miR-218 promotes apoptosis of SW1417 human colon cancer cells by targeting c-FLIP

QINGKAI MENG[1], YUE CHEN[1], BO LIAN[1], YAN SHANG[1] and HONGMEI YANG[2]

Departments of [1]Colorectal Surgery and [2]Medical Oncology, Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, Shenyang, Liaoning 110042, P.R. China

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Abstract. MicroRNAs (miRNAs) are suggested to act as either tumor oncogenes or tumor suppressors in different types of cancer. miRNA-218 (miR-218) is a type of short, non-coding RNA which is involved in gastric cancer development. In the present study, we evaluated the functions of miR-218 in SW1417 human colon cancer cells and its potential mechanisms. Following overexpression of miR-218 in human colon cancer cells, cell viability was determined by CKK-8 assay, cell apoptosis was observed using a TUNEL Kit, the expression of caspase-8, and its inhibitor cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (c-FLIP) was assessed by RT-PCR, western blot analysis and immunohistochemistry. The results indicated that miR-218 and caspase-8 expression was decreased while c-FLIP expression was elevated in human colon cancer tissues. In cultured SW1417 human colon cancer cells, miR-218 overexpression potently inhibited cell viability and promoted cell apoptosis. Furthermore, downregulation of c-FLIP expression and upregulation of caspase-8 expression were detected in miR-218-stimulated SW1417 cells. In addition, following the knockdown of c-FLIP using c-FLIP siRNA, the apoptotic effects of miR-218 on SW1417 cells were significantly reduced. Collectively, the present study demonstrated that miR-218 induced the apoptosis of SW1417 cells by targeting c-FLIP. Therefore, miR-218 may represent a potential therapeutic method for screening and treating colon cancer.

Introduction

Gastric cancer is the third leading cause of mortality throughout the world especially in developing countries (1,2). Among them, colon cancer is one of the most common cancers resulting in cancer-related deaths in China (3-5). Over the past decade, surgery and chemotherapy have been chosen as the main treatments for colon cancer (6,7). Although major advances have been developed in the treatment of colon cancer, the therapeutic efficacy of anticancer agents remains very poor (8). Thus, it is urgent to develop novel, safe and effective therapeutic agents for colon cancer.

miRNAs are a class of 22-25-nucleotide-long non-coding RNA molecules which can exert their functions by inhibiting mRNA translation or promoting mRNA degradation. Increasing evidence has revealed that miRNAs have various biological functions such as regulating cell viability, death and migration in cultured cells (9,10). Notably, miRNAs have been reported to be involved in cancer development through their tumor-suppressing or -activating functions (11-13). Recently, miR-218 was reported to play a tumor-suppressive role in various types of human cancers, such as lung, bladder and gastric cancer, hepatocellular carcinoma, oral cancer and renal cell carcinoma (14-20), indicating that miR-218 may be a potential therapeutic target for the treatment of cancers.

c-FLIP, a death effector domain (DED)-containing anti-apoptotic protein, can suppress cell apoptosis induced by tumor necrosis factor-α (TNF-α), Fas-L, TNF-related apoptosis-inducing ligand (TRAIL), and chemotherapy agents in cancer cells by regulating the activation of caspase-8 and caspase-10. New findings have provided evidence that c-FLIP can be regarded as a critical target for therapeutic intervention due to its inhibition in transcription and post-transcription (21,22). Increased expression of c-FLIP has been found in cell lines from various types of cancers, including colorectal (21,22), head and neck (23), prostate (24), cervical (25), breast (26), lung (27) and hepatocellular cancers (28), and c-FLIP has been demonstrated to be an important indicator in these types of cancers. In our study, we evaluated the roles and underlying mechanisms of miR-218 in human colon cancer cells. It was demonstrated that miR-218 exerted its anti-apoptotic functions in SW1417 colon cancer cells by downregulating c-FLIP and may become a biomarker and therapeutic target in colon cancer treatment.

Materials and methods

Patients and tissue sample collection. A total of 20 patients diagnosed with colon cancer were involved in this study.

Correspondence to: Dr Hongmei Yang, Department of Medical Oncology, Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, 44 Xiaoheyan Road, Dadong, Shenyang, Liaoning 110042, P.R. China
E-mail: hm99_yang@163.com

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Tissue samples were obtained from the tumoral area and adjacent area of patients who had not undergone radiotherapy or chemotherapy prior to surgery at the Cancer Hospital of China Medical University. All the patients that participated in this study provided written informed consent. The protocol of this study was approved by the local Ethics Committee (Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, Shenyang, China. Permit no. 122/17).

Cell culture. Human colon cancer cell line SW1417 was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, and 10 mg/ml streptomycin. Cultures were incubated in 5% CO_2 at 37˚C.

Analysis of cell viability. Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA; St. Louis, MO, USA) was used to determine cell proliferation. Briefly, SW1417 cells at a density of $1.0x10^5$ cells/ml were seeded in 96-well plates in DMEM containing 10% FBS for 24 h. Then, the cells were cultured in a serum-free medium. Following incubation for 24 h, the cells were incubated with miR-218-mimic control, miR-218 mimics, cFLIP-siRNA control, cFLIP siRNA and miR-218 mimics plus cFLIP siRNA in fresh DMEM using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). At 24 and 48 h, cell viability was evaluated according to a previous study (7).

Analysis of cell apoptosis. SW1417 cells at a concentration of $1.0x10^5$ cells/ml were seeded onto a 96-well plate and treated with miR-218 mimic control, miR-218 mimics, cFLIP-siRNA control, cFLIP siRNA and miR-218 mimics plus cFLIP siRNA for 24 or 48 h, and the cell apoptotic rate was assessed using TUNEL Apoptosis Detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions (27). The cell apoptotic rate was assayed using a FACScan flow cytometric apparatus (BD Biosciences, San Jose, CA, USA) (1).

Western blot analysis. Total protein was extracted and the protein expression levels of caspase-8, c-FLIP and GAPDH were performed separately using western blot analysis according to a previous study (29). Antibodies including caspase-8 (1:2,500 dilution; cat. no. MAB704), c-FLIP (1:2,500 dilution; cat. no. MAB8430) and GAPDH (1:5,000 dilution; cat. no. MAB5718) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The protein expression levels were obtained using the chemiluminescence reader, ImageQuant™ LAS 4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and the band density was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (1).

Gene expression analysis. RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to detect the expression levels of c-FLIP, caspase-8, miR-218 and GAPDH in SW1417 cells at indicated time-points. Following the manufacturer's instructions, total cellular RNA was isolated from SW1417 cells cultured in 6-well plates using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). GAPDH expression was used as an internal control. All primer sequences used in this study were synthesized at BioSune Biotechnology Co., Ltd. (Shanghai, China), and the sequences of the primers were as follows: c-FLIP forward, 5'-TAACAT TTCAGCCGGTGGGT-3' and reverse, 5'-ATCCTTTCCAGT GGGGGAGT-3'; caspase-8 forward, 5'-CTGGTCTGAAGG CTGGTTGT-3' and reverse, 5'-CAGGCTCAGGAACTTGAG GG-3'; GAPDH forward, 5'-AACGGATTTGGTCGTATT GGG-3' and reverse, 5'-CCTGGAAGATGGTGATGGGAT-3'; miR-218 forward, 5'-GCGGCTTTGTGCTTGATCTAA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'.

Transfection. miR-218 mimics and their non-specific control miRNAs (miR-218-NC, miR-218-NC-In), c-FLIP siRNA (AAGGAACAGCTTGGCGCTCAA) and c-FLIP-control siRNA (AATTCTCCGAACGTGTCACGT) were all purchased from RiboBio (Guangzhou, China). A total of 3 μg of miR-218-mimics, non-specific control microRNAs, c-FLIP-specific siRNA or non-targeting siRNA was transfected into SW1417 cells using HiPerFect transfection reagent (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocols. After SW1417 cells were cultured in medium for 48 h, RT-PCR and western blot assays were used to determine the efficiency of miR-218 mimics and c-FLIP siRNA, respectively.

Immunohistochemistry. Immunohistochemical analysis of c-FLIP and capspase-8 was performed using Image Analysis System (Leica Microsystems, Wetzlar, Germany) according to the previous study (2). Primary monoclonal antibodies of c-FLIP (1:1,000 dilution; cat. no. MAB8430) and capspase-8 (1:1,000 dilution; cat. no. MAB704) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Biotinylated goat anti-rabbit antibody (dilution, 1:1,000) was provided by Vector Laboratories, Inc. (Burlingame, CA, USA). Positive immunoreactivity was semi-quantitatively scored as 0 for none to trace, 1+ for <10%, 2+ for 10-50%, and 3+ for >50% tumor cells showing positive expression.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Duncan's new multiple range test (MRT) in SPSS 15.0 software was used to compare the statistical differences between the treatment and control groups. $P<0.05$ indicated a statistically significant difference.

Results

Increased c-FLIP expression and decreased miR-218 and caspase-8 expression is revealed in human colon cancer tissues. In human colon cancer tissues and normal colon tissues, the expression of c-FLIP, caspase-8 and miR-218 was assessed and compared. The results revealed that compared with normal colon tissues, miR-218 expression as determined by RT-PCR was significantly reduced in human colon cancer tissues (Fig. 1A). As revealed in Fig. 1B, upregulation of c-FLIP expression was observed, whereas downregulation

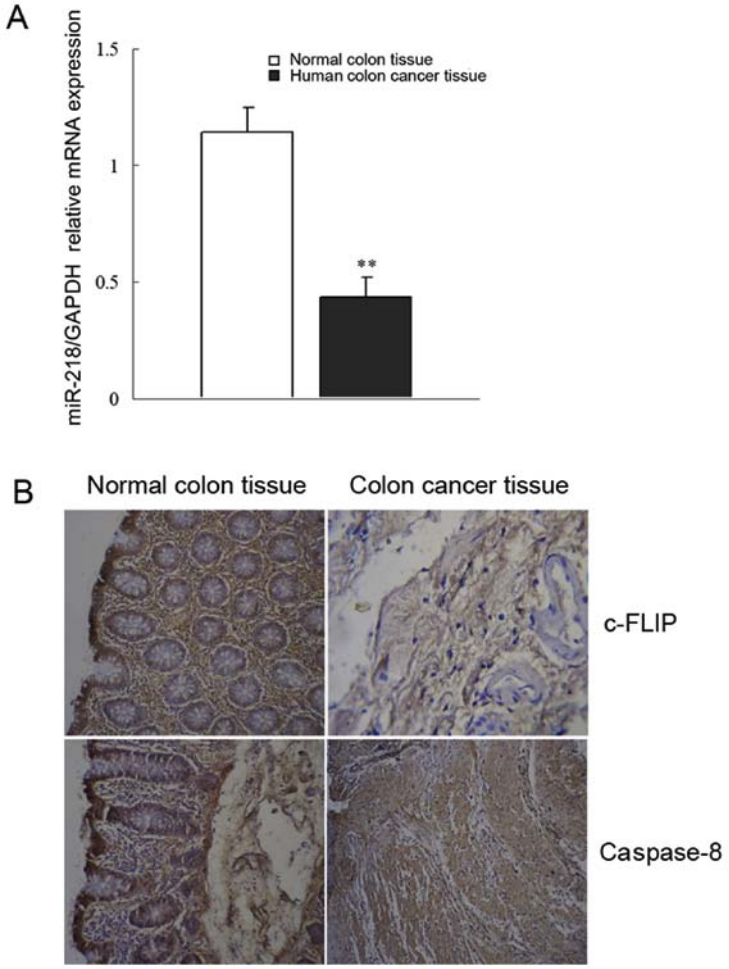

Figure 1. Expression of miR-218, c-FLIP, and caspase-8 in human colon cancer tissues. (A) The expression of miR-218 and (B) c-FLIP and caspase-8 was assessed in human colon cancer tissues and normal colon tissues. *P<0.05 or **P<0.01 indicated significance. miR-218, miRNA-218; c-FLIP, Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein.

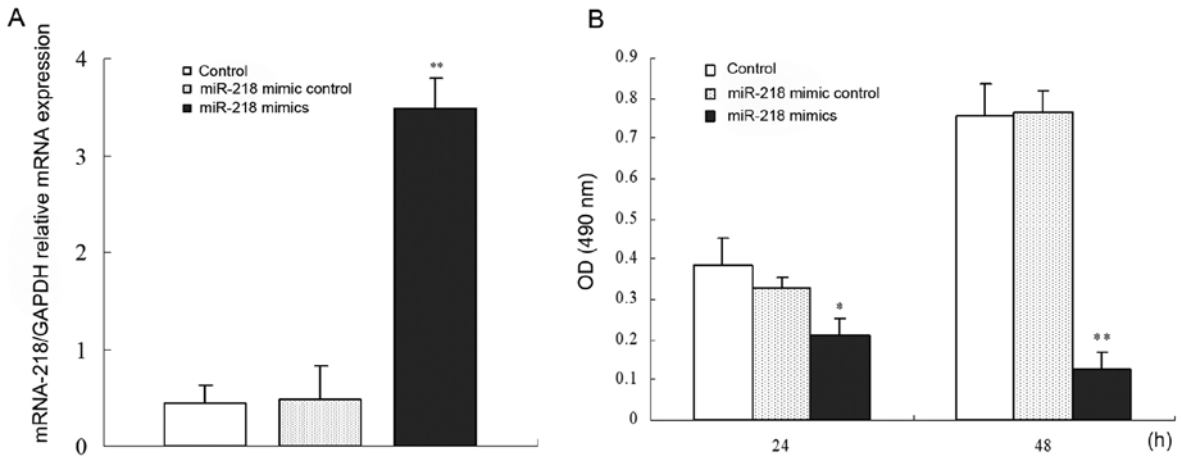

Figure 2. miR-218 overexpression inhibits proliferation of SW1417 cells. (A) The expression of miR-218 was assessed after endogenous miR-218 was overexpressed in human SW1417 colon cancer cells. (B) Following treatment with the control mimic or miR-218 mimics for 24 and 48 h, cell viability of SW1417 cells was determined by CKK-8 assay. *P<0.05 or **P<0.01 indicated significance. miR-218, miRNA-218.

of caspase-8 was found in human colon cancer tissues in comparison with normal colon tissues.

miR-218 overexpression suppresses the proliferation while it promotes the apoptosis of SW1417 cells. Firstly, miR-218 mimics were stably transfected into SW1417 cells to increase the expression level of miR-218. A high expression level of miR-218 was determined by RT-PCR, indicating that miR-218 was successfully introduced into SW1417 cells (Fig. 2A). Next, we ascertained the effects of the transfected miR-218 mimics

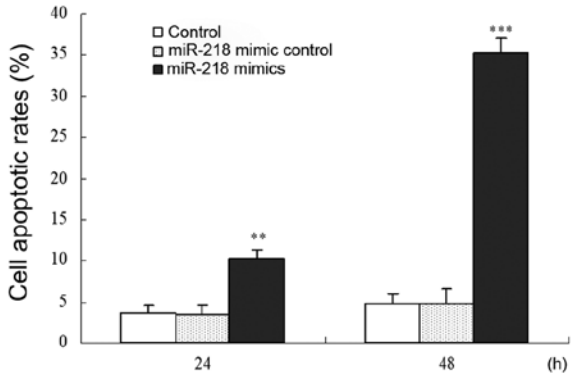

Figure 3. miR-218 overexpression increases apoptosis of SW1417 cells. Following transfection with control mimic or miR-218 mimics for 24 and 48 h, the apoptotic rate of human SW1417 colon cancer cells was assessed by flow cytometry. **P<0.01 or ***P<0.001 indicated significance. miR-218, miRNA-218.

on the proliferation and apoptosis of SW1417 cells. The results revealed that overexpression of miR-218 inhibited the proliferation of SW1417 cells compared with the mock-transfected cells, and the effects were time-dependent (P<0.05) (Fig. 2B). Furthermore, a higher apoptosis rate was found in the miR-218-mimic treated SW1417 cells when compared to cells treated with negative control mimic (P<0.05) (Fig. 3). Therefore, miR-218 is an important molecule in the promotion of the apoptosis of human colon cancer cells.

miR-218 overexpression inhibits the expression level of c-FLIP in SW1417 cells. To determine the molecular mechanism in apoptosis signaling involved in the inhibition of cell apoptosis induced by miR-218, we further assessed the expression of c-FLIP and caspase-8 by western blotting and RT-PCR. In vector-transfected SW1417 cells, c-FLIP mRNA expression was detectable and significantly decreased with miR-218 stimulation. In contrast, mRNA expression of caspase-8 was evidently elevated in miR-218-overexpressing SW1417 cells (P<0.05) (Fig. 4A). Accordingly, compared to the control, the protein expression of c-FLIP was decreased while caspase-8 was increased by miR-218 (Fig. 4B). Thus, miR-218 overexpression inhibited the expression level of c-FLIP and promoted the expression level of caspase-8 in human colon cancer cells.

miR-218-triggered cell apoptosis in SW1417 cells is c-FLIP-dependent. The molecular mechanism of miR-218 on cell apoptosis in human colon cancer cells was further investigated. c-FLIP-targeted siRNA was used to transfect SW1417 cells and western blot analyses indicated that c-FLIP-targeted siRNA efficiently inhibited c-FLIP expression (Fig. 5A). Notably, cell viability assays revealed that transfection with miR-218 mimics or c-FLIP-targeted siRNA decreased the number of SW1417 cells, however the effect of miR-218 mimics was more significant (P<0.05). Moreover, there was no difference in cell viability between thr c-FLIP-targeted siRNA group and c-FLIP-targeted siRNA+miR-218 mimic group (P>0.05) (Fig. 5B). Furthermore, flow cytometric experiments indicated that miR-218 overexpression resulted in a marked increase in cell apoptosis, and c-FLIP knockdown also led to an upregulation of the apoptotic rate in SW1417 cells. A similar effect was observed in c-FLIP-knockdown SW1417 cells and c-FLIP knockdown plus miR-218-mimic SW1417 cells (Fig. 5C). These results revealed that miR-218 induced the onset of apoptosis through the suppression of c-FLIP in human colon cancer cells.

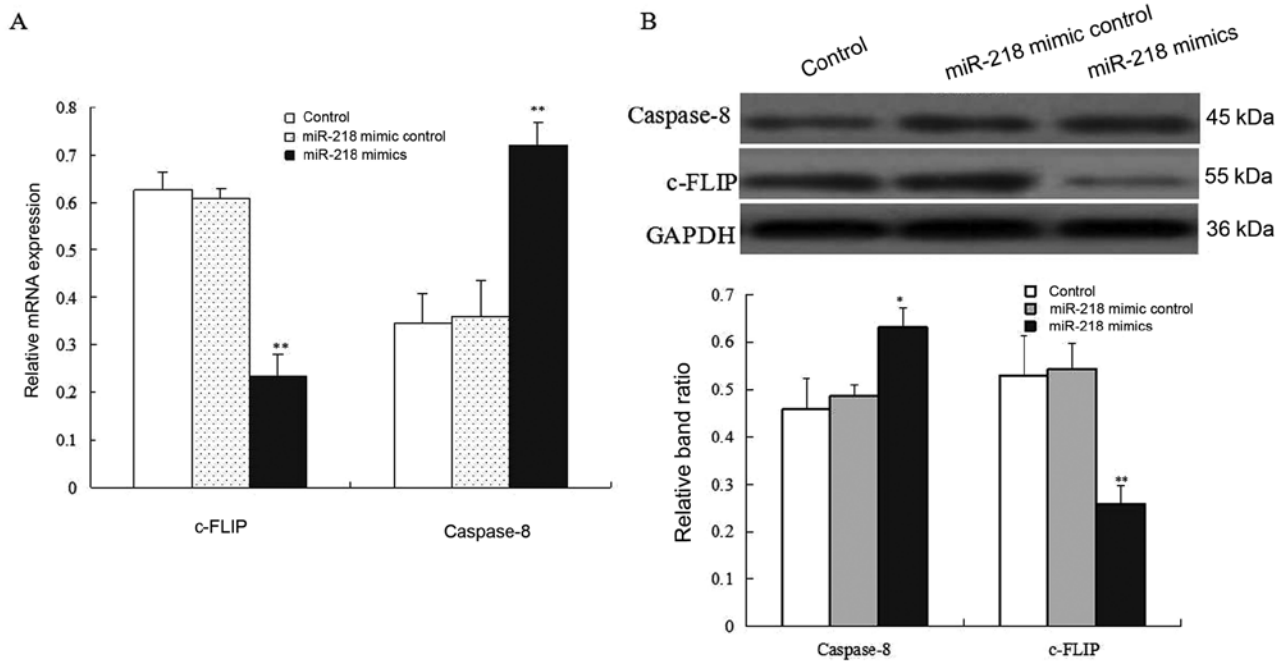

Figure 4. Effects of miR-218 overexpression on c-FLIP and caspase-8 expression in SW1417 cells. (A) After SW1417 cells were treated with control mimic or miR-218 mimics for 48 h, mRNA expression of c-FLIP and caspase-8 was determined by RT-PCR. (B) Protein expression of c-FLIP and caspase-8 was determined by western blotting and band relative ratio was analyzed using ImageJ software. *P<0.05 or **P<0.01 indicated significance. miR-218, miRNA-218; c-FLIP, Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein.

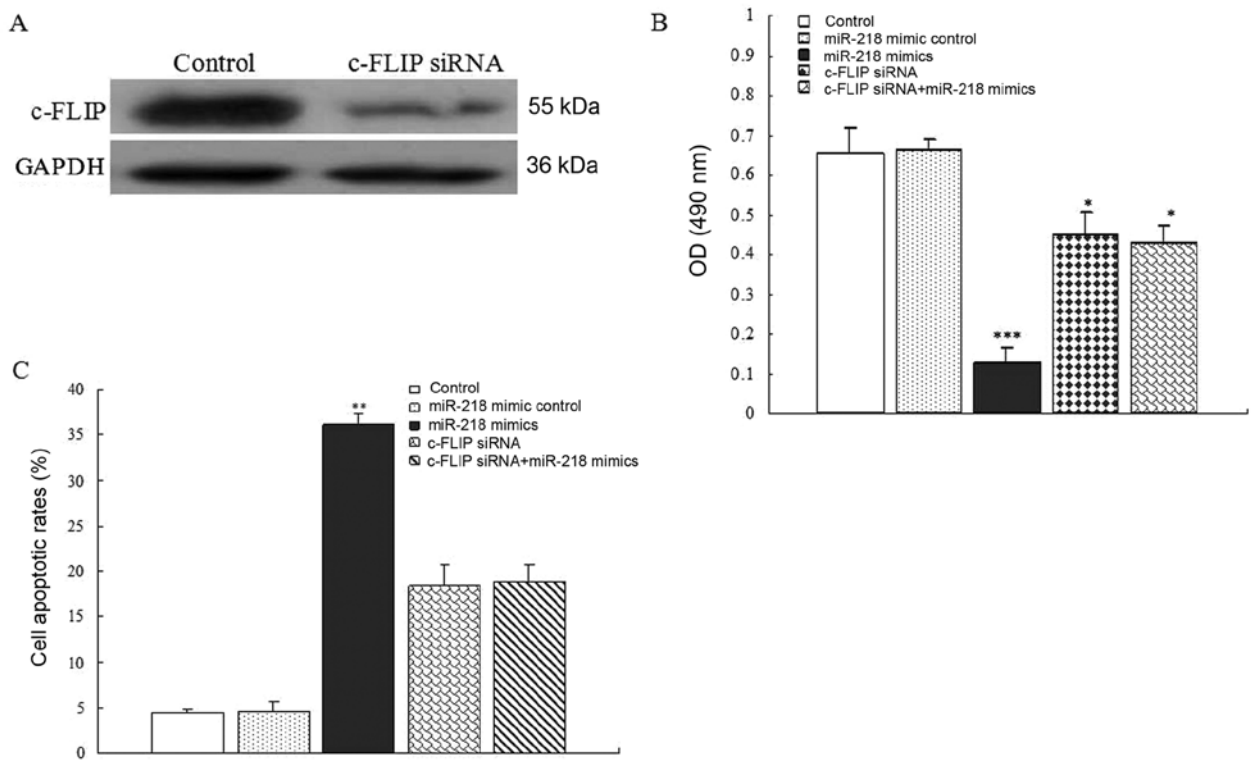

Figure 5. miR-218 overexpression promotes SW1417 cell apoptosis through the suppression of c-FLIP. (A) After SW1417 cells were transfected with c-FLIP siRNA for 48 h, c-FLIP expression was assessed by western blotting. Following knockdown of c-FLIP, SW1417 cells were treated with miR-218 mimics, and then (B) cell viability and (C) the apoptotic rate were determined. *P<0.05 or **P<0.01 or ***P<0.01 indicated significance. miR-218, miRNA-218; c-FLIP, Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein.

Discussion

Recently, miR-218 has been reported to be involved in the proliferation, apoptosis and migration of different types of tumor cells (9,10). The present study demonstrated that the miR-218 expression level was significantly downregulated in colon cancer tissues. Additionally, the results revealed that miR-218 may play a role in the inhibition of colon cancer cell apoptosis through downregulation of c-FLIP expression.

As demonstrated in a previous research, changes in miR-218 expression were associated with cancer development. For instance, compared to normal tissues without cancer, the expression levels of miR-218 were significantly inhibited in lung cancer tissues (15). In cultured gastric cancer cell lines SGC7901 and BGC823, it was revealed that miR-218 mRNA and protein expression were significantly decreased compared to those in normal gastric epithelial cell line GES-1 (30). Furthermore, compared with gastric cancer cell lines MGC80-3 and HGC-27, decreased miR-218 expression levels were also found in the more aggressive gastric cancer cell line NCI-87 (17). Notably, the present study revealed a significant decrease in miR-218 expression in human colon cancer tissues and SW1417 cells.

miR-218 is also regarded as a tumor suppressor due to its function in the inhibition of invasion and growth of nasopharyngeal (31), oral (19), lung (15,32), and bladder (33) cancer cells. By regulating the expression of Ang-2 in gastric cancer cells NCI-87 and HGC-27, miR-218 overexpression suppressed cell proliferation and angiogenesis (17). An MTT assay revealed that miR-218 overexpression markedly suppressed the proliferation of gastric cancer cells and wound scratch assays indicated that miR-218 inhibited cell migration and EMT by targeting WASF3 (30). Another study found that miR-218 could promote cell apoptosis and inhibit cell growth in colorectal cancer cells (34). Notably, Tie *et al* provided the evidence that upregulation of miR-218 could inhibit tumor cell invasion and proliferation in gastric cancer cells by altering miR-218-targeted genes (18). In LoVo colon cancer cells, miR-218 overexpression suppressed the invasion, proliferation migration of cells and the signaling pathways including PI3K/Akt/mTOR and MMP9 were involved in these functions (35). Furthermore, miR-218 played an important role in colon cancer development through inhibition of cell proliferation and promotion of cell apoptosis by targeting BIRC5 (36), BMI-1 (37) and MACC1 (38). In the present study, we also revealed that miR-218 overexpression inhibited cell viability and promoted cell apoptosis in human SW1417 colon cancer cells.

FU is a chemotherapy drug commonly used for the treatment of colon cancer by targeting rpL3 to downregulate p53 (39). miR-218 enhanced 5-FU-induced apoptosis while the suppression of miR-218 expression was associated to the resistance to 5-FU (40). These findings revealed that miR-218 could exert its functions by targeting some molecules. c-FLIP belongs to a family of apoptosis inhibitors, and plays a crucial role in tumor development and progression (41). Studies have revealed that c-FLIP could inhibit apoptosis mediated by death receptors trough binding to Fas-associated death domain and inhibit caspase-8 activation (42). Overexpression of c-FLIP has been revealed in different types of cancers (26,43). c-FLIP

upregulation has also been observed in gastric cancer tissues and cells (44). Using RT-PCR and flow cytometric analyses, the high expression level of c-FLIP in colon carcinoma cell line HT-29 was detected. Furthermore, silencing of c-FLIP with the specific siRNA promoted Fas-mediated apoptosis (45). Knockdown of c-FLIP by siRNA was found to sensitize colon cancer cells to TRAIL-induced apoptosis (46). In the present study, the increased expression of c-FLIP was also observed and knockdown of c-FLIP could enhance cell apoptosis of human colon cancer cell line SW1417. Furthermore, it was demonstrated that miR-218-induced cell apoptosis in this type of cell line was c-FLIP dependent.

In summary, the present study revealed that miR-218 could inhibit the apoptosis of human colon cancer cell line SW1417 by suppressing c-FLIP expression, indicating that the miR-218/c-FLIP axis could be an important target for gastric cancer therapy. However, due to the limited support of funding, we only used colon cancer cell line SW1417 in this study, and only provided evidence that miR-218 inhibited c-FLIP expression using western blotting and RT-PCR methods without a luciferase assay with a 3' UTR region of c-FLIP. Thus, further investigations are warranted to demonstrate the functions of miR-218/c-FLIP in other colon cancer cell lines.

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Availability of data and materials

The data used in the present study are available from the corresponding author YHM upon reasonable request.

Authors' contributions

QM designed the study and wrote the paper. YC, BL and YS performed the experiments and analyzed data. HY reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The protocol of the present study was approved by the local Ethics Committee (Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, Shenyang, China. Permit no. 122/17).

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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