CA916798 Regulates Multidrug Resistance of Lung Cancer Cells

Hai-Jing Wang, He-Ping Yang, Xiang-Dong Zhou*, Xiao-Tian Dai*, Yong-Feng Chen, Wei Xiong

Abstract

Objectives: Multidrug resistance (MDR) significantly reduces the efficacy of chemotherapy for lung cancer. In this study, we characterized the significance of CA916798, a gene up-regulated in cis-dichlorodiamine platinum (CDDP)-resistant lung adenocarcinoma cells, in mediating MDR in lung cancer cells. Methods: CA916798 was stably transfected into H446 cells with low endogenous expression of CA916798, and knocked down in A549/CDDP cells with high endogenous level of CA916798. Expression was confirmed by real-time PCR, Western immunoblotting and immunocytochemistry. Subsequent effects were examined on cellular growth, apoptosis and cell cycle progression. Results: Ectopic expression of CA916798 in H446 cells conferred enhanced resistance to multiple chemotherapeutic agents, while its reduction rendered A549/CDDP cells less resistant to chemotherapeutic agents tested. Further analysis revealed that CA916798 regulates CDDP-induced cell growth, apoptosis and cell cycle progression. Conclusion: CA916798 may be a novel MDR-related target for lung cancer therapy.

Keywords: CA916798 gene - lung cancer - multidrug resistance - cis-dichlorodiamine platinum

Asian Pacific J Cancer Prev, 12, 3403-3408

Introduction

Lung cancer is the leading cause of cancer-related mortality in both males and females (Jemal et al., 2011). Among lung cancer patients, those with good performance status often receive standard treatment with double-agent platinum-based chemotherapy (Schiller, 2002; Metro and Cappuzzo, 2009; Stinchcombe and Sociinski, 2009). Such chemotherapeutic regimens have been shown to extend patient survival, alleviate disease-related symptoms, and improve life quality (Pfister et al., 2004; Song et al., 2010). However, many patients develop resistance to chemotherapeutic drugs over time, and eventually present with recurrent cancer no longer sensitive to the same or similar chemotherapeutic regimens. Studies over the past 40 years have identified multidrug resistance (MDR) as a key reason for the failure of cancer chemotherapy (Patel and Rothenberg, 1994).

MDR refers to the phenomenon whereby tumor cells resistant to one type of chemotherapy are resistant to other drugs that may differ in structure and/or modes of action (Baguley, 2010b; Ozben, 2006). MDR, either inherent to tumor cells or acquired during chemotherapeutic treatment, can be mediated by multiple cellular mechanisms, including defects in drug uptake, intracellular drug distribution and metabolism, drug efflux, and cellular death response (Baguley, 2010b; Gillet and Gottesman, 2010). Yet there may be additional cellular mechanisms underlying MDR that remain to be identified and characterized.

By suppression subtractive hybridization (SSH), we identified 23 cDNA clones differentially expressed between the human MDR lung adenocarcinoma cell line SPC-A-1/CDDP and its drug-sensitive counterpart SPC-A-1 cells (Chen et al., 2001). Further characterization led to the discovery of a full-length cDNA, CA916798 (GenBank: CA916798.3) that showed 99% homology to the BC006151 gene located on human chromosome 19 (Zhou et al., 2002). The full-length cDNA for CA916798 is 1258 bp in length and encodes a protein of 117 amino acids including 47.0% hydrophobic, 30.8% hydrophilic, 12.8% basic and 9.4% acidic amino acids (Zhou et al., 2002). No domains typical for signal peptides, transmembrane spanning or zinc-finger structures could be identified, suggesting CA916798 does not share a common structure with most adenosine triphosphate-binding cassette (ABC) transporter family members. Its amino acid sequence shows no significant homology with any other known human proteins, indicating that CA916798 is a novel protein with unknown functions. The up-regulation of CA916798 in SPC-A-1/CDDP cells, however, suggests its potential involvement in chemo-resistance.

To determine the biological functions of CA916798, we prepared a polyclonal antibody specifically recognizing...
CA916798, with which, the expression of CA916798 was analyzed in multiple human lung carcinoma cell lines. Then loss-of-function and gain-of-function approaches were applied in these cell lines, and the effects of altering CA916798 expression on cellular growth, apoptosis and cell cycle progression in response to chemotherapeutic agents were examined. This study provides the experimental evidence that CA916798 is a novel MDR gene, which may benefit the future therapy of chemoresistant lung cancer.

Materials and Methods

Antibody generation and purification

Full-length CA916798 cDNA was cloned from A549 cDNA library into the pGEM-T vector (Promega, Madison, WI) using the following primers: sense 5’ CGCGGATCCGATGATGATGATAAGATGACATGCTGGAGGCCGTCTTGG and antisense 5’ AGGCTCATCTAAACCAGGACGCTCCCCCAG 3’, where the underlined sequences were confirmed by sequencing. The GST-CA916798 cDNA was then digested by BamHI and SalI and ligated using T4 ligase (Promega, Madison, WI) following the manufacturer’s instructions. The CA916798 cDNA fragment was then transformed into DH5α competent cells and purified using the Plasmid Miniprep kit (Omega Bio-Tek, Norcross, GA) following the manufacturer’s instructions. The CA916798 cDNA fragment was then digested with BamHI and SalI and ligated using T4 ligase (Promega, Madison, WI) into the mammalian expression vector pET-42a(+) (Novagen, Gibbstown, NJ) between an N-terminal GST tag and a C-terminal His tag. The identity of the insert was confirmed by sequencing. The GST-CA916798-His fusion protein was then expressed in BL21 bacterial cells by induction with isopropyl-β-D-thio-galactoside (IPTG) and purified with the His-trap-HP column (Amersham, Pittsburgh, PA) following the manufacturer’s instructions.

The purified GST-CA916798-His protein was injected into two male New Zealand White rabbits to produce a polyclonal antibody as previously described (Hu et al., 2002). Briefly, the purified fusion protein diluted in PBS (600 μg/mL) was emulsified with equal volume of Freund’s Complete Adjuvant (Sigma St Louis, MO), and injected subcutaneously at 10 locations on the back and four limbs of each rabbit (100 μL/location) (initial immunization). The same injections were repeated three days later (secondary immunization) and 25 days later (third immunization). On day seven after the third immunization, 1 mL of venous blood was taken from the ear pinna and the titer of antiserum was determined by indirect ELISA on day 15 thereafter. The rabbits were sacrificed. Otherwise, the rabbits were immunized the fourth time, with the antiserum titer checked again by indirect ELISA as previously described (Shen et al., 2005). If the titer reached 1:16, the rabbits were used as a control (shCtrl), a sequence targeting no known human gene was designed: TGGATATTGTTGCAATCA. The five shRNA duplexes containing a BamHI site in the 5’ end of the sense strand, a HindIII site in the 5’ end of the antisense strand, and a nine-base-pair loop sequence (TTCAGAGA) in between the sense and antisense fragment of the same shRNA targeting sequence were synthesized by Sangon (Shanghai, China), and cloned into the shRNA expressing plasmid pGCsi-U6/Neo/GFP (Genechem, Shanghai, China).

Cell lines and culture

Human adenocarcinoma cells A549 and small cell lung cancer cells H446 were acquired from ATCC (Manassas, VA). The MDR A549/CDDP cell line was established by selection through intermittent administration of high doses of CDDP as previously described (Pan et al., 2009). A549 and A549/CDDP cells were cultured in RPMI-1640 (HyClone, Logan, UT) supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin (North China Pharmaceutical, Shijiazhuang, China). H446 cells were cultured in DMEM (Hyclone) containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. All cells were maintained at 37 °C with 5% CO2 in a sterile incubator.

To establish stable cell lines, H446 and A549/CDDP cells growing in log phase were seeded into 24-well plates at 5 × 10⁴ cells/well. At approximately 85–90% confluence, cells were transfected with pQE-CA916798 or empty pQE vector (for H446 cells, generating H-CA and H-Ctrl cells, respectively), and pGC-shCA916798-1 to 4 or pGC-shCtrl (for A549/CDDP cells, generating A-sh1 to 4 and A-shCtrl cells, respectively) using Lipofectamin-2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. After 48 h, G418 (Sigma) was added to a final concentration of 100 μg/mL for H446 cells and 700 μg/mL for A549/CDDP cells. The G418-containing medium was replaced every two days for a total of 15 to 20 days. Then, the surviving stable cells were maintained in G418 (50 μg/mL for H446 cells and 350 μg/mL for A549/CDDP cells).

Indirect immunocytochemistry and immunofluorescence

Cells growing on glass coverslips were fixed with ice-cold acetone for 10 min, washed with PBS and incubated with the polyclonal anti-CA916798 antibody (1:100) at 4 °C overnight. For immunocytochemistry, cells were
incubated with horse-radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:2000) (Zhongshan Golden Bridge, Beijing, China) at 37 °C for 30 to 60 min, washed with PBS three times, then incubated with ABC complex (Sangon Biotech.) at 37 °C for 30 min. The signal was developed with DAB substrate at room temperature (RT) for 10 to 20 min. Cells were then counter-stained with hematoxylin, dehydrated in ethanol series, cleared in xylene and mounted with permount. For immunofluorescent staining, cells were incubated with FITC-labeled anti-rabbit secondary antibody (1:2000) (Zhongshan Golden Bridge) at 37 °C for 30 to 60 min, then washed twice with PBS and once with distilled water. Cells were mounted with 50% glycerol and observed by light or fluorescence microscopy at 200 × magnification. Five random fields were imaged for each sample and analyzed with Image-Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD).

**Western blot analysis**

Cells were lysed in RIPA buffer (Beyotime, Jiangsu, China) containing PMSF, and protein concentrations determined by the Bradford method. For each sample, 10 μg of total protein was separated on a 10% SDS-PAGE gel and electropherferred to a PVDF membrane (Roche, Madison, WI). Membranes were blocked with 5% skim milk in TBS-Tween 20 at RT for 1–2 h, and incubated with anti-CA916798 antibody (1:100 dilution) or anti-GAPDH antibody (Sigma, 1:1000) at RT for 1 h, followed by HRP-conjugated anti-rabbit secondary antibody (1:2000, Zhongshan Golden Bridge) at RT for 2 h. The signal was then developed with chemiluminescence substrate (Sangon). Densitometric analysis was performed using Image J software.

**Reverse transcription and semi-quantitative polymerase-chain reaction (PCR)**

Total RNA was extracted from cells using Trizol® Reagent (Invitrogen) following the manufacturer’s instructions. Total RNA (0.5 μg) was then reverse transcribed into cDNA using PrimeScript RT enzyme (Takara). The following primers were used for subsequent PCR reactions: CA916798, forward 5’CTTCTCTTCAACCTCGTCC3’, reverse 5’AGGCCCTTGGTAGCCCACTTA3’; amplicon size: 272 bp; β-actin (as internal control), forward 5’GGGATCCACGAGAATTAC3’, reverse 5’AAAGGGTGTGAAAGCACTAA3’; amplicon size: 346 bp. The conditions for PCR included 30 cycles of: 94 °C for 30 sec, 55 to 65 °C for 30 sec and 72 °C for 20 sec. The PCR products were electrophoresed on a 1.5% agarose gels, stained with ethidium bromide, imaged under UV light and quantified by densitometric analysis.

**Giemsa and acridine orange staining**

For Giemsa staining, cells grown on glass coverslips were fixed in methanol for 10 min, air-dried and incubated in Giemsa staining solution (Sigma). When optimal color developed (approximately 10 to 20 min), staining was stopped by rinsing the coverslips in water. Cells were then observed with light microscopy (200 ×), and five random fields were imaged for each sample. The percentage of apoptotic cells, as characterized by deep purple nuclear stain, was quantified from each image and presented as mean ± SD for all five fields.

For acridin orange staining, cells grown on glass coverslips were fixed in methanol for 10 min, air-dried and incubated in 0.1 mg/mL acridine orange staining solution (Chongqing Medicines, Chongqing, China) at RT for 10 min. Cells were then washed in water three times and mounted with glycerol. The cells were imaged by fluorescence microscopy with an excitation wavelength of 502 nm.

**Drug resistance analysis**

Cells were seeded into 96-well plates at 5 × 103 cells/well (in duplicate for each condition) and treated with different chemotherapeutic drugs for 48 h. Cells not treated with any drug were used as control (Ctrl). Cell viability was measured with a CCK-8 kit (Dojindo Laboratories, Tokyo, Japan) following the manufacturer’s instructions. Briefly, 10 μL of CCK-8 solution was added to each well and the plate was incubated at 37 °C for 1–4 h. The plate was then read for absorbance at 450 nm (A450) and % growth inhibition was calculated as (A450Ctrl-A450Drug)/A450Ctrl × 100%. The concentration of a drug that achieved 50% growth inhibition was defined as IC50. The resistance index (RI) of a target cell line was defined as IC50-target line/IC50-ctrl line.

**Flow cytometry**

Cells growing in log phase were lifted, resuspended in PBS, and fixed in 70% ethanol at 4 °C overnight, washed with PBS three times, and incubated in 20 μL RNase A at 37 °C for 30 min. Following staining with 0.1 mg/mL propidium iodine (PI, Sigma) at 4 °C in the dark for 30 min, the cells were analyzed by flow cytometry.

**Statistical analysis**

Statistical analysis was performed using SPSS 10.0 software. All quantitative data were presented as mean ± SD for at least three independent experiments. A P value of < 0.05 was considered statistically significant.

**Results**

**CA916798 is differentially expressed in A549, A549/CDDP and H446 cells**

The endogenous expression levels of CA916798 protein, as determined by western blot, were greater in A549/CDDP cells than in A549 cells (p < 0.05, Figure 1A). Immunocytochemistry revealed that CA916798 mainly distributes in the cytoplasm of A549/CDDP and H446 cells in a diffuse manner and the expression level was also higher in A549/CDDP than in H446 cells (Figure 1B, p < 0.01).

**Ectopic expression of CA916798 in H446 enhances resistance to chemotherapeutic agents**

Stable ectopic expression of CA916798 in H446 cells (H-CA cells) dramatically increased the expression of CA916798 on mRNA (Figure 1C) and protein levels (Figure 1D), as compared to those in parental H446 cells.
and H446 stably transfected with empty vector (H-Control) (p < 0.01). The ectopically expressed CA916798 was mainly detected in the cytoplasm (Figure 1E), consistent with the localization of the endogenous protein (Figure 1B).

Treatment with CDDP inhibited the growth of both H446 and H-Control cells in a dose-dependent manner, resulting in a calculated IC50 of 0.16 ± 0.04 μg/mL (H446) or 0.17 ± 0.01 μg/mL (H-Control) (Table 1). In contrast, CDDP-induced growth inhibition was significantly lower in H-CA cells, leading to a much higher IC50 of 1.25 ± 0.11 μg/mL (H-Control) (p < 0.01, as compared to both parental H446 and H-CA cells) (Table 1) and control H-Control cells, Figure 1F). The IC50 of several other common chemotherapeutic lung cancer drugs was also determined in H-Control and H-CA cells (Table 1). The resistance index (RI) of H-CA cells was calculated using H-Control as control (Table 1). The IC50 of all tested drugs was significantly lower in H-Control than in H-CA cells, and the calculated RI of H-CA cells was greater than 1 for each drug tested (Table 1).

**Ectopic expression of CA916798 in H446 reduces CDDP-induced apoptosis**

As shown in Figure 2A, the morphology of H-Control cells treated with 1.0 μg/mL CDDP for 24 h appeared normal, and cells displayed lightly stained cytoplasmas, oval or round nuclei, and visible nucleoli. In contrast, the morphology of H-Control cells treated with 1.0 μg/mL CDDP for 24 h was characteristic of apoptotic cells, and included shrunken cell bodies and condensed, heavily stained nuclei. By quantification, the percentage of cells undergoing apoptosis in response to CDDP was significantly higher in H-Control than in H-CA cells (Figure 2B). CDDP-induced morphological changes were also observed by acridin orange staining. H-CA cells presented intact uniform morphologies with bright green nuclei, while H-Control cells presented orange nuclei and chromatin condensation (Figure 2C).

PI staining to analyze cell cycle distribution indicated that, following CDDP treatment, the percentage of H446 and H-Control cells (approximately 10%) in the sub-G1 phase was significantly larger than the percentage of H-CA cells (2.73 ± 0.18%, p < 0.01) (Figure 2D). Further analysis revealed that CDDP treatment led to dramatic G2 cell cycle arrest in H446 and H-Control cells, but not in H-CA cells (p < 0.01, Figure 2E).

**Down-regulation of CA916798 in A549/CDDP reduces resistance to chemotherapeutic agents**

CA916798 mRNA in A549/CDDP cells was reduced...
CA916798 Regulates Multidrug Resistance of Lung Cancer Cells

Growth inhibition was greater in A-sh3 cells treated with CDDP (≥ 0.6 µg/mL) than in A-shCtrl cells treated in the same manner (p < 0.01, Figure 3F). Similarly, the IC50 calculated for other chemotherapeutic drugs was significantly lower in A-sh3 cells than in A-shCtrl cells (p < 0.05, Table 2). For each drug, the RI of AD549/CDDP/sh3 cells was less than 1 (Table 2).

**Discussion**

In this study, we examined the functional significance of CA916798 in regulating the resistance of human lung cancer cells to chemotherapeutic drugs. By both gain-of-function and loss-of-function studies, we demonstrated, for the first time, that CA916798 is sufficient and necessary for regulating MDR. Mechanistically, this action appears to involve the modulation of apoptosis and cell cycle progression.

MDR is a principal impediment to the successful chemotherapeutic treatment of many cancers (Patel and Rothenberg, 1994). Multiple cellular mechanisms for MDR have been identified, including membrane transporter proteins that actively pump chemotherapeutic drugs outside of the cells, molecules conferring resistance to apoptosis, and factors essential for the maintenance...
and differentiation of cancer stem cells (Baguley, 2010a; Baguley, 2010b).

In this study, we focused on CA916798, a differentially expressed gene first identified in MDR lung adenocarcinoma cell line SPC-A-1/CDDP (Chen et al., 2001). To facilitate the detection and characterization of CA916798, we first developed a polyclonal antibody that specifically recognizes CA916798. The experiments presented here indicate that CA916798 is differentially expressed in drug-sensitive and MDR lung cancer cells. The diffuse cytoplasmic localization of CA916798 supports the notion that it may not be a typical membrane transporter protein. The differential expression of CA916798 between A549/CDDP and H446 cells provided an optimal experimental model system that covers two distinct histological subtypes of lung cancer and enables us to address its sufficiency as well as necessity in MDR.

Upon stable expression in drug-sensitive H446 cells, CA916798 dramatically enhanced cellular resistance to several common chemotherapeutic drugs. In particular, the cells became more resistant to CDDP. Mechanistic studies revealed that expression of CA916798 prevented CDDP-induced apoptosis and G2 cell cycle arrest, demonstrating its ability to induce MDR. Reduction of CA916798 expression in CDDP-resistant A549 cells by shRNA-mediated gene silencing restored the sensitivity of these cells to cytotoxic effects of a variety of chemotherapeutic agents, with the most increase to CDDP. These experiments also suggest that CA916798 confers MDR, at least partially, through the induction of apoptosis and G2 arrest.

The limited ability to knockdown CA916798 on mRNA and protein levels (approximately 40%, at the best) in A549/CDDP cells could be due to the design of the shRNA constructs. However, as sub-optimal knockdown levels were obtained with all four shRNA constructs, CA916798 might be essential for the survival of A549/CDDP cells; therefore, cells with very low levels of CA916798 would not survive the selection with G418. Although the knock-down of CA916798 by shRNA was not complete, we did observe a significant enhancement of cell sensitivity to chemotherapeutic drugs, indicating the potency of this gene in regulating MDR. Consistent with our finding, Li et al. recently reported that CA916798 essentially regulates chemosensitivity in H446/CDDP cells (Li et al., 2011).

In summary, we have presented evidence that CA916798 is crucial for maintaining MDR in human lung cancer cells. This research suggests that CA916798 may be a target for restoring the sensitivity of tumor cells to chemotherapeutic agents. The experimental system designed here will allow further examination to determine the exact mechanisms by which CA916798 mediates MDR and the methods to overcome this resistance.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30470773; 81102857).

References


