma cell lines were obtained from the American Type Culture Collection (ATCC). Cells were grown in McCoy's 5A medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C. Human CRC tissue specimens were collected from patients who underwent surgery at the Changhai Hospital (Second Military Medical University, Shanghai, China). All patients signed an informed consent statement prior to participating in this study. The study was approved by the Human Research Ethics Committee at the Second Military Medical University. Tissue samples were collected during surgery, immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. The characteristics of the patients included in this study are described in Table I.

DETERMINATION OF IC_{50}

The IC_{50} values of 5-FU (Applichem, Germany) were assessed using a cell counting kit-8 (CCK-8) assay (Dojindo Corp., Japan). HCT116 and HT29 (1.6×10^4) cells were seeded in 96-well microtiter plates, as reported elsewhere [Violette et al., 2002]. 5-FU was added to each plate and the plates were incubated for 24 h (HCT116) or 48 h (HT29) at 37°C. Following incubation, CCK-8 (10 μl/well) was added to each well, and the cultures were incubated for another 2 h at 37°C. The absorbance of formazan at 450 nm was measured using a microplate reader (BIO-RAD, USA). The cytotoxic effect of 5-FU was assessed by analyzing the IC_{50} values [Karasawa et al., 2009]. In the following experiments, the concentrations of 5-FU used were equal to the IC_{50} and the duration of 5-FU treatment was 24 h in HCT116 cells or 48 h in HT29 cells.

CONSTRUCTION OF PLASMIDS

In this study, we used online database, TargetScan (http://www.targetscan.org/), to predict the possible binding target gene of miR-23a. The program searched for complementarity to the miR-23a seed region in the 3′UTR of APAF-1. The 3′-UTR of the APAF-1 gene contains three sites for predicted miR-23a binding targets (positions 238–245, 1,474–1,481, and 2,166–2,173 bp of the APAF-1 3′-UTR). The pMIR-REPORT Luciferase Expression Reporter Vector (Applied Biosystems) was used to identify possible target genes of miR-23a. Using the *Hin*dIII and *Sac*I sites, the three predicted miR-23a target sequences were cloned into the pMIR-REPORT Vector. The PCR primers are shown in Table II.

The miR-23a antisense and negative control expression vectors were constructed as previously described [Zhang et al., 2009]. Oligonucleotides encoding miR-23a antisense and negative control sequences with fold-back stem-loop structures were inserted into the *Age*I–*Eco*RI site of pLKO.1-puro vector (<!--Open Biosystems, USA). Two plasmids, pLKO-miR-23a antisense and pLKO-antisense negative control, were generated. All the oligonucleotide sequences are shown in Table III.

REVERSE-TRANSCRIPTION REACTION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA from HCT116 and HT29 cells and tumor tissue samples were purified using Trizol® (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA using reverse transcriptase (Invitrogen). Expression of mature miR-23a was assayed using stem-loop reverse transcription, followed by real-time polymerase chain reaction (PCR) analysis, as previously described. Real-time PCR was carried out using a standard SYBR Green PCR Kit protocol in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) as previously described [Zhang et al., 2009]. The expression levels of miR-23a relative to the U6 endogenous control were calculated using the $2^{-\Delta\Delta CT}$ method. Real-time PCR of APAF-1

TABLE I. Demographic and Clinical Features of 38 Patients With Colorectal Cancer

Patient no.	Sex	Age	Histological grade	Chemotherapeutic agent
1	M	53	II	5-FU + oxaliplatin + calcium folinate
2	F	52	III	5-FU + oxaliplatin + calcium folinate
3	F	59	IV	5-FU + oxaliplatin + calcium folinate
4	F	77	III	5-FU + oxaliplatin + calcium folinate
5	M	50	III	5-FU + oxaliplatin + calcium folinate
6	F	69	IV	5-FU + oxaliplatin + calcium folinate
7	M	59	II	5-FU + oxaliplatin + calcium folinate
8	M	71	III	5-FU + oxaliplatin + calcium folinate
9	M	63	III	5-FU + oxaliplatin + calcium folinate
10	F	62	IV	5-FU + oxaliplatin + calcium folinate
11	M	59	III	5-FU + oxaliplatin + calcium folinate
12	M	63	III	5-FU + oxaliplatin + calcium folinate
13	F	59	III	5-FU + oxaliplatin + calcium folinate
14	M	52	III	5-FU + oxaliplatin + calcium folinate
15	M	50	II	5-FU + oxaliplatin + calcium folinate
16	M	47	III	5-FU + oxaliplatin + calcium folinate
17	M	50	II	5-FU + oxaliplatin + calcium folinate
18	F	55	IV	5-FU + oxaliplatin + calcium folinate
19	F	57	IV	5-FU + oxaliplatin + calcium folinate
20	M	52	I	No chemotherapy
21	M	68	II	No chemotherapy
22	M	43	I	No chemotherapy
23	F	57	II	No chemotherapy
24	F	60	II	No chemotherapy
25	F	67	II	No chemotherapy
26	M	57	I	No chemotherapy
27	F	52	II	No chemotherapy
28	M	49	I–II	No chemotherapy
29	M	53	II	No chemotherapy
30	F	77	II	No chemotherapy
31	F	69	III	No chemotherapy
32	F	69	III	No chemotherapy
33	M	49	II	No chemotherapy
34	F	71	IV	No chemotherapy
35	M	68	III	No chemotherapy
36	F	42	II	No chemotherapy
37	M	45	I	No chemotherapy
38	M	64	II	No chemotherapy

mRNA expression was quantified and normalized relative to β-actin expression. The real-time PCR and reverse transcription primers are listed in Tables II and III.

TRANSFECTION WITH miR-23a SENSE, miR-23a ANTISENSE AND SMALL INTERFERING RNAs

The miR-23a sense, miR-23a antisense, small interfering RNAs (siRNAs) targeting APAF-1, and their negative-control RNAs were synthesized by GenePharma (Shanghai, China). Transfection was conducted using a Lipofectamine 2000 Kit (Invitrogen) in HT29 and HCT116 cells [Zhang et al., 2009]. For transfection of the RNA oligonucleotides, siRNA (200 pmol/plate) or miRNA-23a sense or antisense oligonucleotides (5 pmol/well and 400 pmol/plate, respectively) were used. The transfection efficiency of miR-23a sense, antisense and their negative-control was done and the expression of mature miR-23a was evaluated using quantitative real-time polymerase chain reaction.

CELL PROLIFERATION AND CYTOTOXICITY ASSAY

Cell proliferation and drug cytotoxicity were assayed using the CCK-8 kit (Dojindo Corp.). HCT116 and HT29 (1.6×10^4) cells were transfected with miR-23a sense, miR-23a antisense and negative controls (5 pmol/well) as reported elsewhere [Violette et al., 2002]. The plates were incubated at 37°C for 2 days for the cell proliferation assays. Drug cytotoxicity assays were carried out according to the manufacturer's instructions. 5-FU was added 10 h after transfection and the cells were incubated for 24 h (HCT116) and 48 h (HT29). Following incubation, CCK-8 (10 μl/well) was added to each well, and the absorbance of formazan at 450 nm was measured using a microplate reader (BIO-RAD).

FLOW CYTOMETRY ANALYSIS

Apoptosis was measured using the annexin V labeled with fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I (Keygen, China). Cells were treated with annexin V–FITC and propidium iodide (PI) according to the manufacturer's protocol, and the fluorescence levels were analyzed using a multicolor flow cytometer (FACSCalibur, BD Biosciences, USA). Briefly, HCT116 and HT29 cells transfected with miR-23a antisense and the negative controls were plated on six-well plates (5×10^5 cells/plate). The cells were detached and resuspended in an annexin-binding buffer. Annexin V–FITC and PI were added, after which the cells were incubated for 15 min at room temperature, and subjected to flow cytometry.

DUAL LUCIFERASE REPORTER ASSAY

HCT116 cells (5×10^4/well) were seeded in a 24-well plate the day before transfection and were transfected with pMIR-REPORT-APAF-1-3′-UTR (500 ng/well), *Renilla reniformis* luciferase control vector (50 ng/well, pRL-TK-Promega), miR-23a sense, miR-23a antisense and their negative controls (15 pmol/well). After 48 h of transfection, the ratio of *Renilla* to firefly luciferase was determined using a dual luciferase reporter assay system (Promega, USA).

WESTERN BLOT ANALYSIS

Total protein was extracted from HCT116 and HT29 cells (transfected with miR-23a sense, antisense and negative controls, or

TABLE II. Primers for Real-Time PCR and PCR

Name	Sense primer (5′-3′)	Antisense primer (5′-3′)
APAF-1	5′ ATGGACACCTTCTTGGACGACAG 3′	5′ TGTGGGGGCGGACAACTAA 3′
β-actin	5′ TGTGTTGGCGTACAGGTCTTTG 3′	5′ GGGAAATCGTGCGTGACATTAAG 3′
miR-23a	5′ GGGGATCACATTGCCAGG3′	5′ AGTGCGTGTCGTGGAGTC 3′
U6	5′ GCTTCGGCAGCACATATACTAAAAT 3′	5′ CGCTTCACGAATTTGCGTGTCAT 3′
3′ UTR of APAF-1 position 238–244 bp	5′ GAGCTCAGCTCTTAATTGTTGTGCAGTATTG 3′	5′ AAGCTTCACAGCCTGCATAACAGTAATAAAT 3′
3′ UTR of APAF-1 position 1,474–1,480 bp	5′ TTGGACACTATTCCTGCTCCCTC 3′	5′ AGGAAAAGAGGATGAGAGAGA 3′
3′ UTR of APAF-1 position 2,166–2,172 bp	5′ GAGCTCAGGGTAAGGGAATAGATCACTCAGA 3′	5′ AAGCTTTATAACAGTCAGTGGCTTTTAATTC 3′

TABLE III. Primers for Reverse Transcription and Oligonucleotide Sequences

Name	Primers or oligonucleotide sequences
miR-23a sense	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAA TTGCACTGGATACGACGGAAAT3′
U6	5′CGCTTCACGAATTTGCGTGTCAT 3′
PLKO.1-miR-23a antisense	Forward oligo: 5′ CCGGTATCACATTGCCAGGGATTTCCCTCGAGGGAAATCCCTGGCAATGTGATTTTTTG 3′ Reverse oligo: 5′AATTCAAAAAATCACATTGCCAGGGATTTCCCTCGAGGGAAATCCCTGGCAATGTGAT A 3′
PLKO.1-antisense negative control	Forward oligo: 5′ CCGGTTTCTCCGAACGTGTCACGTCTCGAGAAGAGGCTTGCACAGTGCATTTTTG 3′ Reverse oligo: 5′ AATTCAAAAATTCTCCGAACGTGTCACGTCTCGAGAAGAGGCTTGCACAGTGCA A 3′

treated with 5-FU) as previously described. Blots were probed using either rabbit polyclonal antibodies against APAF-1 (1:200, Santa Cruz Biotechnology, USA) or mouse monoclonal antibodies against β-actin (1:5,000, Sigma–Aldrich). The secondary antibodies, IRDye™-800-conjugated goat anti-rabbit IgG or IRDye™-700-conjugated goat anti-mouse IgG, were used at a ratio of 1:5,000 (Rockland Inc., P.O. BOX 326, Gilbertsville, PA). The immunological complexes were detected using an Odyssey infrared scanner (Li-Cor; Lincoln, NE).

IMMUNOFLUORESCENT STAINING FOR CYTOCHROME-*c* RELEASE

Immunofluorescence was performed to detect the release of cytochrome-*c* in both HCT116 and HT29 cells, which induces apoptosis via 5-FU. The cells were fixed with 4% paraformaldehyde in PBS. The cell slides were permeabilized with 0.2% Triton X-100 and then blocked for 60 min with PBS containing 10% BSA and 10% normal rabbit serum. The cells were treated overnight at 4°C with primary antibody, rabbit anti-human cytochrome-*c* (1: 50, Santa Cruz Biotechnology), or PBS alone (as a negative control). The secondary antibody was AlexaFluor 555-conjugated goat anti-rabbit antibody (1:500, Invitrogen). As indicated, the cells were stained with the chromatin dye, Hoechst 33342 (5 μg/ml, Sigma), for 5 min. Slides were mounted with antifading media (Dako, Denmark) and visualized with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) equipped with LSM 510 software, version 2.02, and Ar/Kr (458 and 488 nm) and He/Ne (543 nm) lasers. A Plan-Neofluar 63× 1.3 oil lens was used for observation.

DETECTION OF CASPASE-3, -7 AND -9

Caspase-9 activity was detected following the Caspase Colorimetric Assay Kit protocol (Keygen, China) as previously described [Yao et al., 2007]. Briefly, the cells were harvested and lysed for 45 min on ice. Then, 150 μg of protein was diluted in 50 μl Cell Lysis buffer. Next 50 μl of 2× reaction buffer was added to each sample. The treated protein was incubated at 37°C for 4 h. The pNA light emission at 405 nm was quantified using a microplate reader.

A Caspase-Glo® 3/7 Assay Kit (Promega) was used to detect the miR-23a-regulated activation of caspase-3 and -7 in 5-FU-induced apoptosis in HCT116 colon cancer cells. The cells (5×10^3 cells/well) were plated in five replicates in 96-well plates. They were then transfected with miR-23a sense, antisense or negative control. After 10 h of transfection, the cells were treated with 5-FU, incubated for 24 h (at 37°C) and then Caspase-Glo 3/7 Reagent (100 μl) was added to each well. The luminescence of each sample was measured with Xenogen IVIS Lumina II in vivo imaging system (Caliper, USA).

ESTABLISHMENT AND IMAGING OF IN VIVO TUMOR MODEL

Athymic female mice age-matched between 6 and 8 weeks were used for xenograft tumor growth assays. Mice were implanted with either HCT116 or HT29 cells that stably expressed pLKO-miR-23a antisense or pLKO-miR-23a antisense negative control, as well as the bioluminescence plasmid, pGL4.17. Mice were randomly divided into three groups (n = 3–6): pGL4.17 and pLKO-miR-23a antisense co-expressed cells implanted mice that received 5-FU treatment, pGL4.17 and pLKO-miR-23a antisense negative control co-expressed cells implanted mice that received 5-FU treatmemt, and pGL4.17 and pLKO-miR-23a antisense negative control co-expressed cells implanted mice that received PBS injection. The cells (1.0×10^7 cells/mouse) were implanted subcutaneously into the flanks (HCT116) or backs (HT29) of the mice. Xenograft tumor growth was analyzed by monitoring and quantifying noninvasive bioluminescence [Tavazoie et al., 2008]. In vivo bioluminescence images were obtained using Xenogen IVIS Lumina II in vivo imaging system (Caliper). Once the xenograft tumor volume reached 100 mm^3, mice bearing the pGL4.17 and pLKO-miR-23a antisense or pLKO-antisense negative control xenograft tumor received 5-FU (45 mg/kg) intravenously every 7 days [Harris et al., 2005]. As the control group, mice bearing the pGL4.17 and pLKO-antisense negative control received PBS intravenously every 7 days. The tumor bioluminescence was measured at day 7, 21, and 35 after 5-FU or PBS treatment. For analysis of 5-FU-induced miR-23a and APAF-1 mRNA in vivo, xenograft tumors were excised on day 35 and the tissues were homogenized and used for mRNA isolation as described above. The animal studies were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University, Shanghai, China.

STATISTICAL ANALYSIS

A Student's *t*-test was used for comparison between the two groups. The expression of miR-23a in CRC tissue was analyzed using the Mann–Whitney test. The xenograft tumor growth was analyzed by repeated-measures ANOVA. Tukey's HSD post hocs were used to do multiple comparisons if the overall ANOVA was significant. The significant value was set at $P \leq 0.05$. Data are presented as mean ± standard error of (SE). Bioluminescence intensities are given as the mean photons/s/cm^2/sr ± SE. All statistical analysis was performed using Statistica Visual Basic Primer Software (StatSoft, USA).

RESULTS

5-FU-BASED CHEMOTHERAPY UPREGULATED THE EXPRESSION OF miR-23a

We examined the IC_{50} value of 5-FU using a CCK-8 assay kit after the cells were exposed to 5-FU. The IC_{50} of 5-FU for HT29 cells was higher than for HCT116 cells (6.14 ± 0.037 and 4.89 ± 0.071 μg/ml, respectively).

The differential sensitivity of colon cancer cells to 5-FU may be related to 5-FU-induced alterations in miRNA expression levels. We assessed the expression of miR-23a in 5-FU-treated or untreated HCT116 and HT29 cells, using real-time PCR analysis (Fig. 1A,B). The expression of miR-23a was significantly increased by 5-FU treatment in both HCT116 (5.042-fold change, $P < 0.01$) and HT29 (53.86-fold change, $P < 0.01$) cells, and more miR-23a expression was detected in HT29 cells.

To determine whether miR-23a is associated with CRC 5-FU chemoresistance, we profiled miR-23a expression in human CRC tissues treated with 5-FU-based chemotherapy and untreated tissues. Quantitative real-time PCR was conducted on 38 samples of colon cancer tissues obtained from different patients during surgical resection (Fig. 1C). The expression of miR-23a was significantly increased in human CRC tissue treated with 5-FU-based chemotherapy compared to untreated tissues ($P < 0.01$). Together, this data suggests that 5-FU-based chemotherapy up-regulated the expression of miR-23a in CRC cells and tissues.

miR-23a ANTISENSE OVEREXPRESSION INCREASED 5-FU-INDUCED APOPTOSIS IN COLON CANCER CELLS

To determine the transfection efficiency of miR-23a sense, antisense and their negative-control, we assessed the expression of mature miR-23a using quantitative real-time PCR in both HCT116 and HT29 cells. The expression of miR-23a was significantly up-regulated in both HCT116 and HT29 cells transfected with miR-23a sense compared to the negative-control transfected cells (Fig. 2A). The expression of miR-23a was significantly down-regulated in miR-23a antisense transfected HCT116 and HT29 cells compared to the negative-control transfected cells (Fig. 2B).

Cell proliferation, cell viability, and cell apoptosis rate were tested in both HCT116 and HT29 cells to determine the effects of miR-23a sense and antisense on chemosensitivity. In cells transfected with miR-23a sense or antisense, the cell proliferation was not significantly altered compared to the cells transfected with negative controls or untreated control cells (Fig. 2C). Our results indicated that overexpression of miR-23a sense or antisense may not relate to regulation of the proliferation of colon cancer cells. In both HCT116 and HT29 cells, cell viability was significantly decreased by 50% after 5-FU treatment compared to untreated control cells ($P < 0.01$). In cells transfected with miR-23a sense, after 5-FU treatment, cell viability significantly increased by 6.5% (HCT116) and 18.9% (HT29) compared to cells transfected with miR-23a sense negative control ($P < 0.05$). In cells transfected with miR-23a antisense, after 5-FU treatment, cell viability significantly decreased by 13.3% (HCT116) and 10.9% (HT29) compared to the cells transfected with miR-23a antisense negative control ($P < 0.05$; Fig. 2D).

We investigated whether miR-23a played a functional role in 5-FU-induced apoptosis. As shown in Figure 2E,F, miR-23a antisense overexpression increased the 5-FU-induced apoptosis. The apoptosis rate was significantly increased by 24.6% in HCT116 cells and 25.01% in HT29 cells after 5-FU treatment compared to untreated control cells ($P < 0.01$). After transfection with miR-23a antisense and5-FU treatment, the apoptosis rate significantly increased by 19.45% ($P < 0.01$) in HCT116 and 10.89% ($P < 0.05$) in HT29 cells, compared to the cells transfected with miR-23a antisense negative control and treated with 5-FU. These results indicated that miR-23a antisense increased cell apoptosis in both HCT116 and HT29 cells and miR-23a may contribute to chemoresistance in CRC.

APAF-1 AS A TARGET GENE OF miR-23a

We predicted the possible targets of miR-23a by searching the target gene prediction database (TargetScan (http://www.targetscan.org/). The system predicted that APAF-1 was an apoptosis-related target of miR-23a. The three binding targets of miR-23a in 3′-UTR of the APAF-1 gene (positions 238–245, 1,474–1,481 and 2,166–2,173 bp of the APAF-1 3′-UTR) were shown in Figure 3A.

To further explore the mechanisms by which miR-23a controls APAF-1 expression, the 3′-UTR of APAF-1 was cloned into a luciferase construct and a dual luciferase reporter assay was performed. In the HCT116 cells co-transfected with miR-23a sense and APAF-1 3′-UTR expression vectors, the activity of luciferase was

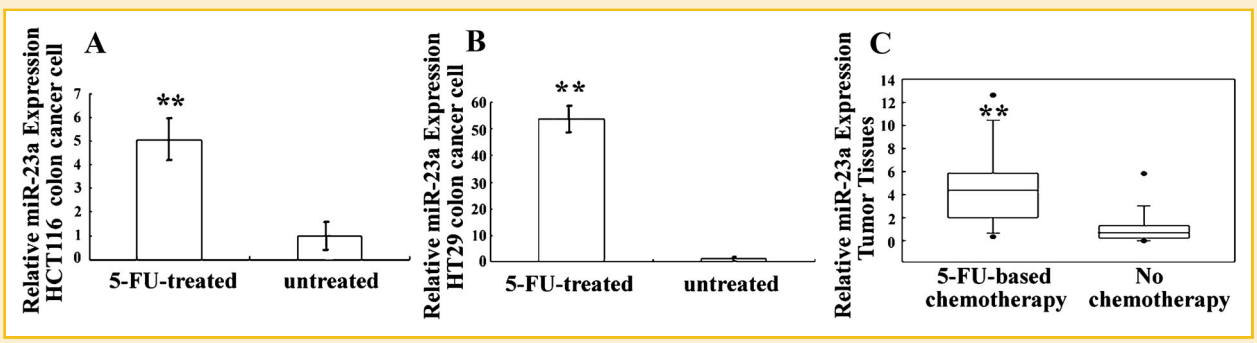

Fig. 1. 5-FU-based chemotherapy upregulated the expression of miR-23a. The relative expression of miR-23a was up-regulated in 5-FU-treated HCT116 (A) and HT29 (B) cells compared to 5-FU untreated cells. C: The miR-23a expression was up-regulated in tissues from colon rectal cancer with 5-FU based chemotherapy compared to 5-FU untreated patients. Error bars represent the standard deviations of at least three independent experiments. $^{**}P < 0.01$

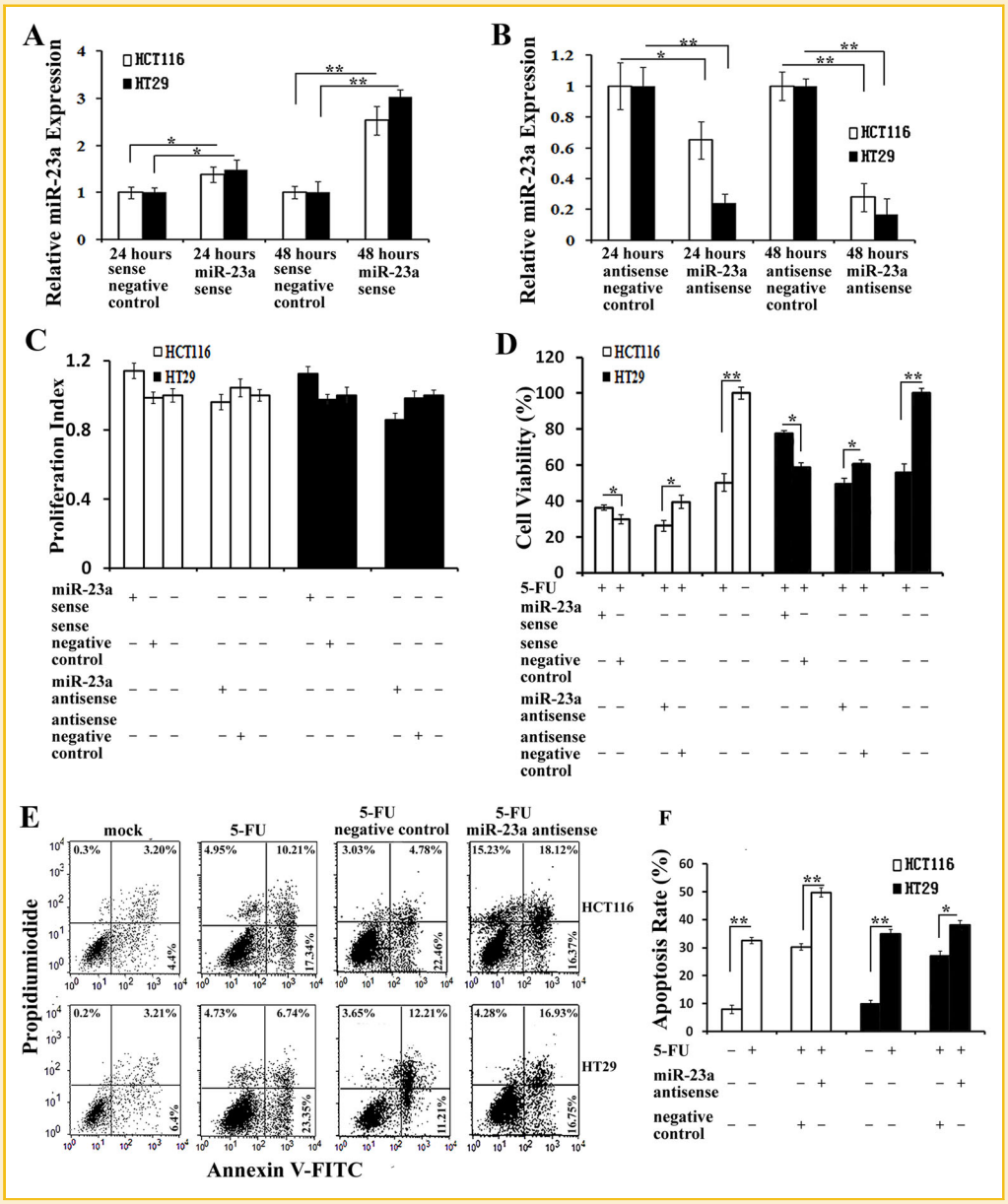

Fig. 2. miR-23a antisense overexpression increased 5-FU-induced apoptosis in colon cancer cells. The transfection efficiency of miR-23a sense (A) antisense (B) and their negative controls in both HCT116 and HT29 cells at 24 or 48 h after transfection. C: Cell proliferation was not significantly changed after transfected with miR-23a sense or antisense in both HCT116 and HT29 cells. D: The cytotoxicity index was the percentage of viable cells. Cell viability was significantly decreased in HCT116 and HT29 cells after 5-FU treatment compared to untreated control cells. In both HCT116 and HT29 cells, cell viability was significantly increased after transfected with miR-23a sense compared to the cells transfected with miR-23a negative control, but significantly decreased after transfected with miR-23a antisense compared to the cells transfected with miR-23a negative control. E: Incidence of apoptotic cells was studied by flow cytometry. The cells that were stained with annexin V–fluorescein isothiocyanate (V-FITC) and counterstained with propidium iodide (PI). The percentage of annexin V-positive versus PI-positive cells in miR-23a-antisense transfected cells treated with 5-FU, shown in the quadrant, was higher than antisense negative control transfected cells treated with 5-FU. Mock: the cells were untreated with miR-23a-antisense, miR-23a-antisense negative control or 5-FU. F: The apoptosis rate was significantly increased in HCT116 and HT29 cells after 5-FU treatment compared to untreated control cells. After transfection with miR-23a antisense and 5-FU treatment the apoptosis rate significantly increased in HCT116 and HT29 cells, compared to the cells transfected with miR-23a antisense negative control and treated with 5-FU. $^{*}P < 0.05$; $^{**}P < 0.01$ when compared to controls.

significantly decreased relative to that in cells transfected with miR-23a sense negative control. The luciferase activity was decreased by 37.16% (APAF-1 3′-UTR238), 34.99% (APAF-1 3′-UTR2166), and 37.3% (APAF-1 3′-UTR1474; $P < 0.01$, Fig. 3B). In the HCT116 cells co-transfected with miR-23a antisense and APAF-1 3′-UTR expression vectors, the expression level of luciferase was significantly increased compared to cells transfected with miR-23a antisense negative control; the luciferase activity was increased by 72.2% (APAF-1 3′-UTR238), 59.17% (APAF-1 3′-UTR2166), and 39.6% (APAF-1 3′-UTR1474; $P < 0.01$; Fig. 3C).

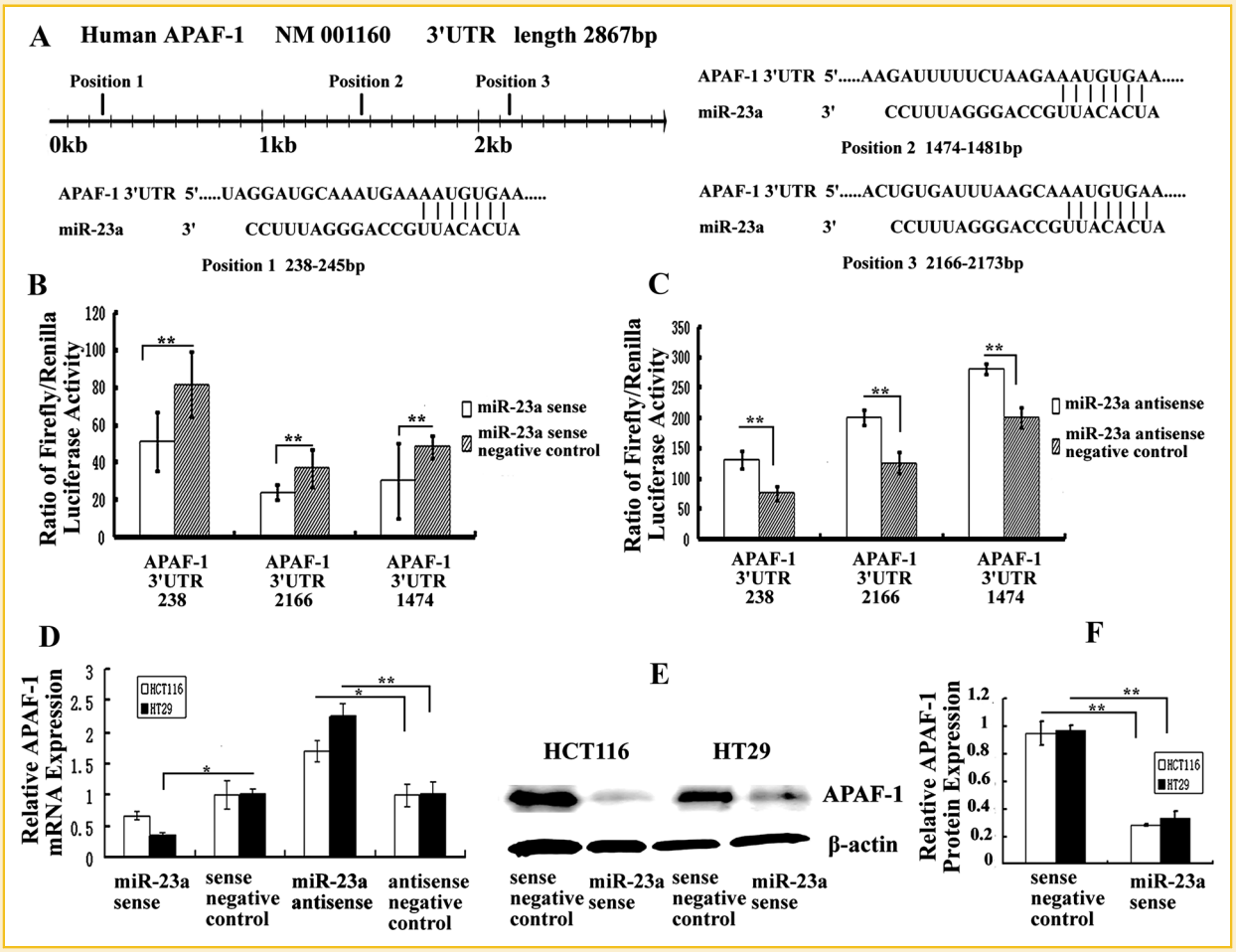

Fig. 3. APAF-1 is a direct target gene of miR-23a. A: Putative conserved target sites in the APAF-1 3′UTR were identified with the TargetScan database. Schematic of conserved (position 2 from 1,474 to 1,481 bp) or poorly conserved (position 1 from 238 to 245 bp and position 3 from 2,166 to 2,173 bp) binding sites for miR-23a. B: In HCT116 cells co-transfected with miR-23a sense and APAF-1 3′-UTR expression vectors, the activity of luciferase was significantly decreased relative to that in cells transfected with miR-23a sense negative control and APAF-1 3′-UTR expression vectors. C: In HCT116 cells co-transfected with miR-23a antisense and APAF-1 3′-UTR expression vectors, the expression level of luciferase was significantly increased compared to cells transfected with miR-23a antisense negative control and APAF-1 3′-UTR expression vectors. D: The expression of APAF-1 mRNA was up-regulated in HCT116 and HT29 cells that were transfected with miR-23a antisense, but down-regulated in HCT116 and HT29 cells that were transfected with miR-23a sense. E: The expression level of APAF-1 protein in HCT116 and HT29 cells transfected with miR-23a sense or sense negative control. F: Statistical analysis is shown that the expression of miR-23a decreased the expression level of APAF-1 protein. $^{*}P < 0.05$; $^{**}P < 0.01$.

To confirm whether APAF-1 is a target of miR-23a, we first determined whether sense and antisense of miR-23a could influence APAF-1 expression in both HCT116 and HT29 cells. First, we examined the expression of APAF-1 mRNA in miR-23a sense- and antisense-overexpressed cells using real-time PCR. We found that the expression of APAF-1 mRNA was up-regulated in both HCT116 cells (1.69-fold change, $P < 0.05$) and HT29 cells (2.25-fold change, $P < 0.01$) transfected with miR-23a antisense, compared to the controls transfected with miR-23a antisense negative control (Fig. 3D). We also found that the expression of APAF-1 mRNA was down-regulated in HT29 cells (0.33-fold change, $P < 0.05$) transfected with miR-23a sense, compared to controls transfected with miR-23a negative control, but no significant changes in HCT116 cells after transfection (Fig. 3D). Second, to determine the effect of miR-23a on the expression of APAF-1 protein, we transfected HCT116 and HT29 cells with miR-23a sense or negative control and determined the level of APAF-1 protein expression by Western blot analysis. The results showed that increasing the expression of miR-23a decreased the level of APAF-1 protein by 70% (HCT116) and 65.5% (HT29; $P < 0.01$; Fig. 3E,F). In summary, these findings suggested that APAF-1 was a target gene of miR-23a.

APAF-1 POSITIVELY REGULATED AND miR-23a NEGATIVELY REGULATED THE 5-FU-INDUCED MITOCHONDRIA-MEDIATED APOPTOSIS

We investigated whether APAF-1 was functionally related to the 5-FU-induced mitochondria-mediated apoptosis pathway. First, we tested whether 5-FU influenced the expression of the APAF-1 protein and the release of cytochrome-*c* from the mitochondria into the cytosol in colon cancer cells. Western blot analysis showed that the

expression of APAF-1 protein was decreased by 28% and 44% in 5-FU-treated HCT116 and HT29 cell lines, respectively, compared to the controls (Fig. 4A,B). To determine the molecular basis of 5-FU-induced apoptosis, the release of cytochrome-*c* from the mitochondria into the cytosol was screened in 5-FU-treated and untreated HCT116 and HT29 cells by immunofluorescence analysis. In 5-FU-treated cells, fluorescence represented the quantity of cytochrome-*c*, and it was distributed throughout the cytosol. In untreated cells, mitochondrial fluorescence showed a clustered, spotty distribution (Fig. 4C).

In the following experiments, we profiled and quantified whether miR-23a antisense can influence the activation of caspase-3, -7, and -

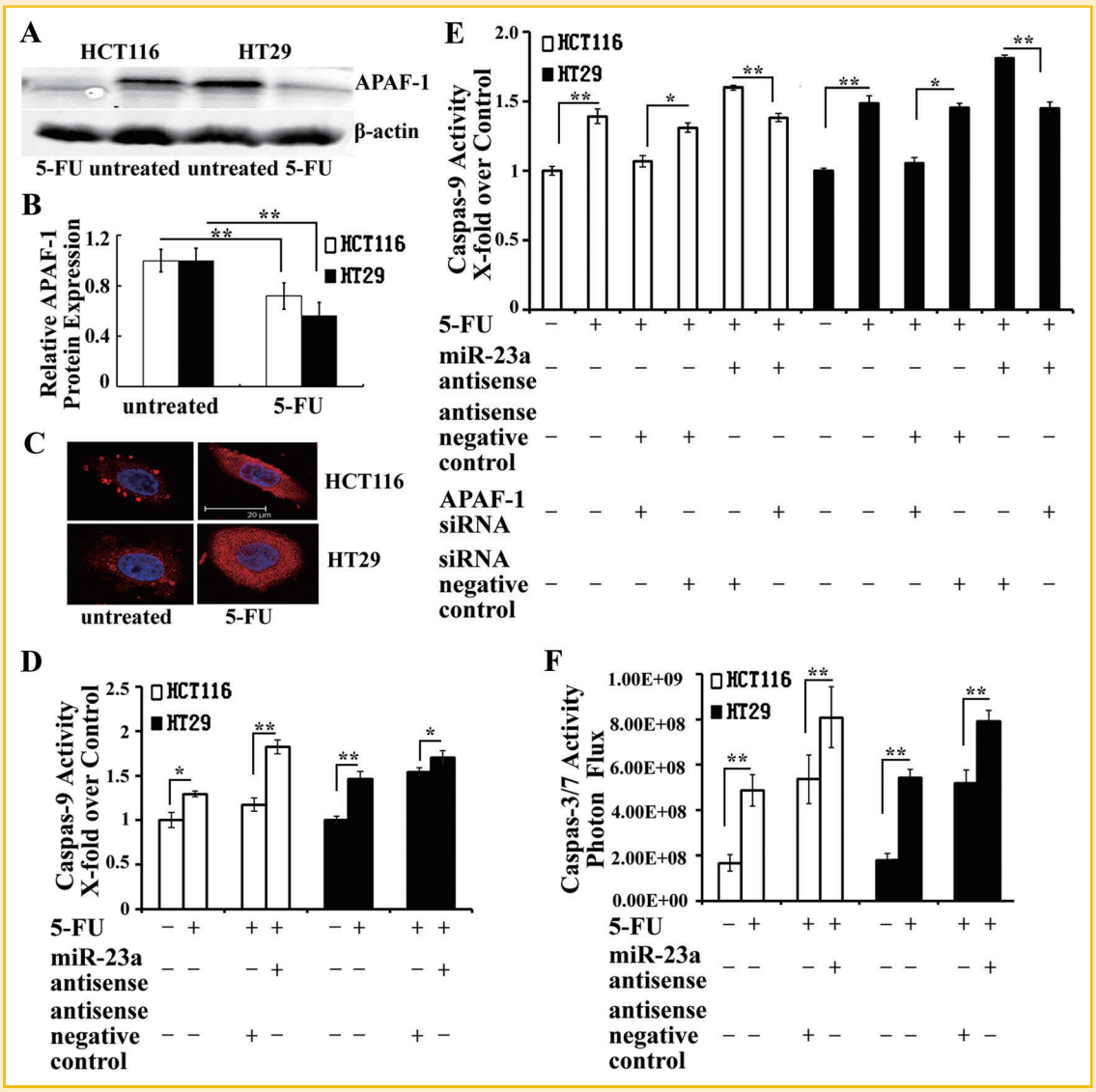

Fig. 4. APAF-1 and miR-23a antisense positively regulated the 5-FU-induced mitochondria-mediated apoptosis. A: APAF-1 protein expression levels in HCT116 or HT29 cells that were 5-FU treated or untreated. B: The expression of APAF-1 protein was significantly decreased in 5-FU-treated HCT116 and HT29 cell lines. C: Immunofluorescence images of HCT116 or HT29 cells double labeled for cytochrome *c* (red) and nuclear (blue). In 5-FU-treated cells, fluorescence was distributed throughout the cytosol. In untreated cells, mitochondrial fluorescence showed a clustered, spotty distribution. Bars represent 20 μm. D: The caspase-9 activity was significantly increased in HCT116 and HT29 cells treated with 5-FU compared to untreated control cells. Following 5-FU treatment, significant increases in caspase-9 activity were detected in both HCT116 and HT29 cells that overexpressed miR-23a antisense compared to the cells transfected with miR-23a antisense negative control E: The caspase-9 activity was significantly increased in HCT116 and HT29 cells treated with 5-FU compared to untreated control cells. After 5-FU treatment, the caspase-9 activity was significantly decreased in HCT116 and in HT29 cells co-transfected with APAF-1 siRNA and miR-23a antisense negative control compared to the cells co-transfected with APAF-1 siRNA negative control and miR-23a antisense negative control. After 5-FU treatment, the caspase-9 activity was significantly decreased in HCT116 and HT29 cells co-transfected with APAF-1 siRNA and miR-23a antisense compared to the cells co- transfected with APAF-1 siRNA negative control and miR-23a antisense. F: In both HCT116 and HT29 cells treated with 5-FU, there were significant increases in caspases-3 and -7 activities compared to untreated control cells. After 5-FU treatment, the activity of caspases-3 and -7 was significantly increased in HCT116 and HT29 cells transfected with miR-23a antisense compared to cells transfected with miR-23a antisense negative control. $^{*}P < 0.05$; $^{**}P < 0.01$. Experiments shown are representative of at least three independent experiments.

9 in colon cancer cells in the 5-FU-induced mitochondria-mediated apoptotic pathway. The caspase-9 activity was significantly increased in HCT116 (1.29-fold change, $P < 0.05$) and HT29 (1.46-fold change, $P < 0.01$) cells treated with 5-FU compared to untreated control cells. Following 5-FU treatment, significant increases in caspase-9 activity were detected in both HCT116 (1.56-fold change, $P < 0.01$) and HT29 (1.1-fold change, $P < 0.05$) cells that overexpressed miR-23a antisense compared to cells transfected with miR-23a antisense negative control (Fig. 4D).

We studied whether APAF-1 knockdown by APAF-1 siRNA adversely affected the enhancement of caspase-9 activity induced by miR-23a antisense following 5-FU treatment (Fig. 4E). The caspase-9 activity was significantly increased by 36.2% ($P < 0.01$) in HCT116 and 45.1% ($P < 0.01$) in HT29 cells treated with 5-FU compared to untreated control cells. After 5-FU treatment, the caspase-9 activity was significantly decreased by 19.1% ($P < 0.05$) in HCT116 and 27.4% ($P < 0.05$) in HT29 cells co-transfected with APAF-1 siRNA and miR-23a antisense negative control compared to the cells co-transfected with APAF-1 siRNA negative control and miR-23a antisense negative control. Moreover, after 5-FU treatment, the caspase-9 activity was significantly decreased by 13.4% in HCT116 and 19.9% in HT29 cells co-transfected with APAF-1 siRNA and miR-23a antisense compared to the cells co-transfected with APAF-1 siRNA negative control and miR-23a antisense ($P < 0.01$).

We also investigated the effects of miR-23a antisense on 5-FU-induced mitochondria-mediated apoptotic pathways by an activation assay on caspases-3 and -7 activities in both HCT116 and HT29 cells (Fig. 4F). In both cell lines treated with 5-FU, there were significant increases in caspases-3 and -7 activities compared to untreated control cells ($P < 0.01$). After 5-FU treatment, the activity of caspases-3 and -7 was significantly increased by 33.7% and 34.6%, respectively, in HCT116 and HT29 cells transfected with miR-23a antisense compared to cells transfected with miR-23a antisense negative control ($P < 0.01$). These findings indicated that APAF-1 and miR-23a antisense positively regulated the 5-FU-induced mitochondria-mediated apoptosis.

ANTITUMOR EFFECTS OF 5-FU IN COMBINATION WITH miR-23a ANTISENSE ON THE HCT116 AND HT29 HUMAN CRC XENOGRAFT MODELS

The effects of miR-23a antisense on 5-FU chemotherapeutic sensitivity in HCT116 and HT29 human CRC xenograft models were investigated. The miR-23a antisense expression vector (pLKO-miR-23a antisense) was used to reduce the expression levels of miR-23a in xenografts. The bioluminescence in each mouse was used to illustrate the tumor size. The tumor size was related to the effect of each therapy on tumor growth in the xenograft models. The bioluminescence-imaging pattern was observed for each group (Fig. 5A,B and Fig. 6A,B). In the mice model of xenograft implanted with HCT116 cells, there was reduced bioluminescence intensity in the mice implanted with cells expressing pLKO-miR-23a antisense or pLKO-miR-23a antisense negative control and having received 5-FU,

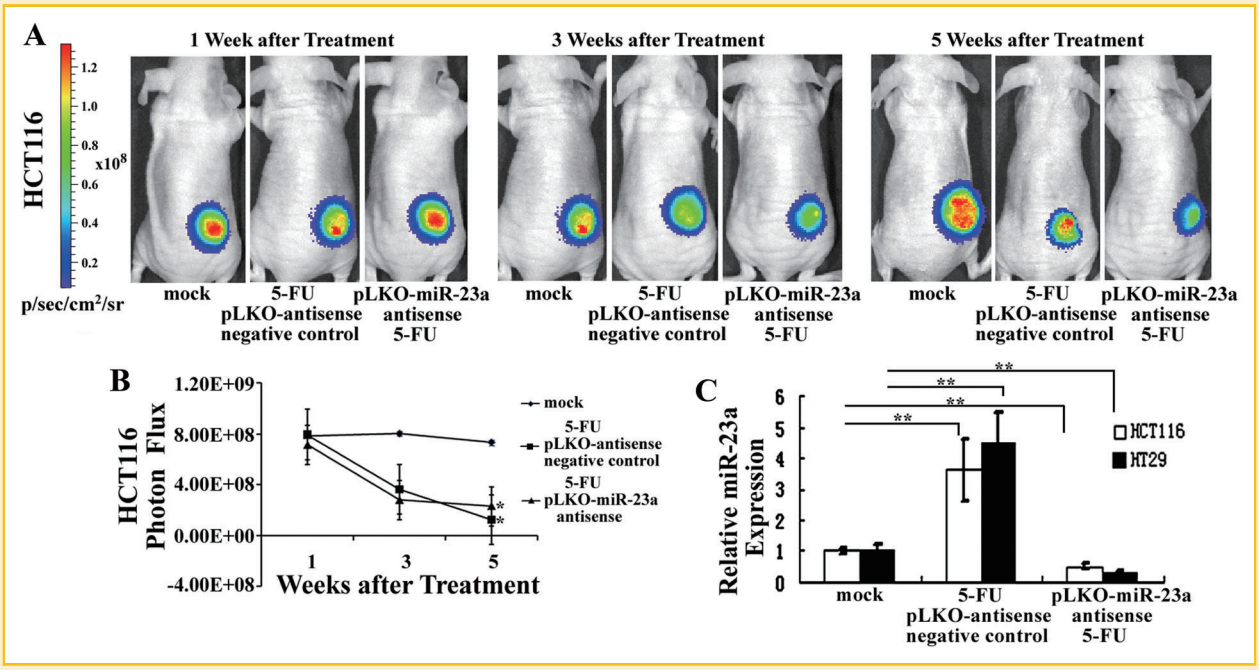

Fig. 5. Antitumor effects of 5-FU combined with pLKO-miR-23a antisense were evaluated in HCT116 human colon carcinoma xenograft models. HCT116 human colon carcinoma xenograft models were established, stably expressing the pLKO-miR-23a antisense or pLKO-antisense negative control, as well as the bioluminescence plasmid, pGL4.17. Mice were injected through the tail vein with 5-FU or PBS. A: Bioluminescence images of colon carcinoma xenografts. B: Xenografts were assessed through bioluminescence, which was quantified as photon flux. C: In 5-FUtreated and miR-23a antisense negative control-expressing xenografts, miR-23a expression was increased, but miR-23a expression was decreased in xenografts expressing miR-23a antisense and treated with 5-FU compared to the mock. Mock: mice xenograft models were stably expressing pLKO-antisense negative control and mice were injected with PBS. $^{*}P < 0.05$; $^{**}P < 0.01$.

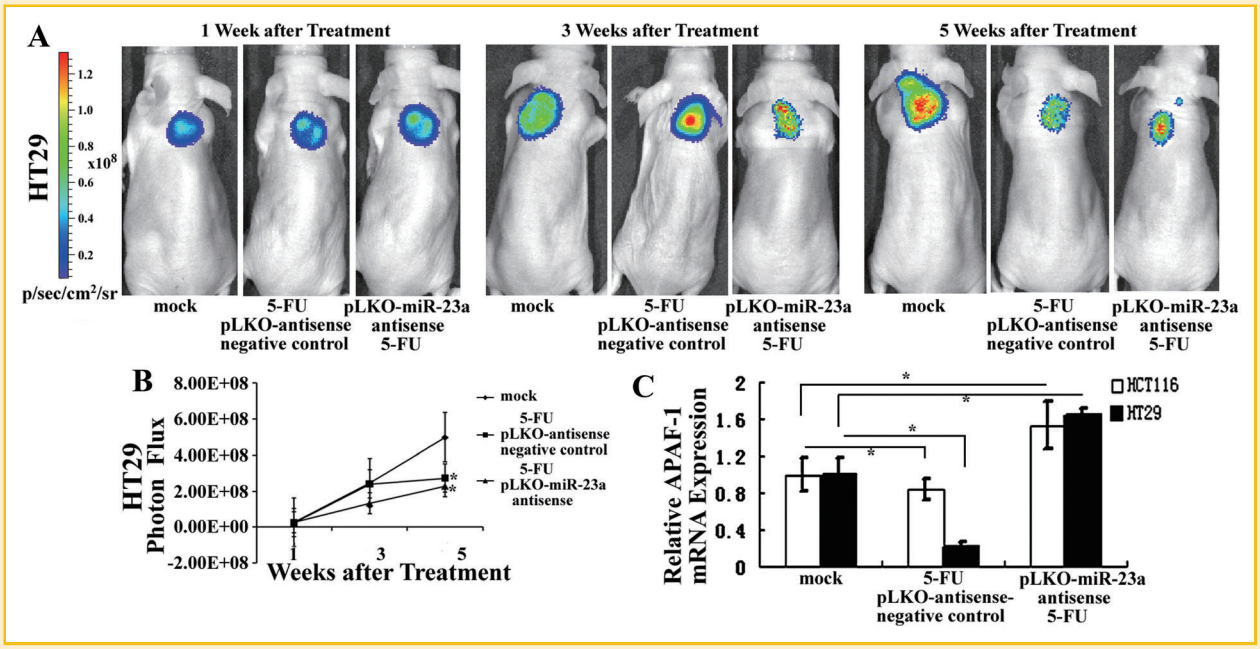

Fig. 6. Antitumor effects of 5-FU combined with pLKO-miR-23a antisense were evaluated in HT29 human colon carcinoma xenograft models. HT29 human colon carcinoma xenograft models were established, stably expressing the pLKO-miR-23a antisense or pLKO-antisense negative control, as well as the bioluminescence plasmid, pGL4.17. Mice were injected through the tail vein with 5-FU or PBS. A: Bioluminescence images of colon carcinoma xenografts. B: Xenografts were assessed through bioluminescence, which was quantified as photon flux. C: APAF-1 was down-regulated in 5-FU-treated and miR-23a antisense negative control-expressing xenografts, but upregulated in xenografts expressing miR-23a antisense and treated with 5-FU compared to the mock. Mock: mice xenograft models were stably expressing pLKO-antisense negative control and mice were injected with PBS. $^{*}P < 0.05$; $^{**}P < 0.01$.

compared to the mice implanted with cells expressing pLKO-miR-23a antisense negative control and having received PBS at week 5 after treatments. However, there was no significant difference of bioluminescence intensity between mice implanted with cells expressing pLKO-miR-23a antisense and mice implanted with cells expressing pLKO-antisense negative control at week 5 after 5-FU treatment by tail vein injection. There was also no significant difference among groups at week 1 and week 3 after 5-FU or PBS treatment by tail vein injection (Fig. 5B). In the mice model of xenograft implanted with HT29 cells, there was reduced bioluminescence intensity in mice implanted with cells expressing pLKO-miR-23a antisense or pLKO-miR-23a antisense negative control and having received 5-FU treatment compared to the mice implanted with cells expressing pLKO-miR-23a antisense negative control and having received PBS treatment at week 5. However, there was no significant difference of bioluminescence intensity between the mice implanted with cells expressing pLKO-miR-23a antisense and the mice implanted with cells expressing pLKO-antisense negative control at week 5 after 5-FU treatment. There was also no significant difference among groups at week 1 and week 3 after 5-FU or PBS treatment (Fig. 6B).

miR-23a ANTISENSE UPREGULATED THE EXPRESSION OF APAF-1 IN 5-FU-TREATED COLON CANCER XENOGRAFTS

To evaluate the biological relevance of alterations in miR-23a expression to chemotherapeutic responses in vivo, we used stably expressing pLKO-miR-23a antisense or pLKO-antisense negative control HCT116 and HT29 cells to establish the xenograft model. The miR-23a antisense expression plasmid, pLKO-miR-23a antisense, was used to reduce miR-23a levels in the xenografts. We also profiled miR-23a expression in xenografts treated with either 5-FU or phosphate buffered saline (PBS) in nude mice. In 5-FU-treated and miR-23a antisense negative control-expressing xenografts, miR-23a expression was increased by 3.63- (HCT116) and 4.5-folds (HT29) compared to the PBS-treated xenograft control ($P < 0.01$; Fig. 5C). In xenografts expressing miR-23a antisense and treated with 5-FU, miR-23a expression was decreased by 49% and 73% in HCT116 and HT29, respectively, compared to the PBS-treated xenograft control ($P < 0.01$; Fig. 5C).

To determine the relationship between APAF-1 and the alteration of miR-23a expression, we evaluated the levels of APAF-1 mRNA in 5-FU-treated and untreated xenografts using real-time PCR. The results showed that APAF-1 mRNA was involved in the dynamic regulatory mechanisms controlling miR-23a expression in tumor xenografts. The expression of miR-23a was increased in 5-FU-treated and pLKO-antisense-negative control-expressing xenografts (Fig. 5C), while APAF-1 was downregulated by 16% (HCT116) and 79% (HT29) relative to the control ($P < 0.05$; Fig. 6C). In contrast, the expression of miR-23a was decreased in miR-23a antisense-expressing and 5-FU-treated xenografts (Fig. 5C), but the expression of APAF-1 mRNA was increased by 1.53- (HCT116) and 1.64-folds (HT29; $P < 0.05$; Fig. 6C).

DISCUSSION

The higher expression of miR-23a in HT29 cells than in HCT116 after 5-FU treatment might be related to the approximately 10 times less restriction of p53 in the cytoplasm in HT29 cells compared to HCT116 cells [Gestl and Anne Bottger, 2012]. This implies that p53 nuclear function may be less limited in HT29 cells. Literature has shown that p53, a key regulator of miRNAs expression, activates miR-23 expression [Wang et al., 2013]. The much less restricted p53 nuclear function in HT29 cells may lead to much higher miR-23a expression. The half inhibitory concentration of 5-FU on HCT116 and HT29 cells is associated with 5-FU induced apoptosis, in which p53-independent pathway is involved, such as the Bax pathway [Nita et al., 1998]. The involvement of the p53-independent apoptotic pathway in HCT116 and HT29 cells after 5-FU treatment may result in fewer differences in IC_{50} in the two colon cancer cell lines. Possible future research could include investigating the difference between IC_{50} and miR-23a expression in colon cancer cells.

The relationship between miRNAs and cellular responses to chemotherapy is complex. Some miRNAs that are modulated by chemotherapeutic agents may prevent the development of chemo-resistance in CRC [Blower et al., 2008; Allen and Weiss, 2010]. We provided new data on the profile of expression of miRNA-23a in colon cancer cell lines and tissues. We found that the expression of miR-23a was consistently altered and dramatically up-regulated in 5-FU-treated human HCT116 and HT29 colon carcinoma cells relative to untreated cells. Moreover, the expression level of miR-23a was significantly increased in 5-FU-based chemotherapy-treated CRC tissues compared to untreated samples. These results were consistent with reports showing up-regulation of miR-23a in human colon cancer cells following exposure to 5-FU in vitro [Rossi et al., 2007; Chen et al., 2008]. One study showed that miR-23a was related to the chemoresistance toward cisplatin administration in tongue squamous cell carcinoma lines [Yu et al., 2010]. The up-regulated expression of miR-23a in 5-FU treated colon cancer cells as well as tissues indicated that miR-23a may be associated with chemoresistance of 5-FU.

Emerging evidence has shown that miR-23a directly binds the 3′ UTR of X-linked inhibitor of apoptosis (XIAP), and miR-23a regulation of XIAP contributes to sex differences in the response to cerebral ischemia [Siegel et al., 2011]. Research has also indicated that miR-23a regulates DNA damage repair and apoptosis in UVB-irradiated HaCaT cells [Guo et al., 2013]. To better understand the functional role of miR-23a in the regulation of 5-FU-induced apoptosis, we performed cell proliferation, cytotoxicity and apoptosis analyses in vitro. These results indicate that miR-23a may suppress cell apoptosis in both HCT116 and HT29 cells. The positive relationship between 5-FU-induced apoptosis and miR-23a antisense expression could be interpreted as miR-23a may negatively regulate other genes involved in the apoptosis process. We identified an apoptosis-related target gene, APAF-1, for miR-23a using luciferase 3′-UTR reporter and Western blot analysis. Our results are consistent with the mechanism in which miRNA negatively regulates target gene expression during various crucial cell processes, such as apoptosis.

Anticancer drugs exert their effects, at least in part, by triggering apoptosis [Lei et al., 2007; Braconi et al., 2010]. Previous studies have demonstrated that a wide range of anticancer agents induce apoptosis in malignant cells in vitro. After treatment with various cytotoxic drugs, including 5-FU, the characteristics of apoptosis in mice with solid tumors have also been described [Kaufmann and Earnshaw, 2000]. Although recent studies have revealed that 5-FU induces cytotoxicity through the Fas/FasL pathway in colon cancer cells [Houghton et al., 1997; Tillman et al., 1999], other studies have shown that 5-FU can cause massive apoptosis in colon cancer cells by activating the apoptotic mitochondrial pathways [Shin et al., 2005]. In addition, recent studies have found that APAF-1 and caspase-9 are important apoptotic biomarkers [Krajewska et al., 2005; Strater et al., 2010]. In the present study, we observed that both the release of cytochrome-*c* from the mitochondria into the cytosol and the expression of APAF-1 were modulated by 5-FU. We also found that miR23a antisense increased the expression of APAF-1 mRNA in both cell lines, but miR-23a sense decreased the expression of APAF-1 protein in HCT116 and HT29 cells. These results indicated that 5-FU-induced apoptosis through the mitochondria-mediated apoptotic pathway in colon cancer cells. Cytochrome-*c*, miR-23a and APAF-1 may be critical members of this pathway. The expression of APAF and/or miR-23a was also related to caspase-3, -7, and -9 activities. In both cell lines, APAF-1 knockdown as well as miR-23a antisense overexpression resulted in significant decrease in caspase-9 activity compared to 5-FU treated alone controls. Our present work also showed that miR-23a antisense significantly increased the activity of caspase-3, -7, and -9 in 5-FU-treated colon cancer cells compared to control cells. Thus, the activity of caspase-3, -7, and -9 was regulated by APAF-1 through miR-23a in the 5-FU-induced apoptosis signaling pathway. These results suggest that miR-23a may inhibit 5-FU-induced apoptosis through the APAF-1/caspase-9 pathway and provide new insight into CRC treatment.

Studies have shown that miRNAs can be effective inhibitors as anti-tumor agents. In this regard, an example such as the antisense-based inhibition of a specific miRNA has been found to be useful due to the enhancement of the corresponding anti-tumor immunity [Tili et al., 2007]. The apoptosis of leukaemic MEG01 could be induced through the reintroduction of miR-15a and miR-16-1, which were shown to inhibit tumor growth in vivo in a xenograft model [Calin et al., 2008]. To explore the potential effects of miR-23a antisense on silence endogenous miR-23a in vivo, we developed a xenograft model by implantation of colon cancer cells stably expressed pLKO-miR-23a antisense or pLKO-antisense negative control alongside a bioluminescence plasmid. The up-regulation of miR-23a was suppressed in these 5-FU treated mice implanted with miR-23a antisenses expressing colon cancer cells. Our data also showed that the APAF-1 mRNA was up-regulated in the 5-FU-treated mice implanted with miR-23a antisense expressing xenografts and the expression of miR-23a is downregulated in these mice. Nevertheless, we did not find that xenografts′ growth was rendered by miR-23a antisense after 5-FU treatment in vivo than the xenografts′ growth in mice treated with miR-23a antisense negative control and 5-FU. Literature suggests that microRNAs are usually found to be clustered on chromosomes [Yuan et al., 2009; Chhabra et al., 2010]. Each target mRNA can be controlled by multiple miRNAs [Kim et al., 2009], indicating that one pathophysiological process (e.g., chemoresisitense) may be regulated by several or many miRNAs. There is a report which shows that miRNAs of a miRNA cluster might work in combination to

accomplish their function [Yuan et al., 2009]. Studies have shown that a miR-23a cluster, the miR-23a–27a–24-2 cluster, may cooperate together to control several process (e.g., cell cycle and proliferation) during health and diseases (reviewed in [Chhabra et al., 2010]). The miR-23a–27a–24-2 cluster encodes primary miRNA transcript composed of 3 miRNAs: miR-23a, miR-27a, and miR-24. miR-27a have been found in oncogenesis and multidrug resistance of cancer [Chhabra et al., 2010]; miR-23a and miR-27a regulate the activation of dendritic cells, NK cells and macrophage cells by inhibition of their target genes and by involvement in anti-tumor immunity [Yu et al., 2013]; miR-24 contributes to anti-apoptosis and tumorigenesis in colorectal cancer [Chhabra et al., 2010]. The miR-23a–27a–24-2 cluster is related to chemosesnsity and chemoresisitance. In this present study, not only miR-23a but other miRNAs may be involved in the process of 5-FU induced chemoresisitence. This may be the reason why only miR-23a knockdown did not improve the chemotherapy effect of 5-FU on xenograft growth. The miR-23a cooperation with other miRNAs in the cluster of miR-23a–27a–24-2 may not only markedly enhance apoptosis induced by 5-FU treatment, but also further activate immune system to be involved in the anti-tumor process. This miRNAs cooperation via drugs or other agents during their transcription might show the potential of miRNAs as therapeutic adjuvant tools to improve the response and overcome resistance.

In conclusion, our study shows that miR-23a expression is negatively associated with 5-FU chemosensitivity in colon cancer cells in vitro. Our present work shows that miR-23a regulates 5-FU-induced apoptosis through APAF-1/caspase-9 apoptoic pathways. The exploration of miR-23a knockdown in HCT116 and HT29 cell xenograft model could be essential data for the improvement of 5-FU-based CRC chemotherapy. Knowledge of specific processes that are regulated by miR-23a and identification of the apoptotic mechanisms involved in the mitochondrial pathway will yield useful information and novel insight into the mechanisms of chemo-resistance of colorectal cancer and other types of cancer to drug administration.

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