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MicroRNA-19a/b regulates multidrug resistance in human gastric cancer cells by targeting PTEN

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ABSTRACT

Multidrug resistance (MDR) is the major cause of failure of gastric cancer chemotherapy. Members of the miR-17-92 cluster, including miR-19a/b, are considered oncomiRs and influence multiple aspects of the malignant phenotype of gastric cancer. However, the role of miR-19a/b in MDR in gastric cancer and its underlying mechanism remain unclear. In this study, we found that miR-19a/b were upregulated in MDR cell lines. Our results also showed that miR-19a/b upregulation decreased the sensitivity of gastric cancer cells to anticancer drugs. We further confirmed that miR-19a/b accelerated the ADR efflux of gastric cancer cells by increasing the levels of mdr1 and P-gp and that miR-19a/b suppressed drug-induced apoptosis by regulating Bcl-2 and Bax. Finally, we verified that PTEN, an inhibitor of AKT phosphorylation, is the functional target of miR-19a/b. Overall, these findings demonstrated that miR-19a/b promote MDR in gastric cancer cells by targeting PTEN.

1. Introduction

Gastric cancer is the second most common cause of death from cancer worldwide, and there has been little improvement in long-term survival over the past decade [1]. Chemotherapy is the most frequently used primary treatment for gastric cancer. However, the main barrier to the success of chemotherapy is multidrug resistance (MDR), which is caused by the development of resistance to multiple chemotherapeutic agents after exposure to a single chemotherapeutic drug [2]. Extensive investigations have indicated that the cellular basis of MDR involves changes in drug uptake, efflux and metabolism, DNA synthesis and repair, cell survival, apoptosis and other cell functions [2–4]. More recently, other factors, including non-coding RNA expression, have been proposed to play an important role in the development of MDR [5].

MicroRNAs (miRNAs) are small non-coding RNAs that bind to the 3′ UTRs of their target mRNAs, resulting in translational inhibition or mRNA degradation [6]. miRNAs are considered to play pivotal roles in the regulation of various biological mechanisms such as the cell cycle, apoptosis and migration, among others. Recent studies have indicated that the drug-induced dysregulation of miRNA function may modulate MDR in cancer cells [7,8]. More interestingly, several studies have shown that the knockdown or re-expression of miRNAs could modulate drug resistance in cancer cells [9,10]. For instance, miR-21 was found to be increased in MDR cancer cells, and the downregulation of miR-21 via transfection with anti-miR-21 oligonucleotides sensitised cells to apoptosis signals [9]. Our previous study also showed that the overexpression of miR-15b or miR-16 sensitised SGC7901/VCR cells to anticancer drugs by targeting the anti-apoptotic gene BCL2 [10].

miR-19 belongs to the miR-17-92 cluster, and genes in this cluster have been validated as widely overexpressed in diverse tumour subtypes [11]. Thus, miR-17-92 cluster miRNAs, including miR-19a/b, are considered oncomiRs. A number of studies have shown that certain tumour suppressors, such as p21 [12] and BIM [11], are direct targets of multiple members of the miR-17-92 cluster. However, the role of miR-19a/b in the MDR development process in gastric cancer and its underlying mechanism remain unclear. Based on our previous results indicating that miR-19a/b is upregulated in MDR gastric cancer cell lines, we hypothesised that miR-19a/b may promote MDR in gastric cancer.

In this study, we report that miR-19 was upregulated in two MDR human gastric cancer cell lines. We also found that the introduction of miR-19a/b could decrease GC cell sensitivity to

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anticancer drugs. More importantly, we found that miR-19a/b promoted MDR in GC cells by accelerating the efflux of chemotherapeutic drugs and inhibiting drug-induced apoptosis. Finally, we demonstrated that miR-19 may play a role in the development of MDR in human gastric cancer cells by targeting PTEN.

2. Materials and methods

2.1. Cell lines and culture

Human gastric adenocarcinoma cell line SGC7901 (obtained from Academy of Military Medical Science, Beijing, China) and its multidrug-resistant variants SGC7901/VCR and SGC7901/ADR (established and maintained in our laboratory) were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37 °C. To maintain the MDR phenotype, vincristine (VCR, final concentration 1 μg/ml) and adriamycin (ADR, final concentration 0.4 μg/ml) were added to the culture media of SGC7901/VCR and SGC7901/ADR cells, respectively.

2.2. Quantitative RT-PCR for miRNA

Total RNA was extracted from the cultured cells using Trizol (Invitrogen), and the concentration of the total RNA was quantified by measuring the absorbance at 260 nm. The expression of mature miRNAs was assayed using stem-loop RT followed by real-time PCR analysis [13]. All reagents for the stem-loop RT were obtained from Applied Biosystems (Foster City, CA). The PCR primers used were as follows: PTEN-forward 5′- CCCAGTCAGAGGCGCTATGTGTAT-3′, PTEN-reverse 5′-GTTCCGCCACTGAACATTGG -3′; mdr1-forward 5′-AAGAAGCCCTGGACAAAGCC-3′, mdr1-reverse 5′-ACAGTCAGAGTTCACTGGCG-3′; GAPDH-forward 5′-ATGTCGTGGAGTCTACTGGC-3′, and GAPDH-reverse 5′-TGACCTTGCCCACAGCCTTG-3′. PCR was performed in triplicate. Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa) and measured in a LightCycler 480 system (Roche, Basel, Switzerland). U6 or GAPDH was used as the internal control. The $2^{-\triangle\triangle CT}$ method was used to calculate the fold-change of the RNA expression of one sample compared to the calibration sample [14].

2.3. Oligonucleotide transfection

The miR-19a/b mimics or inhibitor and corresponding negative control were designed and synthesised by RiboBio (Guangzhou, China). Target cells were transfected with miR-19a/b mimics, inhibitor or the corresponding negative controls at a final concentration of 100 μM (mimics) or 200 μM (inhibitor) using DharmaFect Transfection reagent (Thermo-Fisher) according to the manufacturer's protocol. Cells were collected 24 h after transfection.

2.4. In vitro drug sensitivity assay

The 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 as described previously [15,16]. After 72 h, cell viability was assessed using the MTT assay. The absorbance of each well at 490 nm (A490) was read on a spectrophotometer. The concentration at which each drug produced 50% inhibition of growth (IC50) was estimated by the relative survival curve. Four independent experiments were performed in quadruplicate.

2.5. Cell apoptosis assays

At 24 h after miR-19a/b mimic transfection, cells were incubated with anti-cancer drugs including 5-FU and CDDP for 48 h. Then, the cells were harvested, and the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was used for apoptosis assays. Cells (1×10^6) were stained according to the manufacturer's protocol and sorted using a FACS sorter (BD Biosciences, La Jolla, CA, USA), and the data were analysed using ModFit (BD Biosciences).

2.6. Fluorescence intensity assay of intracellular ADR

The fluorescence intensity of intracellular ADR was determined by FCM as described previously [17]. The ADR release index of the gastric cancer cells was calculated according to the following formula: release index = (accumulation value–retention value)/accumulation value.

2.7. Western blot analysis

Cells were transfected with 200 pmol of miR-19a or miR-19b mimics, inhibitor or negative control. Cells were harvested at 72 h post-transfection and homogenised in lysis buffer. The membrane was probed with primary monoclonal antibodies specific to Bcl-2 (1:200, Santa Cruz Biotechnology, CA), Bax (1:100, ZSGB), Caspase-3 (1:200, ZSGB), P-gp (1:200, Santa Cruz Biotechnology), PTEN (1:500, Cell Signaling Technology), AKT (1:500, Signalway Antibody) or p-AKT (1:500, Santa Cruz Biotechnology); β-actin (1:1000, Sigma, St. Louis, MO) was used as an internal control for protein loading. The bands were scanned using the Chemi-DocXRS + Imaging System (Bio-Rad) and quantified using Quantity One v4.6.2 software (Bio-Rad).

2.8. Statistical analysis

Each experiment was repeated at least 3 times. Numerical data are presented as the mean ± SEM. The difference between means was analysed with Student's t test. All statistical analyses were performed using SPSS 14.0 software (Chicago, IL). Differences were considered significant when $p < 0.05$ (∗) or $p < 0.01$ (∗∗).

3. Results

3.1. miR-19a/b are upregulated in multidrug-resistant gastric cancer cell line

To determine whether miR-19a/b are involved in the development of MDR in gastric cancer cells, we performed quantitative real-time PCR (qRT-PCR) in the multidrug-resistant gastric cancer cell lines SGC7901/ADR and SGC7901/VCR and their parental cell line SGC7901. We found that miR-19a/b were upregulated in SGC7901/ADR and SGC7901/VCR cells compared to SGC7901 cells (Fig. 1A). miR-19a/b have also been proven to promote MDR to chemotherapeutic agents in breast cancer cells by repressing PTEN. Our results demonstrated that miR-19a/b may be involved in the multidrug-resistance of gastric cancer cells.

3.2. miR-19a/b suppress the sensitivity of gastric cancer cells to anticancer drugs

To investigate whether miR-19a/b have a direct function in MDR development or are simply differentially modulated in MDR gastric cancer cells, we studied SGC7901 or SGC7901/VCR cells transfected with a specific miR-19a/b mimic or inhibitor, which express higher or lower levels of miR-19a/b respectively (Fig. 1B and

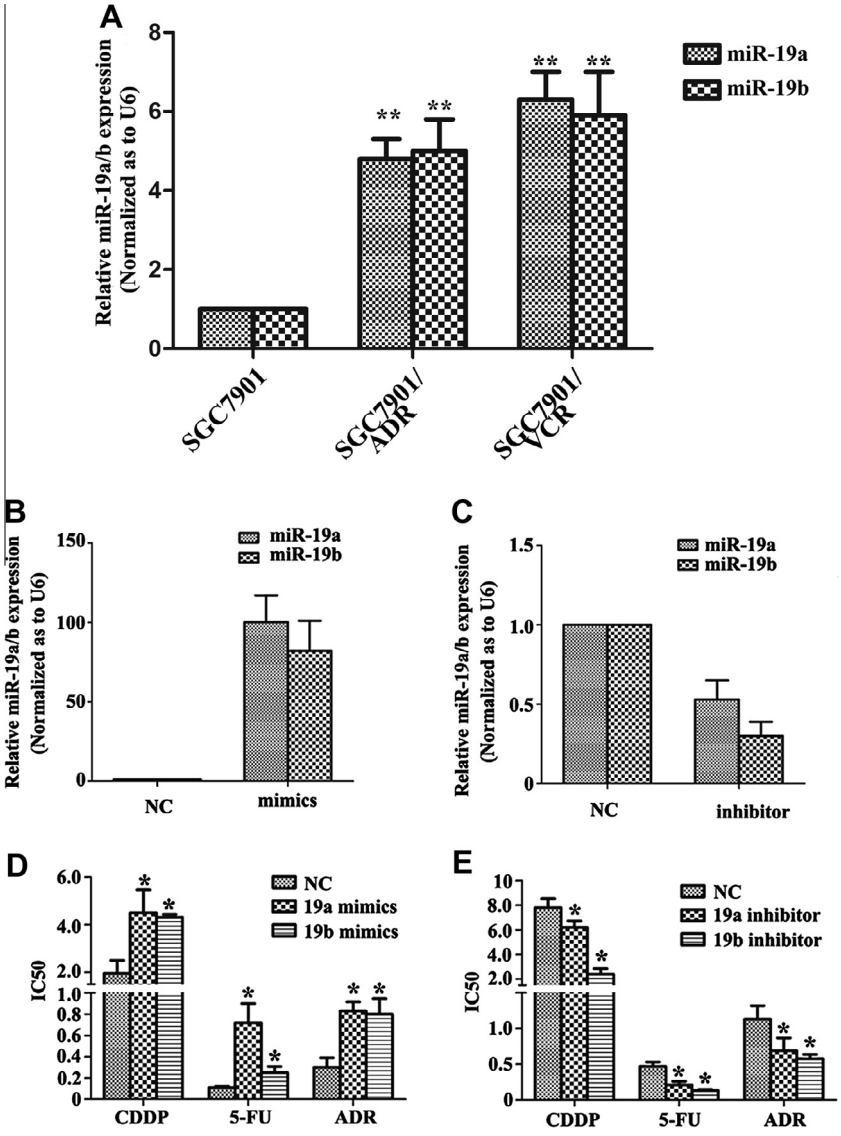

Fig. 1. Expression and function of miR-19a/b in gastric cancer cells. (A) The expression levels of miR-19a/b were examined by qRT-PCR. U6 small nuclear RNA was used as an internal control. (B) SGC7901 cells were transfected with miR-19a/b mimics at a final concentration of 100 nM. miR-19a/b expression was detected by qRT-PCR at 48 h post-transfection. U6 was used as an internal control. (C) SGC7901/VCR cells were transfected with miR-19a/b inhibitor at a final concentration of 200 nM. miR-19a/b expression was detected by qRT-PCR at 48 h post-transfection. U6 was used as an internal control. (D and E) Cells were transfected as in (B and C) and incubated with 3 anticancer drugs for 72 h; then, cell viability was assessed using the MTT assay, and the IC50 value of each drug was calculated. The data are shown as the mean ± SEM ($n = 4$) of one representative experiment. Similar results were obtained in three independent experiments.

C). The MTT assay results revealed that SGC7901 cells transfected with the miR-19a/b mimic exhibited greatly decreased sensitivity to CDDP, 5-FU and ADR, as indicated by the significantly increased IC50 values (Fig. 2D). On the other hand, the suppression of the miR-19a/b level in SGC7901/VCR cells led to an enhanced sensitivity to CDDP, 5-FU and ADR (Fig. 2E). Similar results were observed in a colony-forming assay (data not shown). The above data indicate that the modulation of miR-19a/b expression altered the sensitivity of gastric cancer cells to anticancer drugs.

3.3. miR-19a/b accelerate the efflux of ADR through P-gp upregulation

To confirm whether miR-19a/b play a role in drug efflux in gastric cancer cells, we detected the ADR release index in SGC7901 cells transfected with miR-19a/b mimics. The efflux of ADR was clearly enhanced (Fig. 2A). Consistent with the above data, RT-PCR demonstrated increased mdr1 levels in miR-19a/b-transfected SGC7901 cells (Fig. 2B). The overexpression of P-gp in SGC7901 cells transfected with miR-19a/b was validated by western blot (Fig. 2C). To further investigate whether miR-19a/b promotes MDR in gastric cancer cells partly through increasing P-gp levels and accelerating the drug efflux, we treated transfected cells with the P-gp inhibitor verapamil. The ADR release results showed that the index increased in untreated miR-19a/b mimic-transfected SGC7901 cells but not in the same cells incubated with verapamil (Fig. 2D). In contrast, the ADR release index decreased significantly in SGC7901/ADR cells transfected with the miR-19a/b inhibitor compared with cells transfected with the control inhibitor (Fig. 2E). This indicated that P-gp upregulation may play an important role in the miR-19a/b-induced acceleration of ADR efflux.

3.4. miR-19a/b reduced susceptibility to drug-induced apoptosis

Among the mechanisms responsible for MDR in various cancer cells, the repression of drug-induced apoptosis is particularly important. To investigate whether miR-19a/b affect the drug-in-

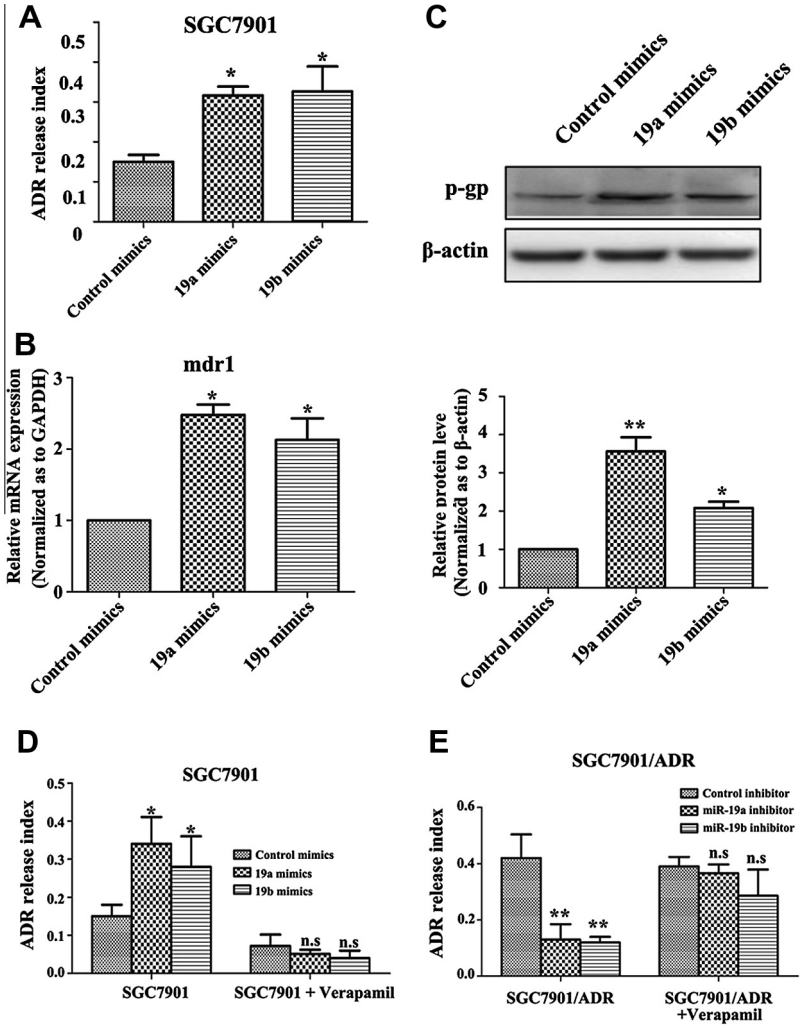

Fig. 2. miR-19a/b accelerate the efflux of ADR through the upregulation of P-gp. (A) The ADR release index of miR-19a/b SGC7901 cells was measured after incubation with 5 g/ml ADR. The ADR release index was calculated as described in the Materials and Methods. (B) Expression of mdr1 in SGC7901 was examined by qRT-PCR after transfection of miR-19a/b mimics. GAPDH mRNA level was used as an internal control. (C) The expression of P-gp in SGC7901 was examined by western blot after transfection with the miR-19a/b mimics. β-actin was used as an internal control. (D) SGC7901 cells were transfected with miR-19a/b mimics and incubated with 5 μg/ml ADR after incubation with verapamil (25 μg/ml) or a negative control for 24 h, and the ADR release index was calculated as in Fig. 3. (E) SGC7901/ADR cells were transfected with miR-19a/b inhibitors and treated as in (D), and the ADR release index was calculated. The data are shown as the mean ± SEM ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments.

duced apoptosis of gastric cancer cells, we analysed the percentage of apoptotic cells after incubation with 5-FU and CDDP. FACS showed that the percentage of cells that underwent apoptosis after 5-FU incubation decreased in SGC7901 cells transfected with miR-19a/b mimics, compared with cells that had been transfected with control mimics (Fig. 3A). Similar results were observed in cells incubated with CDDP (Fig. 3B). Further western blot assays demonstrated the upregulation of Bcl-2 together with the downregulation of Bax and Caspase-3 in SGC7901 cells transfected with the lentiviral vector encoding miR-19a/b compared with cells transfected with the negative control vector (Fig 3C). These results show that miR-19a/b can reduce cells' susceptibility to drug-induced apoptosis.

3.5. miR-19a/b modulate MDR by targeting PTEN

To further investigate the mechanism of apoptosis inhibition induced by miR-19a/b in gastric cancer cells, we examined the expression of PTEN in miR-19a/b–stable-transfected GC cells, which was found to be a direct target of miR-19a/b in breast cancer cells. RT-PCR assays did not show a significant difference in mRNA level (Fig. 4A), whereas western blots showed that PTEN expression was downregulated in miR-19a/b-transfected SGC7901 cells (Fig. 4B). PTEN promotes apoptosis by negatively regulating its downstream element AKT, which plays an important role in the anti-apoptosis pathway. Thus, we examined AKT expression and further found that the expression and phosphorylation of AKT were significantly increased in stably transfected SGC7901 cells (Fig. 4C). These results, along with findings from a recent study indicating the positive effects of AKT on mdr1 expression, demonstrated that miR-19a/b could reduce susceptibility to drug-induced apoptosis and increase drug efflux by regulating the PTEN-AKT pathway.

4. Discussion

MDR is the most frequent cause of treatment failure in gastric cancer chemotherapy. Several recent studies have demonstrated that miRNAs were involved in the development of MDR in gastric cancer [7,18]. Our present data also indicate that miR-19a/b, the most important oncogenic component of the miR-17-92 cluster

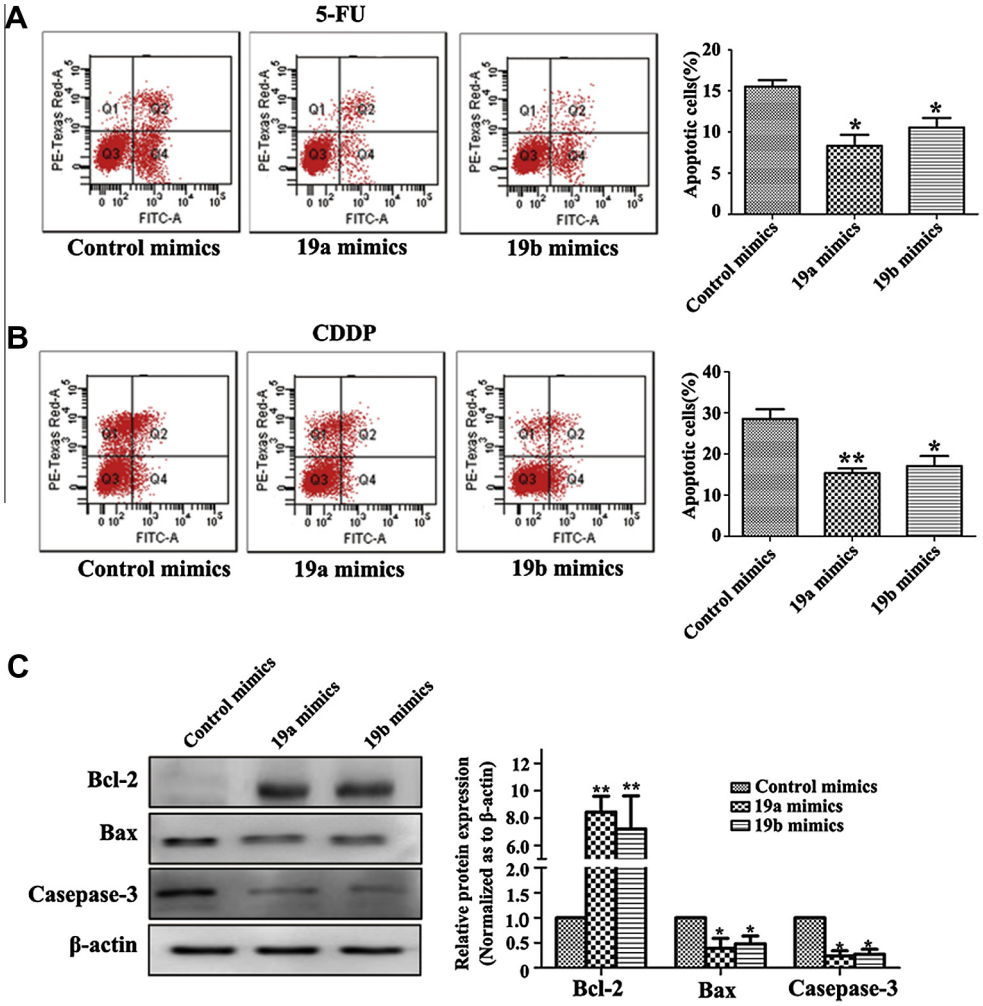

Fig. 3. miR-19a/b reduced cell susceptibility to drug-induced apoptosis. (A) SGC7901 cells were transfected with miR-19a/b mimics and control mimics and incubated with 5-FU for 48 h. The percentage of apoptotic cells was calculated via FACS. (B) SGC7901 cells were transfected as in (A) and incubated with CDDP for 48 h. The percentage of apoptotic cells was calculated by FACS. (C) The expression levels of Bcl-2, Bax and Caspase 3 were examined by western blot after miR-19a/b mimics were transfected into SGC7901 cells. β-actin was used as an internal control. The data are shown as the mean ± SEM (n = 3) of one representative experiment. Similar results were obtained in three independent experiments.

[19], could regulate multidrug resistance in human gastric cancer cells by targeting PTEN.

The multidrug-resistant cell line SGC7901/VCR and SGC7901/ADR, which have been widely employed as in vitro models for the study of MDR in gastric cancer, was derived from the human gastric adenocarcinoma cell line SGC7901 by stepwise selection using VCR as an inducing reagent [20]. Dozens of differentially expressed mRNAs and proteins have been identified between SGC7901/VCR and its parental cell line using subtractive hybridisation [21], differential display [22] and 2-DE [23]. In our study, we observed the upregulation of miR-19a/b in SGC7901/VCR cells as well as in SGC7901/ADR cells. Our further investigations suggest that the modulation of the expression of miR-19a/b could alter the sensitivity of gastric cancer cells to chemotherapeutic drugs including CDDP, 5-FU and ADR. This discovery provides a more comprehensive picture of the molecular network underlying MDR in gastric cancer.

One of the classical causes of drug resistance, the increased efflux of cytotoxic drugs, involves a family of ATP-dependent efflux pumps, known as ATP-binding cassette (ABC) transporters [2]. P-gp is an important member of the ABC transporter family and has been extensively studied in many tumours. The increased expression of P-gp in gastric cancer cell lines and tumours was found to be associated with resistance to chemotherapeutic agents [24]. Our study showed that P-gp, a well-characterised protein with key roles in cytotoxic drug efflux [25], was upregulated significantly after miR-19a/b transfection. We further found that the specific inhibition of P-gp with verapamil abrogated the enhanced ADR increase induced by miR-19a/b, which suggest that P-gp upregulation accounts for the miR-19a/b-induced acceleration of drug efflux. These results indicate that miR19a/b enhances the efflux of cytotoxic drugs by increasing the expression of the ABC transporter P-gp.

Multidrug resistance (MDR) could also result from defects in apoptosis. Changes in apoptosis and survival pathways (such as the Bcl-2, Apaf-1, AKT and NF-kB pathways) may have an impact on the effects of chemotherapeutic agents [26]. Our present study demonstrated that miR-19a/b upregulation inhibits the drug-induced apoptosis of gastric cancer cells. Further study revealed that miR-19a/b affected the expression of key apoptosis-related molecules, which indicates that miR-19a/b may promote MDR in gastric cancer cells partly by reducing the susceptibility of cancer cells to chemotherapeutic drug-induced apoptosis.

Recent studies have demonstrated that PI3K/Akt, crucial effectors of oncogenic signalling, play a key role in the MDR of gastric cancer cells, and the elevation of the expression of PI3K/Akt could

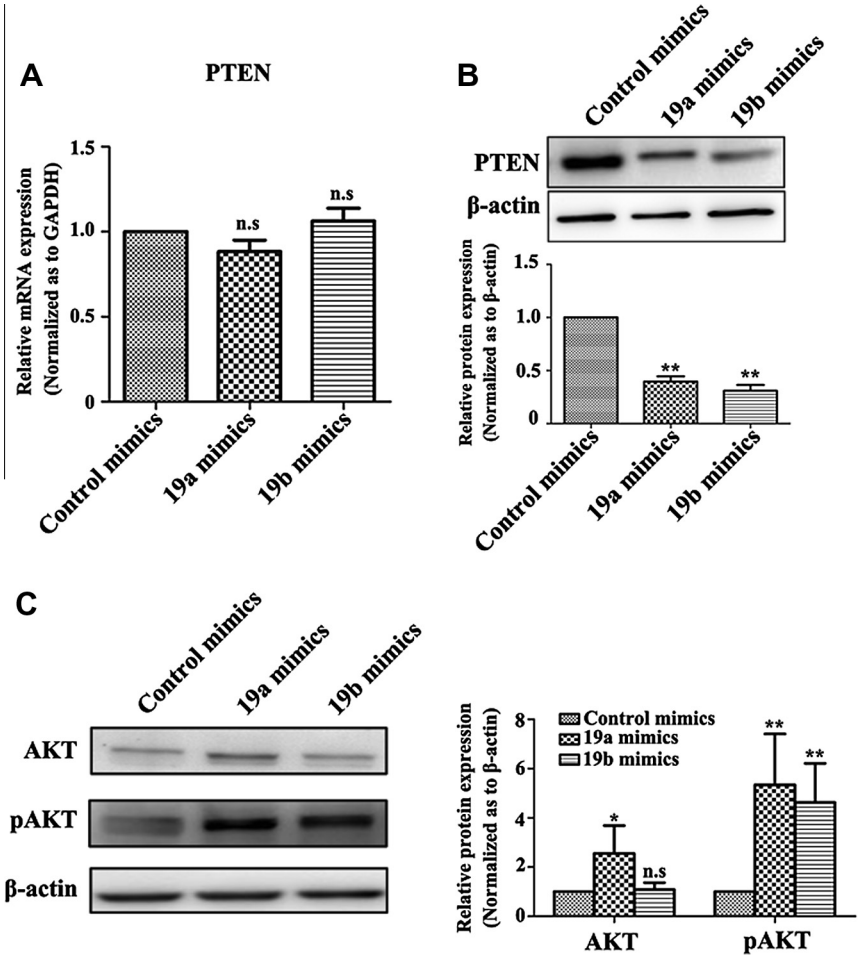

Fig. 4. miR-19a/b modulate MDR by targeting PTEN. (A) The expression of PTEN mRNA was detected using qRT-PCR in SGC7901 cells after transfection with miR-19a/b mimics. GAPDH mRNA was used as an internal control. (B) PTEN protein levels were detected by western blot in SGC7901 cells after transfection with miR-19a/b mimics. β-actin was used as an internal control. (C) The expression and phosphorylation of AKT were examined by western blot in SGC7901 cells after transfection with miR-19a/b. β-actin was used as an internal control. The data are shown as the mean ± SEM ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments.

confer resistance to both P-gp-related and non-related drugs in AGS cells [27,28]. Additional evidence indicated that the altered expression of P-gp, Bcl-2 and Bax might be responsible for the PI3K/Akt-induced drug resistance in AGS cells and was associated with a loss of heterozygosity of PTEN [28–30]. As a previous study had indicated that PTEN was the functional target of miR-19a/b in breast cancer, we further investigated whether miR-19a/b affects PTEN expression in gastric cancer cells. Our study demonstrated elevated expression of AKT and p-AKT, together with the decreased expression of PTEN, in miR-19a/b transfected SGC7901 cells. Considering that reduced or abnormal PTEN expression indirectly stimulates PI3K activity to promote oncogenesis and chemoresistance [31], we suggest that miR-19a/b may help to reduce the susceptibility to drug-induced apoptosis by regulating the PTEN/AKT pathway. Several recent studies have confirmed that the activation of PI3K/AKT has positive effects on mdr1 or P-gp expression [32–34], which suggests that miR-19a/b may also promote drug efflux through the regulation of the PTEN/AKT pathway. Taken together, our data indicate that miR-19a/b regulate MDR in human gastric cancer cells by targeting PTEN.

In summary, our study found that miR-19a/b is upregulated in multidrug-resistant gastric cancer cell lines. Moreover, we found that miR-19a/b decrease the sensitivity of gastric cancer cells to anti-cancer drugs by simultaneously accelerating drug efflux and inhibiting cell apoptosis. Most importantly, we validated PTEN as a functional target of miR-19a/b in gastric cancer cells, demonstrating that miR-19a/b may modulate MDR by regulating the PTEN/AKT pathway in gastric cancer. This novel miR-19a/b-PTEN-AKT axis sheds new light on the mechanisms underlying MDR and may provide future therapeutic targets for the treatment of gastric cancer.

Acknowledgments

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