Silencing of the ABCC4 gene by RNA interference reverses multidrug resistance in human gastric cancer

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Abstract. The identification of genes associated with recurrent drug resistance in gastric cancer and the elucidation of the underlying molecular mechanisms associated with recurrent drug resistance in gastric cancer are important for the effective treatment and prognosis of this cancer. Variations in the expression level of the ATP-binding cassette subfamily C member 4 (ABCC4) gene are correlated with the recurrence, development and chemotherapeutic susceptibility of various types of cancers. In the present study, we demonstrated that the ABCC4 gene was highly expressed in multiple types of gastric cancer cells, and ABCC4 expression was even more prominent in the drug-resistant gastric cancer cells. Conversely, in normal gastric mucosal cells, ABCC4 expression was very low or undetectable. We used RNA interference to decrease the expression of ABCC4 in drug-resistant gastric cancer cells, which resulted in an increase in apoptosis and cell cycle arrest in the G1 phase. In addition, we found that ABCC4 knockdown in 5-fluorouracil (5-FU)-resistant cancer cells restored 5-FU sensitivity, resulting in the inhibition of cell proliferation and tumour growth in nude mice. Our results showed that inhibition of ABCC4 gene expression can inhibit the proliferation of multidrug-resistant gastric cancer cells and can enhance gastric cancer cell sensitivity to chemotherapeutic drugs.

Introduction

Gastric cancer is one of the most common malignant tumours. Despite significant advances in the diagnosis and treatment of gastric cancer, the mortality associated with gastric cancer accounts for over 10% of the total cancer mortalities worldwide and remains the second most common cause of cancer-related mortality after lung cancer (1,2). At present, the most commonly used treatment methods for gastric cancer are surgery, radiotherapy and chemotherapy, and comprehensive treatment depending on the patient-specific disease manifestation. Of the available treatment methods for gastric cancer, chemotherapy represents an extremely important and irreplaceable option. However, since gastric cancer cells are prone to the development of multidrug-resistance phenotypes, many chemotherapeutic drugs are ineffective for gastric cancer.

With technological advances in molecular biology, biological treatments such as gene therapy have become areas of interest for the development of novel cancer therapeutics. The introduction of RNA interference (RNAi) technology offers a way to experimentally determine valid targets for gene therapy, as RNAi can be used to elucidate the mechanisms underlying the occurrence of multidrug-resistance and recurrence in gastric cancer.

Many molecular mechanisms associated with the generation of multiple-drug resistance in gastric cancer exist, of which the abnormal expression of the ATP-binding cassette subfamily C member 4 (ABCC4) protein plays a key role. ABCC4 protein, also known as the multi-drug resistance-associated protein, is an important member of the ATP-binding cassette (ABC) transporter family. ABCC4 was first identified on the basal membrane of prostate epithelial cells (3). ABCC4 controls the redistribution and excretion of many anti-viral drugs, antibiotics, cytostatic drugs, and cardiovascular drugs and plays key roles in the protection of cells and cellular signal transduction pathways (4). In addition, ABCC4 can transport intracellular drugs outside of cells, which is often used by cancer cells as a drug resistance mechanism (5). Recent studies have indicated that ABCC4 expression is closely associated with the occurrence of malignant tumours and drug resistance. Studies have also confirmed that ABCC4 is overexpressed in gliomas, neuroblastomas, retinoblastomas and melanomas as well as colon cancer and colorectal cancer cells (6–9). In addition, ABCC4 expression is correlated with drug resistance in leukaemia and ovarian cancer cells (10,11).

The relationship between ABCC4 gene expression and multidrug-resistance in gastric cancer has not been reported. However, studies have shown that ABCC4 gene expression...
is correlated with proliferation capacity and drug resistance in leukaemia, ovarian cancer and colon cancer cells (10-12). Therefore, we used RNAi to knock down ABCC4 gene expression in multidrug-resistant gastric cancer cells to study the role of ABCC4 gene expression in the occurrence and progression of multiple-drug-resistant gastric cancer.

Materials and methods

Cells, antibodies and reagents. Human gastric mucosalGES-1 cells, human gastric cancer MGC-803 cells, and multidrug-resistant SGC-7901/Fu cells were purchased from American Type Culture Collection (ATCC; Rockefeller, MD, USA). ABCC4, P-gp, Bax, Bcl-2, CDK4 and cyclin D1 antibodies were purchased from Abcam (Santa Cruz, CA, USA). PI was purchased from Sigma (Heracles, CA, USA), siRNA was synthesised by GenePharma (Shanghai, China), and the RT-PCR reagent kit (Takara RNA PCR kit (AMV) version 3.0) was purchased from Takara (Dalian, China).

Cell culture. Human gastric mucosal GES-1 cells, human gastric cancer MGC-803 cells, and human gastric cancer SGC-7901 cells were cultured in RPMI-1640 culture medium (Carlsbad, CA, USA) containing 10% foetal bovine serum. SGC-7901/Fu cells were cultured in identical medium containing 20 µg/ml 5-fluorouracil (5-FU) to maintain 5-FU drug resistance. For all experiments, cells were analysed at the logarithmic phase of growth.

Cell viability assays. SGC-7901/Fu cells were seeded in 96-well culture plates until the cells were at the logarithmic phase of growth. Each well received 20 µl MTT (St. Louis, MO, USA) (0.5 mg/ml) and was incubated at 37°C for 4 h. When the yellow solution appeared with blue crystals, the culture medium was replaced with serum-free RPMI-1640 culture medium containing 10% non-fat dry milk at 4°C. The absorbance value (A490 nm) of each well was detected at 490 nm using a microplate reader. The experiment included time points at 24, 48, 72, 96 and 120 h, and each group consisted of 8 replicate wells.

Cell transfection. Cells were seeded in 6-well culture plates, and transfection was performed when the cells reached 70% confluency. Transfection experiments included a control, N-control, and RNAi treatment groups. Culture medium was replaced with serum-free RPMI-1640 culture medium containing both antibiotics. Cells were inoculated with 4 µl of the appropriate virus solution (concentration of 1.5x10^6 Tu/ml) for transfection in the presence of Polybrene (the recombinant RNAi lentivector construct targeting the human ABCC4 gene, LV-shRNA-ABCC4, was constructed previously in our laboratory). Infection was conducted for 12 h, after which the culture medium was replaced with RPMI-1640 culture medium containing 10% FBS. The efficiency of RNA interference was determined by RT-PCR and western blotting.

RNA extraction and RT-PCR detection. Total RNA from the cells in each treatment group was extracted. The extraction procedure was conducted according to the manufacturer's instructions included with the RNAiso™ Plus kit (Takara, Japan). After the RNA concentrations were determined, RT-PCR reactions were conducted using an RT-PCR reagent kit (Takara) according to the manufacturer's instructions. ABCC4, MDR1 and β-actin primers were synthesised by Invitrogen. The primers for ABCC4 were as follows: upstream, 5’-CGCTGGTCTTCTTCTTGTCGT-3’ and downstream, 5’-CTTTATCCAGACACCTTGC-3’. The primers for MDR1 were as follows: upstream, 5’-CCCATCATTGCAATTAGC AGG-3’ and downstream, 5’-GTTTAAACTTCTGCTCC TGA-3’. The primers for β-actin were as follows: upstream, 5’-CCTGGGACGATGAAAGAAA-3’ and downstream, 5’-AACGAGGCCTGAAGATGC-3’. The PCR reaction contained 50 µl in total volume, and the reaction conditions were as follows: 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 30 sec. The PCR products were subjected to 1.2% agarose gel electrophoresis, and the results were scanned and analysed using a gel documentation system.

Detection of apoptosis. Cells from each treatment group were collected and prepared as a single-cell suspension. Staining for apoptotic cells was performed according to the instruction's manual included in the apoptosis reagent kit (Annexin V-FITC kit; BD Pharmingen, San Diego, CA, USA). Briefly, Annexin V and PI were added separately and stained at room temperature in the dark for 20 min. Detection was performed using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Cell cycle analysis. Cells from each treatment group were collected, and the activity of caspase-3, -8, and -9 was detected according to the instruction's manual included with the caspase activity detection reagent kit (Beyotime, China). The analysis was performed using a fluorescence spectrophotometer at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Western blot analysis. After quantification using the bicinchoninic acid (BCA) method, the samples were loaded on an SDS-PAGE gel and separated. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the semi-dry method. The membrane was blocked overnight in 5% non-fat dry milk at 4°C. After washing the membrane with TBST (Tris-buffered saline with Tween), the primary antibodies were added followed by a 1-h hybridisation at 37°C and TBST washes. Secondary antibodies were then added followed by a 1-h hybridisation at 37°C, a TBST wash, 5 min of the chromogenic reaction, and autoradiography. The optical density values were analysed and determined using the Quantity One software, and the results are expressed as the ratio of the sample optical density value to the optical density value of the internal reference.
Inoculation of nude mice. Our animal experiment protocol was approved by the Medical Ethics Committee of Guilin Medical University. A total of 20 male nude mice between 6 and 8 weeks of age at a body weight of ~25 g were purchased from the Experimental Animal Centre of Guilin Medical University. Animals were randomly divided into 2 groups with 10 animals in each group. Cells (5x10^5) from the N-control and RNAi groups were injected subcutaneously into each animal as appropriate. After 4 weeks of tumour growth, the mice were sacrificed by cervical dislocation, and tumour tissues were removed aseptically for further analysis.

Statistical analysis. Statistical analysis was performed using SPSS 16.0 software. All data are presented as the mean ± standard deviation. Comparisons between groups were examined using one-way analysis of variance (ANOVA) and LSD-t. p-values <0.05 were considered statistically significant.

Results

ABCC4 gene expression is increased in multidrug-resistant gastric cancer SGC-7901/Fu cells. RT-PCR and western blotting were used to detect the expression of ABCC4 mRNA and protein in the GES-1, MGC-803, SGC-7901 and SGC-7901/Fu cells. Our results showed that the expression levels of ABCC4 mRNA in the MGC-803, SGC-7901 and SGC-7901/Fu cells were higher than the ABCC4 expression in the GES-1 cells. Notably, the ABCC4 mRNA expression level was highest in the SGC-7901/Fu cells. In addition, western blotting results for ABCC4 protein expression levels in these 4 cell lines were consistent with the mRNA expression level results. These results revealed that the multidrug-resistant human gastric cancer SGC-7901/Fu cells expressed the highest levels of the ABCC4 transcript and protein (Fig. 1).

Knockdown of ABCC4 gene expression in multidrug-resistant gastric cancer SGC-7901/Fu cells. RNAi was used to silence ABCC4 gene expression in the SGC-7901/Fu cells. The cells were transfected with the recombinant RNAi lentiviral vector targeting the human ABCC4 gene (LV-shRNA-ABCC4) or with a negative control construct, after which the expression levels of ABCC4 mRNA and protein were analysed by RT-PCR and western blotting, respectively. Four days after transfection, the expression level of ABCC4 mRNA was significantly downregulated relative to the control and N-control groups. Consistently, the ABCC4 protein expression level was also decreased in the cells receiving the ABCC4 RNAi construct (Fig. 2).

Silencing of the ABCC4 gene sensitises SGC-7901/Fu cells to 5-FU. Next, we analysed the effect of the silencing
of the ABCC4 gene on the proliferation capacity of the multidrug-resistant gastric cancer cell line SGC-7901/Fu. Our results showed that when compared with the control and N-control groups, at all time points (24, 48, 72, 96 and 120 h) after transfection, the proliferation capacity of the SGC-7901/Fu cells did not change (Fig. 3A). However, upon treatment of the ABCC4-silenced cells with a therapeutic dose of 5-FU, the proliferation capacity of the SGC-7901/Fu cells significantly decreased relative to the controls (Fig. 3B). Our results suggest that silencing of the ABCC4 gene significantly enhanced the inhibition of SGC-7901/Fu cell proliferation by 5-FU.

Silencing of the ABCC4 gene enhances 5-FU-induced apoptosis in SGC-7901/Fu cells. To investigate the molecular mechanisms underlying the increase in 5-FU-induced inhibition of SGC-7901/Fu cell proliferation upon ABCC4 knockdown, we analysed cells for apoptosis by flow cytometry. Upon silencing of ABCC4 expression in the presence of 5-FU, the apoptosis rate in the SGC-7901/Fu cells increased significantly relative to the other treatment groups (Fig. 4A). The expression levels of caspase-3 and -9 increased significantly in the ABCC4-knockdown group (Fig. 4B). Furthermore, western blotting results showed that Bax protein expression was significantly increased, whereas Bcl-2 protein expression was decreased (Fig. 4C and D). These results suggest that silencing of ABCC4 expression could enhance the ability of 5-FU to induce apoptosis in SGC-7901/Fu cells. Furthermore, the increase in apoptosis appears to be associated with the mitochondrial pathway.

ABCC4 knockdown sensitises SGC-7901/Fu cells to 5-FU-mediated cell cycle arrest. To further investigate the molecular mechanisms underlying the enhancement of 5-FU-induced inhibition of SGC-7901/Fu cell proliferation upon ABCC4 knockdown, cell cycle analysis was conducted by flow cytometry. Our results showed that treatment with 5-FU or RNAi alone did not change the distribution of the cell cycle. However, ABCC4 knockdown in the presence of 5-FU resulted in a significant increase in the cell population arrested in the G1 phase (Fig. 5A). In addition, the protein expression levels of the cell cycle proteins cyclin D1 and CDK4 were significantly decreased in the 5-FU+RNAi group (Fig. 5B and C). Since cyclin D1 and CDK4 proteins play indispensable roles in regulation of the G1 phase of the cell cycle, our results indicate that the enhancement of 5-FU-induced inhibition of SGC-7901/Fu cell proliferation upon ABCC4 knockdown was achieved by downregulating cyclin D1 and CDK4 protein expression, thereby arresting the cell cycle in the G1 phase.

Figure 4. ABCC4 knockdown enhances 5-FU-induced apoptosis in SGC-7901/Fu cells. (A) Analysis of the apoptotic population in the SGC-7901/Fu cells upon ABCC4 knockdown by flow cytometry. (B) Detection of caspase-3 and -9 activities upon ABCC4 knockdown. (C and D) Analysis of Bax and Bcl-2 protein expression levels by western blotting after ABCC4 knockdown. *p<0.05, each independent experimental sample was repeated in triplicate.
ABCC4 knockdown inhibits the growth of tumour xeno-grafts. The animal experiment program was approved by the Medical Ethics Committee of Guilin Medical University. The subcutaneous tumour xenograft model in the nude mice was conventionally established. Twenty nude mice were divided into equal treatment groups according to the xenograft implanted. One group received tumour implants that were subjected to ABCC4 knockdown, whereas the other group received tumour implants that were subjected to non-specific control knockdown. Upon 5-FU treatment, we found that at all time points, the tumour volume in the 5-FU+RNAi group was significantly smaller than the tumour volume in the N-control group. In addition, the weight of the surgically removed tumours in the 5-FU+RNAi group was also significantly lower than the tumour weight in the N-control group (Fig. 6). Our results revealed that ABCC4 knockdown in vivo significantly reversed the multidrug-resistance in human gastric cancer.
ABCC4 knockdown restores the sensitivity of SGC-7901/Fu cells to 5-FU through MDR1. The generation of multidrug resistance is closely associated with the permeability glycoprotein (P-gp) transport protein (MDR1). To further investigate the mechanisms responsible for the increase in the sensitivity of the drug-resistant gastric cancer cell line SGC-7901/Fu to 5-FU upon ABCC4 knockdown, the expression levels of the P-gp (MDR1) transport protein were analysed. Our results showed that upon ABCC4 knockdown, MDR1 transcript and protein expression also decreased (Fig. 7). These results indicate that the observed increase in the sensitivity of SGC-7901/Fu cells to 5-FU treatment upon ABCC4 knockdown could be associated with the concomitant downregulation of P-gp (MDR1) expression.

Discussion

ABC transport protein family 4 (ABCC4) is a member of the ABC transport protein family and is also known as multidrug-resistance protein 4 (MRP4). ABCC4 was first identified on the basal membrane of prostate epithelial cells (3). The ABCC4 locus is found on human chromosome 13q32, and the full length of the gene is approximately 312 kb. ABCC4 contains 31 exons and encodes 1325 amino acids (13). ABCC4 lacks an N-terminal transmembrane domain and forms an MSD1-NBD1-MSD2-NBD2 structure. ABCC4 possesses the simplest structure of the proteins in the ABC family (14). ABCC4 can transport many endogenous and exogenous organic anions with various structures outside of the cell. This feature of ABCC4 provides cells with the ability to tolerate various cytotoxic compounds and to protect important tissues from xenobiotic damage (15). In addition, ABCC4 also affects drug metabolism in cells contributing to drug resistance.

The association between ABCC4 and cancer has become an area of interest for many researchers in recent years. Dong et al and Cai et al (16,17) showed that ABCC4 was highly expressed in androgen-induced prostate cancer cells, and subsequent studies further confirmed the presence of a high copy number of ABCC4 mRNA in prostate cancer (18). The purpose of our study was to investigate the expression of ABCC4 in drug-resistant human gastric cancer cells and to assess the potential for ABCC4-targeted inactivation in the treatment of drug-resistant gastric cancer. Our results showed that ABCC4 transcript and protein levels were increased in multiple human gastric cancer cell lines especially a drug-resistant gastric cancer cell line. These results suggest that ABCC4 expression and the development of drug resistance in human gastric cancer are likely related hinting at the potential value of ABCC4 as a therapeutic target for drug-resistant gastric cancer.

To further elucidate whether ABCC4 is closely associated with the occurrence and development of drug resistance in human gastric cancer, we knocked down ABCC4 expression levels in a drug-resistant human gastric cancer cell line and observed the biological changes. Our results showed that upon ABCC4 knockdown, proliferation of the drug-resistant human gastric cancer cell line SGC-7901/Fu was inhibited. Furthermore, these cells displayed an increase in apoptosis and G1 cell cycle arrest. Knockdown of ABCC4 also inhibited the growth of tumour xenografts in nude mice, suggesting that high expression levels of ABCC4 in drug-resistant human gastric cancer cells promote tumour cell proliferation. Previous studies have shown that high ABCC4 expression levels could promote the development of malignant tumours such as prostate cancer and ovarian cancer (11,19,20). Consistently, our results showed that ABCC4 plays an important regulatory role in the proliferation of drug-resistant human gastric cancer cells.

To further investigate the role of ABCC4 in the regulation of the inhibition of drug-resistant human gastric cancer cell proliferation, we analysed apoptosis and cell cycle progression. The effect of ABCC4 expression on apoptosis has not been thoroughly researched. Our results showed that downregulation of ABCC4 increased apoptosis in drug-resistant human gastric cancer cells. A study by Dai et al (21) showed that ABCC4 could regulate the mTOR signalling pathway, thereby regulating apoptosis. Consistently, our results showed that downregulation of ABCC4 is closely associated with the inhibition of the proliferation of drug-resistant human gastric cancer cells and the occurrence of apoptosis. In addition, we also found that downregulation of ABCC4 could arrest the cell cycle of drug-resistant human gastric cancer cells at the G0/G1 phase. These results collectively suggest that high ABCC4 expression levels observed in drug-resistant human gastric cancer cells are necessary for tumour cell proliferation. Furthermore, downregulation of ABCC4 results in the inhibition of proliferation of drug-resistant human gastric cancer cells.

Several previous studies have shown that the mitochondrial pathway and death receptor pathway (22) are the classical apoptotic pathways in many tumour cells. Bcl-2 and Bax play important roles in the mitochondrial apoptosis pathway (23,24). If Bax translocates from the cytoplasm to the mitochondrial membrane, permeability of the mitochondrial membrane changes to promote cytochrome c release from the mitochondria into the cytoplasm thereby activating the caspase cascade culminating in apoptosis (25-27). In this study, we

Figure 7. ABCC4 knockdown restores the sensitivity of SGC-7901/Fu cells to 5-FU through MDR1 downregulation. (A) MDR1 gene expression levels upon ABCC4 knockdown by RT-PCR. (B) P-gp protein expression levels upon ABCC4 knockdown by western blotting. *p<0.05, each independent experimental sample was repeated in triplicate.
found that downregulation of ABCC4 in drug-resistant human gastric cancer cells caused translocation of Bax and Bcl-2 and significantly increased caspase-3 and -9 expression levels. We speculated that during the mitochondrial pathway of apoptosis, release of cytochrome c into the cytoplasm may activate the biological effects of caspase-9 and -3, thereby playing a key role in the activation of apoptosis. These results suggest that downregulation of ABCC4 can induce apoptosis in drug-resistant human gastric cancer cells through the mitochondrial pathway.

The occurrence of drug resistance in malignant tumours is thought to be closely associated with the abnormal expression of MDR1 and P-glycoprotein on the membrane of tumour cells. Therefore, some researchers have proposed that downregulation of these two proteins using a variety of methods could reduce the occurrence of drug resistance in tumour cells (28, 29). Abnormal expression of MDR1 and P-glycoprotein is usually closely associated with the generation of drug resistance in tumour cells. Studies have shown that downregulation of MDR1 and P-glycoprotein in drug-resistant tumour cells could inhibit the generation of drug resistance in tumour cells (30). The present study showed that downregulation of ABCC4 in the presence of 5-FU significantly inhibited the proliferation and cell cycle progression of drug-resistant human gastric cancer cells and inhibited tumour cell growth in nude mice. In addition, the expression levels of MDR1 transcript and protein and P-glycoprotein on the membrane of tumour cells also decreased significantly. These results indicate that downregulation of ABCC4 could alter the expression levels of MDR1 and P-glycoprotein on the cell membrane thus inhibiting proliferation of drug-resistant human gastric cancer cells.

In summary, the present study showed that ABCC4 is highly expressed in drug-resistant human gastric cancer cells. Furthermore, downregulation of ABCC4 increased apoptosis and cell cycle arrest in drug-resistant human gastric cancer cells through the regulation of Bcl-2/Bax in the mitochondrial pathway, thereby restoring the sensitivity of the drug-resistant cancer cells to 5-FU. Our study may contribute to making a case for the targeting of ABCC4 for the clinical treatment of drug resistance in gastric cancer.

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References


