RESEARCH ARTICLE

Knockdown of MDR1 Increases the Sensitivity to Adriamycin in Drug Resistant Gastric Cancer Cells

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Abstract

Gastric cancer is one of the most frequently occurring malignancies in the world. Development of multiple drug resistance (MDR) to chemotherapy is known as the major cause of treatment failure for gastric cancer. Multiple drug resistance 1/P-glycoprotein (MDR1/p-gp) contributes to drug resistance via ATP-dependent drug efflux pumps and is overexpressed in many solid tumors including gastric cancer. To investigate the role of MDR1 knockdown on drug resistance reversal, we knocked down MDR1 expression using shRNA in drug resistant gastric cancer cells and examined the consequences with regard to adriamycin (ADR) accumulation and drug-sensitivity. Two shRNAs efficiently inhibited mRNA and protein expression of MDR1 in SGC7901-MDR1 cells. MDR1 knockdown obviously decreased the ADR accumulation in cells and increased the sensitivity to ADR treatment. Together, our results revealed a crucial role of MDR1 in drug resistance and confirmed that MDR1 knockdown could reverse this phenotype in gastric cancer cells.

Keywords: Gastric cancer cell - MDR1 - drug resistance - adriamycin

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Introduction

Intrinsic or acquired resistance of human tumor to multiple chemotherapeutic agents often results in the failure of the cancer therapy. Multiple drug resistance (MDR) is a phenomenon of that the resistance to one drug is accompanied by cross-resistance to a whole range of drugs with different structures and mechanisms of action (Ozben, 2006). Multiple drug resistance 1/P-glycoprotein (MDR1/p-gp), also known as ABCB1, is a member of ATP-binding cassette (ABC) family of transporters that contribute to drug resistance via ATP-dependent drug efflux pumps (Leonard et al., 2003). Elevated expression MDR1 was found in many resistant tumors, including breast (O'Driscoll and Clynes, 2006a, b), lung (Cole et al., 1992) and gastric cancers (Yamauchi et al., 1992). MDR1 pumped out various different drugs from cells to reduce the intracellular drug concentration and decrease the cytotoxicity of a broad spectrum of anticancer drugs. MDR1 confers resistance to many drugs including anthracyclines (e.g. ADR) (Kroger et al., 1999), vinca alkaloids, podophyllotoxins (Szakacs et al., 2004) and taxanes (Duan et al., 2005). MDR1 expression correlated with poor response to chemotherapy and an overall poorer prognosis in many kinds of cancers (Filipits et al., 1999). Although the mechanisms of MDR development were broadly studied, efficient strategies to overcome MDR are still needed to be developed.

Gastric cancer is one of the most frequently occurring malignancies (Liu et al., 2013) and the second leading cause of cancer mortality in the world (Zhang and Fan, 2007; Li et al., 2011). Surgery and chemotherapy are the major treatment modalities for this cancer and significantly improved overall survival. However, the prognosis of gastric cancer patients has not been improved significantly. This poor prognosis is mainly due to MDR in gastric cancer cells. Many strategies have been developed to overcome MDR in cancer, including modification of chemotherapy regimens (Dong and Mumper, 2010), inactivation o MDR-associated genes(Kruhn et al., 2009; Stege et al., 2010), use of monoclonal antibodies for MDR1 (Goda et al., 2007), new inhibitors of ABC transporters (Dong and Mumper, 2010) and so on. However, there are numerous challenges for these strategies.

In the present study, we use the shRNA of MDR1 to inhibit the expression of MDR1 in a drug resistance gastric cancer cell line, SGC7901-MDR1, which express MDR1 in high level and are resistant to anticancer drugs. The knockdown of MDR1 decreased the accumulation of ADR in gastric cancer cells and increased the sensitivity to ADR. Notably, these data demonstrate that the MDR1 knockdown is an efficient strategy to reverse drug resistance of gastric cancer cells.

Materials and Methods

Cell culture and transfection

Human gastric cancer cell line SGC7901 and drug resistance cell line SGC7901-MDR1 were used in the present study. These cells were maintained in RPMI-1640

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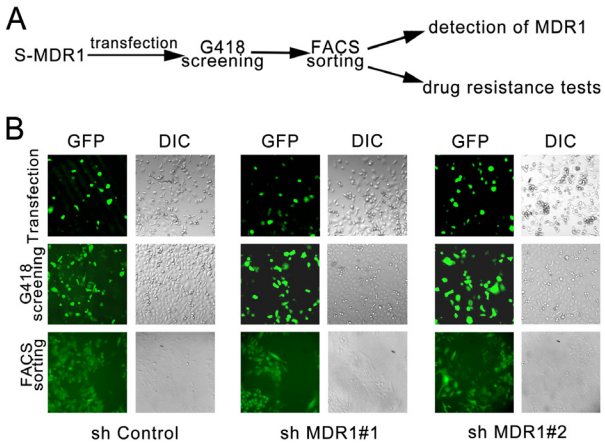

Figure 1. Establishment of MDR1 Knockdown Cell Line. (A) Schematic chart of the establishment of MDR1 knockdown cell line. 0.5μg GFP-labeled control shRNA or MDR1 shRNA#1 or MDR1 shRNA#2 were transfected into SGC7901-MDR1 (S-MDR1) cells for 48h and the cells were selected with G418 (600 μg/ml) for 2 weeks. The selected cells were collected and the GFP-positive cells sorted using FACS for detection of MDR1 and drug resistance analysis. (B) GFP was measured after transfection, G418 screening or FACS sorting using fluorescence microscopy to confirm the efficiency of screening. The corresponding differential interference contrast (DIC) microscopy images are also shown

medium supplemented with 10-15% heat-inactivated fetal bovine serum, glutamine, and antibiotics at 37 °C in 5% CO2. The transfections were performed using the LipofectamineTM 2000 (Invitrogen, USA) following the manufacturer's instruction.

Antibodies and regents

Anti-MDR1 and anti-β-actin rabbit polyclonal antibodies (pAb) were purchased from Santa Cruz Biotechnology (USA). Horseradish peroxidase (HRP)-linked goat anti-rabbit IgG and HRP-linked goat anti-mouse IgG were purchased from Zhongshan Goldenbridge Biotechnology (China). G418 and ADR were brought from Invitrogen (USA) and Sigma (USA) respectively. 3- (4, 5-Dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide (MTT) was purchased from Beyotime (China).

Fluorescence Activated Cell Sorter (FACS)

Transfected cells were collected after G418 screening and GFP-positive cells were sorted using BD FACSAria III cell sorter (BD Biosciences, USA). Cells were treated with ADR (10 μg/mL) for 90min and collected for specific fluorescence analysis. The ADR-positive cells were determined on a BD FacsCalibur flow cytometer (BD Biosciences, USA) and the count was analyzed by ModFit LT software (Verity Software House, USA).

RT-PCR

Total RNA was prepared with Trizol regent (Invitrogen) and cDNA was generated with RT Kit (Tiangen), followed by polymerase chain reaction (PCR) assays. cDNA of cells were examined for their expression of MDR1 and β-actin by PCR. Prime pairs were as follows: human β-actin, forward 5'- TCCTGTGGCATCCACGAA ACT, reverse 5'- GAAGCATTTGCGGTGGA CGAT; human MDR1, forward 5'- TGACTACCAGGCTCGCCAATGAT, reverse 5'- TGTGCCACCAAGTAGGCTCCAAA.

Western blots

Western blot analysis was performed as described (Wang et al., 2013). In brief, whole cell lysates were extracted from cells suspended in radio immune precipitation buffer supplemented with PMSF. The lysates were resolved by electrophoresis on polyacrylamide gels containing 0.1% SDS (SDS-PAGE) and then transferred to 0.45 μm PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris Buffer Saline Tween 20 (TBST) buffer. The blots were incubated with the appropriate primary antibody diluted by TBST and then exposed to the appropriate second antibody conjugated with horseradish peroxidase. The bands on the membrane were visualized and captured using the ECL reagent (Beyotime, China) and X-ray films (Kodak, USA).

MTT assay

Cells were treated with ADR at different concentrations for 48 h. 20 μl MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm.

Data analysis

Statistical significance was determined using the Student's t-test and a p value of <0.05 was regarded as statistically significant. The standard errors were demonstrated by the bar in the figures.

Results

Establishment of MDR1 knockdown cell line.

Many studies have reported the elevated MDR1 and low sensitivity to chemotherapy drugs in gastric cancer cells (Yamauchi et al., 1992; Li et al., 2011). Since SGC7901 cells express low level of MDR1, we established a drug resistance subline of SGC7901, SGC7901-MDR1, which stably expresses MDR1 in high level. SGC7901-MDR1 cells showed low sensitivity to ADR (data not shown), so it is used as a drug resistance gastric cancer cell line. Two GFP-labeled shRNAs were used to knockdown the expression of MDR1 in SGC7901-MDR1 cells to investigate the roles of MDR1 in drug resistance. G418 screening and FACS sorting were performed after transfection with shRNAs of MDR1 to establish MDR1 knockdown cell lines (Figure 1A). Meanwhile, GFP expression was detected to confirm the expression of shRNA and the ratio of shRNA-positive cells. After G418 screening and FACS sorting, above 90% GFP-positive cells were observed (Figure 1B). These cells expressing shRNA were cultured for detection of MDR1 and drug resistance analysis.

Two shRNAs reduced MDR1 expression efficiently

The total RNA and protein were extracted from GFP-positive cells and the MDR1 expression was measured by

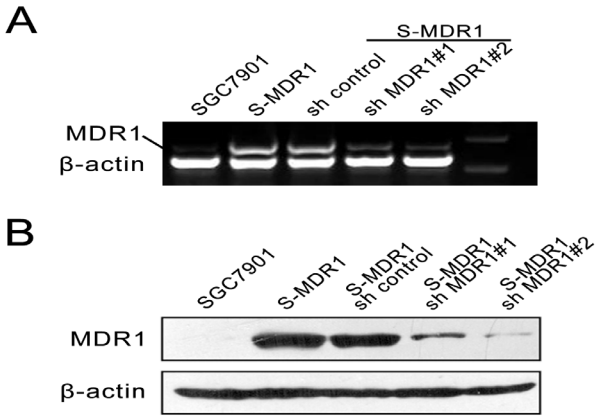

Figure 2. mRNA and Protein Expression of MDR1 Were Decreased in MDR Knockdown Cell Lines. (A) mRNA and (B) protein of MDR1 in SGC7901, SGC7901-MDR1 (S-MDR1), S-MDR1 sh control, S-MDR1 sh MDR#1, S-MDR1 sh MDR#2 cell lines were detected by Western blot analysis. Analysis of β-actin mRNA or protein was included as a loading control

RT-PCR and Western blot analysis. Two MDR1 shRNAs significantly reduced the MDR1 expression in mRNA and protein levels (Figure 2A and B) compare with control shRNA. shRNA#2 of MDR1 suppressed the MDR1 expression more efficiently compared with shRNA#1 (Figure 2B). SGC7901-MDR1 cells express high level of MDR1 and the MDR1 shRNAs works efficiently in this cell line.

Knockdown of MDR1 increased the sensitivity to ADR

shRNAs inhibit the expression of MDR1 in SGC7901-MDR1 cells and the drug resistance of MDR1knockdown cells was evaluated. SGC7901, SGC7901-MDR1, SGC7901-MDR1-sh conrol, -sh MDR1#1, and -shMDR1#2 cells were treated with or without ADR for 90min. Fluorescent cells were measured by FACS and the specific ADR-positive cells were counted (Figure 3A and B). High percentage of ADR cells was observed in SGC7901 cells, whereas MDR1-expressed cell lines, SGC7901-MDR1 and SGC7901-MDR1 sh control, showed low ADR uptake. ADR-positive cells were increased significantly after MDR1 knockdown in SGC7901-MDR1 cells. Half inhibitory concentration (IC_{50}) of ADR was measured in MDR1 knockdown cells to confirm the sensitivity. Two MDR1 knockdown cell lines showed high sensitivity to ADR, whereas SGC7901-MDR1-sh control cells were more sensitive to the drug. These results indicated that MDR1 expression could induce drug resistance and MDR1 knockdown inhibit drug resistance in SGC7901-MDR1 cells.

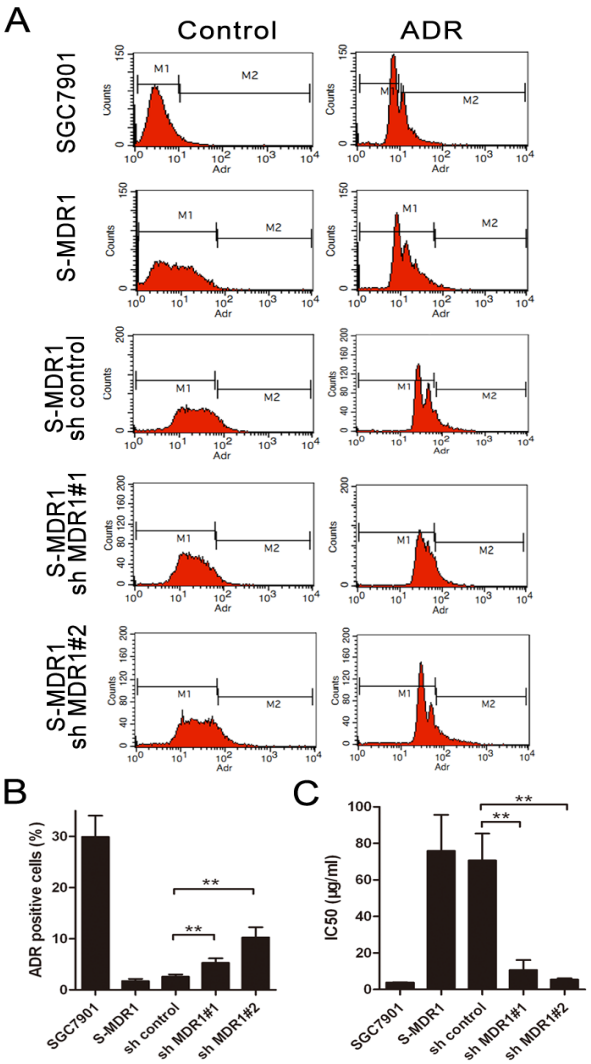

Figure 3. MDR Knockdown in SGC7901-MDR1 (S-MDR1) Cells Increased the Sensitivity to ADR. (A) SGC7901, S-MDR1, S-MDR1 sh control, S-MDR1 sh MDR#1, S-MDR1 sh MDR#2 cells were treated with or without ADR (10 μg/ml) for 90 min and the fluorescence of ADR in cells were measured by FACS. (B) The specific ADR positive cells after ADR treatment were counted and presented as a histogram. (C) SGC7901, S-MDR1, S-MDR1 sh control, S-MDR1 sh MDR#1, S-MDR1 sh MDR#2 cells were treated with ADR at different concentration for 48h and MTT was performed to detect the cell viability. The ADR concentration of IC50 in these cells was presented

Discussion

In the present study, we investigated the consequence of MDR1 knockdown in drug resistance cell line. Our results demonstrated that MDR1 knockdown reverse the drug resistance to ADR in SGC7901-MDR1 cells, which express MDR1 and are resistant to anticancer drugs. These findings indicated the important role of MDR1 expression in multiple drug resistance in gastric cancer cells and that MDR1 knockdown could be used as a strategy to overcome MDR.

Many drug resistance cell lines including SGC7901/VCR (Zhang et al., 2012), SGC7901/DDP (Song et al., 2012), SGC7901/ADM (Huang et al., 2011) have been established to investigate the mechanisms of MDR and to develop the strategies to overcome the MDR in gastric cancer. However, these cell lines were resistant to certain drugs and always lose the property of drug resistance after a few passages. Thus, it's necessary to use a new and stable drug resistance cell line. In the present study, we used SGC7901-MDR1 cells, which express stably MDR1 in high level and are resistant to ADR. SGC7901 cells rarely express MDR1 and are sensitive to ADR (Figure 2 and 3), so SGC7901-MDR1 cells are a better model of

gastric cancer in vitro. Our finding confirms that MDR1 expression could induce drug resistance and MDR1 knockdown could reverse this action.

Many studies showed that knockdown of MDR1 reduce drug resistance of several cancer cells including yolk sac carcinoma L1 cells (He et al., 2011), human colon cancer stem cells (Liu et al., 2009), HePG2/MDR1 cells (Pan et al., 2009), human uterine sarcoma cells (Hua et al., 2005), and Caco-2 cells (Hilgendorf et al., 2005). Decreased MDR1 expression induces an increased accumulation of anticancer drugs, resulting in the increased cytotoxicity to cancer cells. We utilized two shRNAs to suppress the expression of MDR1 and the MDR1 mRNA and protein were reduced significantly in MDR1 knockdown cell lines. In MDR1 knockdown cells, the decreased accumulation of ADR was observed, indicating that the ability of pumping ADR out was inhibited by decreased MDR1.

In summary, two MDR1 knockdown cell lines were established using two efficient shRNAs of MDR1. MDR1 expression was significantly decreased in these cells. MDR1 knockdown reduced the accumulation of ADR in gastric cancer cells and increased the sensitivity to ADR treatment. MDR1 knockdown might be a potential and efficient strategy to overcome MDR in gastric cancer cells.

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