Original Article

Forkhead box O1 targeting replication factor C subunit 2 expression promotes glioma temozolomide resistance and survival

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Background: Additional mechanisms of temozolomide (TMZ) resistance in gliomas remain uncertain. The aim of this study was to identify another DNA repair mechanism involving forkhead box O1 (FoxO1) and replicator C2 (RFC2) in gliomas.

Methods: We established glioma cells against TMZ, U87R, by exposure to TMZ. Proliferation rate Cell counting kit-8 (CCK8) was used, and epithelial-mesenchymal transition (EMT)-related markers were detected by western blot. The association between FoxO1 and RFC2 was analyzed by heat maps and scatter plot, and Real-time reverse transcription polymerase chain reaction (qRT-PCR) and Western blot were used to detect the effect of FoxO1 on the expression of RFC2. The regulation effect of FoxO1 on RFC2 expression was analyzed by luciferase reporter gene assay. Knockdown of FoxO1/RFC2 was achieved via short hairpin RNA (shRNA), the effect of knockdown on the proliferation was determined by CCK8 assay and colony formation assay, and apoptosis was examined by flow cytometry and immunoblotting.

Results: The TMZ-resistant glioma cell line, U87R, was established. The FoxO1 and RFC2 proteins increased significantly in U87R. The expression of FoxO1 and RFC2 were positively related in glioma tissues. We found that FoxO1 contributes to TMZ resistance and cell survival via regulating the expression of RFC2. Moreover, FoxO1 functions as a transcriptional activator to RFC2 by binding to the promoter of RFC2. Furthermore, knockdown of FoxO1/RFC2 suppressed cell proliferation, TMZ resistance, and induced apoptosis in U87R.

Conclusions: The FoxO1/RFC2 signaling pathway promotes glioma cell proliferation and TMZ resistance, suggesting that the FoxO1/RFC2 pathway may be a potential target for TMZ-resistant glioma therapy.

Keywords: Glioma; forkhead box O1 (FoxO1); replication factor C2 (RFC2); temozolomide (TMZ)

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Introduction

Glioma is one of the most common brain tumors and has a poor prognosis. Despite the development of treatment by surgery, radiotherapy, and chemotherapy, the median survival of glioblastoma multiforme (GBM) patients is only 12–14 months (1,2). Temozolomide (TMZ), an effective glioblastoma chemotherapy drug, can easily cross the blood-brain barrier, significantly induce cell apoptosis, effectively inhibit glioblastoma cell proliferation (3). However, Glioblastomas often show resistance to this alkylation agent, the most well-known mechanism of which is the expression of O6-methylguanine-DNA methyltransferase (MGMT), which protects against the mutagenic effects of alkylation agents on the cell genome (4,5). Since MGMT has the function of removing the cytotoxic effect produced by TMZ, it is the main regulator of the sensitivity of glioma cells to TMZ (6,7). However, inactivation of MGMT has been found in almost 40% of all gliomas (8). Such gliomas are still found to be resistant to TMZ, it is revealed that MGMT is not the only factor leading to TMZ resistance (9). Thus, identification of additional mechanisms contributing to TMZ resistance will aid the development of effective therapeutic regimens.

Forkhead box O1 (FOXO1) is a transcription factor in the FOXO family (10). In recent years, the role of FOXOS in physiological activities such as metabolism, angiogenesis, apoptosis, cell cycle arrest and differentiation has been extensively studied (11,12). Previous studies have suggested that FOXO1 is a tumor suppressor. Many cancer types show decreased FOXO1 expression, including the bladder (13) and cervical cancer (14). In addition, FoxO1 inhibited angiogenesis in gastric cancer by inactivating HIF-1/VEGF pathway (15) and inhibitory glioma cell growth and TMZ resistance (16). However, the exact expression pattern and mechanism of FOXO1 in glioma remain unclear.

Replication factor C (RFC) is an ATPase that plays an important role in DNA Replication, DNA repair, and checkpoints related to DNA metabolism (17,18). In mammals, the RFC1 subunit contains N-terminal and C-terminal extensions outside the homologous region with four other subunits, including RFC2, RFC3, RFC4, and RFC5. Recently, 3 protein complexes similar to RFC have been described, which are involved in maintaining genome stability and controlling DNA damage checkpoints (19). Respectively, studies have reported that up-regulation of RFC3 can promote metastasis of triple-negative breast cancer, suggesting a poor prognosis via epithelial-mesenchymal transition (EMT) (20). Also, RFC4 levels are associated with tumor progression and prognosis in colorectal cancer (21). In addition, RFC5 expression promoted TMZ resistance in glioma (22). However, it is unclear whether RFC2 plays an important role in glioma progression and TMZ resistance.

In this study, TMZ-resistant glioma cell line U87R was established by long-term exposure of human glioblastoma U87 cells to TMZ.DNA repair and drug-related proteins FoxO1 and DNA repair genes RFC2, ABCG2 and MDR-1 were upregulated in TMZ drug-resistant glioma cells. We discovered that knockdown of FoxO1 markedly inhibited the promoter activity and expression of RFC2. We confirmed that FoxO1 is able to directly activate RFC2 expression in a transcriptional process by binding to the promoter of RFC2. Moreover, RFC2 over-expression rescued the inhibition of cell viability caused by FoxO1 knockdown in U87R glioma cells. Furthermore, FoxO1 knockdown inhibited colony formation and induced apoptosis in U87R cells, and increased expression of apoptosis-related markers, Cleaved caspase3 and Cleaved poly-ADP ribose polymerase (PARP). Therefore, we demonstrate the important role of FoxO1/RFC2 gene regulatory networks in TMZ resistance and survival of TMZ resistant glioma cells. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-21-1523).

Methods

Cell lines and culture

Human glioblastoma U87 cells were purchased from American type culture collection (ATCC manassas, VA, USA), human TMZ against malignant glioma U251 cells (U251R) from the Hebrew name buy culture collection (BNCC, Jiangsu, China), and on the dole beca modified eagle medium (DMEM) with 10% fetal bovine serum (America's full-backs, Gibco, carlsbad, CA), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, A), and standard culture conditions were maintained.

Antibody and reagents

FoxO1 (C-9/H128), MDR-1 (D-11), ABCG2 (M-70), caspase-3 (H-277), PARP (B-10), Cleaved PARP (H-125), Vimentin (V9), N-cadherin (G-10), E-cadherin (D-4), Snailin (H-130), Slig (A-7), β-actin (C-2) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (G-9) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against

RFC-2 (AB88502) was purchased from AbCAM (Cambridge, MA, USA). Cleaved caspase-3 (Asp175) antibody #9661 was purchased from Cell Signaling Technology (Shanghai, China). TMZ (16435643) Regent from Sigma-Aldrich (St. Louis, Missouri, USA). Double strands of interfering RNA plasmid (shRNA) targeting FoxO1, RFC2, and an untargeted shRNA (shRNA-NC) were purchased from Gene Pharma (Shanghai, China). FoxO1 expression plasmid was purchased from Gene Copocia (Guangzhou, China).

Cell proliferation viability assay

CCK-8 (Sigma-Aldrich, Shanghai, China) was used to measure cell viability. Cells were implanted into 96-well plates (1×10^3 cells/well), incubated overnight at 37 ℃, and then treated with TMZ for the specified time. At the end of treatment, 10 L CCK-8 solution was added to each well and incubated for 4 hours. A Microplate reader (Bio-Rad Laboratory, Hercules, CA, USA) was used to measure the absorbance at 450 nm.

RNA extraction and qRT-PCR

Total RNA was extracted from cell lysates using RNA isolation kits (Gentra, Minneapolis, MN, USA). The isolated RNA was treated with DNA enzymes using a DNA-free kit (Ambion, Austin, TX, USA). CDNA of RNA was reverse transcribed with 1 μg mRNA and 250 U AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The qRT-PCR instrument (Roche Diagnostics, Indianapolis, IN, USA) was used to quantitatively analyze the expression of RFC2 mRNA. The following specific primers were used to amplify 198 bp fragment corresponding to RFC2. Meaning: 5'-GGGGGATCCATGCTGGGCTACTCACCAGAAG-3', 5'-GGGCTCGAGCTAACTGGCCACCGGGGCC-3' antisense. Then, the cDNA was standardized with β-actin as 5'-AAGTCCCTCACCCTCCCAAAAG-3' and antisense 5'-AAGCAATGCTGTCACCTTCCC-3'. DNA amplification was monitored using the quick-start SYBR Green kit (Roche Diagnostics) according to manufacturer's instructions (23). The smelting curve is then analyzed to verify the purity of the product. The increase in mRNA levels was calculated by the intersection deviation between the treatment group and the control group.

Colony formation assay

The treated cells were implanted into 6-well plates (400 cells/well) and cultured for 12–14 days with 10 μM TMZ or without TMZ to form colonies. More than 50 cell colonies were observed with crystal violet staining and photographed using an image analysis system (Invitrogen iBright CL750). All experiments were repeated three times.

Western blotting

Cells were collected in the sample buffer, boiled for 5 min and analyzed by polyacrylamide gel electrophoresis (PAGE) (24). Standard Western blot using the aforementioned antibodies.

Flow cytometry analysis

Cell apoptosis was analyzed by flow cytometry with Annexin V binding. For flow cytometry analysis, cells (4×10^6) were implanted into a 3.5 cm culture dish for overnight culture, and then treated with TMZ, FoxO1 shRNA and RFC2 shRNA at the specified concentrations. Cells were collected, washed twice with PBS, re-suspended in a binding buffer and stained with Annexin V-FITC/PI kit according to manufacturer's instructions. The cells were incubated in the dark for 30 minutes and then analyzed. The data were processed using FACS Cell Quest software (Becton, Dickinson and Co., Franklin Lakes, NJ, USA).

Statistical analysis

All results were expressed as mean ± standard deviation (SD), and the experiment was performed at least 3 times. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Inc., La Jolla, CA, USA) software. Student T test (2-tailed) is used to evaluate whether the difference is significant, $P < 0.05$ indicates a significant difference.

Results

Establishment of the TMZ-resistant glioma cell line U87R

In order to investigate the molecular mechanism of TMZ drug resistance in glioma, we first exposed parent U87 cells to a gradually increasing TMZ concentration for 6 months, and established TMZ drug resistant glioma cell line U87R. The proliferation ability and morphological characteristics of TMZ resistant cells and their parents were compared by cell activity analysis and optical microscopy. As shown in *Figure 1*, TMZ drug-resistant
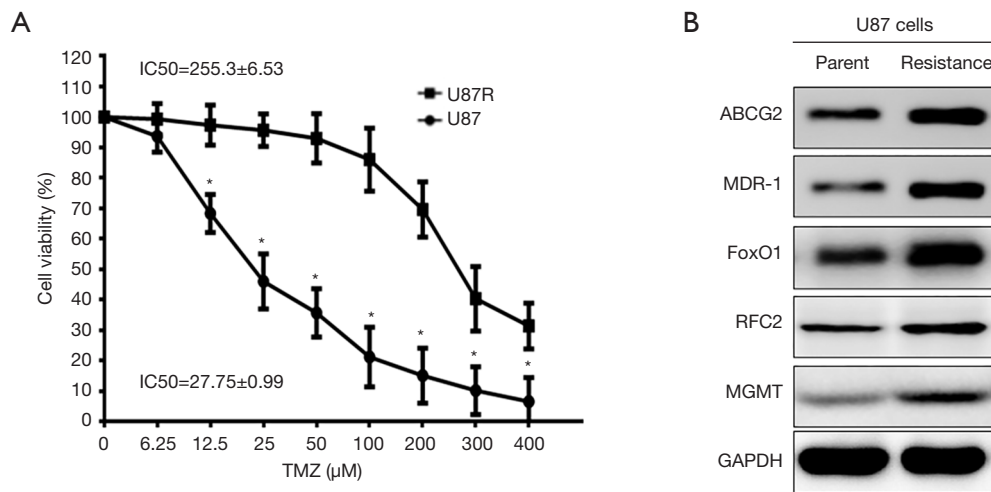
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Figure 1 Sensitivity of the parental glioma cells (U87) and its TMZ resistant cell lines (U87R) to TMZ. Cell lines were cultured with various concentrations (0.0, 6.25, 12.5, 25, 50, 100, 200, 300, and 400 μM) of TMZ for 72 h. Cell proliferation activity was detected by CCK-8 assay. *P<0.05. The TMZ resistant cell lines U87R were more resistant to TMZ than the parent cell lines. (A) Cell proliferation activity of the parental glioma cell U87 and its TMZ-resistant cell lines, U87R, were detected by CCK-8 analysis. The IC50 value of the parental U87 cell and TMZ-resistant U87R cell. (B) Drug resistance related proteins (ABCG2, MDR-1, and MGMT) and DNA damage repair related proteins (FoxO1 and RFC2) were detected by western blot. GAPDH was used as a loading control. TMZ, temozolomide; CCK-8, cell counting kit-8; IC50, half inhibitory concentration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FoxO1, forkhead box O1; RFC2, replication factor C subunit 2.

cells and parent cells were treated with 6.25, 12.5, 25, 50, 100, 200, 300 and 400 μM TMZ for 72 h, respectively, and cell viability was analyzed by cell counting kit-8 (CCK-8). The results showed that the cell viability of both TMZ resistant cells and parent cells decreased in a dose-dependent manner (*Figure 1A*). When the dose was higher than 50–100 μM TMZ, U87 cells were more sensitive to TMZ than U87R cells. The half inhibitory concentration (IC50) of the cells against TMZ was calculated. We found that the IC50 value of U87R was higher than that of U87 (*Figure 1A*). Our data suggest that the U87R cell line has anti-TMZ potential. In addition, TMZ drug-resistant related proteins ABCG2 and MDR-1 expression were significantly increased in multiple cancer cells in TMZ drug-resistant cell lines (24-26) (*Figure 1B*). We also detected the expression of MGMT protein in U87 and U87/TR by Western blotting. We observed that the expression level of MGMT in U87R cells was higher than that in U87 cells (*Figure 1B*). We also found that the expression of DNA repair related proteins FoxO1 and RFC2 was enhanced in drug-resistant cell lines (*Figure 1B*). Our data confirm the successful establishment of TMZ resistant glioma cell line U87R.

U87R cells exhibited slower proliferation rate and EMT property

CCK-8 was used to detect the proliferation of U87 and U87R cells at different time points. We found that on day 4, the number of cells in both cell lines increased significantly, but U87 cells proliferated more actively than U87R cells (P<0.05) (*Figure 2A*). Morphological changes of these cell lines were also observed. The results showed that the appearance of U87R cells and U87 cells was obviously different under the light microscope, the cell polarity was lost, the spindle shape was formed, the intercellular separation was increased, the intercellular adhesion disappeared, and the pseudopod formation was increased (*Figure 2B*). The nucleus of U87R cells was larger than that of U87 cells (*Figure 2B*). To determine whether the establishment of TMZ resistance included specific molecular changes associated with EMT, Western blotting was performed on cell lysates of U87R and U87. The expression of epithelial adhesion molecule E-cadherin in U87R cells was decreased, and the expression of mesenchymal markers N-cadherin and vimentin was significantly increased. In addition, compared with U87
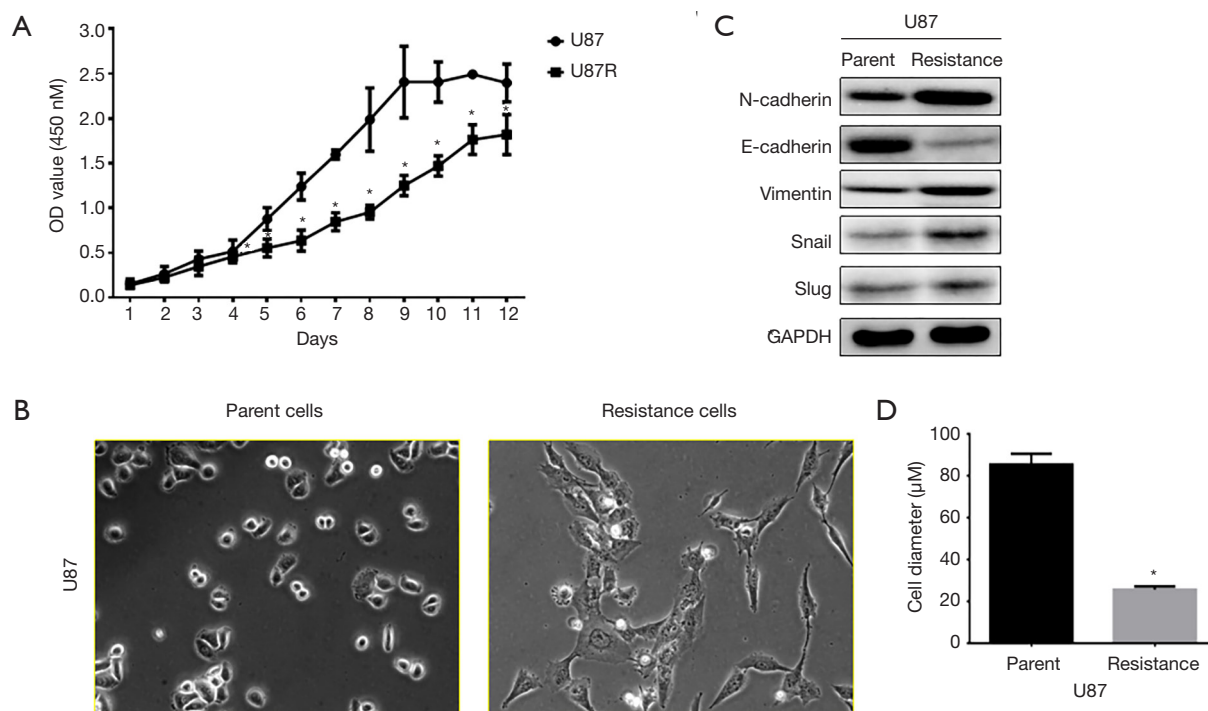
Figure 2 TMZ resistant glioma cell line U87R cells exhibited slower proliferation rate and EMT property. (A) Growth curve of the TMZ resistant glioma cells U87R and its parental cell U87 with was detected by CCK-8. The data are presented as the mean ± SD. *P<0.05. N=3. (B) Morphological changes of the TMZ resistant glioma cells U87R compared with their parent cell lines U87 by optical microscope. Magnification, 400×. (C) EMT related protein markers (N-cadherin, E-cadherin, Vimentin, Snail1, and Slug) were assayed by western blot. (D) Cell diameter was quantitatively analyzed between the parent and the TMZ-resistant U87 glioma cells. The data are presented as the mean ± SD. *P<0.05. N=10 fields. TMZ, temozolomide; EMT, epithelial-mesenchymal transition; CCK-8, cell counting kit-8; SD, standard deviation.

cells, the expression of EMT-related transcription factors Snail and SLUG was also enhanced in U87R cells (*Figure 2C*). In addition, our results showed that the average diameter of U87R cells was longer than that of U87 cells (*Figure 2D*).

FoxO1 regulates RFC2 expression in glioma cells U87R

FoxO1 and RFC2 are DNA damage-associated proteins that protect against alkylating agent-induced cell death by promoting DNA damage repair (18,25,26). Studies have shown that RFC2 and PCNA are crucial for the extension of the primer DNA template. The function of RFC2 is a DNA-dependent ATPase, which plays an important role in DNA replication and repair (27). However, the role of FoxO1 in the initiation of DNA replication or repair in response to TMZ treatment of glioma is unclear.

Therefore, we first analyzed the relationship between FoxO1 and RFC2 expression levels in glioma specimens. As shown in *Figure 3A,B*, we studied molecular subtypes in 539 cohorts and 483 cohorts using gene expression data from the Tumor Genome Atlas (TCGA) Glioma Database (https://genome-cancer.ucsc.edu). A heat map of FoxO1 and RFC2 gene expression showed a high correlation between the expression of FoxO1 and RFC2 in glioma tissues (*Figure 3A*). The scatter plot also showed a positive correlation between the expression of RFC2 and FoxO1 in 483 glioma tissues (*Figure 3B*).

Next, we studied the effect of FoxO1 on the expression of RFC2 in glioma cell lines by real-time reverse transcription polymerase chain reaction (qRT-PCR) and Western blot. FoxO1 knockdown significantly decreased mRNA (mRNA) levels of RFC2 in U87R cells compared to control cells (*Figure 3C*), while FoxO1 overexpression significantly increased RFC2 expression in U87R cells (*Figure 3D*). Western blot analysis also confirmed the previous results
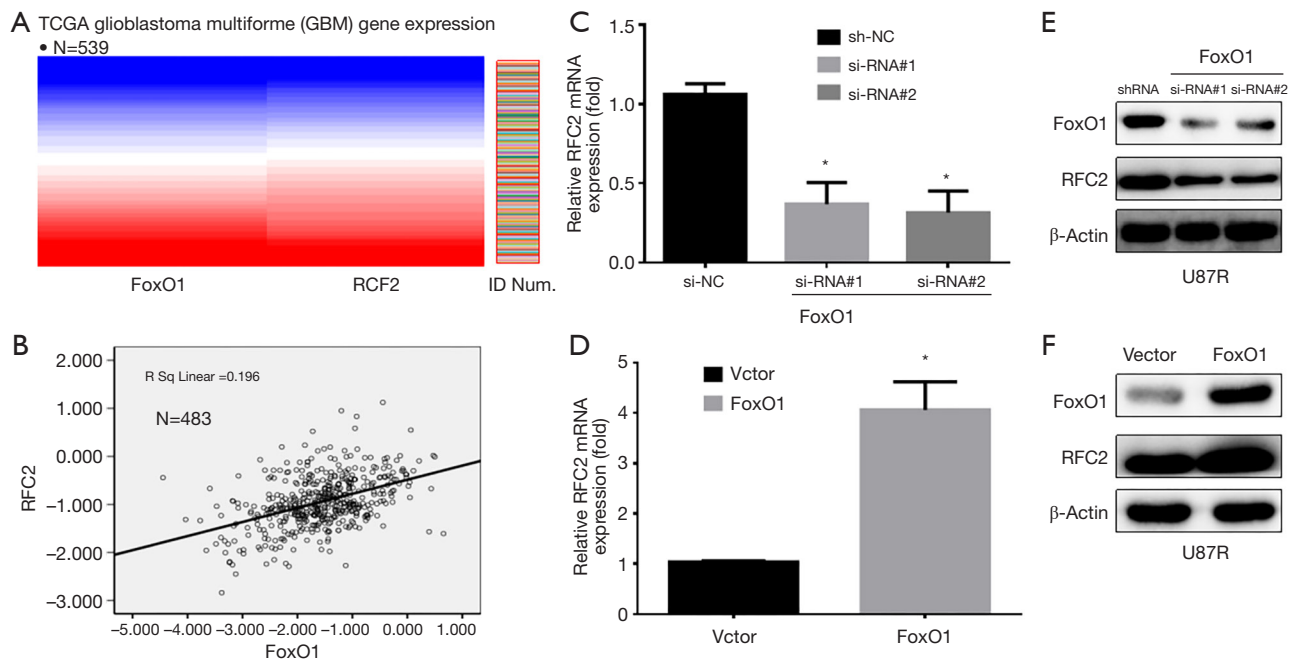
Figure 3 FoxO1 regulates RFC2 expression in glioma TMZ-resistant cell line U87R. (A) The heat map shows that the RFC2 expression is positively associated with FoxO1 expression in 539 cases of glioma tumor tissues. Data are from the TCGA database (B) Scatter plot shows that the RFC2 expression is positively associated with FoxO1 expression in 483 cases of glioma tumor tissues. Data from the TCGA database. (C) U87R cells were transfected with 40 nM shRNA targeting FoxM1 (shRNA#1 and shRNA#2) or control shRNA (sh-NC). The mRNA expression of RFC2 by qRT-PCR. *$P<0.05$. (D) U87R cells were transfected with pcDNA-3.1 (vector) or pcDNA-3.1-FoxO1 (FoxO1). The mRNA expression of RFC2 by qRT-PCR after 48 h. *$P<0.05$. (E) and (F) Western blot analysis of the expression of FoxO1 and RFC2 in U87R cells, β-Actin was used as a loading control. FoxO1, forkhead box O1; RFC2, replication factor C subunit 2; TMZ, temozolomide; TCGA, The Cancer Genome Atlas; shRNA, short hairpin RNA; qRT-PCR, quantitative real-time polymerase chain reaction.

(*Figure 3E,F*), FoxO1 overexpression significantly increased the expression of RFC2 protein in U87R cells. These results suggest that FoxO1 positively regulates the expression of RFC2 in TMZ drug-resistant glioma cells.

FoxO1 targets RFC2 expression through direct binding to RFC2 promoter

Using JASPAR (http://jaspar.genereg.net), we discovered the foxo1, 5'-acctgctttct-3' a potential combination of primitives, located in the upstream RFC2 translation initiation site—to 247-237 bp (*Figure 4A*). From the wild-type RFC2 promoter, we generated mutant reporters of the RFC2 promoter at different truncations and binding motifs. All fragments were cloned into the pGL3-basic luciferase plasmid (*Figure 4A*). Luciferase assay showed that FoxO1 overexpression significantly enhanced RFC2 promoter activity in U87R cells (*Figure 4B,C*). In contrast, mutations

or deletions at the FoxO1 binding sites significantly reduced RFC2 promoter activity (*Figure 4B*), suggesting that these binding sites are critical to FoxO1-mediated RFC2 promoter activity. In addition, FoxO1 overexpression significantly enhanced RFC2 promoter activity in a dose-dependent manner (*Figure 4C*). FoxO1 knockdown by short hairpin RNA (shRNA)#1 and shRNA#2 significantly inhibited RFC2 promoter activity (*Figure 4D*). These results suggest that FoxO1 regulates RFC2 expression through the FoxO1 binding site on the RFC2 promoter.

Knockdown of FoxO1-RFC2 gene regulatory network inhibited glioma cell proliferation and colony formation in glioma cells and TMZ resistance cells U87R

Next, we determined whether knockout of the FoxO1/ RFC2 signaling pathway could re-sensitize U87R and U251R cells to TMZ. As expected, we investigated the effect
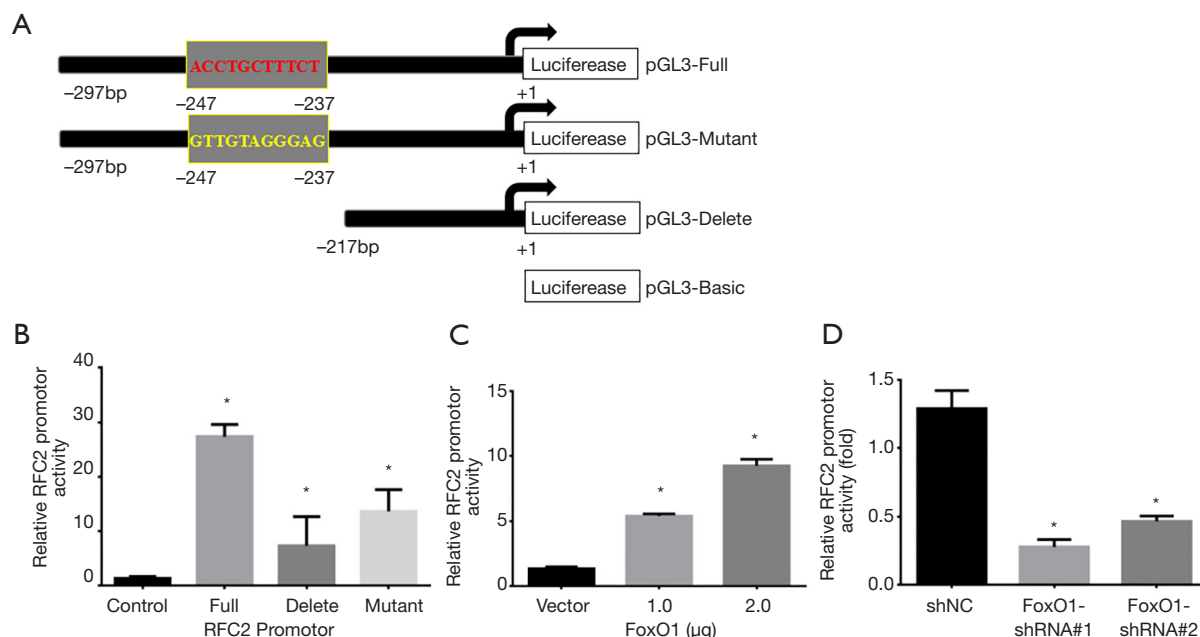
Figure 4 FoxO1 directly regulates RFC2 expression through binding to RFC2 promoter. (A) Potential FoxO1 binding sites in the RFC2 promoter and schematic diagram of different luciferase reporter expression vectors. (B) U87MG cells at the pcDNA3.1-FoxO1 expression plasmid. *P<0.05 *vs.* Basic. (C) U87R cells in 24-well plates were transfected with the pGL3-RFC2-FULL luciferase reporter along with different amounts of pcDNA3.1 vector or with 1.0 and 2.0 μg plasmids of pcDNA3.1-FoxO1 using Lipofectamine 2000. *P<0.05. (D) U87R Cells were transfected with 2.0 μg pGL3-RFC2-Full luciferase reporter unite with 40 nM shRNA targeting FoxO1 (FoxO1 shRNA#1 and FoxO1 shRNA#2) or control shRNA (shRNA-NC). *P<0.05. FoxO1, forkhead box O1; RFC2, replication factor C subunit 2.

of shRNA of FoxO1 and RFC2 on TMZ resistance and cell proliferation. U87R and U251R cells were transfected with shRNA of FoxO1 and RFC2 either alone or together, and then treated with 100 μM TMZ for 72 h. Knockdown of FoxO1 and RFC2 individually or together significantly reduced the proliferation rate of U87R cells (*Figure 5*). Furthermore, when RFC2 expression was restored and transfected RFC2 plasmid was knocked down in U87R FoxO1, the inhibition of cell proliferation was also restored (*Figure 5B*). Next, as expected, the colony formation assay (*Figure 5C,D*) showed that shRNA knockout of FoxO1 or RFC2 inhibited colony formation in TMZ-resistant cell line U87R under 100 μM TMZ treatment, suggesting that FoxO1/RCF2 knockout may be a potential target for the reversal of TMZ-resistant glioma cells.

Knockdown of FoxO1-RFC2 gene regulatory network promotes TMZ-induced apoptosis in U87R cells

Apoptosis is one of the main mechanisms of drug-induced cell death. Next, we investigated the effect of knockout of FoxO1/RFC2 signaling pathway on TMZ-induced apoptosis of U87R cells. We treated U87R cells with FoxO1 shRNA, RFC2 shRNA, and TMZ shRNA, flow cytometry, and Western blotting. Flow cytometry showed that the combination of FoxO1 shRNA and RFC2 shRNA increased apoptosis compared to FoxO1 knockout or TMZ treatment alone (*Figure 6A,B*).Concordant with previous results, Western blotting analysis also showed that inhibition of RFC2 or FoxO1 expression in combination with TMZ treatment of caspase-3 or PARP was stronger than TMZ treatment alone (*Figure 6C*). Our results indicate that inhibition of FoxO1/RFC2 signaling pathway can inhibit the growth of TMZ drug-resistant U87R cells and improve the sensitivity of TMZ drug-resistant U87R cells to TMZ.

Discussion

Although MGMT levels are not associated with TMZ resistance in specific subpopulations of gliomas, it has been suggested that additional DNA repair mechanisms may also contribute to TMZ resistance (7,9,28). In this study, in order
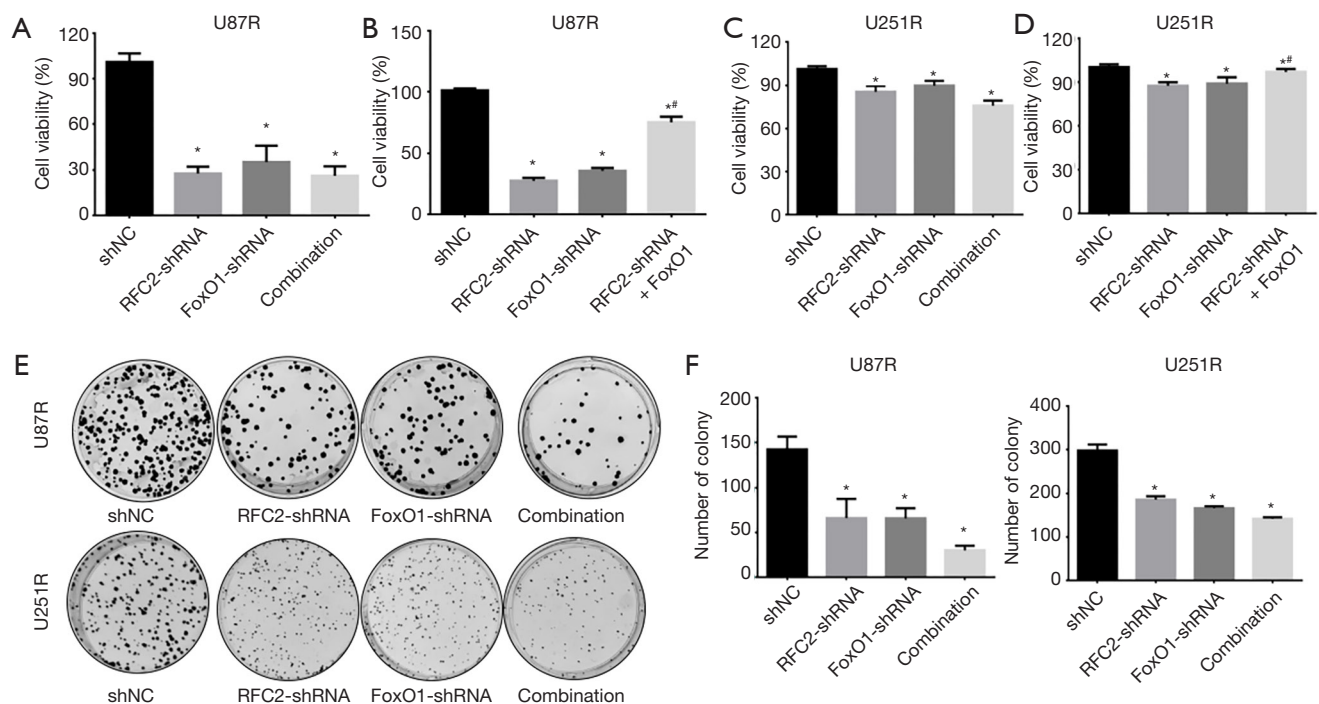
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Figure 5 Knockdown FoxO1/RFC2 signaling pathway inhibited cell growth and colony formation in TMZ resistance cell U87R and U251R. (A) U87R cells were transfected with 40 nM shRNA targeting FoxO1 (FoxO1-shRNA), RFC2 (RFC2-shRNA), FoxO1 plus RFC2 (Combination), or the control shRNA (shNC). After 3 days of transfection, the cell proliferation activity was determined by CCK-8 assay. (B) U87R cells were transfected with 40 nM shRNA targeting FoxO1 (FoxO1-shRNA), RFC2 (RFC2-shRNA), RFC2 (RFC2-shRNA2) plus FoxO1 plasmids (C2+O1), or the control shNC group. After 3 days of transfection, the cell proliferation activity was determined by CCK-8 assay. (C) U251R cells were transfected with 40 nM shRNA targeting FoxO1 (FoxO1-shRNA), RFC2 (RFC2-shRNA), or FoxO1 plus RFC2 shRNA (Combination), or the control shRNA (shNC). After 3 days of transfection, the cell proliferation activity was determined by CCK-8 assay. (D) U251R cells were transfected with 40 nM shRNA targeting FoxO1 (FoxO1-shRNA), RFC2 (RFC2-shRNA), the combination, or the control shRNA (shNC). After 3 days of transfection, the cell proliferation activity was determined by CCK-8 assay. (E) Colony formation assay of U87R and U251R cells transfected with 40 nM shRNA targeting FoxO1 (FoxO1 shRNA), RFC2 (RFC2 shRNA), FoxO1 plus RFC2 (Combination), or the control shRNA (shNC). Culture was for 2 weeks. The quantification of colonies is shown in (F), respectively. *P<0.05 *vs.* shRNA group. #P<0.05 *vs.* RFC2-shRNA group. FoxO1, forkhead box O1; RFC2, replication factor C subunit 2; TMZ, temozolomide; CCK-8, cell counting kit-8.

to clarify the molecular mechanism of TMZ drug resistance involved in glioma, we successfully established TMZ drug resistant glioma cell line U87R.Our data showed that the proliferation rate of these drug-resistant cells was much lower than that of their parent cells, and the expression of MDR-1 and ABCG2 was significantly increased in these drug-resistant cells. Our data also showed that U87R cells had EMT characteristics, and EMT-related molecular markers such as vimentin, E-cadherin, Slug, Snail were significantly increased, suggesting that EMT characteristics may lead to TMZ drug resistance. Our current results also suggest that TMZ-resistant glioma cells can be established by long-term exposure to progressively increased TMZ concentrations. Furthermore, the expression of DNA repair related proteins FoxO1 and RFC2 was significantly increased in TMZ resistant cells, suggesting that these proteins may be involved in TMZ drug resistance in glioma cells. Therefore, we used the University of California, Santa Cruz (UCSC) database to investigate the expression of FoxO1 and RFC2 in clinical glioma specimens. We found that FoxO1 and RFC2 expression were positively correlated in glioma tissue, suggesting that FoxO1 and RFC2 may play an important role in the occurrence and development of glioma.
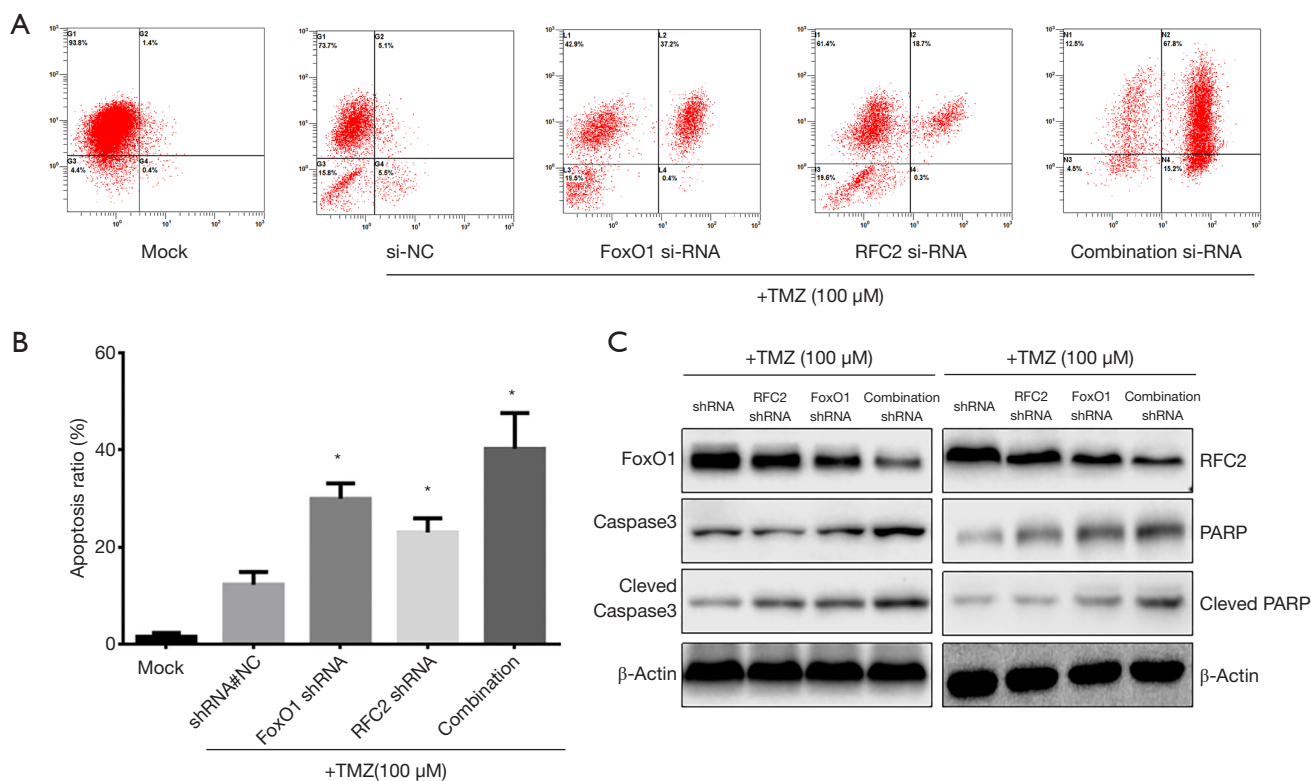
Figure 6 Knockdown of FoxO1 or RFC2 increases TMZ-induced apoptosis in TMZ resistance glioma cell line U87R. (A) U87R cells were transfected with 40 nM shRNA targeting FoxO1 and RFC2 (FoxO1 shRNA and RFC2 shRNA) or control shRNA (shRNA#NC). After 24 h of transfection, the cells were treated with 100 μM TMZ for another 48 h. Cell apoptosis was detected by FACS analysis. (B) Histogram showing the apoptosis ratio of previous group. *P<0.05 *vs.* sh-NC. (C) Western blot analysis expression of FoxO1, RFC2, caspase-3, PARP, Cleaved caspase-3 and cleaved PARP; β-actin was used as a loading control. FoxO1, forkhead box O1; RFC2, replication factor C subunit 2; TMZ, temozolomide; PARP, poly-ADP ribose polymerase.

The FOX family of transcription factors is commonly increased in various cancers (25,29,30). Much recent evidence suggests that targeting FOXO1 is a practical strategy for the development of novel antitumor drugs, as overexpression of FOXO1 contributes to chemotherapy resistance (31,32). It has been shown that FoxO1 can mediate drug resistance by regulating the expression of multiple genes. For example, FoxO1 can mediate insulin resistance by inducing TOR activity (33). FoxO1 has been shown to reduce alkylating agent-induced DNA damage in tumor cells by regulating DNA repair related genes such as CDK2 and NOS (26,34,35). However, the role of FoxO1 in TMZ resistance in glioma cells remains to be clarified. As mentioned above, FoxO1 and RFC2 are both elevated in U87R cells, and we further demonstrate that FoxO1 promotes TMZ resistance and cell survival in glioma cells by regulating RFC2 expression. It is widely believed

that both FoxO1 and RFC2 can effectively activate DNA damage repair (36-38). The main DNA repair mechanisms for TMZ resistance include base excision repair and DNA mismatch repair (39).

Our data suggest that the FoxO1/RFC2 signaling pathway may be involved in the base-excision repair process. We know that RFC2 plays an important role in DNA replication and tumor cell survival (17,40,41), but its upstream regulatory role in tumor cells is unknown. Our results not only indicate that FoxO1 regulates the expression of RFC2, but also that FoxO1 acts as a transcriptional activator of RFC2 by interacting with specific binding sites in the RFC2 promoter. However, this interaction may be direct binding to DNA or indirect binding to FoxO1-bound proteins, and binding to the promoter. Finally, shRNA down-regulation of FoxO1 or RFC2 inhibited colony formation and cell growth of U87R and U251R

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cells, leading to increased TMZ-induced apoptosis. This suggests that the FoxO1/ RFC2 signaling pathway may be a potential therapeutic target.

In conclusion, our results suggest that FoxO1/RFC2 signaling pathway may be involved in TMZ drug resistance of glioma cells, and shRNA knockout of FoxO1/RFC2 signaling pathway can significantly inhibit proliferation and TMZ drug resistance of glioma cells. FoxO1/RFC2 signaling pathway may be a potential therapeutic target for TMZ resistant gliomas. Future work will focus on how to develop inhibitors of the FoxO1/RFC2 signaling pathway.

TMZ drug-resistant glioma cell line U87R was successfully established after long-term exposure to progressively increasing concentrations of TMZ. FoxO1 and RFC2 were significantly increased in TMZ drug-resistant glioma cells, and the expression of FoxO1 and RFC2 was positively correlated in glioma tissues. FoxO1 is involved in TMZ resistance and cell survival in glioma cells by regulating the expression of RFC2. In addition, FoxO1 acts as a transcriptional activator of RFC2 by binding to the promoter of RFC2. In addition, knockdown of FoxO1/RFC2 signaling pathway inhibits proliferation and induces apoptosis of TMZ-resistant glioma cells. We demonstrate the important role of the FoxO1/RFC2 signaling pathway in TMZ resistance and cell survival of glioma cells. Therefore, the FoxO1/RFC2 signaling pathway may be a potential target for the treatment of TMZ-resistant gliomas.

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Footnote

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References

1. Davis ME. Glioblastoma: Overview of Disease and Treatment. Clin J Oncol Nurs 2016;20:S2-8.
2. Tosoni A, Franceschi E, Poggi R, et al. Relapsed Glioblastoma: Treatment Strategies for Initial and Subsequent Recurrences. Curr Treat Options Oncol 2016;17:49.
3. Zhang Z, Wang Y, Chen J, et al. Silencing of histone deacetylase 2 suppresses malignancy for proliferation, migration, and invasion of glioblastoma cells and enhances temozolomide sensitivity. Cancer Chemother Pharmacol 2016;78:1289-96.
4. Kim C, Kim HS, Shim WH, et al. Recurrent Glioblastoma: Combination of High Cerebral Blood Flow with MGMT Promoter Methylation Is Associated with Benefit from Low-Dose Temozolomide Rechallenge at First Recurrence. Radiology 2017;282:212-21.
5. Li H, Li J, Cheng G, et al. IDH mutation and MGMT promoter methylation are associated with the pseudoprogression and improved prognosis of glioblastoma multiforme patients who have undergone concurrent and adjuvant temozolomide-based chemoradiotherapy. Clin Neurol Neurosurg 2016;151:31-6.
6. Okada M, Sato A, Shibuya K, et al. JNK contributes to temozolomide resistance of stem-like glioblastoma cells via regulation of MGMT expression. Int J Oncol 2014;44:591-9.
7. Vlachostergios PJ, Hatzidaki E, Papandreou CN. MGMT repletion after treatment of glioblastoma cells

with temozolomide and O6-benzylguanine implicates NFkappaB and mutant p53. Neurol Res 2013;35:879-82.

8. Melguizo C, Prados J, Gonzalez B, et al. MGMT promoter methylation status and MGMT and CD133 immunohistochemical expression as prognostic markers in glioblastoma patients treated with temozolomide plus radiotherapy. J Transl Med 2012;10:250.

9. Gaspar N, Marshall L, Perryman L, et al. MGMT-independent temozolomide resistance in pediatric glioblastoma cells associated with a PI3-kinase-mediated HOX/stem cell gene signature. Cancer Res 2010;70:9243-52.

10. Murtaza G, Khan AK, Rashid R, et al. FOXO Transcriptional Factors and Long-Term Living. Oxid Med Cell Longev 2017;2017:3494289.

11. Kim CG, Lee H, Gupta N, et al. Role of Forkhead Box Class O proteins in cancer progression and metastasis. Semin Cancer Biol 2018;50:142-51.

12. Coomans de Brachène A, Demoulin JB. FOXO transcription factors in cancer development and therapy. Cell Mol Life Sci 2016;73:1159-72.

13. Lloreta J, Font-Tello A, Juanpere N, et al. FOXO1 down-regulation is associated with worse outcome in bladder cancer and adds significant prognostic information to p53 overexpression. Hum Pathol 2017;62:222-31.

14. Prasad SB, Yadav SS, Das M, et al. Down Regulation of FOXO1 Promotes Cell Proliferation in Cervical Cancer. J Cancer 2014;5:655-62.

15. Kim SY, Ko YS, Park J, et al. Forkhead Transcription Factor FOXO1 Inhibits Angiogenesis in Gastric Cancer in Relation to SIRT1. Cancer Res Treat 2016;48:345-54.

16. Tang H, Bian Y, Tu C, et al. The miR-183/96/182 cluster regulates oxidative apoptosis and sensitizes cells to chemotherapy in gliomas. Curr Cancer Drug Targets 2013;13:221-31.

17. Shiomi Y, Nishitani H. Control of Genome Integrity by RFC Complexes; Conductors of PCNA Loading onto and Unloading from Chromatin during DNA Replication. Genes (Basel) 2017;8:52.

18. Kim J, MacNeill SA. Genome stability: a new member of the RFC family. Curr Biol 2003;13:R873-5.

19. Vijayakumar S, Dziegielewska B, Levin DS, et al. Phosphorylation of human DNA ligase I regulates its interaction with replication factor C and its participation in DNA replication and DNA repair. Mol Cell Biol 2009;29:2042-52.

20. He ZY, Wu SG, Peng F, et al. Up-Regulation of RFC3 Promotes Triple Negative Breast Cancer Metastasis and is Associated With Poor Prognosis Via EMT. Transl Oncol 2017;10:1-9.

21. Xiang J, Fang L, Luo Y, et al. Levels of human replication factor C4, a clamp loader, correlate with tumor progression and predict the prognosis for colorectal cancer. J Transl Med 2014;12:320.

22. Peng WX, Han X, Zhang CL, et al. FoxM1-mediated RFC5 expression promotes temozolomide resistance. Cell Biol Toxicol 2017;33:527-37.

23. Tang H, Peng F, Huang X, et al. Neoisoliquiritigenin inhibits tumor progression by targeting GRP78-beta-catenin signaling in breast cancer. Curr Cancer Drug Targets 2018;18:390-9.

24. Xipell E, Aragón T, Martínez-Velez N, et al. Endoplasmic reticulum stress-inducing drugs sensitize glioma cells to temozolomide through downregulation of MGMT, MPG, and Rad51. Neuro Oncol 2016;18:1109-19.

25. Bullock M. FOXO factors and breast cancer: outfoxing endocrine resistance. Endocr Relat Cancer 2016;23:R113-30.

26. Huang H, Regan KM, Lou Z, et al. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science 2006;314:294-7.

27. Tomida J, Masuda Y, Hiroaki H, et al. DNA damage-induced ubiquitylation of RFC2 subunit of replication factor C complex. J Biol Chem 2008;283:9071-9.

28. Yi GZ, Liu YW, Xiang W, et al. Akt and beta-catenin contribute to TMZ resistance and EMT of MGMT negative malignant glioma cell line. J Neurol Sci 2016;367:101-6.

29. Carbajo-Pescador S, Mauriz JL, Garcia-Palomo A, et al. FoxO proteins: regulation and molecular targets in liver cancer. Curr Med Chem 2014;21:1231-46.

30. Matkar S, Sharma P, Gao S, et al. An Epigenetic Pathway Regulates Sensitivity of Breast Cancer Cells to HER2 Inhibition via FOXO/c-Myc Axis. Cancer Cell 2015;28:472-85.

31. Zhao F, Lam EW. Role of the forkhead transcription factor FOXO-FOXM1 axis in cancer and drug resistance. Front Med 2012;6:376-80.

32. Gao J, Yang X, Yin P, et al. The involvement of FoxO in cell survival and chemosensitivity mediated by Mirk/Dyrk1B in ovarian cancer. Int J Oncol 2012;40:1203-9.

33. Luong N, Davies CR, Wessells RJ, et al. Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity. Cell Metab 2006;4:133-42.

34. Yamaza H, Komatsu T, Wakita S, et al. FoxO1 is involved in the antineoplastic effect of calorie restriction. Aging

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Qiu et al. FoxO1/RFC2 target for TMZ-resistant in glioma

Cell 2010;9:372-82.

35. Hughes KJ, Meares GP, Hansen PA, Corbett JA. FoxO1 and SIRT1 regulate beta-cell responses to nitric oxide. J Biol Chem 2011;286:8338-48.

36. Castro GN, Cayado-Gutierrez N, Zoppino FC, et al. Effects of temozolomide (TMZ) on the expression and interaction of heat shock proteins (HSPs) and DNA repair proteins in human malignant glioma cells. Cell Stress Chaperones 2015;20:253-65.

37. Castro GN, Cayado-Gutierrez N, Zoppino FC, et al. Effects of temozolomide (TMZ) on the expression and interaction of heat shock proteins (HSPs) and DNA repair proteins in human malignant glioma cells. Cell Stress Chaperones 2015;20:253-65.

38. Knizhnik AV, Roos WP, Nikolova T, et al. Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-

induced DNA damage. PLoS One 2013;8:e55665.

39. Papait R, Magrassi L, Rigamonti D, et al. Temozolomide and carmustine cause large-scale heterochromatin reorganization in glioma cells. Biochem Biophys Res Commun 2009;379:434-9.

40. Guan Q, Yue X, Zeng H, et al. The protein phosphatase RCF2 and its interacting partner NAC019 are critical for heat stress-responsive gene regulation and thermotolerance in Arabidopsis. Plant Cell 2014;26:438-53.

41. Strogolova V, Furness A, Robb-McGrath M, et al. Rcf1 and Rcf2, members of the hypoxia-induced gene 1 protein family, are critical components of the mitochondrial cytochrome bc1-cytochrome c oxidase supercomplex. Mol Cell Biol 2012;32:1363-73.

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