Silencing of TGIF sensitizes MDA-MB-231 human breast cancer cells to cisplatin-induced apoptosis

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Abstract. The present study was designed to explore the sensitivity of MDA-MB-231 cells to cisplatin after silencing the expression of TG-interacting factor (TGIF) protein. Cell viability was measured using an MTT assay. Cell apoptosis was detected by the annexin V and dead cell assay and the Hoechst staining assay. Protein expression was analyzed using western blot analysis. A colony formation assay was also performed. It was observed that cisplatin reduced the expression of TGIF protein in a dose- and time-dependent manner. Silencing TGIF significantly suppressed the cell proliferation and colony formation in MDA-MB-231 cells with the treatment of cisplatin. Results indicated that silencing TGIF could dramatically increase the cisplatin-induced apoptosis rate in MDA-MB-231 cells. The expression of PARP and caspase-3 proteins was correlated with the effect that silencing TGIF enhanced cisplatin sensitivity in MDA-MB-231 cells. The present data showed that silencing TGIF promoted apoptotic sensitivity that was induced by cisplatin in MDA-MB-231 human breast cancer cells and suggested that TGIF might be a therapeutic target for improving the chemotherapy response in triple-negative breast cancer.

Introduction

Triple-negative breast cancer (TNBC) is one breast cancer subtype that is characterized by the lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (1,2). TNBC accounts for 9-21% of all breast cancers and has higher metastatic and recurrence rates and a poorer prognosis than other breast cancer subtypes (2-5). Currently, chemotherapy is the only option for treating malignant breast cancer because of the lack of targeted molecules for disease treatment (6,7). Among the chemotherapy drugs, platinum agents have received much attention in the treatment of TNBC (8). However, the growing chemotherapy resistance and side effect problems restrict its clinical application (9,10). Therefore, exploring the underlying mechanisms of drug resistance and seeking a new target for TNBC chemotherapy is needed.

TG-interacting factor (TGIF), a member of the three-amino-acid loop extension (TALE) subfamily of atypical homeodomain proteins, is involved in multiple signaling pathways (11-17). Since TGIF was identified to be involved in holoprosencephaly development (18), it has been implicated in diverse biological and pathological functions. Recently, numerous studies have focused on the potential roles of TGIF in tumor development, invasion and migration in various types of cancers, including lung cancer (19-21), liver cancer (22,23), ovarian cancer (24), leukemia (25), gastric carcinoma (26), upper urinary tract urothelial carcinoma (27) and esophageal squamous cell carcinoma (28). However, research regarding the correlation of TGIF protein with TNBC is limited. Prof. Atfi's laboratory revealed that TGIF expression was much higher in TNBC patients compared with normal tissue or other breast cancer subtypes. Moreover, the elevated levels of TGIF correlated with a high relapse and mortality rate of TNBC patients (29,30). Kwon *et al* reported that targeted interference of SIN3A-TGIF function by SID decoy treatment inhibited Wnt signaling and invasion in TNBC cells (31). Together, previous papers suggested that TGIF protein might be a target for TNBC chemotherapy. To the best of our knowledge, there is no publication focusing on the relationship between TGIF silencing and cisplatin-induced apoptotic sensitivity in TNBC cells. In the present study, we used the representative TNBC cell line of MDA-MB-231 to observe the effects of TGIF silencing on cisplatin-induced apoptosis.

Materials and methods

Cell culture and cell transfection. The MDA-MB-231 cell line was maintained in our laboratory and cultured in DMEM

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supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum (FBS), and 2 mM L-glutamine in a humidified atmosphere that contained 5% CO_2 at 37˚C. TGIF shRNA human (h) lentiviral particles (sc-36659-V) and control shRNA lentiviral particles-A (sc-108080) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The shRNA lentiviral particles against TGIF were infected into MDA-MB-231 cells according to the manufacturer's instructions. Next, the stable clones expressing shRNA were initially selected by 10 μg/ml of puromycin for three weeks (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The TGIF expression level of the infected cells was detected by western blot to confirm the transfection efficiency. Cells that were stably transfected with the TGIF shRNA (h) lentiviral particles and control shRNA lentiviral particles were named MDA-MB-231-shRNA-TGIF cells and MDA-MB-231-shRNA-control cells, respectively.

Western blot analysis. Cell lysates were prepared in a RIPA buffer (Pierce; Thermo Fisher Scientific, Inc.), and a BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.) was conducted to quantify the protein concentration. The samples were then separated on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE) and proteins were transferred onto a nitrocellulose (NC) membrane. After blocking with 5% bovine serum albumin (BSA)/Tris-buffered saline Tween-20 (TBST) for 1 h, the membrane was incubated with primary antibody overnight at 4˚C, followed by adsorption to peroxidase-coupled protein G (ZSGB-BIO, Beijing, China) for 1 h at room temperature. Antibodies against TGIF (sc-9084) and p21 (sc-397) were purchased from Santa Cruz Biotechnology, Inc., and antibodies against PARP (no. 9532S), Bax (no. 2772S), caspase-3 (no. 9665S) and caspase-9 (no. 9508S) were obtained from Cell Signaling Technology. Immunoreactive bands were visualized with a Bio-Rad Clarity™ western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Antibody to β-actin (sc-47778; Santa Cruz Biotechnology, Inc.) was used as a loading control.

MTT assay. Cell viability was determined by MTT assay. We collected the MDA-MB-231-shRNA-TGIF cells and MDA-MB-231-shRNA-control cells at a density of $5x10^4$/ml and then plated cells in 96-well plates at a density of $5x10^3$ cells per well (6-well per group). After incubation in culture medium for 24 h, the culture medium was replaced with the following concentrations of cisplatin at 0, 2.5, 5.0, 7.5 and 12.5 μg/ml and maintained for 48 h. Four h before the cisplatin treatment finished, 10 μl of 5 mg/ml MTT were added to each well. Then, 150 μl of DMSO were added to each well and the absorbance was determined on a micro-plate reader (Multiskan Ascent;. Thermo Labsystems; Thermo Fisher Scientific, Inc.) at 492 nm.

Annexin V and dead cell assay. Annexin V and dead cell assay was used to determine the cell populations in the apoptosis stage, including the early apoptotic cells and the late apoptotic cells. For the induction of apoptosis, cells were seeded in 60-mm plates and cultured for 24 h at 37˚C and then incubated for 48 h with cisplatin (12.5 μg/ml). Cells were trypsinized and resuspended in at least 1% FBS. The cell samples were incubated with Muse™ annexin V and dead cell reagent for 20 min at room temperature in the dark. The apoptosis rate was measured by the Muse™ Cell Analyzer with the Muse™ annexin V and dead cell software module.

Cell apoptosis analysis by hoechst 33258. The Hoechst staining kit (Beyotime Institute of Biotechnology, Haimen, China) was used to detect the state of nucleus condensation. Cells were seeded onto cover slides in 6-well plates overnight at 37˚C and then treated with 12.5 μg/ml of cisplatin for 48 h followed by adding 0.5 ml of fixation fluid for 30 min. After washing with phosphate-buffered saline (PBS) twice, 0.5 ml of Hoechst 33258 were added to the plate and incubated for 5 min. The stained cells were washed with PBS twice and then the nuclear morphology was observed under a fluorescence microscope (Zeiss AG, Oberkochen, Germany). The apoptotic cells showed condensed and fragmented nuclei.

Colony formation assay. A total of 400 cells were plated in 60-mm plates. After plating for 72 h, cells were treated with 2.5 μg/ml of cisplatin for 4 h and washed with HANKs three times. Cells were maintained in an incubator of 5% CO_2 at 37˚C for 18 days. The culture medium was changed every three days. At the end of the experiments, cells were washed with PBS and fixed with methanol for 30 min at room temperature and then stained with Giemsa for 30 min at room temperature. Colonies with more than 50 cells were counted under an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. All data were expressed as the mean ± standard deviation (SD). A Student's t test was applied to evaluate the difference of relative cell viability between groups at each dose of cisplatin treatment (0, 2.5, 7.5 and 12.5 μg/ml) and one-way ANOVA (LSD for post-hoc test) was applied to evaluate the difference among groups in additional experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cisplatin decreased the expression of TGIF protein in MDA-MB-231 cells. As shown in Fig. 1, the expression of TGIF protein was suppressed by cisplatin treatment from 48 h in MDA-MB-231 cells (Fig. 1A). We also tested the level of TGIF protein expression under different concentrations of cisplatin; Fig. 1B shows that cisplatin treatment suppressed TGIF protein expression in a dose-dependent manner in MDA-MB-231 cells.

TGIF silencing efficiency in MDA-MB-231 cells. Fig. 2A shows that the level of TGIF protein expression in shRNA-TGIF infected cells was markedly decreased compared with control cells. This suggested a successful construction of a stable TGIF-silenced MDA-MB-231 cell line.

Silencing TGIF increased the cytotoxicity of MDA-MB-231 cells to cisplatin. Fig. 2B and C demonstrated the effects of TGIF silencing on cisplatin-induced cytotoxicity. Our data

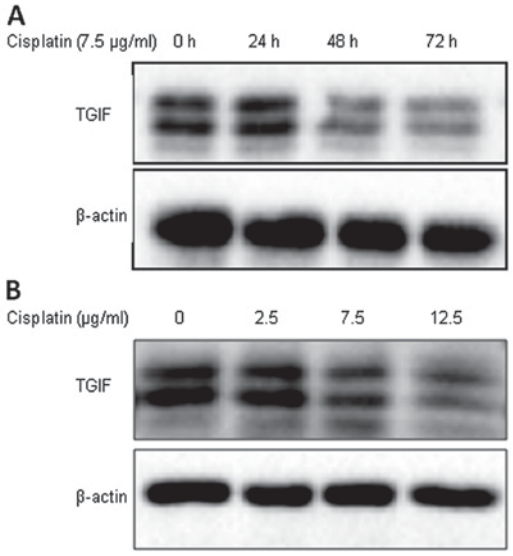

Figure 1. Expression level of TGIF protein in MDA-MB-231 cells treated by cisplatin. (A) The MDA-MB-231 cells were treated with 7.5 μg/ml cisplatin for 0, 24, 48 and 72 h. (B) The MDA-MB-231 cells were treated with increasing concentrations of cisplatin (0, 2.5, 7.5 and 12.5 μg/ml). Expression level of TGIF protein was then determined by western blot analysis. β-actin was used as a loading control. TGIF, TG-interacting factor.

showed that TGIF silencing significantly impeded cell proliferation in MDA-MB-231 cells compared with the control groups in the presence of cisplatin (Fig. 2C). Furthermore, we observed that there were significantly decreased colonies in the MDA-MB-231-shRNA-TGIF group compared with the MDA-MB-231-shRNA-control group in the presence of cisplatin (Fig. 2B).

Silencing TGIF increased the apoptotic sensitivity of MDA-MB-231 cells to cisplatin. Fig. 3 indicated the effects of TGIF silencing on cisplatin-induced apoptosis in MDA-MB-231 cells by annexin V and dead cell assay. Our results showed that the percentage of apoptosis in MDA-MB-231-shRNA-TGIF cells was much higher than that in MDA-MB-231-shRNA-control cells with the treatment of 12.5 μg/ml cisplatin (Fig. 3). Similar results were obtained from the Hoechst 33258 staining assay. The MDA-MB-231-shRNA-TGIF cells treated with cisplatin contained more cells in the apoptotic morphological stages, while the control cells treated with cisplatin were uniformly stained. After counting the cells, a significantly increased percentage of apoptotic monocytes was observed in MDA-MB-231-shRNA-TGIF cells compared with MDA-MB-231-shRNA-control cells with the treatment of cisplatin (Fig. 4).

Silencing TGIF regulated the expression of cell apoptosis-related proteins with the treatment of cisplatin. As illustrated in Fig. 5, the increased expression of cleaved PARP, p21 and Bax proteins and decreased expression of caspase-3 and pro-casepase-9 proteins were observed in MDA-MB-231-shRNA-control cells compared with the cisplatin-untreated group with the treatment of 12.5 μg/ml cisplatin. However, only PARP and caspase-3 were correlated with the cisplatin-induced apoptosis mediated by TGIF protein in MDA-MB-231 cells.

Discussion

Recently, several documents have shown that TGIF might mediate a critical feature of chemoresistance in response to the chemotherapy drugs of arsenic trioxide (As_2O_3, ATO) and gemcitabine. Published studies showed that ATO induced cell apoptosis via the ERK1/2 signaling and the TGF-β/Smad pathway, and enhanced $p21^{WAF1/CIP1}$ (p21) expression in human keratinocytes and hepatocellular carcinoma cells. However, this effect could be inhibited by ATO-induced TGIF expression through the recruitment of the HDAC1/TGIF complex to the p21 promoter (32,33). In addition, among the patients with upper tract urothelial carcinoma treated with the gemcitabine and cisplatin systemic chemotherapy, increased expression of TGIF was significantly associated with worse oncological outcomes. Higher disease progression and cancer-related death rates in the TGIF positive group were 100 and 83.3%, respectively, compared with 63.6 and 27.3% in the TGIF negative group (34).

Cisplatin, also called cis-diamminedichloroplatinum (II), is a representative of the platinum drugs. Since its cytotoxic properties were discovered in the 1960s, it has gained attention in the systemic treatment of cancer cells and now it has been one of the most compelling chemotherapy drugs (6,35). Cisplatin crosslinks with the purine bases on the DNA in the nucleus and yields four main cisplatin-DNA adducts, including interstrand crosslink, 1,2-intrastrand cross-link, 1,3-intrastrand crosslink and DNA-protein cross-links (36,37). Cisplatin-DNA adducts can be recognized by several families of proteins and then can interfere with DNA repair mechanisms, induce cell cycle arrest and DNA damage, and subsequently induce cell apoptosis in cancer cells (38).

Previous studies have shown that ectopic expression of several genes is associated with cisplatin therapeutic sensitivity in MDA-MB-231 cells. Zhou *et al* reported that silencing eIFE4 enhanced the chemosensitivity of MDA-MB-231 cells to cisplatin (39). Yang *et al* showed that knockdown of HAX-1 via RNA interference decreased the IC_{50} level of cisplatin by 70.91% in MDA-MB-231/CR cells (40). Hong *et al* demonstrated that knockdown of BCL2L12 and BCL2L12A dramatically inhibited cisplatin-induced apoptosis in MDA-MB-231 cells (41). Zhang *et al* indicated that MiR-363 sensitized cisplatin-induced apoptosis targeting in Mcl-1 in MDA-MB-231 cells; moreover, cells transfected with Mcl-1 expression plasmid abolished the sensitization effects of MiR-363 to cisplatin-inducing cytotoxicity (42). Wu *et al* showed that MiR-153 promoted MDA-MB-231 cell apoptosis treated with cisplatin by targeting HECTD3. Moreover, stable overexpression of HECTD3 abrogated the sensitization effects of MiR-153 to cisplatin treatments (43). Together, combined treatment with gene therapy and chemotherapeutic drugs might effectively enhance chemotherapy-induced cytotoxicity in TNBC cells and may have great clinical significance.

In the present study, we explored the correlation of TGIF silencing with cisplatin chemosensitivity in MDA-MB-231 cells. Our data indicated that TGIF protein expression was markedly decreased in a time- and dose-dependent manner in MDA-MB-231 cells with the treatment of cisplatin, however, we did not observe cisplatin had obvious effects on the expression of TGIF mRNA (0, 2.5, 7.5 and 12.5 μg/ml for

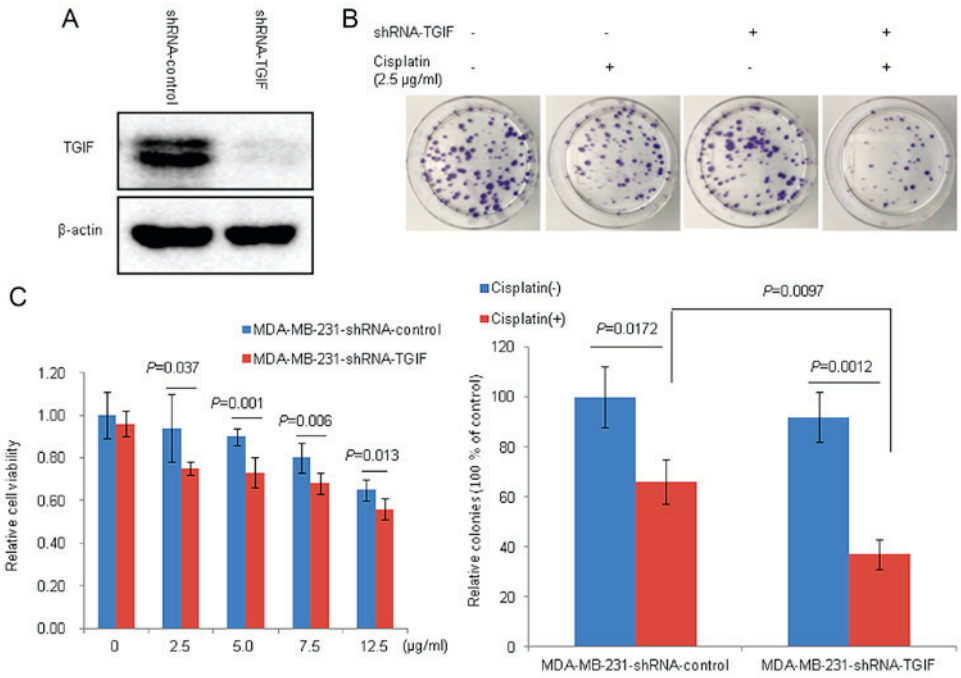

Figure 2. Silencing efficiency of shRNA targeting TGIF and the effects of silencing TGIF on cell proliferation and colony formation in MDA-MB-231 cells treated by cisplatin. (A) TGIF expression level of the infected cells was detected by western blot analysis to confirm the transfection efficiency. The level of TGIF protein in MDA-MB-231-shRNA-TGIF cells was markedly decreased compared with MDA-MB-231-shRNA-control cells. (B) There were significantly decreased colonies in the MDA-MB-231-shRNA-TGIF group compared with the MDA-MB-231-shRNA-control group in the presence of cisplatin. (C) The cell viability of transfected MDA-MB-231 cells with the treatment of different concentrations of cisplatin (0, 2.5, 5.0, 7.5 and 12.5 μg/ml) for 48 h was measured by MTT analysis. The plots showed that TGIF silencing significantly impeded cell proliferation in MDA-MB-231-shRNA-TGIF cells in the presence of cisplatin compared with the control groups. TGIF, TG-interacting factor.

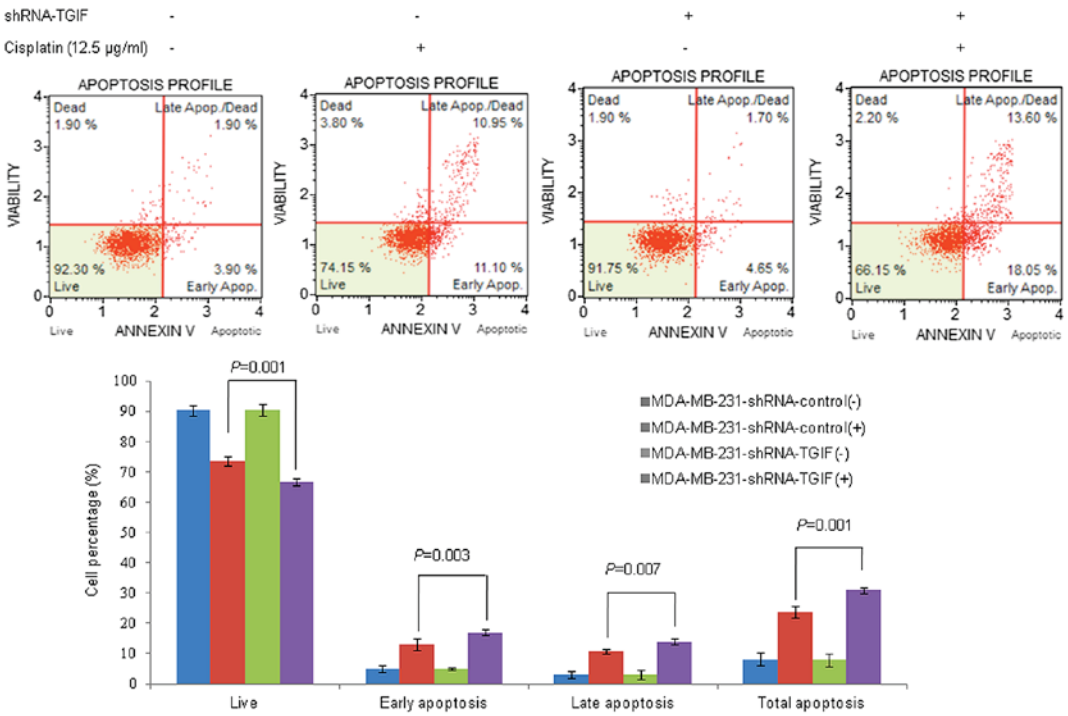

Figure 3. Effects of TGIF silencing on cisplatin-induced apoptosis detected by annexin V and dead cell assay. The MDA-MB-231-shRNA-TGIF cells and the control cells were treated with or without 12.5 μg/ml cisplatin for 48 h. The cell populations of apoptosis were detected by annexin V and dead cell assay. Cisplatin markedly induced cell apoptosis and this effect was further enhanced by TGIF silencing in MDA-MB-231 cells. A higher rate of apoptotic cells was observed in the MDA-MB-231-shRNA-TGIF group than that in the MDA-MB-231-shRNA-control group in the presence of cisplatin. TGIF, TG-interacting factor.

48 h or 7.5 μg/ml for 0, 24, 48 and 72 h; data not shown). The mechanism underlying cisplatin repressing TGIF expression should be explored in future studies. We observed that cell proliferation was significantly inhibited in TGIF-silenced

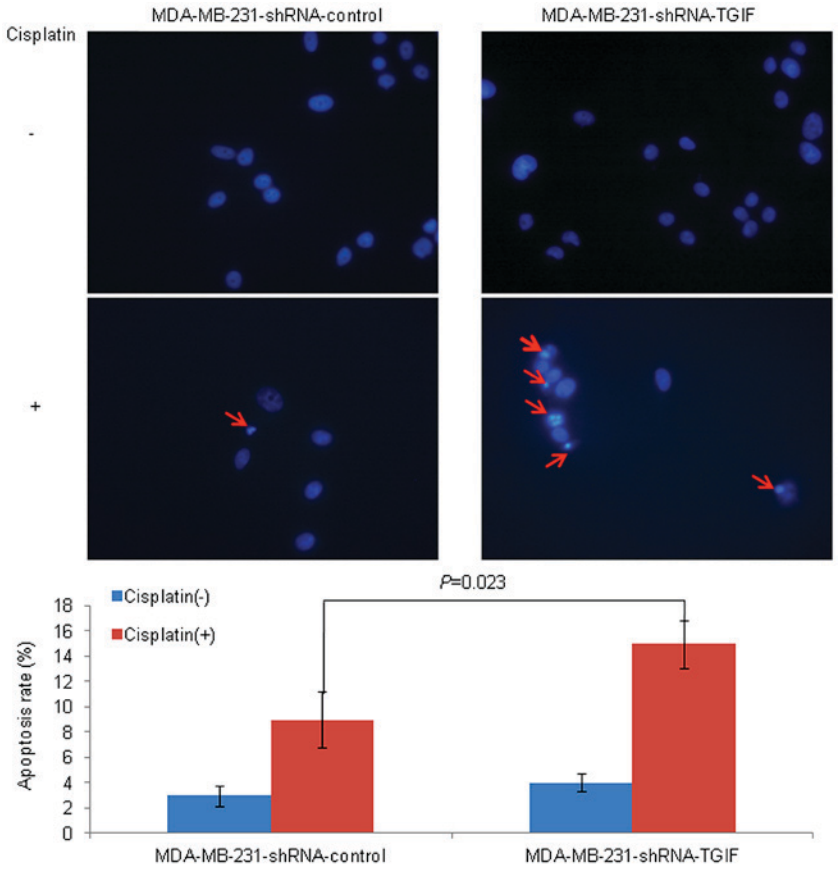

Figure 4. Effects of TGIF silencing on cisplatin-induced apoptosis detected by Hoechst staining. The cells were treated with 12.5 μg/ml cisplatin for 48 h and then stained with Hoechst 33258. Morphological changes were observed under a fluorescence microscope. Representative results were shown; the arrows indicated condensed and fragmented nuclei of apoptotic cells. The apoptosis rate of MDA-MB-231-shRNA-TGIF cells was significantly higher than that in control cells in the presence of cisplatin. Magnification, 10x20. TGIF, TG-interacting factor.

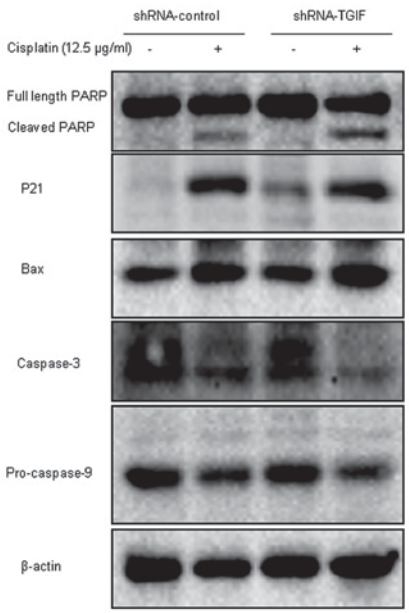

Figure 5. Silencing TGIF modulated the expression of cell apoptosis-related proteins induced by cisplatin. MDA-MB-231 cells were stably transfected with TGIF shRNA human lentiviral particles or control shRNA lentiviral particles, followed by treatment with or without 12.5 μg/ml cisplatin for 48 h. Western blot analysis showed an increased expression of cleaved PARP, p21 and Bax proteins and a decreased expression of caspase-3 and pro-casepase-9 proteins on cisplatin induction. Only PARP and caspase-3 were correlated with the cisplatin-induced apoptosis mediated by TGIF protein in MDA-MB-231 cells. TGIF, TG-interacting factor.

cells treated by cisplatin. Furthermore, silencing TGIF reduced the formation of colonies of MDA-MB-231 cells in the presence of cisplatin compared with controls. In addition, TGIF silencing dramatically increased the cisplatin-induced apoptosis rate as measured by flow cytometry assay as well as the Hoechst staining assay. These data revealed that TGIF might be a potential therapeutic target for cisplatin treatment in MDA-MB-231 cells. For further investigation, the expression of apoptosis-related proteins was measured by western blot analysis. The expression of PARP and caspase-3 proteins was involved in the event that silencing TGIF protein enhanced cisplatin-induced apoptosis.

As is well-known, caspase-3 is the key molecule in the execution phases of apoptosis (44,45). Caspase-3 is usually present in the cytoplasm in the form of an inactive 32-kDa precursor; after the stimulation of the apoptosis signal, it can produce 17 kDa (p17) and 12 kDa (p12) subunits and later form a mature enzyme that is responsible for the proteolytic cleavage of many key proteins. PARP is one of the main cleavage targets of caspase-3 *in vivo*; the cleavage is typically a marker of cells undergoing apoptosis. When a cell is engaged in cell apoptosis, autoribosylated PARP is cleaved by caspases into the 89 kDa C-terminal fragment and the 24 kDa N-terminal fragment; these fragments interact with intact PARP and block the function of PARP on DNA repair and the maintenance of genomic stability (46,47). Presently, we found that caspase-3 and PARP cleavage were involved in cisplatin-induced apoptosis

mediated by TGIF protein. However, the mechanisms that are involved should be elucidated further.

We should acknowledge that there are some limitations in our present study. First, only one cell line of MDA-MB-231 human TNBC has been applied to investigate the effects of TGIF knockdown on cisplatin-induced apoptosis; other breast cancer cell lines representing different types of breast cancer should be used to verify our current results. Second, *in vitro* experiments, such as cell proliferation assay, colony formation assay and apoptosis assay, were performed to observe the effects of TGIF silencing on cytotoxicity and apoptosis in MDA-MB-231 cells induced by cisplatin; a tumor xenograft model should be performed in future studies to confirm our findings. Third, it would be interesting to investigate the expression of TGIF in breast cancer patients who develop resistance to cisplatin in clinical treatment. Fourth, a TGIF-overexpressed cell line should be constructed in future studies to observe whether TGIF overexpression could result in breast cancer cells being resistant to cisplatin; this may verify our present data in other aspects.

In summary, our results showed, for the first time, that silencing TGIF by RNA intervention promoted cell apoptosis and cell cytotoxicity to cisplatin treatment in MDA-MB-231 cells. In addition, this effect might be attributed to caspase-3 protein and its cleavage substrate PARP. Given the association of TGIF protein with a chemotherapeutic response in MDA-MB-231 TNBC cells, targeted therapies directed at TGIF may be a potential novel strategy for improving the chemotherapy response in TNBC.

Acknowledgements

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