

Effect of ZEB2 silencing on cisplatin resistance in gastric cancer

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Abstract. – OBJECTIVE: To investigate the effect of ZEB2 silencing on cisplatin resistance in gastric cancer.

MATERIALS AND METHODS: The resulting cell line, SGC7901/DDP, was transfected with ZEB2 siRNA, non-specific siRNA, or vehicle control. The effectiveness of ZEB2 silencing was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot. MTT viability assay was used to determine the cisplatin-sensitivity of cells. Cell apoptosis was measured by flow cytometry.

RESULTS: A significant decrease in ZEB2 in mRNA and protein level was seen in cells transfected with ZEB2 siRNA, compared to that in cells transfected with non-specific siRNA or vehicle. Transfection with ZEB2 siRNA in cisplatin-resistant SGC7901/DDP cells resulted in a significant decrease in cell viability in response to the cisplatin treatment, and cell viability decreased with increasing cisplatin concentrations. A higher apoptotic rate was also seen in cells transfected with ZEB2 siRNA under cisplatin treatment.

CONCLUSIONS: ZEB2 silencing can effectively make gastric cells sensitive to cisplatin treatment *in vitro*.

Key Words:

ZEB2, Gastric cancer, Cisplatin, Resistance, siRNA.

chemotherapy drugs for gastric cancer. Unfortunately, initial cisplatin treatment frequently leads to recurrence of cancer, which unrelentingly retaliates with fast spreading and drug resistance². Thus, the acquisition of cisplatin-resistance seriously hampers the effectiveness of chemotherapy, and is associated with poor patient prognosis and survival. Although some drugs have been applied in combination to cisplatin to gastric treatment cancer, the effectiveness of these regimens to counter chemoresistance is still limited³. Therefore, it is imperative to elucidate how gastric cancer acquires cisplatin-resistance and develop therapeutic strategies to reverse chemoresistance. Epithelial-to-mesenchymal transition (EMT) has been associated with chemoresistance in various cancers⁴. During EMT, cells lose cell-cell adhesions and gain cell-matrix interaction, acquiring traits linked to enhanced invasion and migration abilities⁵. EMT also generates cancer stem cells (CSC), which are capable of initiating metastases in secondary organs⁴. Recent research⁶ provided additional evidence about the association between HOXA13 upregulation and gastric cancer progression. Also, it showed that HOXA13 contributes to invasion and EMT of gastric cancer cells via the TGF- β signaling pathway. The low expression of miR-195 played important roles in the pathogenesis and development of gastric cancer, possible by influencing the proliferation and growth of gastric cancer cells. In clinical practice, the detection of miR-195 played a certain role in guiding the treatment and prognosis of patients with gastric cancer⁷. Reportedly, miR-138 sensitized NSCLC cells to ADM through regulation of EMT regulator ZEB2, these findings provided new insight into the mechanism responsible for the chemoresistance in human NSCLC and implied that miR-138 may serve as a potential

Introduction

Gastric cancer is the second leading cause of cancer-related death worldwide, with the highest incidence rate in Eastern Asia¹. While surgery is primarily used for the treatment of early-stage gastric cancer, many patients develop advanced-stage cancers, or experience relapse after surgery, and therefore, require chemotherapy. Currently, cisplatin is one of the first-line

therapeutic candidate in drug-resistant NSCLC patients⁸. Zinc finger E-box-binding homeobox 2 (ZEB2) is a transcription factor that intracellular promotes EMT by inhibiting E-Cadherin expression. It has been reported that ZEB2 overexpression is clinically associated with the poor survival of patients with colorectal cancer⁹, prostate cancer¹⁰, pancreatic cancer¹¹, etc. It also maintains the stemness of cancer cells. Therefore, suppressing ZEB2 activation is a promising approach for suppressing cancer by inhibiting EMT. This possibly deprives cancer of chemoresistance. RNA interference using small interfering RNA (siRNA) is an effective method for gene silencing. These specifically designed double-stranded RNAs interfere the expression of target genes that possess a homologous sequence with the siRNAs. RNA interfering with siRNA has used to develop novel cancer therapies whereby conventional treatments lack efficacy¹². Previously, a lot of efforts have been devoted to silencing ZEB1 with siRNA, in which reversal of cancer EMT characteristics has been observed¹³⁻¹⁵. This also holds promise to re-sensitize cancer cells to chemotherapy. Despite the functional role of ZEB2 has been revealed, few researches have focused on ZEB2 silencing in cancers, particularly gastric cancer. Herein we set force to explore the effectiveness of ZEB2 siRNA silencing to sensitize cisplatin-resistant human gastric cancer cells SGC7901/DDP. The silencing efficiency was evaluated and the effects on sensitivity to cisplatin and cell apoptosis were demonstrated *in vitro*. The results of this study justified the use of ZEB2 siRNA in combination with cisplatin to treat gastric cancer.

Materials and Methods

Cell Culture and Establishment of Cisplatin-resistant Gastric Cancer Cell Line

The human gastric cancer cell line, SGC7901 was purchased from (Kaiji Biotechnology, Nanjing, China). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Siji Pharmaceutical Co., Ltd., Hangzhou, China), 100 µg/mL penicillin and 100 µg/mL streptomycin (Huabei Pharmaceutical Co., Ltd., Shijiazhuang, China). Cells were placed in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) maintained at 37°C and 5% CO₂. SGC7901 was continuously

cultured and passaged in medium containing cisplatin (Qilu Pharmaceutical Co., Ltd., Jinan, Shandong, China) at concentrations increasing from 0.06 µg/mL to 2 µg/mL over six months. The resultant cisplatin-resistant cells, SGC7901, demonstrated normal growth in 2-µg/mL cisplatin, and were kept in cisplatin-containing medium during further studies.

Cell Viability Analysis

MTT cell viability kit (Sigma-Aldrich, St. Louis, MO, USA) was used to characterize the effect of ZEB2 silencing on the cisplatin-sensitivity of SGC7901/DDP cells. Cells were treated with 5 µg/mL, 10 µg/mL, 15 µg/mL and 20 µg/mL of cisplatin. After treatment for 72 h, MTT was added to cells followed by dissolving formazan crystals formed in cell with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), and the resulting solution was analyzed by measuring absorbance in 490 nm using a plate-reader (Thermo Fisher, Waltham, MA, USA). The inhibitory rate (IR) was calculated using the following equation: $IR = (OD_{control} - OD_{model}) / OD_{control}$, in which OD_{control} and OD_{model} represent absorbance of solution from control group and model group, respectively.

Cell Apoptosis Assay

Cells were digested using trypsin (Sigma-Aldrich, St. Louis, MO, USA), centrifuged and resuspended to the concentration of 1×10⁶ cells/mL. The suspension of 100 µL was transferred to a 5 mL test tube. FITC Annexin V (Biyuntian Biotechnological Institute, Shanghai, China) of 5 µL and propidium iodide (PI) (Biyuntian Biotechnological Institute, Shanghai, China) of 5 µL were added to the solution, and mixed by gentle shaking. The mixture was incubated in the dark at room temperature for 15 min. Annexin V binding buffer (400 µL) were then added followed by incubation for 20 min at 4°C. Annex-V and PI markers were analyzed using a flow cytometer (Beckman Coulter, Mississauga, ON, Canada).

Transfection of ZEB2 siRNA

The cisplatin-resistant SGC7901/DDP cells were divided into the following groups: (1) mock group, which received treatment with empty Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); (2) negative control (NC) group, which received nonspecific siRNA delivered by Lipofectamine 2000; (3) model group, which received ZEB2-siRNA delivered by lipofectamine 2000.

The ZEB-2 siRNA were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection was performed by adding 20 μ L Lipofectamine 2000 mixed with siRNA into 230 μ L serum-free medium, followed by adding 8 μ L plasmid DNA into 250 serum-free medium and rested at room temperature for 5 min. The DNA- and lipofectamine-containing medium were then mixed and incubated at room temperature for 20 min. The mixture was then added to the culture dishes with cells. After 6 h, RPMI medium containing 10% fetal bovine serum (FBS) was added. After 72 h, cells were collected for further studies.

RT-PCR

Total RNA from cells was isolated and purified using the Trizol kit (Thermo Fisher, Waltham, MA, USA). Synthesis of cDNA was performed using SuperScript II Cells Direct cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA), and 1 μ L of cDNA was used for qRT-PCR with SYBR Green Master kit according to manufacturer's recommendations. Amplified cDNA was electrophoresed in agarose gel, which was further stained with ethidium bromide (EB) for band visualization under UV light. Gel images were captured using a digital camera and ImageJ was used for image analysis. Band intensities were normalized to β -actin levels. Taq, dNTP, DL2000 DNA Marker, plasmid extraction kit, and DNA gel extraction kit used in this study were acquired from Dalian Bao Biotechnology (Dalian, Liaoning, China). PCR primers were synthesized by Shanghai Biotechnology (Shanghai, China) using following sequences: ZEB2 sense: 5'-AGGAGCAGGTAATCG-3'; anti-sense: 5'-TGGGCACTCGTAAGG-3'; β -actin: sense: 5'-TTGTTACCAACTGGGACG-3'; anti-sense: 5'-GGCATAGAGGTCTTTACGG-3'.

Western Blot Analysis

Cells were collected by trypsinization and centrifugation. The cell pellet was lysed and the protein content was quantified by BCA assay. Protein lysates of 30 μ g were boiled in 50 μ L 2 $\times$ laemmli buffer for 5 min, and was further loaded onto 10%-20% ready gel. Electrophoresis was performed at 20 V for 3 h using apparatus acquired from Bio-Rad (Hercules, CA, USA). Blotting was performed with polyvinylidene fluoride (PVDF) membranes at 20 V for 50 min and under dry conditions. The membranes were then washed and blocked with 5% non-fat milk. Primary antibody

against ZEB2 was then used for incubation of the membrane at 1000 dilution, at 4°C overnight. The membranes were then washed three times and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody at 4°C overnight. Antibodies were diluted according to manufacturer's guidelines. After washing for three times, chemiluminescence substrates were then added for band visualization. Protein expression was quantified based on band intensities normalized to β -actin levels. The rabbit anti-human ZEB2 antibody was acquired from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit IgG and rabbit anti-human β -actin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical Analysis

SPSS19.0 (IBM, Armonk, NY, USA) was used to analyze differences between groups. All data were represented in the format of mean $\pm$ SD. Differences were considered significant if $p < 0.05$.

Results

We first evaluated the knockdown efficiency of ZEB2 siRNA transfection. The DDP-resistant cell lines, SGC7901/DDP were subjected to transfection with Lipofectamine vehicle control (mock group), non-specific siRNA carrying Lipofectamine (NC group) and ZEB2 siRNA carrying Lipofectamine. As a result, a significant decrease in ZEB2 mRNA and protein levels was seen in cells that received ZEB2 siRNA transfection ($p < 0.05$ for comparison to other groups, Figures 1 and 2). In contrast, transfection with non-specific siRNA did not induce significant downregulation of ZEB2 (Figures 1 Figure 2). We next examined if ZEB2 silencing affected the cisplatin sensitivity in SGC7901/DDP. SGC7901/DDP cells were exposed to 5 μ g/mL to 20 μ g/mL of cisplatin and evaluated for inhibition ratio (IR). In overall, all groups exhibited higher IR in response to increasing concentrations of DDP ($p < 0.05$). Particularly, cells that receive ZEB2 siRNA transfection exhibited a much greater IR compared with those that received vehicle or non-specific siRNA transfection ($p < 0.05$) (Figure 3). However, when cisplatin concentration reached certain level, the difference was not significant. This result indicated that ZEB2 silencing could effectively sensitize SGC7901/DDP cells to cisplatin. To verify the increased cisplatin sensitivity due to ZEB2

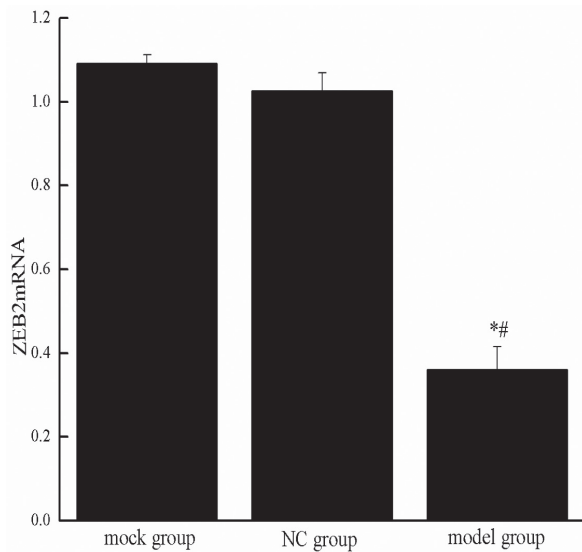


Figure 1. RT-PCR analysis of ZEB2 mRNA levels in SGC7901/DDP cells that received Lipofectamine (mock group), non-specific siRNA (NC group), or ZEB2 siRNA (model group). β -actin mRNA level was used as an internal control to normalize ZEB2 expressions.

silencing, we also performed apoptosis analysis using Annexin V-PI staining and flow cytometry. As shown in Figure 4, the cell population with high Annexin V and PI expression increased after ZEB2 silencing, indicating a higher apop-

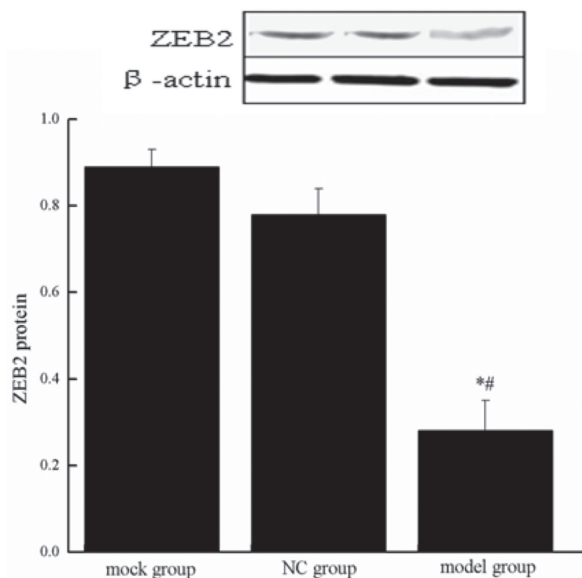


Figure 2. Western blotting analysis of ZEB2 protein expression in SGC7901/DDP cells that received Lipofectamine (mock group), non-specific siRNA (NC group), or ZEB2 siRNA (model group). ZEB2 band intensities were normalized to β -actin levels.

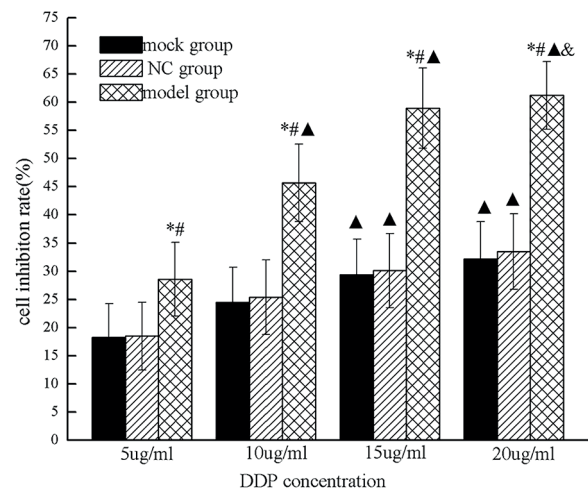


Figure 3. Effect of ZEB2 silencing in the DDP resistance in SGC7901 cells.

totic activity in these cells. Taken together, these data suggested that ZEB2 silencing is an effective strategy for sensitizing gastric cancer cells to DDP treatment.

Discussion

The vast majority of people are suffering from cancers^{16,17}. The siRNAs are a novel class of biopharmaceutical therapeutics with great potential for cancer therapy. With proper design, a wide range of cancer-related proteins can be silenced by siRNAs to suppress cancer progression^{18,19}. This has permitted rapid development of siRNA-based therapeutics. A few siRNA candidates have already entered clinical trials¹⁹. Due to the important role of EMT in cancer progression, EMT markers are commonly used as targets for gene therapy, among which transcription factors have generated particular interest^{20,21}. Inhibition of EMT markers has been widely utilized to attenuate chemoresistance in multiple cancers. Recent evidence indicated that ZEB2 overexpression was closely related to multi-drug resistance in lung cancer²², bladder cancer²³, and ovarian cancer²⁴. As a transcription factor that inhibits E-cadherin, ZEB2 was considered as master EMT activator and was associated with the malignant phenotypes of cancers. Nevertheless, no therapeutic strategies for gastric cancer based on ZEB2 silencing have been developed. Current studies are primarily focusing on the inhibition of ZEB family using microRNAs (miRNAs).

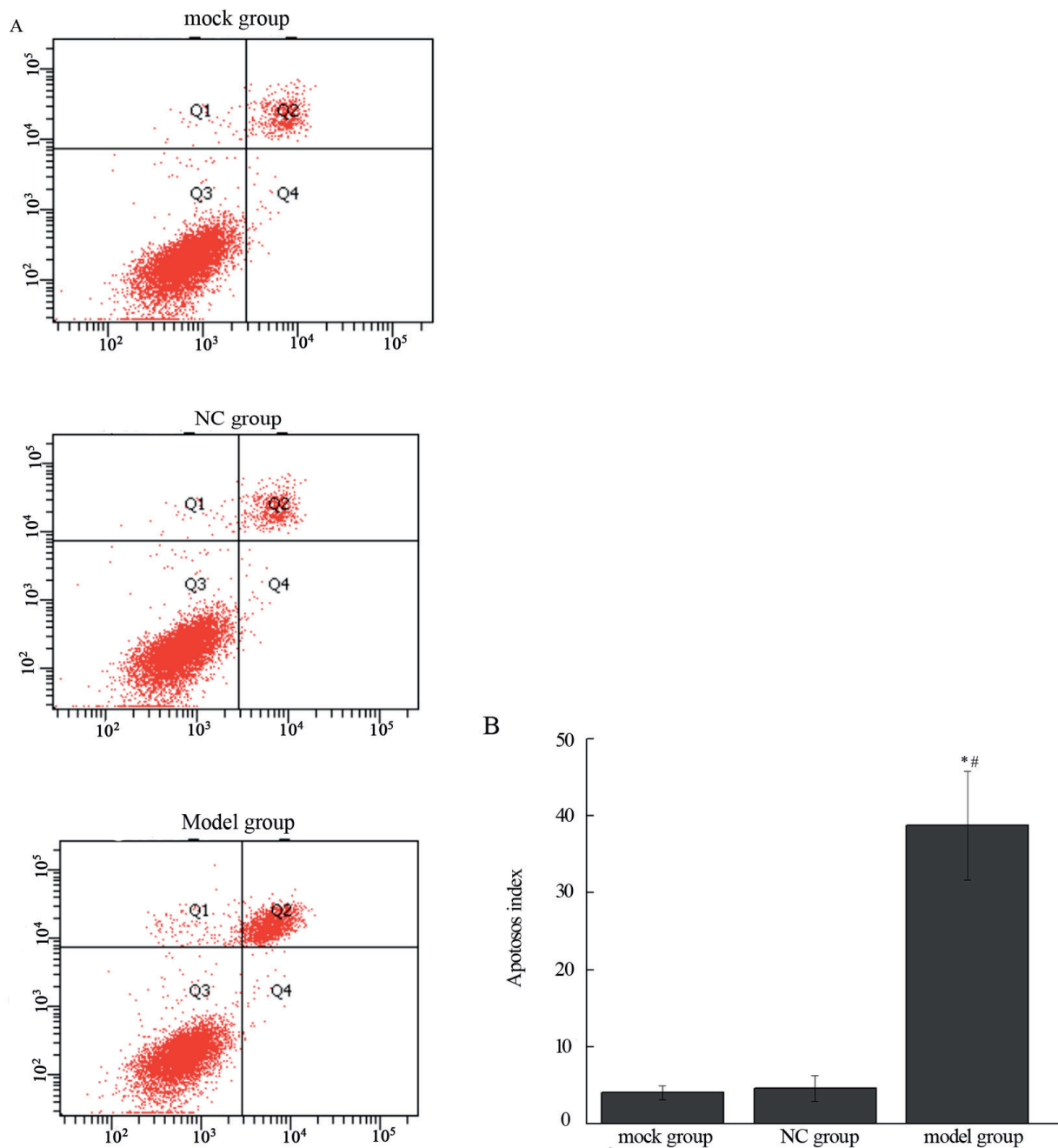


Figure 4. Effect of ZEB2 silencing in the DDP-induced cell apoptosis. **A**, Representative images of Annex V-PI flowcytometry showing apoptotic cell populations. Cells possessing high Annexin V FITC and PI markers were associated with high apoptotic activities. **B**, Bar graph showing the quantification of apoptosis in all groups.

MiRNAs are a class of non-coding RNAs that post-transcriptionally regulate multiple mRNAs to exert biological function. Specifically, the miR-200 family are considered the major regulators of ZEB1 and ZEB2²⁵. However, as miRNAs are broad-spectrum regulators of multiple genes, the effect of specific inhibition of ZEB2 needs to be

investigated. In the present study, we evaluated the effect of ZEB2 silencing using siRNA in modulating cisplatin resistance in gastric cancer cells. A cisplatin-resistant gastric cell line, SGC7901/DDP was first constructed by continuously exposing SGC7901 to cisplatin treatment. This cell line was then subjected to transfection with ZEB2

siRNA, and the effect on cisplatin sensitivity was monitored. Consistent with the role of ZEB2 in chemoresistance, we showed that ZEB2 silencing reduced cisplatin resistance. Considering that chemoresistance of gastric cancer, and subsequent unstoppable cancer growth and metastasis, are the major causes of cancer mortality, this RNA interference technology can potentially lead to complement existing chemotherapies to improve clinical outcomes.

Conclusions

Our preliminary results potentiated the use of siRNA technology in the treatment of gastric cancer. Enabled by siRNA delivery technologies²⁶, ZEB2 silencing could achieve by specifically delivering ZEB2 siRNA to malignant tumors to suppress cancer progression. Further, *in vivo* studies on ZEB2 silencing are warranted to verify the clinical utility of this technology.

Ethical Approval

The research was conducted in accordance with the Declaration of Helsinki and the United National Institutes of Health.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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