Int J Clin Exp Pathol 2015;8(6):6397-6406
www.ijcep.com /ISSN:1936-2625/IJCEP0008996

Original Article

miR-218 inhibits multidrug resistance (MDR) of gastric cancer cells by targeting Hedgehog/smoothened

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Received April 11, 2015; Accepted May 27, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: Multidrug resistance (MDR) is the main obstacle to successful chemotherapy for patients with gastric cancer. The microRNA miR-218 influences various pathobiological processes in gastric cancer, and its down-regulation in this disease raises the question of whether it normally inhibits MDR. In this study we observed that two MDR gastric cancer cell lines showed lower expression of miR-218 compared with their chemosensitive parental cell line. Overexpressing miR-218 chemosensitizes gastric cancer cells, slowed efflux of adriamycin, and accelerated drug-induced apoptosis. We identified the *smoothened* (*SMO*) gene as a functional target of miR-218, and found that *SMO* overexpression counteracts the chemosensitizing effects of miR-218. These findings suggest that miR-218 inhibits MDR of gastric cancer cells by down-regulating *SMO* expression.

Keywords: Gastric cancer, hedgehog, hsa-miR-218, multi-drug resistance, SMO

Introduction

Although the incidence of gastric cancer (GC) is decreasing, the prognosis remains poor because of the majority of GC shows distant metastasis at the time of diagnosis. In China, it ranks third in morbidity and accounts for 300,000 deaths per year [1, 2]. Little improvement in long-term survival has been made over the past few decades, partially due to the multidrug resistance (MDR) that frequently renders chemotherapy ineffective [3]. MDR occurs when tumor cells become resistant to multiple chemotherapeutic agents [4]. Several mechanisms have reported account for MDR, including efflux by ATP-binding cassette (ABC) transporters such as P-gp, also known as MDR1 [5-8].

MicroRNAs (miRs) are endogenous, small, non-coding RNAs that inhibit translation or induce mRNA degradation by binding to 3'-untranslated regions (UTRs) with imperfect complementarity [9]. They have been proposed to play an important role in MDR development. Up- or down-regulation of miR-27a, miR-137 and miR-218 can modulate MDR in various types of cancers [10-15]. In diverse cancers, miR-218 is down-regulated, and low expression is associated with clinical aggressiveness of gastric cancer [16]. However, whether miR-218 has an implication in MDR of gastric cancer remains unclear.

To examine this possibility, we overexpressed miR-218 in two MDR gastric cancer cell lines and found that this overexpression substantially increased their sensitivity to various anticancer drugs and promoted cell apoptosis. Using software analysis, we identified, *smoothened* (*SMO*), which encodes a seven-membrane-spanning receptor that is an important component of the Hedgehog (Hh) signaling pathway, as a potential target of miR-218. We then tested the hypothesis that miR-218 acts via Hh/SMO to activate gastric cancer cell apoptosis and inhibit MDR.

Methods

Cell lines and culture

The SGC7901 human gastric adenocarcinoma cell line, maintained in the Surgical Laboratory at Guangzhou Medical University and the MDR variants SGC7901/ADM and SGC7901/L-OHP, established and maintained in our laboratory as described previously [17], were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were grown in 25-mL flasks (Corning, New York, USA) in a humidified incubator at 37°C with 5% CO_2 (Thermo Fisher, MA, USA). To maintain the MDR phenotype, SGC7901/ADM cells were cultured in the presence of 0.4 μg/ml adriamycin (ADM), and SGC7901/L-OHP cells were cultured in 1 μg/ml oxaliplatin (L-OHP).

RNA extraction and quantitative real-time PCR

The miRNA Extraction Kit (Tiangen, Beijing, China) was used to extract miRNAs from SGC7901, SGC7901/ADM and SGC7901/L-OHP. The miRNAs were subjected to stem-loop reverse transcription (RT) using reagents purchased from Applied Biosystems (Foster City, CA, USA), followed by real-time PCR. To measure *SMO* expression, total RNA was extracted from cultured cells using Trizol (Invitrogen) and analyzed by real-time PCR. Quantitative real-time PCR was performed using the following primers: P-gp-forward, 5'-AAGAAGCCCTGGACAAAGCC-3'; P-gp-reverse, 5'-ACAGTCAGAGTTCACTGGCG-3'; GAPDH-forward, 5'-ATGTCGTGGAGTCTACTGGC-3'; and GAPDH-reverse, 5'-TGACCTTGCCCACAGCCTTG-3'. Amplification reactions were performed in triplicate using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) and measured in a LightCycler 480 system (Roche, Basel, Switzerland). GAPDH was used as the endogenous control. The $2^{-\Delta\Delta CT}$ method was used to calculate the fold-change in mRNA expression. All experiments were performed twice.

Transfection

Pre-miR-218 precursor (pre-miR-218) and anti-miRNA-218 inhibitor (anti-miR-218) were purchased from Invitrogen (Carlsbad, California, USA). Pre-miR Precursor Molecules-Negative Control (Invitrogen, AM17110) and anti-miR Inhibitors-Negative Control (Invitrogen, AM-17010) were used as control miRNAs, for pre-miR-218 and anti-miR-218, respectively. These oligonucleotides were then transfected into SGC7901, SGC7901/ADM and SGC7901/L-OHP cells using siPORT NeoFX Transfection Agent (Ambion, Austin, USA) according to the manufacturer's protocol. After 24 h of incubation, RNA and total cellular protein were extracted and subjected to qRT-PCR and Western blot analysis, respectively.

In vitro drug sensitivity assay

The gastric cancer cell line named SGC7901 and the gastric cancer multi-drug cell lines named SGC7901/ADM and SGC7901/L-OHP were seeded into 96-well plates (6×10^3 cells/per well) and maintained overnight. ADM, L-OHP, and 5-fluorouracil (5-Fu) were freshly prepared before experiments. Sensitivity of the gastric cancer cells to anticancer drugs was evaluated using a colony-forming assay and the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance of each well at 490 nm (A490) was read on a spectrophotometer. The concentration of each drug at 50% growth inhibition (IC_{50}) was estimated using relative survival curves. Three independent experiments were performed in triplicate.

Cell apoptosis assay

At 24 h after transfection with pre-miR-218, anti-miR-218 or the corresponding negative controls, SGC7901 cultures were incubated for 48 h with ADM, L-OHP or 5-Fu. Cells were then harvested and analyzed for apoptosis using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, NY, USA). Cells (1×10^6) were stained according to the manufacturer's protocol and sorted using a FACS sorter (BD Biosciences, La Jolla, CA, USA). Data were analyzed using ModFit (BD Biosciences). Experiments were performed in triplicate.

Analysis of intracellular ADM and L-OHP concentrations

Cells were seeded into 6-well plates (1×10^6 cells/per well) and cultured overnight at 37°C. ADM (5 μg/ml) was added to cultures and cells were further incubated for 1 h. Cells were then either collected to detect ADM accumulation or maintained in drug-free medium for another 3 h and analyzed for ADM retention. The fluores-

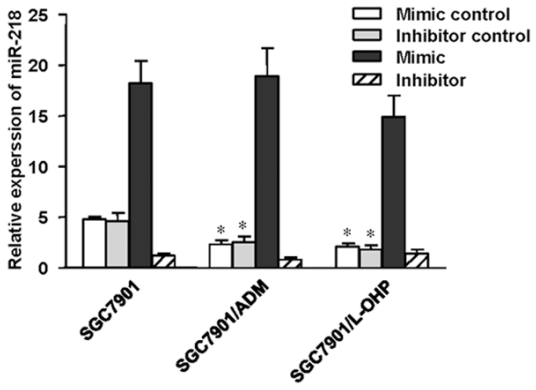

Figure 1. miR-218 is down-regulated in MDR gastric cancer cell lines. Levels of miR-218 are lower in two MDR gastric cancer cells than in the chemosensitive parental cell line (SGC7901vesus SGC7901/ADM or SGC7901/L-OHP; *, $P < 0.05$). Tansfection of mimics increases miR-218 levels, while inhibitor decreases miR-218 levels. Results shown are from three independent experiments.

cence intensity of intracellular ADM was measured using fluorescence confocal microscopy at an excitation wavelength of 488 nm and emission wavelength of 575 nm [18]. ADM and L-OHP release indices were calculated according to the following formula: release index = (accumulation value-retention value)/accumulation value. Experiments were performed in triplicate.

Construction of SMO expression plasmids and luciferase assay

The human SMO 3'-UTR sequences predicted to interact with miR-218 were amplified using the following primers: 5'-CCGCTCGAGGCCTGCAGAGCAGGACCTGGG-3' (forward) and 5'-ATAAGAATGCGGCCGCATACAAAAACCTTTTATTGACTGTATTTCTTCTC-3' (reverse). Mutated 3'-UTR sequences predicted to lack the miR-218 binding site were amplified using the primers 5'-CAGCAGGAAGCCACTGGGTTCCAGGTTATG-3' (forward) and 5'-CATAACCTGGAGAGCGCTATGGCTTCCTGCTG-3' (reverse). PCR products were cloned into the psi-CHECK2 vector (Promega, Beijing, China), amplified, and confirmed by sequencing. These vectors were then named psi-CHECK2-SMO and psi-CHECK2-mut SMO. For the luciferase assay, SGC7901 cells were cultured in 24-well plates and transfected with 0.5 μg of either psi-CHECK2-SMO vector or psi-CHECK2-mut SMO vector together with 50 nM miR-218 mimic or pre-miR Precursor Molecules-Negative Control using siPORTTM NeoFX Transfection Agent. At 40 h after transfection, cells were harvested and analyzed using a Dual-Luciferase Reporter Assay System Kit (Promega, Beijing, China). Experiments were performed in triplicate and repeated twice.

Protein extraction and Western blotting

SGC7901/ADM and SGC7901/L-OHP were seeded in 6-well plates (5×10^5 cells/well), cultured in DMEM overnight, and then transiently transfected with miR-218 mimic, SMO siRNA, or control oligonucleotide (5'-TGCTGCTGCTTG:CAAGCAGCTTGAT-3'). Two days after transfection, total cellular protein was extracted using a cell lysis buffer containing 150 mM NaCl, 10 mM Tris at pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA. Protein concentration was measured with a Bio-Rad protein assay kit (BioRad, Hercules, CA, USA). A total of 30 μg of each protein sample was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). Next the membrane was incubated in 5% non-fat dry milk in Tris-buffered saline at room temperature for 1 h to block non-specific binding. The membrane was probed with primary monoclonal antibodies against Bcl-2 (1:200; Santa Cruz Biotechnology, CA, USA), Bax (1:100; Santa Cruz), P-gp (1:200; Santa Cruz), and SMO (1:500, Cell Signaling Technology, Boston, USA). Antibody against GAPDH (1:1000; Sigma, St. Louis, USA) was used as an internal control for protein loading. After washing with Tris-buffered saline Tween-20 (TBST) for 10 min, bound proteins in the membrane were detected using an ECL chemiluminescent detection system (Pierce, Rockford, IL, USA) followed by exposure to X-ray film. Blots were scanned and band density was quantitated using the Gene Gnome Western blot imaging system and GeneSpring software (Agilent, Palo Alto, CA, USA).

Data analysis

Data were presented as mean ± SD and analyzed using SPSS 13.0 (IBM, Chicago, USA). Differences/correlations between groups were calculated using Student's *t* test, the chi-squared test, or Pearson's correlation. A *P*

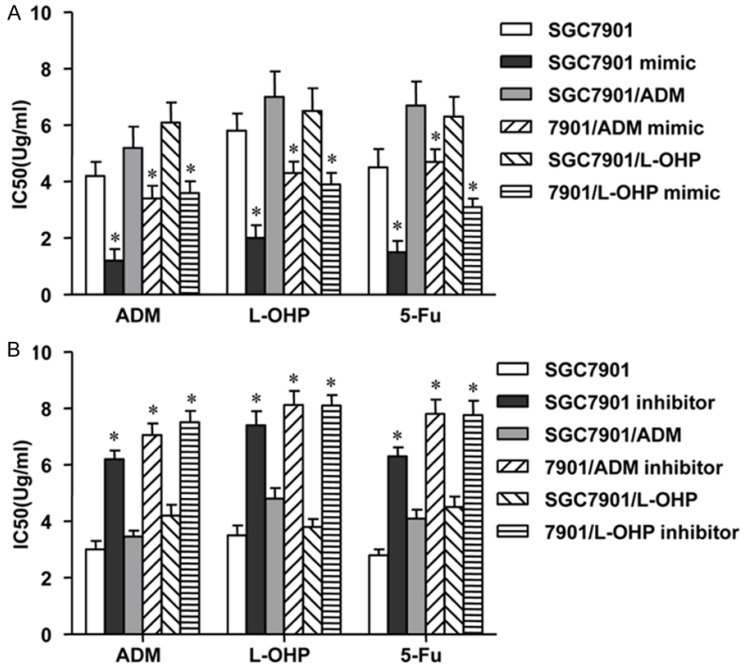

Figure 2. Expression of miR-218 increases sensitivity of gastric cancer cells to anticancer drugs (ADM, L-OHP, 5-Fu). A. IC_{50} values of SGC7901, SGC7901/ADM and SGC7901/L-OHP cells following transfection with miR-218 mimic or with negative control. B. IC_{50} values of SGC7901, SGC7901/ADM and SGC7901/L-OHP cells following transfection with anti-miR-218 inhibitor or without transfection. $*P < 0.05$, compared with the negative control group.

value less than 0.05 was considered statistically significant.

Results

miR-218 is down-regulated in MDR gastric cancer cell lines

To determine whether miR-218 is involved in MDR development in gastric cancer cells, its expression levels were determined by qRT-PCR in the MDR lines SGC7901/ADM and SGC7901/L-OHP as well as in the chemosensitive parental cell line SGC7901. Expression of miR-218 was down-regulated in the two MDR lines compared to the parental line (**Figure 1**). These results imply an association between miR-218 underexpression and MDR in gastric cancer cells. To verify that our cell culture system is available for us to examine the effects of changing miR-218 expression, we showed that transfecting SGC7901 cells with pre-miR-218 significantly increased miR-218 levels, whereas transfecting the cells with anti-miRNA-218 significantly reduced it (**Figure 1**).

miR-218 increases sensitivity of gastric cancer cells to anticancer drugs

To investigate whether miR-218 can affect existing MDR in gastric cancer, we transfected SGC7901, SGC7901/ADM and SGC7901/L-OHP cells with miR-218 mimic or inhibitor. After transfection with miR-218 mimic, all three cell lines showed greatly enhanced sensitivity to ADM, L-OHP, and 5-Fu as demonstrated by MTT assay, reflected in significantly lower IC_{50} values than cells transfected with negative control **Figure 2A**). Conversely, after transfection with anti-miR-218, all three cell lines showed decreased sensitivity to ADM, L-OHP, and 5-Fu (**Figure 2B**). These results indicate that miR-218 expression is closely related to the sensitivity of gastric cancer cells to chemotherapeutic drugs.

miR-218 inhibits efflux of ADM by down-regulating P-gp

To determine the role of miR-218 in drug efflux from gastric cancer cells, we examined the ADM release index in SGC7901 cells transfected with miR-218 mimic or negative control. ADM efflux was significantly decreased after transfection with miR-218 mimic compared with the negative control (**Figure 3A**). Consistent with the ADM release index, SGC7901 cells transfected with miR-218 mimic resulted in reduced mRNA and protein levels of P-gp in relative to the negative control (**Figure 3B**).

To further investigate whether miR-218 may inhibit MDR in gastric cancer cells by decreasing P-gp levels and thereby inhibiting drug efflux, we transfected SGC7901/ADM and SGC7901/L-OHP cultures with miR-218 mimic or anti-miR-218 and incubated them in the presence or absence of the P-gp inhibitor verapamil. Transfection with miR-218 mimic led to lower ADM and L-OHP release index and this effect was enhanced by verapamil treatment (**Figure 3C**, **3D**). Conversely, transfection with miR-218 inhibitor led to significantly higher ADM and L-OHP release index and this effect

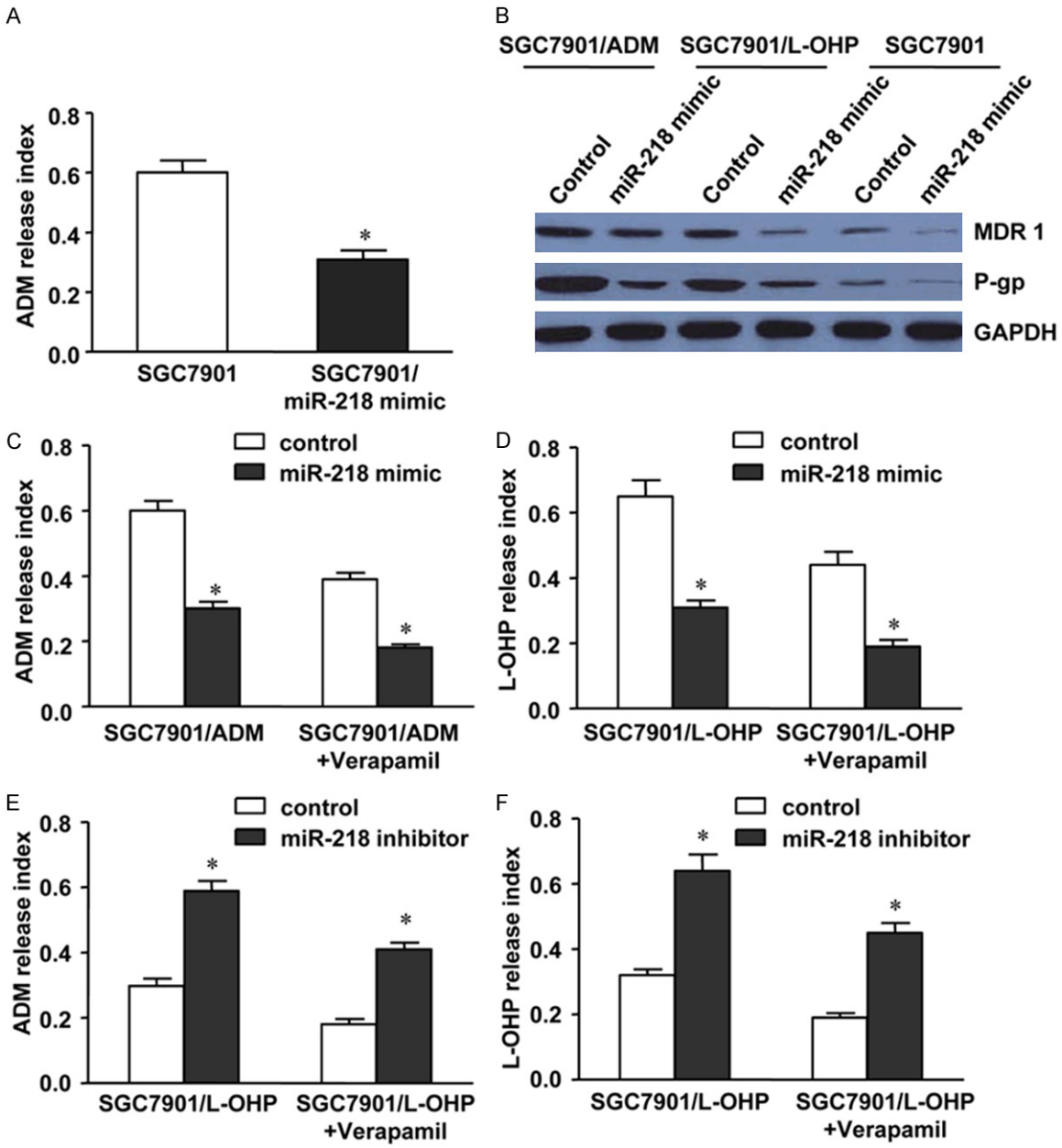

Figure 3. Down-regulation of P-gp by miR-218 inhibits efflux of ADM. (A) The ADM release index was measured in SGC7901 cells transfected with miR-218 mimic and incubated with 5 μg/ml ADM for 24 h. (B) Expression of P-gp was measured by qRT-PCR in SGC7901, SGC7901/ADM and SGC7901/L-OHP cells at 48 h after transfection with miR-218 mimic. At the same time, P-gp protein expression was measured by Western blot. SGC7901/ADM and SGC7901/L-OHP cells were transfected with miR-218 mimic and then cultured for 24 h in the presence or absence of verapamil (2.5 μg/ml) as well as 5 μg/ml ADM or L-OHP. Release index was calculated for (C) L-OHP and (D) ADM. SGC7901/ADM and SGC7901/L-OHP cells were transfected with anti-miR-218 inhibitor, and release indices were calculated for (E) L-OHP and (F) ADM. Results are shown from three independent experiments. *$P < 0.05$, compared with the negative control group.

was partially blocked by verapamil treatment (**Figure 3E**, **3F**). These results suggest that miR-218 may inhibit efflux of ADM and L-OHP by down-regulating P-gp expression.

miR-218 accelerates drug-induced apoptosis

Among the mechanisms responsible for MDR, the suppression of drug-induced apoptosis is

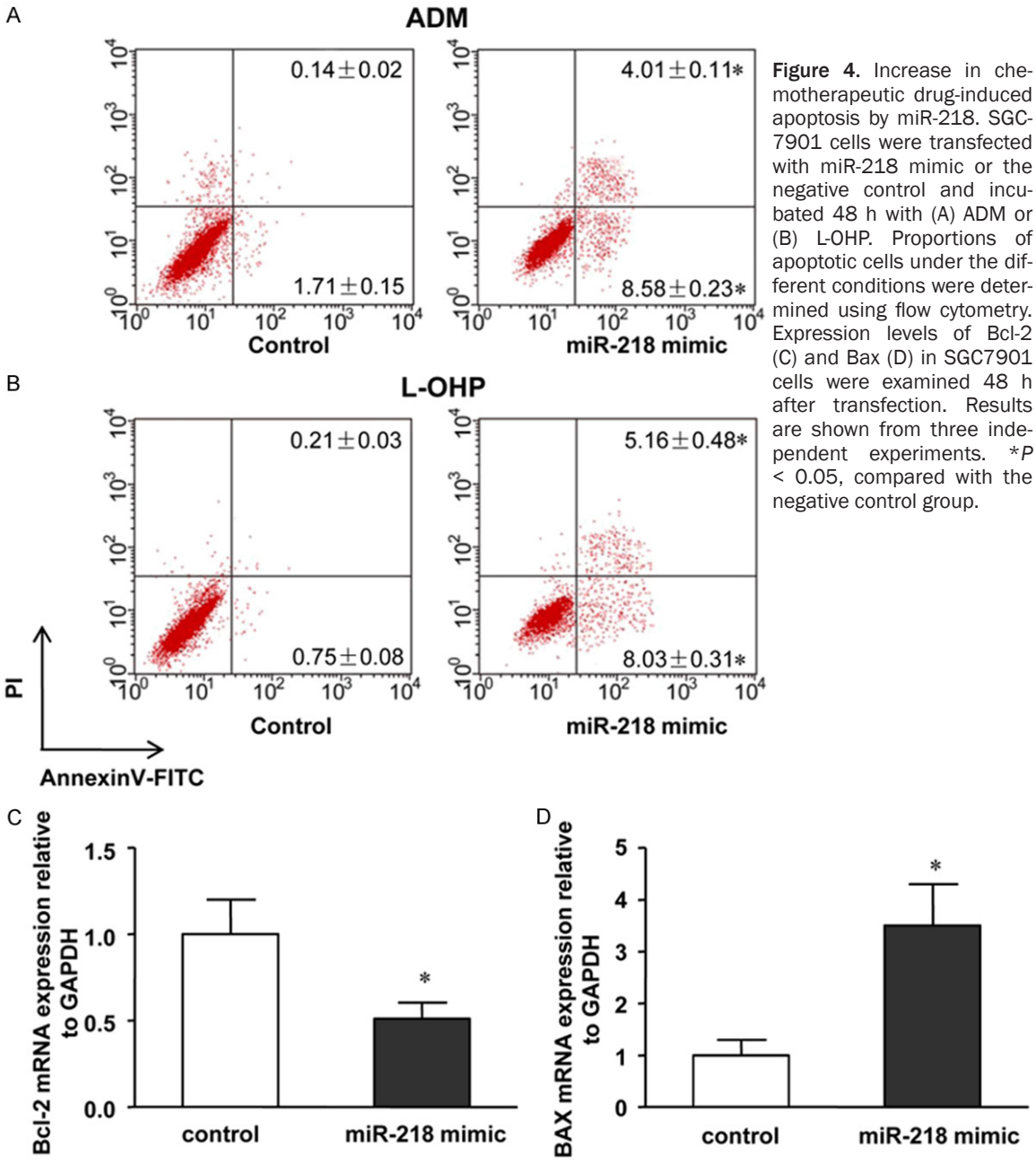

Figure 4. Increase in chemotherapeutic drug-induced apoptosis by miR-218. SGC-7901 cells were transfected with miR-218 mimic or the negative control and incubated 48 h with (A) ADM or (B) L-OHP. Proportions of apoptotic cells under the different conditions were determined using flow cytometry. Expression levels of Bcl-2 (C) and Bax (D) in SGC7901 cells were examined 48 h after transfection. Results are shown from three independent experiments. *$P < 0.05$, compared with the negative control group.

particularly important. Therefore we investigated the effects of miR-218 on drug-induced apoptosis in gastric cancer cells. After transfection with miR-218 mimic or negative control, cultures of SGC7901 cells were incubated with ADM or L-OHP, and apoptosis levels were measured by flow cytometry. The proportion of apoptotic cells was significantly higher in cultures transfected with mimic than in cultures transfected with negative control, for both ADM (**Figure 4A**) and L-OHP treatment (**Figure 4B**). Moreover, cells transfected with mimic showed lower levels of Bcl-2 (**Figure 4C**) and higher levels of Bax than cells transfected with negative control (**Figure 4D**). These results suggest that miR-218 may sensitize SGC7901 cells to drug-induced apoptosis.

miR-218 modulates MDR by inhibiting SMO expression

Using MirSNP [19] and TargetScan Human 6.2 [20], we determined the *SMO* mRNA is a potential target of miR-218, and its binding site in the

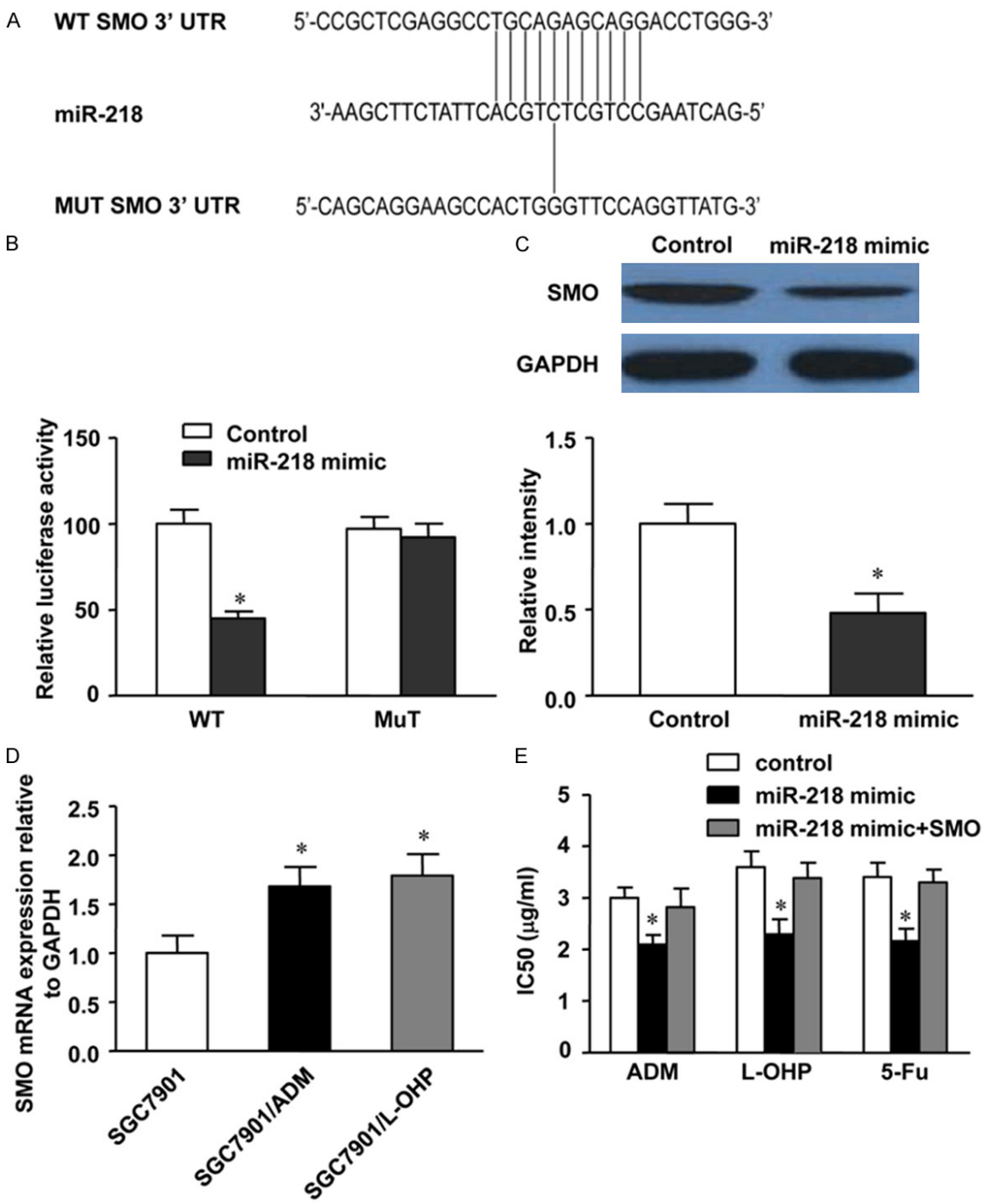

Figure 5. Down-regulation of SMO by miR-218 inhibits MDR. A. The predicted miR-218 target site in the wild-type SMO 3' UTR (WT) and the corresponding mutated 3' UTR sequence (Mut) are shown. B. SGC7901 cells were co-transfected for 48 h with luciferase construct and the miR-218 mimic or the negative control. Luciferase activity was measured. C. SGC7901 cells were transfected with miR-218 mimic, and SMO expression was analyzed at 48 h after transfection. D. SMO expression was measured in SGC7901, SGC7901/ADM and SGC7901/L-OHP cells. E. SGC7901, SGC7901/ADM and SGC7901/L-OHP cells were cotransfected with SMO and miR-218 mimic, then incubated for 72 h with ADM, L-OHP or 5-Fu. Cell viability was assessed using the MTT assay, and IC_{50} values were calculated. Results are shown from three independent experiments. *$P < 0.05$, compared with the negative control group.

3'-UTR was predicted (**Figure 5A**). We then constructed two luciferase reporter constructs to examine whether miR-218 targets the *SMO* transcript in both constructs. The luciferase

open reading frame was fused to the wild-type (WT) or mutated (Mut) 3'-UTR of SMO. Each of these constructs was cotransfected into SGC7901 cells with miR-218 mimic or the negative control. Luciferase activity was significantly decreased after miR-218 mimic treatment in cells cotransfected with WT UTR but not Mut UTR (**Figure 5B**). Consistent with the luciferase activity assays, Western blotting showed that levels of SMO protein were significantly lower in cells cotransfected with miR-218 mimic than in cells cotransfected with negative control (**Figure 5C**).

To examine more directly whether down-regulating SMO mediates the chemosensitizing effects of miR-218 in gastric cancer, we examined SMO expression levels in the MDR lines SGC7901/ADM and SGC7901/L-OHP, as well as in the chemosensitive parental line SGC7901. Expectedly, SMO levels were significantly higher in the two MDR lines (**Figure 5D**). Overexpressing SMO counteracted the chemosensitizing effect of miR-218 (**Figure 5E**). These results strongly suggest that miR-218 inhibits MDR in gastric cancer cells by down-regulating SMO expression.

Discussion

MDR is one of the most frequent causes of treatment failure in gastric cancer chemotherapy, and several studies have implicated the roles of miRNAs in MDR development in gastric cancer [5-8, 21-27]. In the present study, through comparing MDR gastric cancer cell lines with their chemosensitive parental line, our findings suggest that miR-218 inhibits MDR by down-regulating SMO expression.

The MDR cell lines SGC7901/ADM and SGC7901/L-OHP are widely employed as *in vitro* models for studying MDR in gastric cancer. We found that miR-218 levels are lower in these two MDR cell lines than in the chemosensitive parental line. This suggests that miR-218 levels correlate directly with sensitivity of gastric cancer cells to chemotherapeutic drugs including ADM, L-OHP and 5-Fu.

One of the primary mechanisms of drug resistance is ATP-dependent efflux by ABC transporters, which efficiently ship cytotoxic drugs out of the cell [8, 21, 23]. P-gp is an important member of the ABC transporter family and has been extensively studied in numerous tumor types. Inhibition of P-gp expression has been shown to reverse MDR [24, 25]. Our results showed that miR-218 transfection efficiently inhibits P-gp expression, rendering cells sensitive to ADM and L-OHP. We observed a similar chemosensitizing effect when we inhibited P-gp directly using verapamil. These findings suggest that miR-218 chemosensitizes gastric cancer cells by down-regulating P-gp, thereby reducing drug efflux.

Another mechanism of MDR is avoiding the apoptosis typically induced by chemotherapeutic drugs. We found that treatment with miR-218 mimics increased levels of drug-induced apoptosis in MDR and parental gastric cancer cells of SGC7901. Consistent with this pro-apoptotic effect, we also showed that higher miR-218 levels increased the level of Bax and reduced the level of Bcl-2. These results suggest that miR-218 may reverse MDR in gastric cancer cells in part by increasing their susceptibility to drug-induced apoptosis.

We then identified SMO, a fundamental component of the Hh signaling pathway and an important anti-cancer drug target, as a candidate target of miR-218 [26-28]. Upon activation, SMO triggers a series of intracellular events that end in activation of zinc-finger transcription factors, which induce the expression of target genes that regulate proliferation, adhesion, and apoptosis [29-32]. Increased expression of SMO has been observed in a variety of cancers including breast cancer, prostate cancer, and pancreatic cancer [33]. In addition, point mutations in the SMO gene have been observed in certain type of human cancers [34]. These observations suggest that SMO may function as an oncogene in several human cancers. Guo et al. reported that SMO acts as a tumor activator and renders pancreatic cancer cells resistant to chemotherapeutic drugs by promoting expression of Bcl-2 and Cyclin D2 [35]. It makes sense, then, that miR-218 modulates MDR in gastric cancer cells at least in part by down-regulating SMO in current study.

In summary, we have shown that miR-218 levels are lower in MDR gastric cancer cells than in chemosensitive parental cells. Treatment with miR-218 mimic increases the sensitivity of gastric cancer cells to chemotherapeutic drugs by simultaneously inhibiting drug efflux and accel-

erating apoptosis. SMO is a functional target of miR-218 in gastric cancer cells, and overexpressing SMO counteracts the chemosensitizing effects of the miR-218. These findings suggest that miR-218 may modulate MDR by downregulating SMO. This work identifies a new mechanism underlying MDR and suggests the miR-218/SMO axis may act as a therapeutic strategy to treat gastric cancer.

Acknowledgements

This work was supported by Guangdong Province Natural Science Foundation (S20130-10016662), Health Bureau of Guangdong Province (A2014224 and B2014196), Science and Technology Planning Project of Guangdong Province (2013B021800284) and the National Natural Science Foundation of China (8120-1932 and 81372493).

Disclosure of conflict of interest

None.

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