FoxO3a induces temozolomide resistance in glioblastoma cells via the regulation of β-catenin nuclear accumulation

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Abstract. Glioblastoma multiforme (GBM), the most common malignant brain tumor, is currently treated with temozolomide (TMZ), but GBM often exhibits resistance to TMZ. Although several mechanisms underlying GBM resistance to TMZ have been identified, these mechanisms are yet to fully explain how GBM gains resistance to TMZ. Our previous work has shown that FoxO3a, a member of the FoxO subfamily of transcription factors, promotes glioma cell proliferation and invasion. In this study, we sought to determine whether FoxO3a participates in TMZ resistance in GBM cells. Parental cell lines (also designated as sensitive cell lines) U87-MG and U251-MG, as well as the corresponding resistant cell lines U87-TR and U251-TR (generated by repeated TMZ treatments), were subjected to western blot analysis. Our results showed that the resistant cells (both U87-TRand U251-TR) exhibited higher levels of FoxO3a and β-catenin relative to their corresponding sensitive counterparts. Depletion of FoxO3a in the resistant cells enhanced the cytotoxic effect of TMZ. Further investigation showed that FoxO3a depletion did not affect the total protein level of β-catenin, but otherwise markedly reduced the nuclear β-catenin level. Taken together, these findings strongly support that FoxO3a renders GBM cells resistant to TMZ treatment, at least in part, through the regulation of β-catenin nuclear accumulation.

Introduction

Glioblastoma multiforme (GBM) is the most common and lethal primary malignant brain tumor. Even with intensive multimodality treatment, including surgical resection combined with radiation and chemotherapy, the prognosis of GBM patients remains very poor, with a median survival of less than 15 months (1). Chemoresistance to alkylating agents and to temozolomide (TMZ) in particular has been identified as a major cause of treatment failure (2). Understanding the mechanisms of TMZ resistance in GBM may contribute to improving the efficacy of the conventional chemotherapeutic agents. Researchers have conducted multiple investigations into the mechanisms of TMZ resistance. Among these investigations, most have focused on O6-methylguanine-DNA methyltransferase (MGMT), primarily because MGMT directly mediates TMZ-induced cytotoxicity. Glioblastoma cells with a high level of MGMT are resistant to TMZ, whereas a low level or the absence of MGMT sensitizes glioblastoma cells to TMZ (3-5). However, MGMT alone does not fully account for the chemoresistance of GBM to TMZ, as >40% of glioblastomas with a low level of MGMT remain resistant to TMZ. These lines of evidence suggest that a high MGMT level is merely one of the possible mechanisms of TMZ resistance (6-8). In addition to MGMT, several genes have been reported to be involved in TMZ resistance. Nevertheless, these data combined are still unable to fully elucidate the mechanisms of TMZ resistance, which prompted us to search for other undefined mechanisms that may contribute to TMZ resistance in GBM.

β-catenin, a subunit of the cadherin protein complex, serves as a fundamental mediator in the Wnt signaling pathway. In particular, nuclear β-catenin is the hallmark of active Wnt/β-catenin signaling (9-11). Multiple studies have shown that β-catenin contributes to glioblastoma inception and progression. For instance, β-catenin is positively correlated with the grade of glial neoplasms (12,13) and has been identified as a marker of poor prognosis in glial neoplasms (14). In addition, compared to membrane-localized β-catenin, nuclear β-catenin is associated with a worse prognosis in cancer (15), indicating that β-catenin subcellular localization is linked to prognostic differences (16-18). Not only does β-catenin play a part in cancer development and progression but it also contributes to chemoresistance. Nuclear β-catenin mediates the activation of the Wnt/β-catenin pathway and confers doxorubicin (DoxR)-resistance in neuroblastoma (NB) (19). β-catenin

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activation by the glycogen synthesis kinase-3 inhibitor induces chemoresistance to the interferon (IFN)-α/5-fluorouracil (5-FU) combination therapy for hepatocellular carcinoma (HCC) (20). Importantly, the active β-catenin (nuclear β-catenin) significantly abrogated the efficacy of three chemotherapeutic agents, TMZ, cisplatin and doxorubicin (21). By contrast, β-catenin depletion sensitized resistant glioblastoma cells to chemotherapy (22).

FoxO3a, a Forkhead box O (FoxO) protein member of the Forkhead family, plays an important role in the regulation of cell differentiation, proliferation, and survival (23). Although FoxO3a has been defined as a ubiquitous tumor suppressor, as it induces cell apoptosis (24,25), emerging evidence indicates that FoxO3a is associated with poor clinical outcomes in specific cancer types (26-28). Consistent with this evidence, our previous work (unpublished data) has shown that in GBM cells, FoxO3a leads to slow cell proliferation, possibly by regulating its target genes such as cyclin D1 and p21. It is noteworthy that the dysregulation of genes involved in survival signal, such as p21, and cell cycle progression, such as cyclin D1 (2,29), results in the development of chemoresistance in malignant glioblastoma. Additionally, Tenbaum *et al* showed that in colon cancer, FoxO3a acting in concert with β-catenin conferred drug resistance in cancer cells (28). Although there is accumulating evidence on the critical role played by FoxO3a in tumorigenesis and cancer progression and on the FoxO3a potential connection with β-catenin and chemoresistance, to the best of our knowledge, whether FoxO3a is involved in conferring TMZ chemoresistance in GBM is still unknown. Therefore, the present study was designed to investigate whether FoxO3a contributes to TMZ resistance in GBM cells and to understand its molecular mechanism.

Materials and methods

Cell lines and cell culture. The glioma cell line U251-MG was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and U87-MG was obtained from the American Type Culture Collection. The two human glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) containing 2 mM glutamine, 10% fetal calf serum (FBS), 100 U/ml penicillin (Sigma, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma). Cells were maintained at 37˚C in a 5% CO_2 incubator.

Generation of temozolomide-resistant glioblastoma cell lines. The parental U251-MG cells and U87-MG cells were exposed to 400 μM TMZ for 3 weeks to generate TMZ-resistant colonies. Initially, we cultured the two cell lines in 6-well plates separately and allowed them to adhere during overnight incubation at 37˚C. TMZ treatment was repeated every 24 h for 5 consecutive days, and the cells were then exposed to fresh TMZ every 3 days for a total of 3 weeks. The majority of the cells died, but a small population survived and propagated. The surviving colonies were selected and established as TMZ-resistant U251 (U251-TR) and U87 (U87-TR) cell lines.

Lentivirus production and transduction. The following short hairpin (sh) RNA against FoxO3a (FoxO3a-knockdown) was used: 5'-GCATGTTCAATGGGAGCTTGGA-3'. Another construct expressing shRNA against irrelevant gene luciferase (shRNA-NC) was used as a negative control. These constructs were co-transfected with packaging plasmids into HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions; the viral particles were harvested 48 h later. U87-TR and U251-TR cells were infected with the lentivirus with 6 μg/ml polybrene (Sigma).

Cell viability assay. Cell viability was determined using the Cell Counting Kit (CCK)-8 assay. Cells were seeded in 96-well plates at a density of $3x10^3$ cells/well. After overnight incubation, the cells were transduced with lentivirus and then treated with various concentrations of TMZ (ranging from 200 to 2000 μM) for 1-5 days. After a 2 h incubation with 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamato, Japan), cell viability was detected at 490 nm using a microplate reader (BioTek, Winooski, VT, USA). The survival rate of untreated cells was set at 100% and used to calculate the half-maximal inhibitory concentration (IC_{50}). Each experiment was conducted in triplicate.

Real-time PCR. Total RNA was extracted from the TMZ resistant cell lines and the GBM parental cell lines. cDNA was prepared using 1 μg of total RNA from each sample, using specific primers (Applied Biosystems). Six nanograms of cDNA were then used for real-time PCR analysis in a final reaction volume of 20 μl. Samples were analyzed in triplicate, and statistical analysis was performed using the t-test.

Western blotting. Total protein was extracted and separated by gel electrophoresis. Protein was then transferred to nitrocellulose membranes and probed overnight using the appropriate primary antibodies. The antibodies used were against N-cadherin (Cell Signaling Technology, 4061), β-catenin (Cell Signaling Technology, 8480), and Histone H3 (Abcam, ab1791). Nuclear and cytoplasmic fractions of total protein were separated using a NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific, Rockford, IL, USA) and then subjected to western blotting.

Statistical analysis. Data shown in the graphs represent the mean values ± SDs of three independent experiments. The difference among groups was determined by ANOVA analysis, and the difference between two groups was analyzed by Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

FoxO3a and β-catenin protein levels are enhanced in U87-TR and U251-TR cells, compared with their parental GBM cells U87-MG and U251-MG. To investigate the cytotoxic impact of TMZ on GBM cells, we analyzed the response of the parental cell lines U87-MG and U251-MG (also designated as sensitive cell lines) and their corresponding TMZ-induced drug-resistant cell lines (U87-TR and U251-TR) to TMZ treatment. Dose titrations of TMZ in the sensitive and resistant cell lines were performed in parallel to show the effects of TMZ on cell viability. The survival of the sensitive cells was significantly reduced within a relatively low TMZ concentration gradient (50-500 μM). By

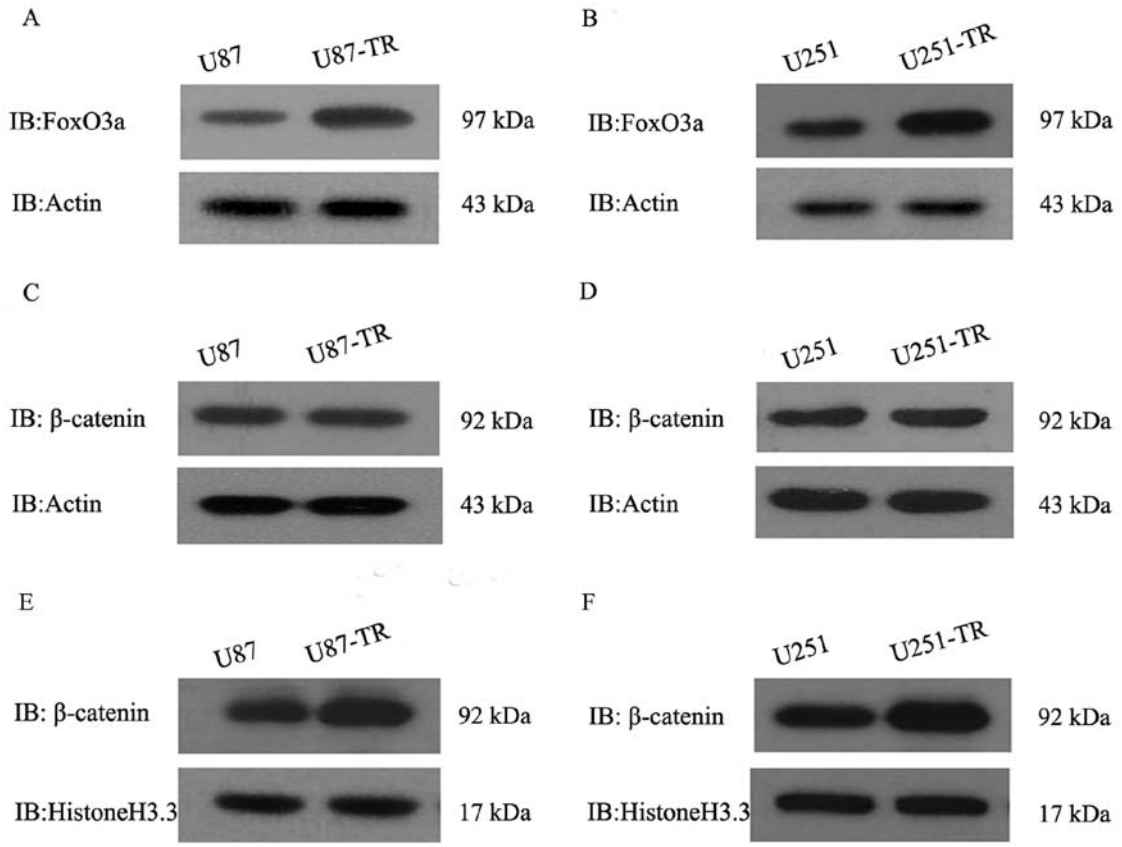

Figure 1. Expression of FoxO3a and β-catenin in U87 and U251 glioma cell lines. As a loading control, β-actin was also visualized in the same blot. (A and C) Western blot analysis showing the levels of FoxO3a and β-catenin in U87 parental and resistant cell lines. (B and D) Western blot analysis showing the levels of FoxO3a and β-catenin in U87 and U251 parental and resistant cell lines (U87-TR and U251-TR). (E and F) Cell fractionation analysis showing the abundance of nuclear β-catenin in the two glioma parental and resistant cell lines. Histone H3.3 was used as a nuclear marker.

contrast, this concentration gradient did not affect the survival of the resistant cells (data not shown); however, a higher TMZ concentration gradient resulted in the reduction of their cell viability. To determine whether FoxO3a and β-catenin were differentially expressed between the resistant cells and their sensitive counterparts, we measured the FoxO3a and β-catenin expression levels in the sensitive cells and their corresponding resistant counterparts. Fig. 1 shows elevated protein levels of FoxO3a and β-catenin in U87-TR cells compared with those in U87 cells. Consistently, the levels of these two proteins were higher in U251-TR cells than in U251 cells, suggesting that the protein levels of FoxO3a and β-catenin were indeed enhanced in the resistant cells generated by repeated exposure to TMZ.

FoxO3a depletion increases the chemosensitivity of U87-TR and U251-TR cells to TMZ. To show that FoxO3a has a critical role in TMZ resistance in glioma cells, we transduced the resistant cells expressing a relatively high level of FoxO3a with either lentivirus FoxO3a-specific shRNA or non-specific control shRNA and studied the response of these cells to TMZ treatment. As shown in Fig. 2, FoxO3a depletion by lentivirus-mediated FoxO3a shRNA treatment in U87-TR cells resulted in a marked reduction of cell viability following TMZ treatment, and a similar result was observed in U251-TR cells. Moreover, the dosage of TMZ that could cause a 50% inhibition of cell growth (IC_{50}) in U87-TR was reduced to 432 μM (nearly 41%) after FoxO3a depletion. Similar to this finding, the depletion of FoxO3a in U251-TR cells also led to a significant reduction in the IC_{50} of TMZ (817.6 μM vs. 449.1 μM).

Considering the fact that β-catenin has been implicated in TMZ resistance, coupled with the potential connections between FoxO3a and β-catenin as revealed by several studies (28,30-32), we speculated that the functional contribution of FoxO3a to the glioma cell resistance to TMZ may be due to its modulating effect on the expression or the subcelluar distribution of β-catenin. We found that the depletion of FoxO3a in U87-TR cells did not lead to any change in β-catenin expression, nor did FoxO3a depletion in U251-TR cells (data no shown), indicating that the part FoxO3a plays in GBM cell resistance is not by regulating β-catenin expression.

β-catenin nuclear accumulation is the hallmark of active β-catenin, which has a role in chemotherapeutic resistance (31,33), we therefore investigated whether FoxO3a had the ability to affect the subcellular distribution of β-catenin. The depletion of FoxO3a led to a significantly reduced nuclear level of β-catenin but no change in its total level, suggesting that the functional contribution of FoxO3a to TMZ resistance in GBM cells may depend on it regulating the nuclear accumulation of β-catenin (Fig. 3), but not its expression. Since β-catenin activation could induce chemoresistant characteristics by cleaving N-cadherin, with a consequential effect on β-catenin nuclear accumulation *per se*, we tested whether the resistant GBM cells depleted of FoxO3a could display differential localization of N-cadherin relative to their

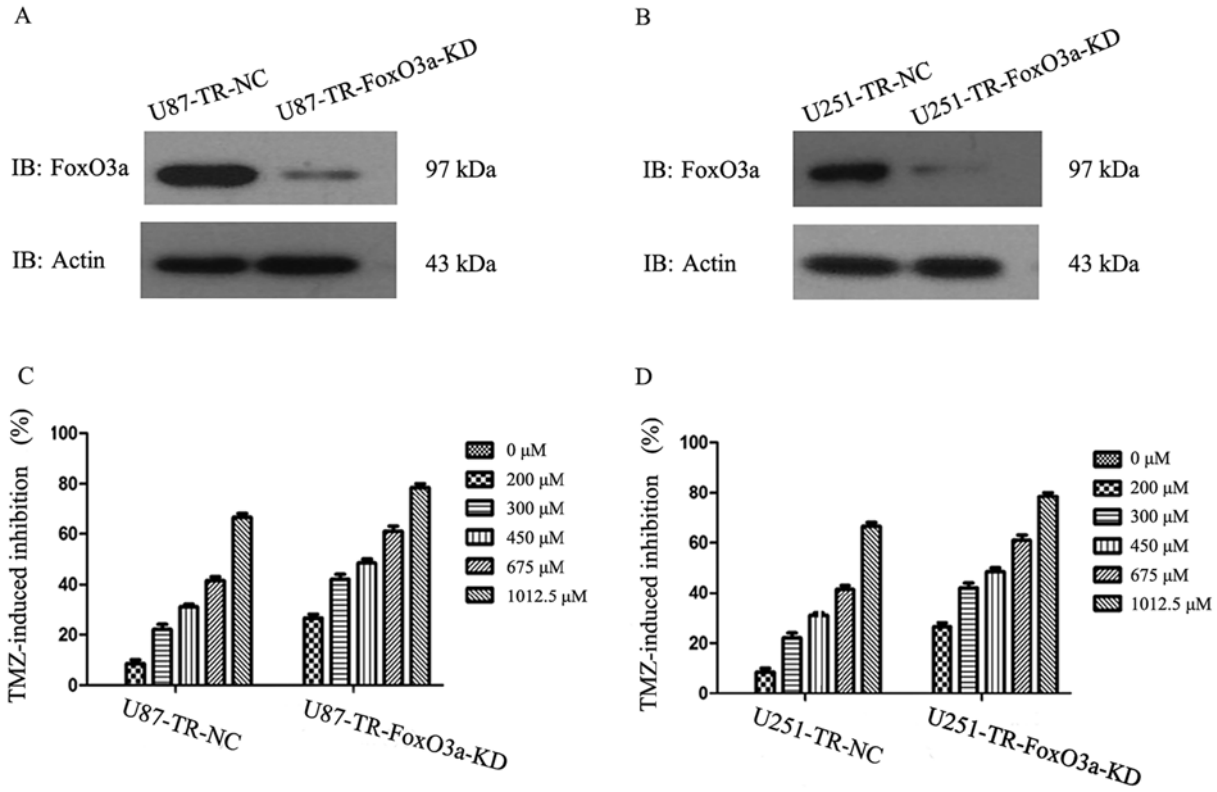

Figure 2. Effect of the depletion of FoxO3a on cell viability in U87 and U251 resistant cells (U87-TR and U251-TR) in response to TMZ treatment. (A and B) FoxO3a were fully depleted in U87-TR and U251-TR cells as determined by western blot analysis. (C) U87-TR cells transduced with lentiviral FoxO3a-specific shRNA or non-specific control shRNA were treated with TMZ, and cell viability after TMZ treatment was analyzed using the CCK8 assay. The IC_{50} value of TMZ in U87-TR (733 μM) was reduced to 432 μM upon FoxO3a depletion. (D) U251-TR cells transduced with lentiviral FoxO3a-specific shRNA or non-specific control shRNA were treated with TMZ, and cell viability after TMZ treatment was analyzed using the CCK8 assay. The IC_{50} value of TMZ in U251-TR (817.6 μM) was reduced to 449.1 μM upon FoxO3a depletion.

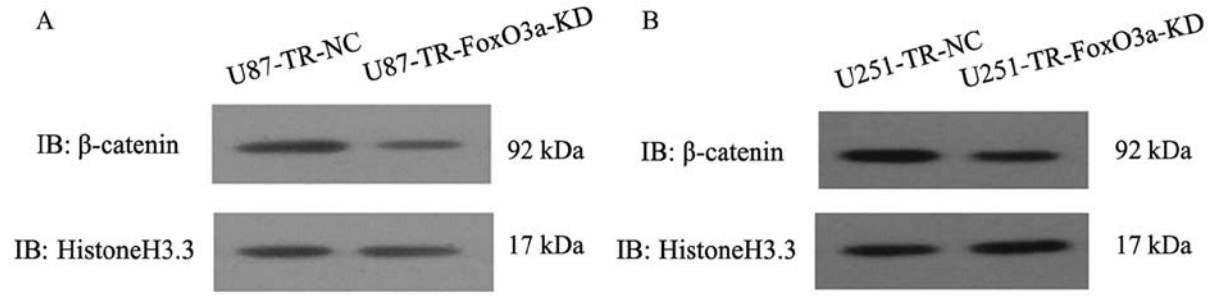

Figure 3. Western blot analysis of the nuclear levels of β-catenin in the U87-TR and U251-TR cells upon FoxO3a depletion. Histone H3.3 was examined as a nuclear marker.

untreated counterparts. N-cadherin localization was affected by FoxO3a depletion, further supporting that FoxO3a-mediated chemoresistance is dependent on the nuclear level of β-catenin (Fig. 4).

Discussion

Drug resistance is a crucial clinical feature that determines the rate of tumor relapse and patient survival. TMZ is a widely used GBM chemotherapeutic agent, and TMZ chemoresistance has been identified as a main cause of treatment failure (34,35). To address this challenge, researchers have studied the molecular basis of this chemoresistance in GBM. Since the DNA repair system has been associated with the chemoresistance, there has been intensive investigation into genes involved in the DNA repair system, particularly MGMT (5,7). However, these published findings combined are still unable to fully explain how GBM cells gain resistance to TMZ.

Herein, we found that FoxO3a, a novel factor for the known mechanisms of TMZ resistance, may be required for the TMZ-resistant phenotype in glioblastoma cells. Using the TMZ-sensitive glioma cell lines U87 and U251, as well as the TMZ-resistant cell lines U87-TR and U251-TR, we showed that TMZ-resistant cells exhibited higher expression levels of FoxO3a and β-catenin than their corresponding parental cells (also designated as sensitive cells). The depletion of FoxO3a in U87-TR cells resulted in significantly reduced growth upon TMZ treatment, relative to the U87-TR control (treated with

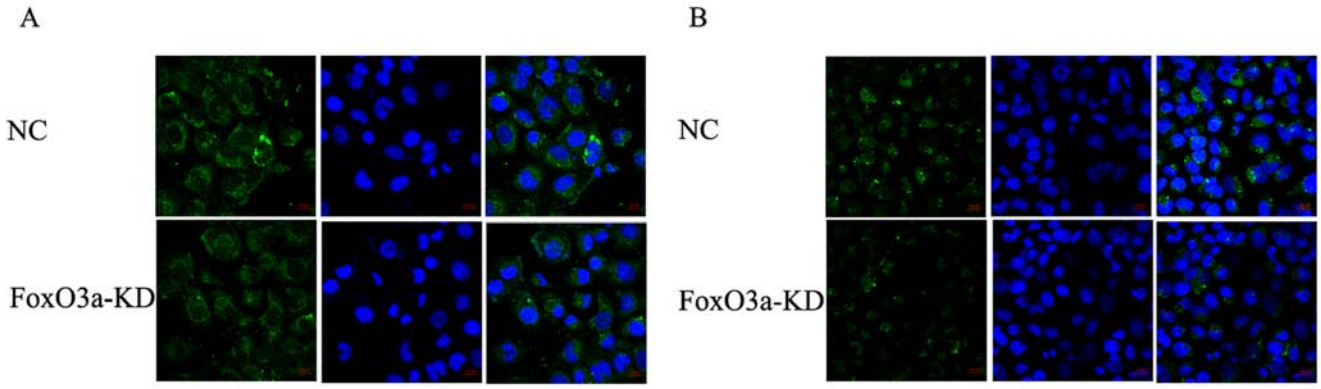

Figure 4. Effect of FoxO3a depletion on N-cadherin distribution in U87-TR and U251-TR cells. (A and B) Immunofluorescence staining of N-cadherin in U87-TR and U251-TR cells. N-cadherin was partially localized at cell-cell contacts when FoxO3a was not depleted. By contrast, N-cadherin showed a diffuse localization pattern after FoxO3a depletion.

irrelevant shRNA). Parallel studies performed using U251-TR cells and U251-TR cells depleted of FoxO3a showed a similar result. These results indicated that FoxO3a depletion increased the sensitivity of the resistant glioma cells to the TMZ treatment. In addition, FoxO3a depletion reduced the abundance of nuclear β-catenin in the resistant cell lines. Taken together, these findings suggest that the regulation of nuclear β-catenin accumulation by FoxO3a could be a novel mechanism by which glioma cells gain resistance to TMZ treatment.

Although a number of studies have shown that FoxO3a functions as a tumor suppressor, there is emerging evidence that correlates FoxO3a function with metastasis or a poor prognosis of cancer, suggesting that FoxO3a can be either oncogenic or tumor suppressive, presumably depending on the cell types and the specific context. In glioma cells, we have previously demonstrated that FoxO3a leads to slow cell proliferation and promotes cell invasion. A study by Osuka *et al* showed that the nuclear level of FoxO3a was greater in the radiological-resistant glioma cells (generated by repeated radiation treatments) than their sensitive counterparts, indicating that FoxO3a promotes radioresistance in glioma (36).

Moreover, Tenbaum *et al* found that FoxO3a, together with β-catenin, confers chemoresistance in colon cancer (28). This background and, in particular, the evidence for the important role of FoxO3a in chemoresistance in colon cancer cells led us to hypothesize that FoxO3a contributes to TMZ chemoresistance. The depletion of FoxO3a significantly reduced the chemoresistance of U87-TR to TMZ treatment as revealed by a combination of gradually reduced cell growth upon increasing doses of TMZ treatment and a reduction in the IC_{50} dosage of TMZ in U87-TR cells depleted of FoxO3a relative to the U87-TR control cells. A similar result was observed in the U251-TR cells and the FoxO3a-depleted U251-TR cells (Fig. 2). These findings indicate that FoxO3a contributes to cancer cell survival under TMZ-driven chemotherapeutic stress, which is consistent with our hypothesis. Given the critical role of FoxO3a in TMZ resistance in glioma cells, the combined use of synthetic FoxO3a-siRNA and TMZ may potentially in part abrogate the TMZ dose-limiting side effect, which is a main disadvantage of TMZ administration, and improve the efficacy of TMZ. Notably, the resistant cells that had FoxO3a shRNA treatment (more than 90% FoxO3a had been depleted) were not as susceptible to TMZ as their sensitive parental cells (U87 and U251), suggesting that other mechanisms may contribute to this pathological process. As U87 and U251 are known to be MGMT-negative cell lines (37), it is unlikely that MGMT is involved in the chemoresistance. However, FoxO3a depletion led to a 41% reduction in U87-TR cell growth upon TMZ treatment as evaluated by IC_{50} analysis and a 45% reduction in U251-TR cell growth, suggesting that some other mechanisms might exist in both of these glioma cell lines. In our future investigations, we will attempt to identify these other mechanisms contributing to the TMZ resistance in the glioma cells.

β-catenin is a fundamental canonical Wnt/β-catenin signaling effector, and its preferential nuclear accumulation is the hallmark of the activation of this pathway. Notably, the importance of β-catenin has been well established in glioma tumorigenesis. Studies from independent research groups have shown that β-catenin is highly expressed in gliomas and is associated with the poor prognosis and short survival of GBM patients (13,15). The depletion of β-catenin using the RNA interference approach resulted in a significant inhibition of glioma cell proliferation. Moreover, cytoplasmic ATRA (all-trans retinoic acid)-induced β-catenin retention led to a reduced growth of glioma cells (30), indicating that the pharmacological or genetic inhibition of the expression or nuclear accumulation of β-catenin impairs the glioma cell growth.

Additionally, the repression of β-catenin by sulforaphane promoted TMZ-induced cell apoptosis (33), and the blockade of nuclear β-catenin translocation by FH535, a β-catenin inhibitor, enhanced the antitumor function of TMZ, establishing a critical role for β-catenin in TMZ chemoresistance in glioma. Prompted by these published data, we tested whether TMZ sensitivity induced by FoxO3a depletion is associated with the level of expression or the nuclear accumulation of β-catenin. Of note, we only observed a reduced nuclear β-catenin level and concomitant increased sensitivity to TMZ in U87-TR and U251-TR cells, whereas the depletion of FoxO3a did not affect β-catenin expression at the total protein level (Figs. 2 and 3). As β-catenin functions are dependent on its nuclear accumulation and transcriptional activity, it is plausible that β-catenin nuclear accumulation alone may be sufficient for

conferring the chemoresistance. Our findings, together with the previously published studies, corroborate that nuclear β-catenin sufficiently causes the chemoresistance of GBM cells.

Although it is known that β-catenin nuclear accumulation is important for glioma tumorigenesis, the mechanism of this molecular event has been surprisingly difficult to define. Nevertheless, an important mechanism for β-catenin nuclear translocation via binding to FoxM1, a forkhead box (Fox) transcription factor, has been discovered (38). Given the antagonistic action between FoxO3a and FoxM1 and the observation that FoxO3a activation and the concomitant FoxM1 down-regulation increased chemosensitivity, it is unlikely that FoxO3a mediates β-catenin nuclear accumulation and the resulting chemoresistance in glioma cells by regulating FoxM1. It is important to note that increased cellular oxidative stress promotes FoxO3a nuclear accumulation and facilitates the binding of FoxO3a and β-catenin (32). This finding suggests that FoxO3a could directly bind to β-catenin and mediate its nuclear import. Taken together, although we observed FoxO3a depletion resulted in enhanced chemosensitivity and a reduction in β-catenin nuclear abundance, experimentally comprehending the molecular basis of FoxO3a-mediated chemoresistance in glioma cells is still challenging. In our future studies, we will perform intensive investigation into this mechanism.

In conclusion, we provide direct evidence of the critical role of FoxO3a in TMZ resistance in glioma cells. We show that the repression of FoxO3a using lentivirus-mediated RNA interference renders glioma cells more susceptible to TMZ treatment and reduces the nuclear β-catenin level (without affecting its total level). Our findings reveal a previously unknown role of FoxO3a as an inducer of TMZ resistance in glioma cells and provide a novel potential target for chemotherapeutic drug development, as well as new diagnostic and predictive biomarkers.

Acknowledgements

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