

Overexpression of microRNA-15 increases the chemosensitivity of colon cancer cells to 5-fluorouracil and oxaliplatin by inhibiting the nuclear factor- κ B signalling pathway and inducing apoptosis

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Abstract. Overcoming chemoresistance is a challenge in clinical treatment. It has been reported that microRNAs (miRNAs) are involved in regulating chemosensitivity. Therefore, the present study aimed to identify the effect and mechanism of miR-15 on colon cancer chemotherapy. Reverse transcription-quantitative polymerase chain reaction was performed to measure miR-15 level in 62-paired colon cancer and para-cancerous colon tissues. The overexpression of miR-15 in HCT116 cells was induced by transfection. The effect of miR-15 on the chemosensitivity of colon cancer cells to 5-fluorouracil (5-FU) and Oxaliplatin (OX) was determined using a luminescent cell viability assay. Flow cytometry, dual-luciferase assay and western blot analysis were used to determine the potential mechanism of miR-15. The results suggested that the expression of miR-15 was decreased in tumour tissues and that overexpression of miR-15 increased the chemosensitivity of colon cancer cells to 5-Fu and OX. miR-15 promoted apoptosis in colon cancer cells treated with 5-Fu and OX by inhibiting the expression of p50, which repressed the expression of B cell lymphoma-2 and B cell lymphoma-extra large; two direct target genes of nuclear factor- κ B with anti-apoptotic functions. Thus, the current study demonstrated that miR-15 increased the chemosensitivity of colon cancer cells to 5-FU and OX by inhibiting the NF- κ B signalling pathway and inducing apoptosis.

Introduction

Colon cancer is one of the most common (1). In recent years, improvements in early screening techniques and treatment strategies has largely decreased the morbidity and mortality of

colon cancer in the United States (1). However, these regions do not include developing countries such as China. Furthermore, the incidence is increased in younger patients (2). Previous studies have focussed on early diagnosis and clinical treatment of colon cancer (3,4), however, the mechanism of action remains unknown. Therefore, investigating the mechanism of colon cancer has important practical significance.

microRNAs (miRNAs) are a class of small non-coding RNAs that are 19-25 nucleotides long (5). They regulate gene expression by binding to their target mRNAs, resulting in the degradation of mRNA or repression of mRNA translation (6). Previous studies have reported that miRNAs are involved in the development of cancer (7) and serve a role in the development of chemotherapy resistance in different tumours (8,9). Therefore, targeting miRNAs may be developed as a novel method of treating cancer and improve the responses of patients to chemotherapy.

miR-15 is a miRNA that has been extensively studied. miR-15 is aberrantly regulated in various types of cancer and is associated with cell proliferation, angiogenesis and metastasis (10). However, the role of miR-15 in cancer remains unclear. Some studies have suggested that miR-15 functions as an oncogene, whereas others regard it as a tumour suppressor (11,12). The effects of miR-15 on chemotherapy are also inconsistent (13,14) and to the best of our knowledge, there have been no studies investigating the potential effect of miR-15 on colon cancer chemotherapy.

In the current study, miR-15 was detected in matching colon cancer and para-carcinoma tissues, the effect of miR-15 on colon cancer chemotherapy was analysed and its associated mechanism of action was investigated. These results may improve understanding of the process of chemotherapy resistance and the search for potential intervention targets.

Materials and methods

Patients. In the present study, miR-15 was detected in 62 paired colon cancer and para-cancerous colon tissues collected from patients who underwent surgical resection to treat colon cancer at Linyi People's Hospital (Shandong, China) between May 2011 and December 2014. The mean range of the enrolled patients was 50.8 \pm 16.4 years old (range, 34-65 years) and the male-female ratio was 42:20. The final diagnosis for each patient was confirmed by two double-blinded pathologists.

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Following resection, the tissues were rapidly frozen and stored in liquid nitrogen. All procedures performed in the present study that involved human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The current study was approved by the Ethics Committee of Linyi People's Hospital (Linyi, China) and informed consent was obtained from all participants.

Cell culture and transfection. Human colorectal carcinoma HCT116 cells (Cell Center of Institute of Basic Medical Science, Chinese Academy of Medical Science, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (Hyclone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

HCT116 cells were plated in 6-well plates (1x10⁵/per well) and the chemosynthetic miR-15 mimics (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were transfected into cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific Inc.) following the manufacturer's protocol. The scrambled sequence was used as negative control (Invitrogen; Thermo Fisher Scientific Inc.). The concentration of miR-15/NC was 50 nmol/l in each well.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from frozen tissues or cells using TRIzol® (Invitrogen; Thermo Fisher Scientific Inc.) and reverse-transcribed into cDNA. In brief, 5x RT buffer (4 μ l), primer (0.1 μ g), 10 mM dNTPs (1 μ l), RNA sample (1 μ g), transcriptase (1 μ l), and RNAase-free water (20 μ l) were mixed. The mixture was incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and then stored at 4°C. All above reagents were supplied by Takara Biotechnology Co. Ltd, (Dalian, China) SYBR Green PCR Mastermix (Takara Biotechnology Co. Ltd) was used to perform qPCR. PCR conditions were as follows: denaturation at 96°C for 3 min, 35 cycles of amplification at 94°C for 10 sec/cycle and then 58°C for 30 sec. miR-15 expression was normalized to that of U6, whereas levels of nuclear factor- κ B (NF- κ B), B cell lymphoma-2 (BCL-2) and B cell lymphoma-extra large (BCL-XL) mRNA were normalized to that of GAPDH. Expression level was analyzed using the 2^{- $\Delta\Delta$ C_q} method (15). The primers (Takara Biotechnology Co. Ltd) for PCR are presented in Tables I and II.

Luminescent cell viability assay. The cells transfected with miR-15/NC (1x10⁴/per well) were seeded into a 96-well plate and treated with 5, 10, 20, 40 and 80 μ g/ml 5-Fluorouracil (5-Fu, Tjkingyork company, Hangzhou, China) or 2.5, 5, 10, 20, 40 μ g/ml Oxaliplatin (OX, Zhejiang Hisun Pharmaceutical Co., Ltd, Zhejiang, China) for 48 h at 37°C. Cellular viability was measured using Cell Titer-Glo™ (Promega Corporation, Madison, WI, USA) following the manufacturer's protocol.

Measurement of apoptosis by flow cytometry. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to

detect the apoptosis of cells transfected with miR-15 or-NC and treated with 5-Fu or OX. The apoptosis kit was obtained from BD Biosciences. Propidium iodide and Annexin V-fluorescein-isothiocyanate stains were used to measure late and early apoptosis, respectively. The stains were incubated for 15 min at room temperature in the dark.

Plasmid construction and dual-luciferase assay. To generate the luciferase reporter with NF- κ B activity, the canonical NF- κ B recognising sequence (GGGRNNYYCCGGGRN NYYCC) was synthesized and inserted into the promoter region of the pGL3 basic vector (Promega Corporation) lined with *Mlu*I and *Xho*I. To measure the interaction between miR-15 and its target mRNAs, the sequence containing predicted target sites within the p50 or p65 mRNAs was synthesized and cloned downstream of the firefly luciferase coding region in the pGL3 control vector (Promega Corporation) lined with *Sac*I and *Hind*III. The plasmids and miR-15 or NC were co-transfected into HCT116 cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific Inc.) following the manufacturer's protocol. At 24 h post-transfection, cells were lysed and luciferase activity was measured using a dual-luciferase reporter test kit (Promega Corporation, Madison, WI, USA). The normalization was completed by comparison with *Renilla* luciferase activity. The primers (Takara Biotechnology Co. Ltd, Dalian, China) were shown in Table III.

Western blot analysis. Protein was extracted from cells transfected with miR-15 or NC using radioimmuno-precipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was determined using the BCA assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (20 μ g/per lane) were loaded onto 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk powder at 37°C for 2 h. Then, membranes were incubated with primary antibodies (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), including mouse anti-human p50 (cat no. sc-53744, 1:200), mouse anti-human BCL-2 (cat no. sc-509, 1:200), rabbit anti-human BCL-XL (cat no. sc-7195, 1:200) and mouse anti-human β -actin (cat no. sc-130300, 1:500) at 4°C overnight and they were subsequently incubated with horse radish peroxide-conjugated bovine anti-mouse/rabbit secondary antibody (Santa Cruz Biotechnology, Inc., 1:2,000; cat no. sc-2371/2370) at 37°C for 2 h. An immunoblot was generated using the ECL western blotting detection system (Thermo Fisher Scientific Inc.).

Statistical analysis. All data were presented as the mean \pm standard deviation. The gene expression level, apoptotic cells percent, NFKB and luminescence activity between two groups were compared using Student's t test. The chemosensitivity was compared by two-way analysis of variance. Multiple comparisons between the groups was completed using the Student-Newman-Keul's method. SPSS software was used to analyse data (version 10.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was regarded as indicating a statistically significant difference.

Table I. Primers for reverse transcription.

Primer names	Primer sequence (5'-3')
U6	AAAATATGGAACGCTTCACGAATTTG
miR-15	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCACAAA
miR-15, microRNA-15.	

Table II. Primers for qPCR.

Primer names	Primer sequence (5'-3')
miR-15	F: TAGCAGCACATAATGGTTTGTG R: GTCGTATCCAGTGCAGGGTCCGAGGT
U6	F: CTCGCTTCGGCAGCACATATACT R: ACGCTTCACGAATTTGCGTGTC
GAPDH	F: ATGGGGAAGGTGAAGGTTCG R: GGGGTCATTGATGGCAACAATA
BCL2	F: GGTGGGGTCATGTGTGTGG R: CGTTTCAGGTACTCAGTCATCC
BCL-XL	F: GAGCTGGTGGTTGACTTTTCTC R: TCCATCTCCGATTCAGTCCCT

Table III. Primers for reporter genes sequence.

Primer names	Primer sequence (5'-3')
pMIR-reporter-P50	F: CCATGTTTGCTGCTGCTGGTGA R: AGCTTCACCAGCAGCAGCAA ACATGGAGCT
pMIR-reporter-P65	F: CCGAGTTTCAGCAGCTGCTGAA R: AGCTTTCAGCAGCTGCTGA AACTCGGAGCT
NF-κB activity reporter	F: CGCGTGGGACTTTCCGGGGAA CCCCC R: TCGAGGGGGTTCCCCGAAAG TCCCA

The primer sequences for pMIR-reporter-P50 and pMIR-reporter-P65 were used to synthesize the DNA fragment containing miR-15b binding sites within P50 and P56 mRNA, respectively. Whereas the primer sequence for NF-κB activity reporter were used to synthesize DNA fragment containing NF-κB binding sites, to detect the activity of NF-κB transcriptional activity. miRNA-15, microRNA-15; F, forward; R, reverse; BCL-2, B cell lymphoma-2; BCL-XL, B cell lymphoma-extra large; NF-κB, nuclear factor-κB.

Results

miR-15 is significantly downregulated in colon cancer. To determine the role of miR-15 in colon cancer chemotherapy, the expression of miR-15 in cancerous and para-cancerous

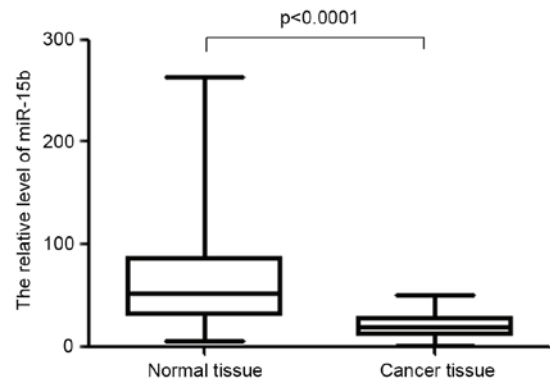


Figure 1. miR-15 expression in colon cancer tissues compared with para-cancerous tissues, as detected by reverse transcription-quantitative polymerase chain reaction. Values are presented as the mean \pm standard deviation. miR-15, microRNA-15.

tissues from 62 patients with colon cancer was measured. The results demonstrated that in the majority of cases (56/62), miR-15 was significantly decreased in cancerous tissue compared with matched para-cancerous tissue ($P < 0.0001$, Fig. 1). The decrease in miR-15 expression in colon cancer tissue may affect the sensitivity of colon cancer tissue to chemotherapy.

miR-15 improves the chemosensitivity of colon cancer cells.

To determine the association between chemotherapeutics and miR-15 expression, HCT116 cells were treated with 5-Fu and OX, two reagents commonly used to treat colon cancer, and the change in expression of miR-15 was assessed (Fig. 2). The results demonstrated that miR-15 expression was significantly upregulated in HCT116 cells treated with 5-Fu and OX ($P < 0.01$; Fig. 2A and $P < 0.05$; Fig. 2C).

The effect of miR-15 on the response of HCT116 cells to 5-Fu and OX was detected using a luminescent cell viability assay. The results indicated that miR-15 significantly increased the inhibitory effect of 5-Fu or OX on HCT116 cell viability (both $P < 0.01$; Fig. 2B and D). The half maximal inhibitory concentrations (IC_{50}) for 5-Fu in NC and miR-15 transfected cells were 289.6 μ M and 93.99 μ M respectively, whereas the IC_{50} for OX in NC and miR-15 transfected cells were 50.27 μ M and 20.07 μ M, respectively. These results suggest that miR-15 increases the sensitivity of colon cancer cells to 5-Fu and OX chemotherapy.

miR-15 promotes 5-Fu- or OX-induced apoptosis in colon cancer cells.

To determine the possible mechanism of miR-15 increasing chemosensitivity, the level of apoptosis induced by 5-Fu or OX in HCT116 cells transfected with miR-15 was measured. The results demonstrated that the rate of apoptosis was significantly increased in miR-15 transfected cells compared with control cells ($P < 0.05$; Fig. 3) following treatment with OX or 5-Fu. This indicates that miR-15 promotes apoptosis induced by chemotherapeutic agents in HCT116 cells.

miR-15 targets NF-κB and inhibits the expression of its target gene. The activation of NF-κB promotes the transcription of several anti-apoptotic factors including BCL-2 and

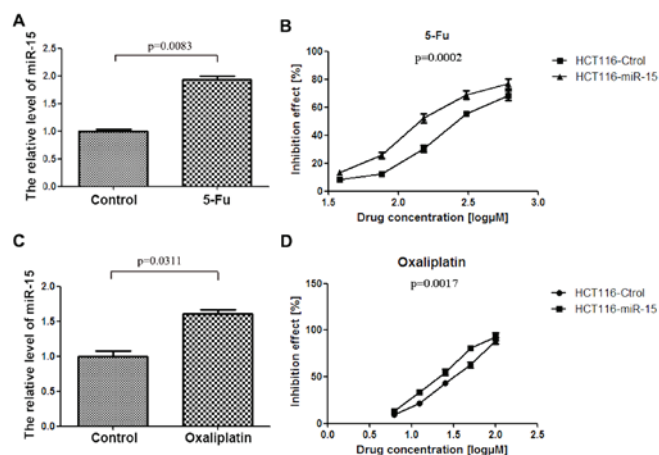


Figure 2. miR-15 affects the chemosensitivity of colon cancer cells to 5-Fu and OX. (A) 5-Fu induced the expression of miR-15 as determined by RT-qPCR. (B) Overexpression of miR-15 increased the chemosensitivity of colon cancer cells to 5-Fu, as determined by the cell viability assay. (C) OX induced the expression of miR-15, as determined by RT-qPCR. (D) Overexpression of miR-15 increased the chemosensitivity of colon cancer cells to OX as determined by the cell viability assay. Values are presented as the mean \pm standard deviation. miR-15, microRNA-15; 5-FU, 5-fluorouracil; OX, oxaliplatin; RT-qPCR, quantitative reverse transcription polymerase chain reaction; Ctrol, control.

BCL-XL (16) and it has been demonstrated that miR-15 inhibits the expression of BCL-2 (17). Thus, the current study investigated whether miR-15 inhibited the activation of NF- κ B in colon cancer cells. It was demonstrated that the transcriptional activity of NF- κ B was significantly decreased in miR-15 transfected cells compared with control cells ($P < 0.01$; Fig. 4A). Following transfection with miR-15, levels of BCL-2 and BCL-XL mRNA, two direct targets of NF- κ B exhibiting anti-apoptotic functions, were also significantly decreased (both $P < 0.01$; Fig. 4B). These results suggest that miR-15 inhibits the activation of NF- κ B in colon cancer. The potential binding sites for miR-15 within p50 and p65, two subunits of the transcription factor NF- κ B, were screened and a potential binding site was identified within the open reading frame of either p50 or p65, which is located between 2,470 and 2,476 nucleotides (nt) and between 1,231 and 1,236 nt in the mRNA of p50 and p65. The results of the dual-luciferase assay demonstrated that miR-15 significantly inhibits the expression of p50 but not of p65 (Fig. 4C). The decrease in the expression of p50 protein following miR-15 transfection was confirmed by western blot analysis (Fig. 4D). Furthermore, the expression of BCL-2 and BCL-XL protein were also decreased in miR-15 transfected cells (Fig. 4D). Taken together, these results indicate that miR-15 increased the chemosensitivity of colon cancer cells to 5-Fu and OX by repressing the NF- κ B signalling pathway and promoting apoptosis.

Discussion

Chemotherapy is an important method of treating cancer. A large number of patients with cancer have benefitted from tumour regression and increased survival following the development of various chemotherapeutic agents (18). However, the development of chemoresistance, in which cancer cells exhibit little or no response to chemotherapy drugs, poses a challenge to the treatment of cancer (19).

Previous studies have identified some of the mechanisms of chemoresistance, which include increased drug efflux (20),

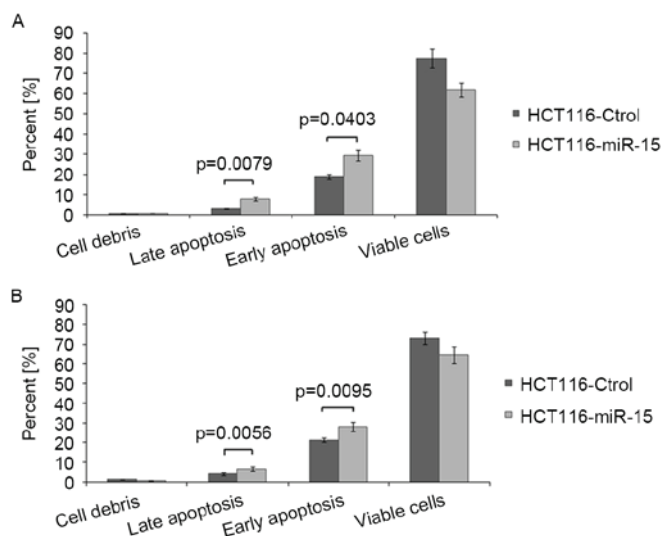


Figure 3. Upregulation of miR-15 promotes the apoptosis of colon cancer cells induced by chemotherapeutics, as determined flow cytometry. (A) Induction by 5-Fu. (B) Induction by OX. Values are presented as the mean \pm standard deviation. miR-15, microRNA-15; 5-FU, 5-fluorouracil and OX, oxaliplatin.

decreased drug activation (21) and increased DNA repair activity (22). **Recently, several studies have indicated the regulatory role of miRNAs in response to chemotherapy (23-25).** For example, miR-214 induces cisplatin resistance in certain types of ovarian cancer by targeting the phosphatase and tensin homolog/phosphoinositide 3-kinase/Akt pathway (26). In addition, high levels of miR-378 may reverse the chemoresistance of lung adenocarcinoma cells to cisplatin by inhibiting the expression of secreted clusterin (27). miRNA is therefore an appealing target to increase effectiveness of various drugs.

It has been demonstrated that miR-15 serves an important role in the tumorigenesis of colorectal cancer (10). However, its expression and function remain unclear. Xi *et al* (28) determined that miR-15 was significantly overexpressed in colorectal cancer tissues and thus may function as an

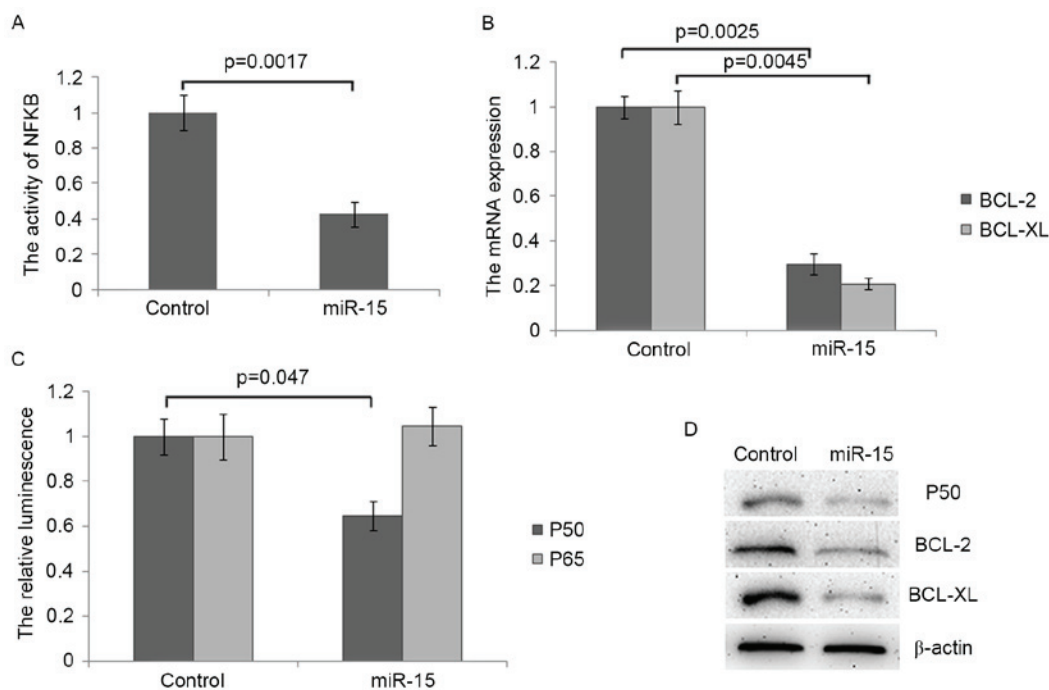


Figure 4. miR-15 inhibits activation of the NF- κ B signalling pathway. (A) miR-15 decreased the transcriptional activity of NF- κ B. (B) Levels of BCL-2 and BCL-XL mRNA were decreased in miR-15 upregulated colon cancer cells as determined by RT-qPCR. (C) miR-15 inhibited the expression of p50 as determined by the dual-luciferase assay. (D) The expression of p50, BCL-2 and BCL-XL was decreased in miR-15 upregulated colon cancer cells as indicated by the results of western blot analysis. Values are presented as the mean \pm standard deviation. miR-15, microRNA-15; NF- κ B, nuclear factor κ B; BCL-2, B cell lymphoma-2; BCL-XL, B cell lymphoma-extra large; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

oncogene. By contrast, Wang *et al* (29) identified that miR-15 was downregulated in colorectal cancer tissues and may act as a tumour suppressor. In the present study, the expression of miR-15 was decreased in the majority of colon cancer tissues compared with para-cancerous tissues, supporting the role of miR-15 as a tumour suppressor in colon cancer.

miR-15 is able to regulate chemoresistance in addition to regulating cell proliferation, apoptosis, angiogenesis and metastasis (30-33). Xia *et al* (34) identified that miR-15 may enhance the sensitivity of gastric cancer cells to anticancer drugs by targeting BCL2 and promoting apoptosis. Reduced levels of miR-15 are also associated with chemotherapeutic resistance in human tongue cancer cells by targeting BMI1 (14). However, it has also been identified that increased expression of miR-15 is associated with decreased sensitivity to cisplatin and the poor prognosis of patients with lung adenocarcinoma by suppressing the expression of phosphatidylethanolamine-binding protein 4 (13). The results of the present study indicate that the upregulation of miR-15 may increase the sensitivity of colon cancer cells to 5-Fu and OX by promoting apoptosis.

To determine the action of miRNA and the role of apoptosis in the development of colon cancer, the target mRNAs of miR-15 associated with apoptosis were detected and analysed. The results demonstrated that NF- κ B was a target of miR-15 and that miR-15 inhibits the activation of NF- κ B by repressing the expression of p50. Previous studies have demonstrated that NF- κ B inhibits apoptosis by transcriptionally activating anti-apoptotic proteins (35,36). This includes BCL-2 and BCL-XL, members of the BCL-2 family of apoptosis regulators (37,38). In the present study, it was demonstrated that the

downregulation of NF- κ B decreased the expression of BCL-2 and BCL-XL, which may contribute to the induction of apoptosis in colon cancer cells by miR-15.

In conclusion, the current study demonstrated that miR-15 was downregulated in colon cancer tissues and may act as a tumour suppressor. Exogenous overexpression of miR-15 may increase the response of colon cancer cells to 5-Fu and OX by inhibiting the NF- κ B/BCL-2/BCL-XL signalling pathway and inducing apoptosis.

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