Downregulation of osteopontin enhances the sensitivity of glioma U251 cells to temozolomide and cisplatin by targeting the NF-κB/Bcl-2 pathway

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Abstract. Glioma is resistant to the apoptotic effects of chemotherapy and the mechanism underlying its chemoresistance is not currently understood. In a previous study, we reported that osteopontin (OPN) was overexpressed in glioma tissues and had an important anti-apoptotic effect. Furthermore, overexpression of OPN was observed following chemotherapy. To elucidate whether OPN plays a role in chemotherapy resistance and to investigate its downstream signaling pathway, this study used small interfering RNA (siRNA) to silence the expression of OPN in U251 human neuronal glioma astrocytoma cells. OPN downregulation in U251 cells enhanced the apoptotic effects induced by temozolomide (TMZ) and cisplatin (DDP). Furthermore, OPN siRNA suppressed the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation and B cell lymphoma 2 (Bcl-2) expression that was induced by chemotherapy. Taken together, these results demonstrated that the expression levels of OPN are involved in glioma chemoresistance. Knockdown of OPN through siRNA enhanced the effects of TMZ and DDP chemotherapy by targeting the NF-κB/Bcl-2 pathway.

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Introduction

Glioma is one of the most lethal types of cancer in the world and is a major cause of morbidity. Current treatment of glioma predominantly focuses on standard procedure options, including surgical intervention, radiotherapy, chemotherapy or a combination of these methods; however, there has been no significant improvement in prognosis (1). Glioma has proved to be resistant to chemotherapy (2), yet the molecular basis underlying this chemoresistance has not been fully understood. Novel treatment strategies for reducing chemoresistance are therefore required.

Osteopontin (OPN) is a secreted, integrin-binding phosphoglycoprotein that is overexpressed in several primary tumors. OPN has been shown to have numerous functions, including roles in cell migration and survival and manipulation of tumor phenotype (3). It has been reported that OPN expression is elevated in glioma tissues (4-6), which is consistent with the results of our previous study (7); however, less definitive evidence has been reported to explain the chemoresistance of glioma cells.

The nuclear factor (NF)-κB family contains p65/RelA, RelB, c-Rel, NF-κB1/p50 and NF-κB/p52, which dimerize and are held in the cytoplasm by specific proteins. NF-κB may be activated by the canonical or non-canonical pathway (8). The canonical pathway depends on the NF-κB essential modulator, I-kappa-B kinase (IKK)β activation and nuclear localization of RelA/p50 dimers; whereas the non-canonical pathway depends on IKKα activation, probably by the upstream NF-κB-inducing kinase nuclear localization of p52/RelB dimers (9). Activation of NF-κB has been reported to be associated with numerous types of cancer. It has been demonstrated to influence cancer initiation, promotion, and progression of lung (10,11), breast (12) and pancreatic cancer (13,14). In glioma, NF-κB was shown to have higher expression levels in glioblastoma (GBM) tissue, as compared with non-GBM tissue (15). McFarland *et al* (16) reported that NF-κB-induced interleukin-6 ensured STAT3 activation and tumor aggressiveness in glioblastoma. Bonavia *et al* (17) demonstrated that NF-κB was implicated in

the promotion of epidermal growth factor variant III, in glioma angiogenesis and growth.

In the present study, it was found that treatment with cisplatin (DDP) and temozolomide (TMZ) could induce overexpression of OPN in human neuronal glioma astrocytoma (U251) cells. Specific downregulation of OPN using RNA interference (RNAi) enhanced the sensitivity of glioma U251 cells to DDP and TMZ via targeting the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)/B cell lymphoma 2 (Bcl-2) pathway. This study has confirmed that OPN plays an important role in chemoresistance. Additionally, this is the first study, to the best of our knowledge, indicating that downregulation of OPN by siRNA can sensitize glioma cells to chemotherapy by suppressing the NF-κB/Bcl-2 signaling pathway.

Materials and methods

Reagents. Cell culture media and supplements were purchased from Gibco Life Technologies (Grand Island, NY, USA). Antibodies against GAPDH and Bcl-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The electrophoretic mobility shift assay (EMSA) kit was obtained from Beyotime Institute of Biotechnology (Shanghai, China). TMZ and DDP were purchased from Sigma (St. Louis, MO, USA). The terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture and treatment. The U251 human glioma cell line was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). U251 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and maintained in a 37°C, 5% CO_2 incubator and routinely passaged at two- to three-day intervals. U251 cells ($1x10^6$) were seeded in a 100-mm petri dish with 10 ml growth medium and were treated with TMZ (100 μM) and DPP (100 μM) for 24 h on the third day as indicated.

Lentiviral vector construction and infection. A lentivirus-based vector for small interfering RNA (siRNA) sequences targeting human OPN was constructed with technical support from Shanghai GeneChem (Shanghai, China). A short hairpin RNA (shRNA) template was designed and cloned into a lentivirus vector containing a U6 promoter upstream of the cloning restriction sites (*Age*I and *Eco*RII). The oligonucleotides encoding the shRNA were annealed and inserted between the *Age*I and *Eco*RII sites of the plasmid. The OPN siRNA vector (pGC-LV) and packaging vectors (pHelper 1.0 and pHelper 2.0) (Invitrogen Life Technologies, Carlsbad, CA, USA) were cotransfected into 293FT cells cells (Cyagen Biosciences Inc., Santa Clara, CA, USA) using Lipofectamine® 2000 (Invitrogen, Grand Island, NY, USA). The culture supernatants were collected, concentrated and stored at -70˚C. U251 cells were infected with lentiviral vectors at a multiplicity of infection of five in the presence of polybrene (10 μg/ml).

Western blotting. To determine the level of Bcl-2 expression, total protein was isolated in lysis buffer (137 mM NaCl, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 15 mM ethylene glycol tetra-acetic acid, 15 mM $MgCl_2$, 25 mM 3-morpholinopropane-1-sulfonic acid, 20 μM leupeptin and 100 μM phenylmethylsulfonyl fluoride, adjusted to pH 7.2). Equal amounts of protein (30 μg) were loaded into the sample wells and separated on a 12% SDS-PAGE gel. The electrophoresed proteins were transferred to a polyvinylidene fluoride Immobilon-P membrane (Millipore, Watford, UK) and subjected to immunoblot analysis with anti-Bcl-2 antibody. GAPDH was used as an internal control.

Quantitative polymerase chain reaction (qPCR). Total RNA was purified from U251 glioma cells using TRIzol® reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. In order to validate the differential relative expression of OPN, TaqMan-based qPCR was performed using a SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan) on the ABI Prism 7300 HT Sequence Detection system (Applied Biosystems Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. Primers used were: 5'-CTG TGC CAT ACC AGT TAA-3' (forward) and 5'-GAT GTC AGG TCT GCG AAA-3' (reverse), designed by Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA). The amplification of β-actin with primers 5'-AAG ACC TGT ACG CCA ACA CAG T-3' (forward) and 5'-AGA AGC ATT TGC GGT GGA CGA T-3' (reverse) was taken as an internal control. In order to determine the relative amounts of the products, the comparative Ct (threshold cycle) method was used according to the instructions supplied by Applied Biosystems Life Technologies.

EMSA. The procedure for the EMSA was followed as previously described (18). Briefly, nuclear proteins were isolated from U251 cells in each group. A total of 20 μg nuclear extract was preincubated for 10 min in binding buffer (1 μg poly(deoxyinosinic-deoxycytidylic) acid, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol and 1 μg/μl bovine serum albumin) on ice and consecutively reacted with $1x10^5$ dpm of γ-^{32}P- (Amersham Biosciences, Sunnyvale, CA, USA) labeled probe containing the NF-κB binding site (5'-AGT TGA GGG GAC TTT CCC AGG C-3') for 30 min at room temperature. The nuclear extracts were electrophoresed on a 6% polyacrylamide gel.

TUNEL. Groups of U251 cells were fixed with 4% paraformaldehyde and washed in phosphate-buffered saline. The TUNEL detection reagent was prepared according to the manufacturer's instructions (Beyotime Institute of Biotechnology). The U251 cells were incubated with the TUNEL detection reagent at 37˚C for 60 min and then examined using a fluorescence microscope (Axio Imager A1; Carl Zeiss AG, Oberkochen, Germany). DAPI staining was used as a control in the corresponding microscope field.

Statistical analysis. Experimental procedures were repeated three times and analyzed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to determine statistically significant differences at a threshold of $P<0.05$.

Results

TMZ and DDP induce OPN overexpression and NF-κB activation. To examine the protein and mRNA expression of OPN

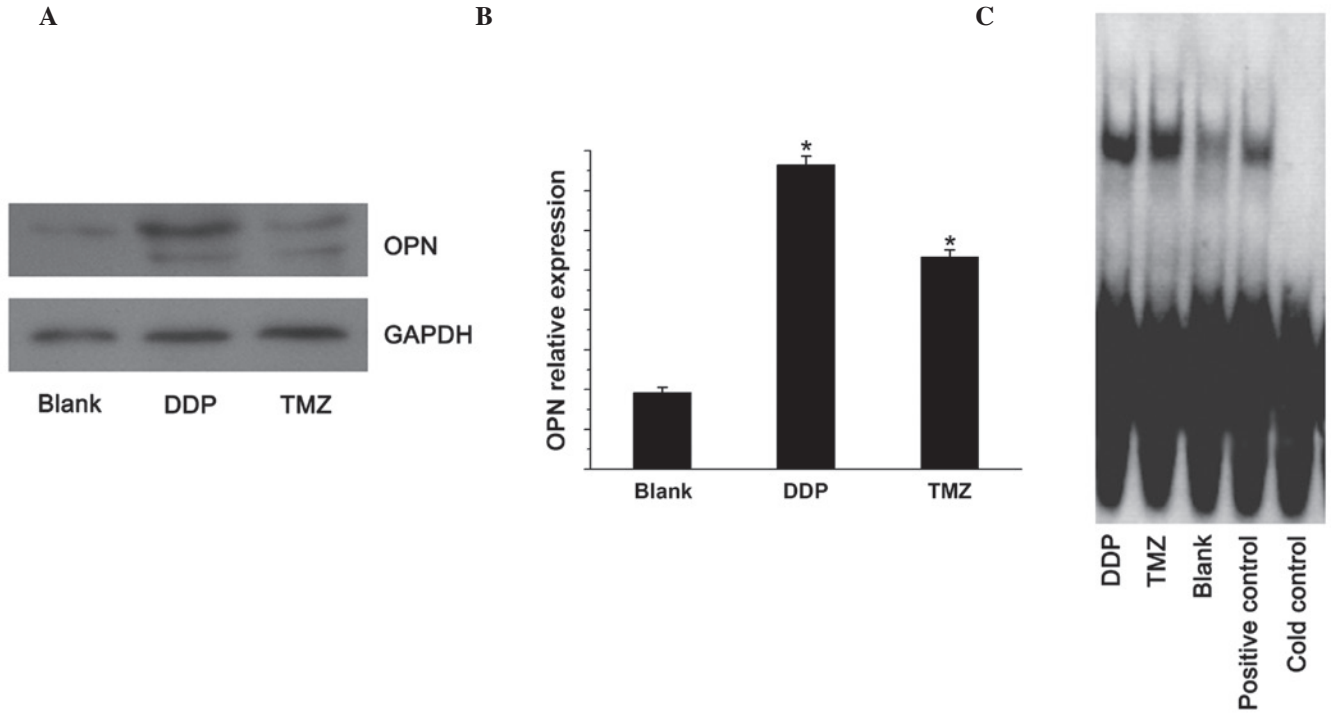

Figure 1. Expression of OPN was measured by (A) western blot analysis and (B) quantitative polymerase chain reaction. Cells treated with TMZ or DPP showed increased expression of OPN. The data represent the means ± standard deviation. (C) The activity of NF-κB was assessed by electrophoretic mobility shift assay. OPN and NF-κB were activated by chemotherapy. *P<0.05 versus blank group. OPN, osteopontin; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; TMZ, temozolomide; DPP, cisplatin.

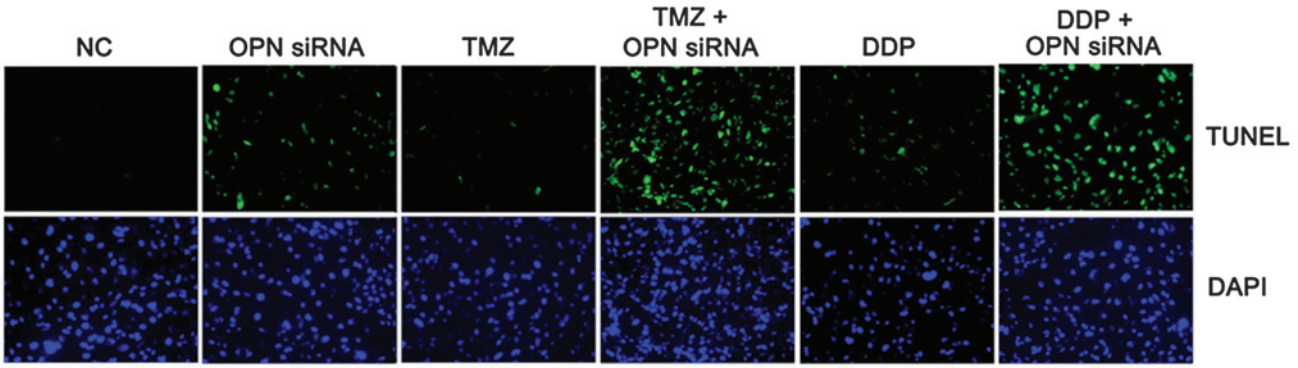

Figure 2. Evaluation of apoptosis in U251 cells by TUNEL assay. U251 cells were treated with TMZ or DDP, separately or in combination with OPN siRNA. The TUNEL assay was performed using an *in situ* cell death detection kit. DAPI staining was used as a control in the corresponding microscope field. Combining TMZ or DDP and OPN siRNA markedly induced apoptosis. OPN, osteopontin; TMZ, temozolomide; DPP, cisplatin; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; siRNA, small interfering RNA; NC, negative control.

in U251 cells treated with TMZ and DDP, western blotting was performed using specific antibodies for OPN and GAPDH (used as an internal control), and qPCR was conducted using specific primers for OPN and β-actin (used as an internal control), respectively. The levels of OPN protein and mRNA in U251 cells treated with TMZ and DDP were significantly higher than those in the negative controls (Fig. 1A and B). EMSA was subsequently used to measure the state of NF-κB activation. TMZ and DDP were shown to induce significant NF-κB activation (P<0.05 vs the blank control group; Fig. 1C).

OPN siRNA enhances apoptosis induced by TMZ and DDP in U251 cells. It was next assessed whether OPN contributed to the resistance of U251 cells to chemotherapy. Cells were infected with either OPN or control siRNA and then incubated with TMZ and DDP. It was found that U251 cells exhibited increased apoptosis when OPN was specifically knocked down by lentivirus-mediated OPN siRNA (Fig. 2). These findings suggested that OPN contributed to the resistance of glioblastoma cells to TMZ and DDP.

OPN siRNA enhances the sensitivity of glioma U251 cells to TMZ and DDP by targeting the NF-κB/Bcl-2 pathway. Constitutive NF-κB activation was evaluated by EMSA. TMZ and DDP induced significant activation of NF-κB as well as Bcl-2 expression in U251 cells. In addition, lentivirus-mediated OPN siRNA was shown to block NF-κB activation and Bcl-2 expression induced by TMZ and DDP (P<0.05; Fig. 3).

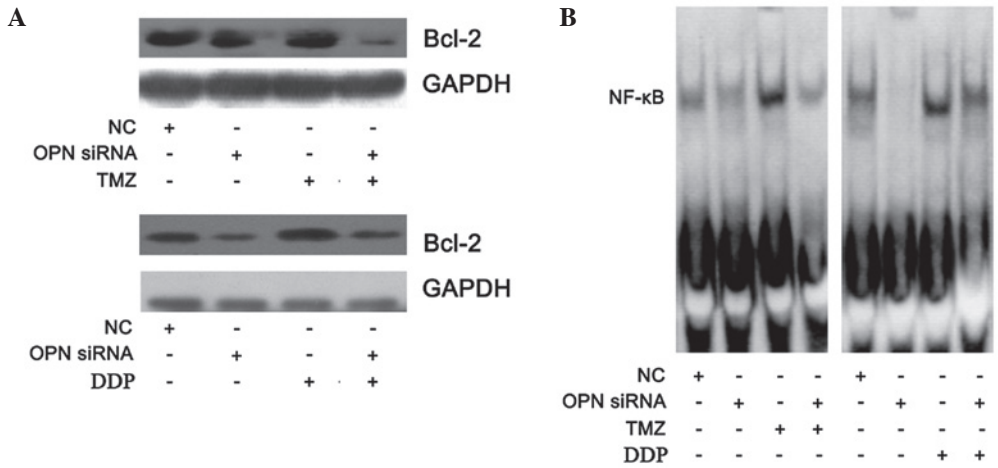

Figure 3. (A) Bcl-2 expression was induced by TMZ or DDP and downregulated by OPN siRNA. Plasmosin of U251 cells treated with TMZ or DDP, separately or in combination with OPN siRNA, was extracted for western blot analysis of Bcl-2. GAPDH was used as a loading control. (B) NF-κB activity was induced by TMZ and DDP, but downregulated by OPN siRNA. Nuclear protein was used for electrophoretic mobility shift assay of NF-κB. OPN, osteopontin; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; TMZ, temozolomide; DPP, cisplatin; Bcl-2, B cell lymphoma 2; NC, negative control.

Discussion

It is well known that glioma cells are resistant to chemotherapeutic drugs (19). However, the molecular mechanisms of chemoresistance in glioma cells are not fully understood. OPN is a secreted non-collagenous phosphoglycoprotein that has been shown to be commonly overexpressed in several types of tumor (20). It has been reported that OPN inhibits apoptosis of adherent endothelial cells deprived of growth factors (21) and prevents curcumin-induced apoptosis (22). In our previous study (7), it was found that OPN siRNA induced apoptosis of glioma cells. In the present study, it was found that TMZ and DDP could induce OPN overexpression. The aim of this study was to determine whether OPN was involved in chemoresistance in glioma cells and to identify novel targeted strategies to enhance chemosensitivity through specific OPN inhibition. The dysfunction of apoptosis represents a critical step in tumorigenesis. Cancer cells commonly show resistance to radiotherapy and chemotherapy, and the overexpression of various anti-apoptotic proteins in cancer cells contributes to chemoresistance (23). In the present study, it was observed that OPN was significantly upregulated in DDP- and TMZ-treated U251 cells. Additionally, lentivirus-mediated OPN siRNA greatly enhanced DDP and TMZ-induced apoptosis in U251 cells. This suggests that DDP and TMZ treatments, in combination with lentivirus-mediated OPN siRNA, may provide a novel approach to sensitizing glioma cells to chemotherapy.

The transcription factor NF-κB serves as a principal mediator of resistance to chemotherapy in several types of tumor. Constitutive activation of NF-κB facilitates cancer cell survival and reduces sensitivity towards chemotherapeutic drugs (24). It has been reported that NF-κB activation in human colon cancers diminishes the level of apoptosis induced by chemotherapy, whilst inhibition of NF-κB has been shown to enhance the sensitivity to anti-neoplastic-induced apoptosis *in vitro* and *in vivo* (25-27). Bcl-2 overexpression has been shown to inhibit apoptosis *in vitro* in response to several chemotherapeutic agents (28), and resistance to chemotherapy in glioblastoma has been linked to the expression of genes of the Bcl-2 family (29,30). Targeting Bcl-2 may provide a novel therapeutic approach to overcome chemoresistance, as proposed for small-cell lung cancer (31). In the present study, U251 cells were examined by EMSA to determine changes in DNA binding of NF-κB in response to TMZ and DDP. It was found that treatment with TMZ and DDP could induce NF-κB activation. In addition, TMZ and DDP treatment increased Bcl-2 expression. It has previously been shown that NF-κB activates Bcl-2 expression in t(14;18) lymphoma cells (32). Furthermore, the NF-κB/Bcl-2 signaling pathway has been suggested to function in chemoresistance (33). In the present study, TMZ and DDP, in combination strategies with specific inhibition of OPN by siRNA, led to inhibition of NF-κB activation and Bcl-2 expression, which resulted in enhanced chemosensitivity. These data demonstrated that chemotherapy induced NF-κB activity in glioma cells, and specific inhibition of OPN blocked this activation of NF-κB and led to enhanced tumoricidal responses. The identification of OPN targets in chemoresistance may lead to novel target-directed strategies in the treatment of glioma cells to improve therapeutic responses.

In conclusion, the present study demonstrates for the first time, to the best of our knowledge, that treatment with DDP and TMZ can induce increased OPN expression in U251 cells, which results in activation of NF-κB and an increase in Bcl-2 expression. Specific downregulation of OPN with RNAi enhanced the sensitivity of glioma U251 cells to DDP and TMZ. These results demonstrate that lentiviral-mediated OPN siRNA can sensitize U251 cells to chemotherapy by targeting the NF-κB/Bcl-2 pathway.

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