Knockdown of PDIA6 Inhibits Cell Proliferation and Enhances the Chemosensitivity in Gastric Cancer Cells

Chao Yan1,*, Xiaolei Song2,*, Su Wang3, Jinhai Wang4, Lu Li4

1Department of Radiation Oncology, Qilu Hospital (Qingdao), Cheiloe College of Medicine, Shandong University, Qingdao 266035, People’s Republic of China; 2Intervention Department, Qilu Hospital (Qingdao), Cheiloe College of Medicine, Shandong University, Qingdao 266035, People’s Republic of China; 3Department of General Surgery, Qingdao Chengyang People’s Hospital, Qingdao 266109, People’s Republic of China; 4Gastroenterology Department, The Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710004, People’s Republic of China

*These authors contributed equally to this work

Background: Protein disulphide isomerase A6 (PDIA6), a member of the disulphide isomerase (PDI) family, has been reported to be closely associated with progression of various cancers. However, the specific effects of PDIA6 on gastric cancer (GC) remain unclear. In this study, we investigated the expression pattern and biological functions of PDIA6 in GC.

Materials and Methods: The CCK-8 assay was carried out to examine cell proliferation and cisplatin cytotoxicity. The Western blot analysis was used to measure the protein expression of PDIA6, Wnt3a and β-catenin. The xenograft tumor assay was performed to evaluate the in vivo effect of PDIA6 on GC cell proliferation and chemoresistance.

Results: PDIA6 was significantly elevated in GC tissues and cell lines. Down-regulation of PDIA6 inhibited GC cell proliferation and chemoresistance to cisplatin while up-regulation of PDIA6 promoted the proliferation and chemoresistance of GC cells. Besides, PDIA6 regulated the chemosensitivity of GC cells to cisplatin in vivo. Mechanically, PDIA6 served as a regulator of the Wnt/β-catenin signaling pathway by affecting the protein expression of Wnt3a and β-catenin in GC cells. Additionally, Wnt activator reversed the inhibitory effect of PDIA6 knockdown on cisplatin resistance in GC cells.

Conclusion: These findings provided new insight into the potential role of PDIA6 as a promising target for drug resistance in GC chemotherapy.

Keywords: PDIA6, proliferation, chemosensitivity, gastric cancer

Introduction

Gastric cancer (GC), one of the most common and fatal malignancies, is the third leading cause of cancer-associated death in the world.1 With development of therapeutic approaches, the mortality of GC patients has fallen dramatically over the past decades.2,3 But, due to atypical symptoms at an early stage, a majority of GC patients are diagnosed at a late stage, resulting in an unfavorable prognosis.4 In this case, chemotherapy is considered a main therapeutic strategy to improve the average survival and quality of life in GC patients.5,6 However, chemoresistance, a major obstacle for cancer therapies, becomes common during treatment.7 Thus, it is urgently required to explore novel chemotherapeutic strategies for enhancement of chemosensitivity.

Protein disulphide isomerase (PDI), the first folding catalyst isolated from rat livers, abounds in many tissues.8 So far, 21 members have been identified in the PDI family and these proteins have different enzymatic properties.9,10 PDI could be induced under endoplasmic reticulum stress and is capable of isomerizing, forming
and rearranging protein disulphide bonds.\textsuperscript{11-13} Besides, increasing evidence has demonstrated an important role of PDI in both the physiology and pathophysiology of disease states such as neurodegenerative conditions and the pathogen entry in infectious diseases.\textsuperscript{14,15} Recently, many studies have reported an association between the PDI family and cancer progression. For example, PDIA6, a member of the PDI family, was found serving as an oncogene in cervical cancer.\textsuperscript{16} In addition, PDIA6 was demonstrated to be a mediator of chemoresistance in lung adenocarcinoma.\textsuperscript{17} However, the specific effects of PDIA6 on GC remain unclear.

In this study, we investigated the biological functions of PDIA6 in GC. We observed that PDIA6 was overexpressed in GC tissues and cell lines. Knockdown of PDIA6 inhibited GC cell proliferation in vitro and in vivo. In addition, we found that PDIA6 could affect chemoresistance in GC cells partly via the Wnt/β-catenin pathway.

Materials and Methods
Patients and Tissue Samples
A total of 48 patients from Qilu Hospital, Cheeloo College of Medicine (Qingdao, China) were enrolled in this study. All patients received no preoperative therapies and provided written informed consent. GC tissues and adjacent normal tissues were collected and frozen in liquid nitrogen and stored at $-80^\circ$C. The study was approved by the Ethics Committee of Qilu Hospital (Qingdao), Cheeloo College of Medicine.

Cell Lines and Cell Culture
GC cell lines (BGC-823 and MKN-45) and normal gastric epithelial cell line GES-1 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 U/mL penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO$_2$.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)
Total RNA was isolated from tissues or cells using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using PrimeScript\textsuperscript{TM} RT Reagent Kit (Takara Biotecnology, Dalian, China). The following primers were used: PDIA6, 5’-TGGATCCACAAAAACAGACC-3’ (forward) and 5’-CTCAAGCGGACATCTACAAT-3’ (reverse); GAPDH, 5’-ACCACGTCCATGCCCATCAC-3’ (forward) and 5’-TCCACACCTGTTGCTGT-3’ (reverse). The thermocycling conditions were: 96°C for 10 min, 45 cycles of 96°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec. The relative mRNA expression level was normalized to GAPDH and calculated using the $2^{-\Delta\DeltaCT}$ method.

Western Blot Analysis
Cells were lysed in RIPA buffer on ice and centrifuged at 15,000 g for 15 min. Protein concentration was measured using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). An equal amount of protein was separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking in 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against PDIA6, Wnt3a, β-catenin and GAPDH. Subsequently, the membranes were washed three times with TBST and incubated with appropriate secondary antibody for 1 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) and quantified by BandScan5.0.

Cell Transfection
Short hairpin RNA (shRNA) against PDIA6 (shPDIA6) and control shRNA (shNC) were synthesized by Ribobio (Guangzhou, China). The pcDNA3.1-PDIA6 expression vector and the empty vector were purchased from GeneCopoeia (Rockville, MD, USA). Cells were seeded in a 24-well plate and cultured for 24 h. Subsequently, cells were transfected with shPDIA6 or pcDNA3.1-PDIA6 using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Forty-eight hours later, the knockdown or overexpression efficiency was confirmed by the RT-qPCR and Western blot analysis.

CCK-8 Assay
Cell proliferation was detected using the CCK-8 assay. After transfection, cells were seeded in a 96-well plate at a density of $2\times10^5$ cells/well and incubated for different times. Then, CCK-8 solution (Dojindo, Japan) was added to each well at different time points and cells were further incubated for 2 h. The OD value was measured at 450 nm using a microplate reader.
The cytotoxicity assay was used to evaluate the effect of cisplatin. After overnight incubation, transfected cells were treated with different concentrations of cisplatin and cultured for 48 h. Subsequently, CCK-8 solution was added to each well. Cell viability was detected at 450 nm using a microplate reader. The survival of control cells was set at 100% and IC₅₀ was calculated using CalcuSyn software.

In vivo Xenograft Tumor Assay
Female BALB/c nude mice, aged 4 to 5 weeks old, were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and maintained under a specific pathogen-free condition in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Qilu Hospital, Cheeloo College of Medicine. Transfected BGC-823 cells (5 × 10⁶) were subcutaneously injected into left flank of mice. After palpable tumors were formed (tumor volume reached about 100 mm³), the mice were randomly divided into four groups (n=4) and intraperitoneally injected with or without cisplatin (5 mg/kg) twice a week. Tumor size was measured every week. Tumor volume was calculated by the following formula: V= length × width² × 0.5. Five weeks later, mice were euthanized and tumors were stripped and weighed.

Statistical Analysis
Data were shown as means ± standard deviation (SD) and evaluated using SPSS 19.0 software. Comparison of different groups was performed using Student’s t-test or one-way ANOVA. Graphs were generated using GraphPad Prism 5.0 software. P < 0.05 was considered statistically significant.

Results
The Expression of PDIA6 is Increased in GC Tissues and Cell Lines
To elucidate the role of PDIA6 in GC, we first determined the expression of PDIA6 in GC tissues using RT-qPCR and Western blot analysis. The results showed that PDIA6 had a higher expression in GC tissues at both mRNA and protein levels than in corresponding normal tissues (Figure 1A and B). We also examined the expression levels of PDIA6 in GC cell lines (BGC-823 and MKN-45). We found that PDIA6 was overexpressed in BGC-823 and MKN-45 cell lines in comparison with the normal gastric epithelial cell line GES-1 (Figure 1C and D).

Down-Regulation of PDIA6 Inhibits GC Cell Proliferation
To further evaluate the specific effects of PDIA6 on cell proliferation, PDIA6 in GC cells was knocked down via shRNA transfection. The transfection efficiency was assessed by the RT-qPCR and Western blot analysis. The results showed that PDIA6 expression was significantly reduced in BGC-823 and MKN-45 cells at both mRNA and protein levels after shRNA transfection (Figure 2AD). Cell proliferation was measured using the CCK-8 assay. As shown in Figure 2E, the proliferative rate of BGC-823 cells was significantly reduced by PDIA6 knockdown in comparison with the control cells. We obtained similar results in MKN-45 cells (Figure 2F).

Up-Regulation of PDIA6 Promotes GC Cell Proliferation
To verify the tumor-promoting effect of PDIA6, the expression of PDIA6 was increased in GC cells via plasmid transfection. The overexpression efficiency was confirmed by the RT-qPCR and Western blot analysis. As expected, the mRNA and protein expression of PDIA6 were obviously up-regulated in BGC-823 and MKN-45 cells after plasmid transfection (Figure 3AD). The CCK-8 assay was performed to evaluate cell proliferation. The results showed that PDIA6 up-regulation obviously accelerated the proliferation of BGC-823 and MKN-45 cells in comparison with corresponding control cells (Figure 3E and F).

Down-Regulation of PDIA6 Inhibits Chemoresistance of GC Cells to Cisplatin
We also investigated the potential role of PDIA6 in chemoresistance of GC cells to cisplatin. The sensitivity of shPDIA6-transfected BGC-823 and MKN-45 cells to cisplatin was assayed 48 h after drug treatment. As shown in Figure 4A and B, knockdown of PDIA6 markedly enhanced the effect of cisplatin on BGC-823 cells and decreased the IC₅₀ value of cisplatin in BGC-823 cells in comparison with the corresponding control cells. We obtained similar results in MKN-45 cells (Figure 4C and D).

Up-Regulation of PDIA6 Promotes Chemoresistance of GC Cells to Cisplatin
Then, we explored the effect of PDIA6 up-regulation on chemoresistance of GC cells to cisplatin. The sensitivity of PDIA6-overexpressed BGC-823 and MKN-45 cells to cisplatin was measured 48 h after drug treatment. The results
showed that PDIA6 up-regulation obviously blocked the effect of cisplatin on BGC-823 and MKN-45 cells and increased the IC_{50} value of cisplatin in these cells in comparison with corresponding control cells (Figure 5AD).

**PDIA6 Serves as a Regulator of the Wnt/β-Catenin Signaling Pathway**

Previous studies have shown that the Wnt/β-catenin signaling pathway plays an essential role in chemoresistance of GC cells,\(^1\),\(^8\) thus we examined whether this pathway was associated with the promoting effect of PDIA6 knockdown on chemosensitivity of GC cells to cisplatin. The results showed that the protein expression levels of Wnt3a and β-catenin were remarkably decreased by PDIA6 knockdown in BGC-823 and MKN-45 cells (Figure 6A and B). In contrast, PDIA6 up-regulation increased the protein expression of Wnt3a and β-catenin in BGC-823 and MKN-45 cells (Figure 6C and D). These observations suggested PDIA6 as a regulator of the Wnt/β-catenin signaling pathway.

**Wnt Activator Reverses the Inhibitory Effect of PDIA6 Knockdown on Cisplatin Resistance in GC Cells**

To further investigate the involvement of Wnt/β-catenin pathway in PDIA6-regulated chemoresistance of GC cells, we used the Wnt pathway activator LiCl for the rescue experiments. The results showed that LiCl significantly restored the expression of Wnt3a and β-catenin in PDIA6-knockdown BGC-823
Figure 2 Down-regulation of PDIA6 inhibits GC cell proliferation. (A–D) PDIA6 knockdown in BGC-823 and MKN-45 cells was measured by the RT-qPCR and Western blot analysis after transfection. (E and F) BGC-823 and MKN-45 cell proliferation were measured using the CCK-8 assay. *p < 0.05.
Figure 3 Up-regulation of PDIA6 promotes GC cell proliferation. (A–D) PDIA6 overexpression in BGC-823 and MKN-45 cells was verified by the RT-qPCR and Western blot analysis after transfection. (E and F) The CCK-8 assay was performed to assess BGC-823 and MKN-45 cell proliferation. *p < 0.05.
and MKN-45 cells (Figure 7A and B). Furthermore, we found that LiCl partially reversed the inhibitory effect of PDIA6 knockdown on BGC-823 and MKN-45 cell proliferation (Figure 7C and D). We also observed that the suppressive effect of PDIA6 knockdown on cisplatin resistance in BGC-823 and MKN-45 cell was weakened by LiCl while the promoting effect of PDIA6 overexpression on cisplatin resistance was enhanced after LiCl treatment (Figure 7EH).

**PDIA6 Regulates the Chemosensitivity of GC Cells to Cisplatin in vivo**

To verify the tumor-promoting effect of PDIA6 in vivo, the xenograft tumor assay was performed. BGC-823 cells transfected with shPDIA6 or shNC were subcutaneously injected into nude mice. After formation of palpable tumors, the mice were treated with or without cisplatin. Tumor size was measured every week. Five weeks later, the mice were euthanized and tumors were stripped and weighed. As shown in Figure 8A, PDIA6 knockdown significantly inhibited tumor growth and meanwhile potentiated the suppressive effect of cisplatin. Consistently, PDIA6 down-regulation markedly reduced tumor weight and simultaneously enhanced the effect of cisplatin (Figure 8B).

**Discussion**

Accounting for a high morbidity and mortality in the world, GC remains a focus of public health issues. The absence of specific symptoms makes early diagnosis of
difficult, generally resulting in a poor prognosis.\textsuperscript{21,22} This circumstance is worsened by development of chemotherapeutic resistance.\textsuperscript{7} Therefore, elucidation of the molecular mechanism underlying chemoresistance in GC will deliver significant benefits to GC treatment.

PDIA6, a member of the PDI family, is ubiquitously expressed in diverse human tissues and could be induced under ischemic and hypoxic stress.\textsuperscript{23} Recently, PDIA6 has been reported to be closely associated with progression of various cancers. For example, Ramos et al demonstrated overexpression of PDIA6 in breast cancer and suggested PDIA6 as a prognostic marker of aggressiveness.\textsuperscript{24} In this study, we found that the expression of PDIA6 was elevated in GC tissues and cell lines. In addition, knockdown of PDIA6 inhibited GC cell proliferation in vitro and in vivo. Like us, Gao et al reported that overexpression of PDIA6 increased the proliferative ability of cervical cancer cells accompanied with accelerated cell cycle progression.\textsuperscript{16} These observations proposed the role of PDIA6 as a tumor promoter in cancer progression.

As a first-line chemotherapeutic agent, cisplatin could cause DNA damage to eliminate proliferating cancer cells.\textsuperscript{25–27} But this anti-cancer effect is frequently

**Figure 5** Up-regulation of PDIA6 promotes chemoresistance of GC cells to cisplatin. BGC-823 (A and B) and MKN-45 (C and D) cells were treated with different concentration of cisplatin, followed by detection of cell viability and sensitivity to cisplatin. *p < 0.05.
limited by development of chemoresistance, finally resulting in therapeutic failure. 28–30 A previous study reported that PDIA6 could regulate the chemosensitivity to cisplatin in lung adenocarcinoma cells, 17 suggesting that PDIA6 may have an association with chemoresistance. In this study, we further examined the correlation between PDIA6 expression and chemosensitivity to cisplatin in GC cells in vitro and in vivo. The results showed that the IC_{50} value of cisplatin in GC cells was markedly decreased by PDIA6.

Figure 6 PDIA6 serves as a regulator of the Wnt/β-catenin signaling pathway. The protein expression of Wnt3a and β-catenin was detected by the Western blot analysis, after PDIA6 was knockeddown (A and B) or overexpressed (C and D) in BGC-823 and MKN-45 cells. *p < 0.05.
Figure 7. Wnt activator reverses the inhibitory effect of PDIA6 knockdown on cisplatin resistance in GC cells. BGC-823 and MKN-45 cells were transfected with shNC or shPDIA6 for 48 h in the presence of LiCl (20 mmol/L). (A and B) The protein expression of Wnt3a and β-catenin was detected by the Western blot analysis. (C–F) Cell viability was determined using the CCK-8 assay in the presence or absence of cisplatin. *p < 0.05 vs shNC; †p < 0.05 vs shPDIA6. BGC-823 and MKN-45 cells were transfected with pcDNA3.1 or pcDNA3.1-PDIA6 for 48 h in the presence of LiCl (20 mmol/L). (G and H) Cell viability was measured by the CCK-8 assay in the presence or absence of cisplatin. *p < 0.05 vs pcDNA3.1; †p < 0.05 vs pcDNA3.1-PDIA6.

Figure 8. PDIA6 regulates GC cell proliferation and chemoresistance in vivo. Mice were injected with shPDIA6- or shNC-transfected BGC-823 cells and then treated with or without cisplatin after formation of palpable tumors. Tumor volume (A) was observed every week and tumor weight (B) was measured after five weeks of injection. *p < 0.05.
knockdown. Furthermore, the sensitivity to cisplatin was significantly enhanced after PDIA6 knockdown in GC cells.

The Wnt/β-catenin signaling pathway is known as an important regulator of various crucial cellular processes. Moreover, the pathway has been demonstrated to play an essential role in chemoresistance of GC cells. Notably, PDIA6 was found interacting with the Wnt/β-catenin pathway to affect cancer progression. In this study, we showed that PDIA6 knockdown remarkably decreased the protein expression of Wnt3a and β-catenin in GC cells. Furthermore, we used the Wnt pathway activator LiCl to further investigate the involvement of Wnt/β-catenin pathway in PDIA6-regulated chemoresistance of GC cells. The results showed that LiCl significantly restored the expression of β-catenin in PDIA6-knockdown GC cells and meanwhile reversed the inhibitory effect of PDIA6 knockdown on cisplatin resistance in GC cells.

Conclusion
This study revealed that PDIA6 was overexpressed in GC tissues and cell lines. Down-regulation of PDIA6 inhibited GC cell proliferation and chemoresistance to cisplatin while up-regulation of PDIA6 promoted the proliferation and chemoresistance of GC cells. Besides, PDIA6 regulated the chemosensitivity of GC cells to cisplatin in vivo. Mechanically, PDIA6 served as a regulator of the Wnt/β-catenin signaling pathway by affecting the protein expression of Wnt3a and β-catenin and Wnt activator reversed the inhibitory effect of PDIA6 knockdown on cisplatin resistance in GC cells. These findings provided new insight into biological functions of PDIA6 and suggested it as a potential target for drug resistance in GC chemotherapy.

Disclosure
The authors declare that they have no conflicts of interest.

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