Characterizing the molecular mechanisms of acquired temozolomide resistance in the U251 glioblastoma cell line by protein microarray

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Abstract. Acquired chemoresistance refers to tumor cells gradually losing their sensitivity to anticancer drugs during the course of treatment, resulting in tumor progression or recurrence. This phenomenon, which has deleterious outcomes for the patient, has long been observed in patients with glioblastoma receiving temozolomide (TMZ)-based radiochemotherapy. Currently, the mechanisms for acquired TMZ chemoresistance are not fully understood. In the present study, a TMZ-resistant cell line U251R with a 4-fold 50% inhibition concentration compared with its TMZ-sensitive parent cell line was isolated by incremental long-time TMZ treatment in the human glioblastoma cell line U251. Fluorescence-activated cell sorting analysis indicated G2/M arrest and a lower proportion of cells in the S phase, accompanied by a decreased apoptosis rate in the U251R cell line compared with the parental U251 cell line. In addition, a sphere-formation assay indicated an increased self-renewal capacity in U251R cells. Furthermore, a high-throughput protein microarray unveiled more than 200 differentially expressed proteins as potential molecular targets accounting for acquired TMZ resistance. Subsequent bioinformatics analysis illustrated the molecular and signaling networks and revealed the central role of SRC. Immunoblotting and reverse-transcription quantitative polymerase chain reaction analysis further confirmed the expressional upregulation of SRC family kinases. Moreover, SRC knockdown led to partial reversal of TMZ resistance in the U251R cell line and sensitization in the U373 cell line. These data helped to develop a comprehensive understanding of survival strategies, particularly with respect to pro-stemness regulation, which could be potential targets for overcoming TMZ resistance.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain malignancy, and is characterized by high invasiveness, therapeutic resistance and recurrence (1,2). Due to the difficulty of total surgical resection, temozolomide (TMZ)-based chemotherapy has been the standard first-line adjuvant treatment for patients with GBM, targeting residual tumor cells with the aim of extending the progression-free and overall survival times (3,4).

TMZ belongs to a group of alkylating agents that are able to cross the blood-brain barrier (BBB) and reach therapeutic concentrations in the activated form of compound 5-3-(methyl)-1-(triazen-1-yl) imidazole-4-carboxamide (MTIC), which can cause DNA lesions mainly by methylating the O^6-guanine. The subsequent mismatch repair (MMR) response in DNA replication, alerted by the misrepair of methylated guanine and thymine (instead of cytidine), causes either double-strand breaks or critical recombinogenic lesions.

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This eventually induces apoptosis and inhibits proliferation of malignant cells (5,6).

Despite fundamental clinical benefits, chemoresistance is an inevitable limitation of TMZ administration. Mechanistically, chemoresistance can be divided into two categories: Intrinsic and extrinsic. The former can mostly be attributed to inherent gene characteristics at the beginning of therapy, such as lack of MGMT gene methylation. Extrinsic chemoresistance, also referred to as acquired resistance, is conferred by tumor cells that gradually develop genetic alterations to antagonize TMZ toxicity. In many cases, the response to TMZ is still poor even if MGMT promoter methylation is detected; therefore, there is a greater interest in acquired chemoresistance, which is more likely to be reversible and targetable (7). In order to uncover the mechanisms of acquired chemoresistance, we first established a variant glioblastoma cell line to simulate the refractory tumor bulk under clinical treatment. Then, we performed a protein microarray, which can provide more reliable evidence directly from interactions among expressed proteins and activities, as compared with cDNA microarray (8), in order to characterize potential molecular mechanisms.

Characteristics of cell stemness are thought to be associated with the tumorigenic capacity and migratory nature of malignant glioblastoma cells, an area that is gaining more interest in the field of cancer treatment. In accordance with results from a number of laboratories indicating that GSCs have an innate ability to resist the effects of therapeutic agents (9,10), the pathway investigation in the present study uncovered stem-like signaling in the TMZ-resistant U251 cell line, which highlighted the central role of SRC in the networks. SRC belongs to a family of non-receptor tyrosine kinases (SFKs), which contain a unique N-terminal sequence, followed by four SRC homology (SH) domains and a C-terminal negative regulatory sequence. Dephosphorylation at Y530 in the C-terminus by protein tyrosine phosphatases (PTPs), and the subsequent autophosphorylation at Y416 triggers the interaction of SFKs with multiple downstream factors involved in cell adhesion, migration, invasion, proliferation and angiogenesis. In addition, several SFK members have been reported to be involved in cell stemness and epithelial-mesenchymal transition (EMT) phenotypes of multiple cancer types, including GBM (37). In the present study, we also examined the role of SRC in TMZ-resistance. Collectively, the present study presents a comprehensive insight into the defense strategies of glioblastoma cells, which may provide potential targets for improving TMZ-based chemotherapy.

Materials and methods

Experimental procedure
Cell culture and transfection. The human glioblastoma cell lines U251 and U373 were purchased from the Chinese Academy of Sciences. The glioblastoma cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 1.5 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified 5% CO_2/95% air incubator, and harvested for passage with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) when they had reached confluence.

For sphere culture and self-renewal assays, U251 cells were cultured in DMEM/F-12 supplemented with N2, B27 supplements (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA) in a low-adhesive plate for 10 days to allow spheres to form. Cells were plated at a density of ~1x10^4 live cells/dish, and the medium was changed every 3-4 days. After obvious spheres were observed (~2 weeks later), these spheres were disaggregated with Accutase (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), followed by repetitive pipetting up and down, and then plated for secondary culture and maintained in aforementioned GSC medium for a further 10 days. When the majority of spheres reached ≥50 μm in diameter, they were viewed under a phase-contrast microscope for morphological comparison. The neurosphere diameters of each cell line were measured for a total of 15 fields in triplicate experiments.

The GV358-RNAi vector, which co-expressed the GFP protein and specific short hairpin RNA (shRNA) against human SRC (cat. no. 54150) or a non-targeting scramble shRNA (both from Shanghai GeneChem Co., Ltd., Shanghai, China) as a control, were commercially synthesized and constructed according to the manufacturer's instructions. The GV358-RNAi vectors containing SRC-specific and control shRNAs were transfected into 293FT cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Complete lentiviral expression vectors were generated and denoted as Si-SRC and Si-Scr lentivirus, respectively. The lentiviral vectors were transfected into glioblastoma cells in the presence of 5 μg/ml polybrene and cultured before they were subjected to cell viability and sphere-formation assays.

Isolation of a TMZ-resistant cell line. For the isolation of resistant cells, TMZ (Tasly Pharmaceutical Co., Ltd., Tianjin, China) was dissolved in DMSO (12.5 mg/ml stock solution) and diluted to the desired concentrations. Parental U251 cells were consecutively re-exposed to an incremental TMZ pulse from initiation of 1 μg/ml to reach the desired concentration for a period of 6 months. Briefly, cells were plated in 25 cm^2 flasks in their usual medium and allowed to attach overnight, following which the medium was replaced with TMZ containing medium of double concentration followed by TMZ-free fresh medium after 2 h, for an additional 10-12 days of regrowth. When there was no obvious cell loss observed, cells were collected and subjected to an MTT assay.

TMZ sensitivity and cell proliferation assays. Cell growth was determined by an MTT colorimetric assay. Cells were plated onto 96-well plates (5x10^5 cells/well) in culture medium overnight to allow adherence. The medium was then replaced with fresh medium containing increasing concentrations of TMZ (0-128 μg/ml). After 120 h, the medium was replaced with MTT for 4 h in the dark at 37˚C, and then shaken in DMSO for 15 min to fully dissolve the formazan crystals. Absorbance (A) of cells was measured at 590 nm with a microplate reader (Tecan Group, Ltd., Männedorf, Switzerland). A total of 3 independent experiments were performed in quadruplicate wells (plus control). The inhibition ratio was calculated as follows: P = (1 - A in treated cells/A in control cells) x 100%.

The 50% inhibitory concentration (IC_{50}) of all experimental groups was obtained.

For the cell proliferation experiment, MTT was added in each well absent of TMZ at the beginning of cell plating (0 h), and at 24, 48, 72, 96 and 120 h afterwards. The absorbance of each well was measured according to the aforementioned procedure, and the relative number of viable cells, which represented the proliferation rate, was calculated as: Absorbance value at each time/absorbance value at 0 h.

Cell cycle and apoptosis assay. Subconfluent cell cultures were incubated with 48 μg/ml TMZ for 72 h, then trypsinized, washed, collected and centrifuged (1,000 rpm) for 5 min, fixed in 1.5 ml 75% ice-cold ethanol and stored at 4˚C overnight. After resuspension in cold PBS, the cell suspension was incubated in 0.2 mg/ml propidium iodide (PI) containing 0.1% Triton X-100 and RNase A (1 mg/ml; Sigma-Aldrich) in the dark for 30 min at 4˚C. Cell cycle distribution was determined using fluorescence-activated cell sorting (FACS) analysis (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA), with excitation at 488 nm and emission at 630 nm. For the apoptosis experiment, the cell suspension was evaluated using a PI/Annexin apoptosis detection kit, according to the aforementioned procedure.

Protein screening and bioinformatics analysis
Grouping. In the present study, TMZ-induced U251 cells were denoted as the treatment group while parental U251 cells were denoted as the control group.

Construction of the protein chips. Proteins from U251 and U251R cells were extracted and assessed to meet the concentration criterion. SET100 Protein Microarrays (Full Moon BioSystems, Inc., Sunnyvale, CA, USA), containing 1,358 antibody probes, were constructed for the two groups according to the manufacturer's instructions. Protein-mixed microarrays were then subjected to signal scanning (GenePix 4000B, Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA).

Protein investigation. Protein signals were acquired using Genepix Pro 6.0 (Axon Instruments; Molecular Devices, LLC) repeatedly with GAPDH as the internal control. Relative protein expression was calculated as follows: Average/GAPDH. Acquired TMZ resistance-related differentially expressed proteins were defined by the threshold as ±1.5-fold change.

Gene Ontology (GO) and pathway analysis. The significant GOs of the genes corresponding to differentially expressed proteins were analyzed using the GO database (http://www.geneontology.org) and online software DAVID (http://david.abcc.ncifcrf.gov) for gene annotation and hierarchical categorization. All signaling pathways were analyzed using data from KEGG (http://www.genome.jp/kegg) and NCBI (http://www.ncbi.nlm.nih.gov) databases. P-values for the differentially expressed genes in all GO categories and pathways were calculated using the two-sided Fisher's exact test to obtain the enrichment value as -log10 (P-value).

TMZ resistance-related signal network. The Human Protein Reference Database was used to search for differentially-expressed proteins detected via microarray. A TMZ resistance-related signal transduction network was constructed using Cytoscape software (http://www.cytoscape.org). The significance of molecules in the network was represented by the area of each node and determined by the value of betweenness centrality, which was calculated according to the number of effective interactions through a particular molecular node. The greater the betweenness value, the more control it possesses in interactive pathways. Upregulated molecules were marked red; downregulated were marked blue; and pink was used to mark the stem and EMT-like molecules.

Protein extraction and western blotting. Proteins (50 μg) were extracted from subconfluent cultures and loaded onto polyacrylamide-SDS gels, separated by electrophoresis, and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk in PBST for 1 h at room temperature, the membranes were blotted with primary antibodies overnight at 4˚C, followed by incubation with peroxidase-conjugated secondary antibodies for 50 min at room temperature. Bands on the membranes were visualized using enhanced chemiluminescence (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary antibodies, including those against SRC (1:1,000; cat. no. ab47405), YES (1:1,000; cat. no. ab109265), LYN (1:1,000; cat. no. ab1890) and GAPDH (1:1,000; cat. no. ab9485) were all purchased from Abcam (Cambridge, MA, USA). GAPDH was used as the internal control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNAs were extracted from two experimental cell lines, using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). First-strand cDNA was reverse transcribed from 1 μg total RNA using the SuperScript First-Strand cDNA system and amplified by Platinum SYBR Green qPCR SuperMix-UDG (both from Invitrogen; Thermo Fisher Scientific, Inc.). For each PCR reaction, a Master mix was prepared that included Platinum SYBR-Green qPCR SuperMix-UDG, forward primer, reverse primer, and 10 ng template cDNA. GAPDH was used as the internal control. The thermocycling conditions were as follows: 95˚C for 2 min for the first step; 40 cycles of 95˚C for 10 sec, 55˚C for 15 sec and 72˚C for 10 sec for the second step; 95˚C for 1 min, 60˚C for 30 sec and 95˚C for 30 sec for the last step. Data were analyzed from three independent experiments and presented as the mean ± SD. The fold-change quantification of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method. Primer pairs used to detect the mRNA levels of genes were as follows: YES forward, 5'-GCCTGTCAGTACAAGTGTGAG-3' and reverse, 5'-AAAGGCGTTACCCCTGAGGAT-3'; SRC forward, 5'-TGGCAAGATCACCAGACGG-3' and reverse, 5'-GGCACCTTTCGTGGTCTCAC-3'; LYN forward, 5'-TTCTGGTCTCCGAGTCACTCA-3' and reverse, 5'-GCCGTCCACTTAATAGGGAACT-3'; GAPDH forward, 5'-CATGAGAAGTATGACAACAGCCT-3' and reverse, 5'-AGTCCTTCCACGATACCAAAGT-3'.

Statistical analysis. Data analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). The α level for type I error was set at 0.05 for rejecting the null hypothesis. Descriptive data are presented as the mean ± standard deviation (SD). The survival rate at
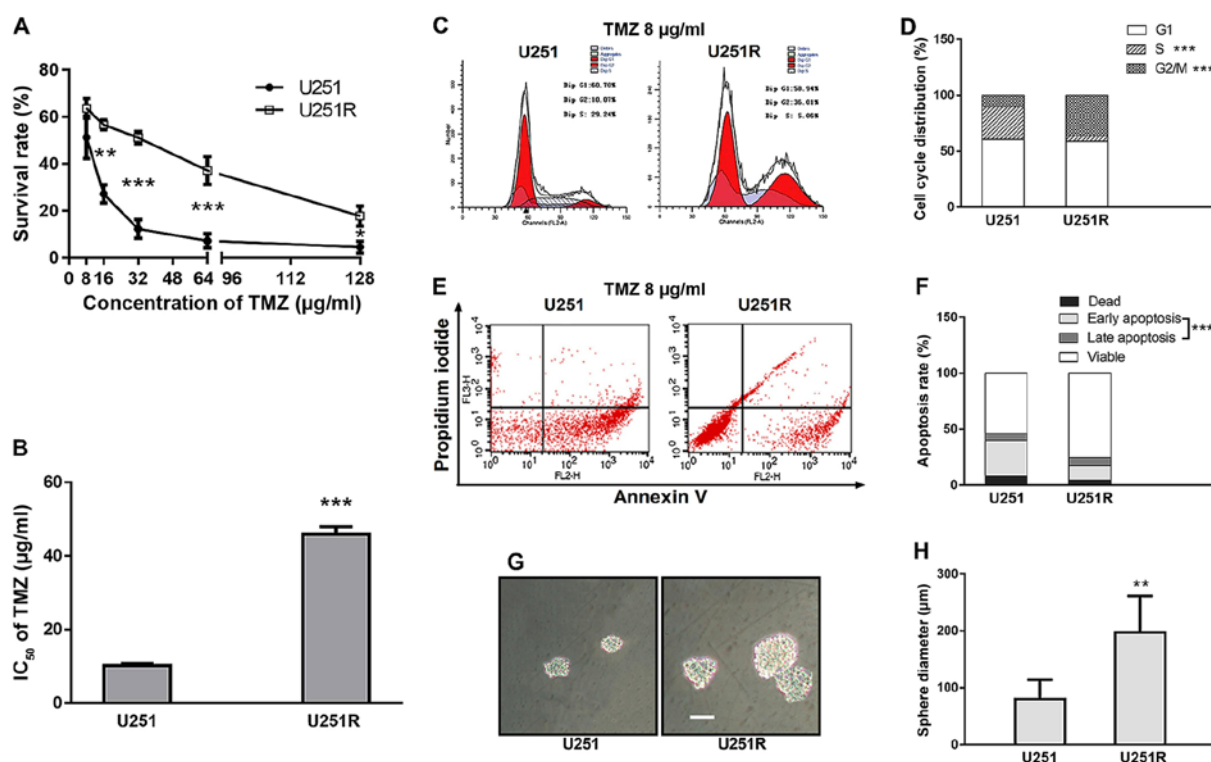
Figure 1. Characteristic changes after long-time re-exposure to TMZ indicate a stemness phenotype in U251R cells. (A) Both U251 and U251R cell lines exhibited TMZ concentration-dependent survival suppression, which was more notable in the resistant U251R group. (B) The IC_{50} value for TMZ in U251R cells was significantly higher than that in parental cultures, indicative of acquired resistance of U251R cells undergoing incremental TMZ exposure. (C and D) Cell cycle distribution following TMZ treatment was revealed by FACS. It was clearly noted in histograms that resistant U251R cells possessed a larger G2/M fraction and a smaller percentage of S-phase cells compared with the U251 cells. (E and F) PI/Annexin V double-staining demonstrated a significant reduction of both early and late apoptotic glioblastoma cells in U251R cell variants compared with sensitive U251 cells. (G and H) Self-renewal capacity of U251R cells was notably greater compared with their counterparts by measurement of secondary sphere-formation diameters. Data are presented as the mean ± SD of triplicate experiments. **P<0.01; ***P<0.001 between groups. The calibration bar in G=100 μm. TMZ, temozolomide; FACS, fluorescence-activated cell sorting.

each TMZ concentration, cell cycle distribution and apoptosis rate for the two glioblastoma cell lines were analyzed with the Student's t-test. P-value <0.05 was considered to indicate a statistically significant difference.

Results

Acquisition of TMZ resistance in the U251 glioblastoma cell line. After initial killing of a large majority of cells at each TMZ concentration period, the remaining cells survived and retained a normal growing state. Finally, a stable resistant phenotype was observed. MTT results revealed that both parental U251 cells and TMZ-induced U251 cells exhibited a prominent and concentration-dependent decrease in the viable cell number. Notably, a 4-fold increase in the IC_{50} value was observed in the TMZ-induced U251 cell line, compared with that in the parental cells (45.19 and 10.89 μg/ml, respectively) (Fig. 1A and B). After 10 passages in TMZ-free medium, and storage for 1 year at -80˚C after the last exposure to TMZ, the resistance characteristics remained constant. The subline isolated from the U251 cells was referred to as U251R.

TMZ induction causes G2/M arrest, duplication inhibition, apoptosis attenuation and increases self-renewal ability in chemoresistant U251 cells. Cell cycle distribution was deter-

mined by FACS analysis. With 8 μg/ml TMZ exposure, U251R cells exhibited a higher proportion of G2/M phase arrest and a lower proportion of S phase compared with the parental U251 cells (36.22 vs. 10.36%, P<0.001 and 4.97 vs. 29.13%, P<0.001, respectively) (Fig. 1C and D), whereas the percentage of G0/G1 phase remained unaltered between the two cell cultures. Assuming that prolonged G2/M distribution was likely to be linked with cell survival, we further assessed cell apoptosis by Annexin V/PI staining. As expected, apoptosis was significantly attenuated in TMZ-resistant U251R cells compared with that in the control group (19.95 vs. 37.83%, P<0.001) (Fig. 1E and F), which supports the link between clonogenic survival and G2/M arrest. Another assumption was that reduced S-phase proportion indicated a slower growth rate. Indeed, there was a trend of reduced proliferation in the U251R cell line compared with that in the U251 cell line, although the difference was not significant (P-value of the group according to time interaction was 0.089, data not shown).

In order to characterize the potential effect of TMZ-induction on the self-renewal activity of tumor cells, we performed a sphere-formation assay with both U251 and U251R cell lines. TMZ-resistant U251R cells grew with more notable generation of spheres in serum-free cultures compared with U251 cells. The average diameter of the neurospheres formed from U251R cells was 198 μm, whereas that of U251 cells was 80 μm (Fig. 1G and H). This observation is notable,
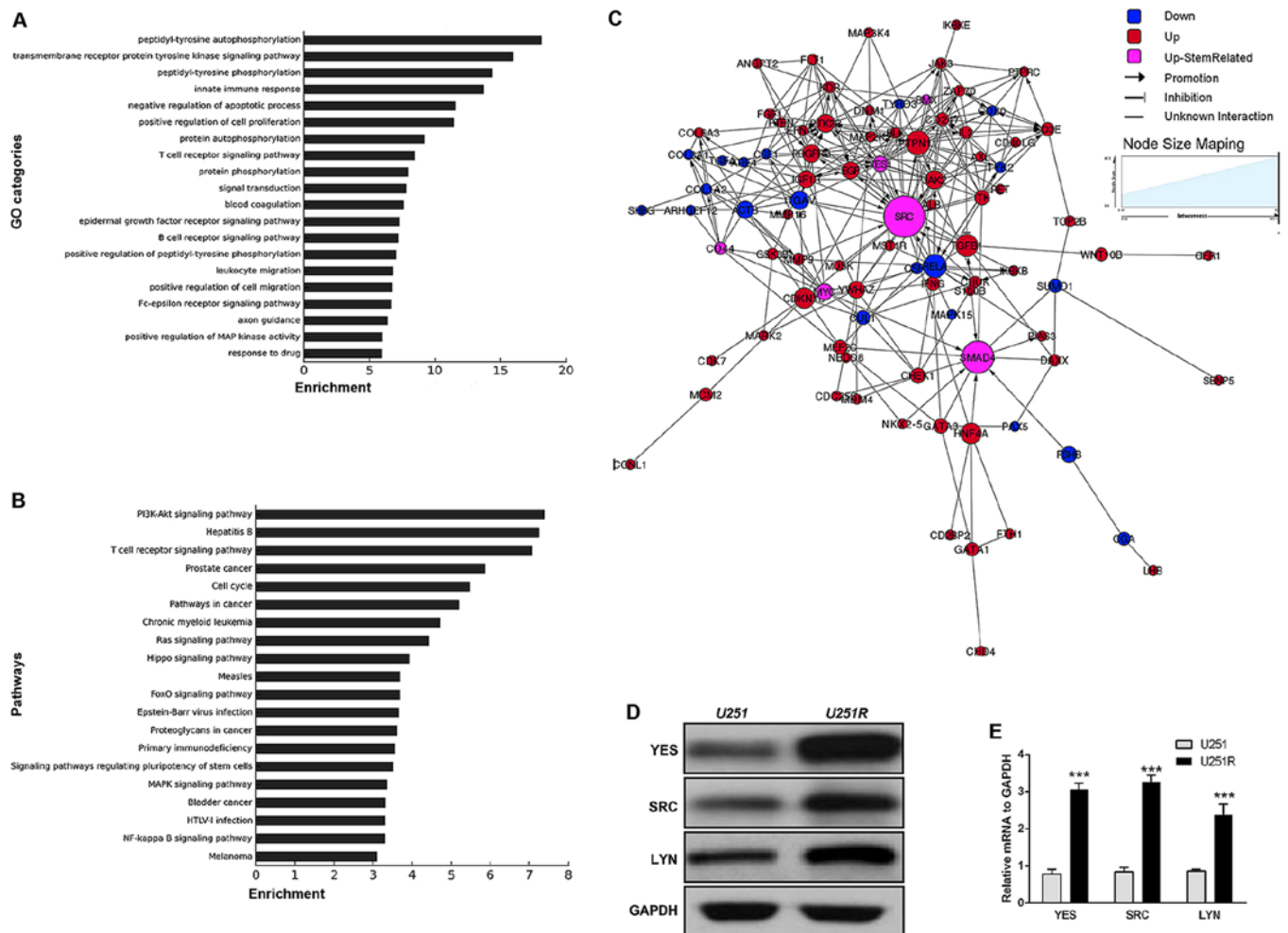
Figure 2. Bioinformatics analysis and verification of protein microarray. (A) Significant GOs of differentially expressed proteins. (B) Significant pathways of differentially expressed proteins. The transverse axis represents enrichment of each GO and pathway. (C) Signaling networks of differentially expressed molecules that are related to acquired TMZ resistance. The area of each node represents the value of betweenness centrality. Red indicates the upregulated proteins, blue indicates the downregulated, and pink indicates the stemness- and EMT-related proteins. (D) Protein levels of cell SFK family members YES, SRC and LYN were notably overexpressed in TMZ-resistant U251R cells compared with parental U251 cells, of which YES and SRC expression was more notable. (E) After normalization, the relative mRNA levels of YES, SRC and LYN were significantly upregulated in U251R cells compared with U251 cells. Data are presented as the mean ± SD of triplicate experiments. ***P<0.001 between groups. GO, Gene Ontology; SFK, SRC family kinase; TMZ, temozolomide.

since recent research indicated that cancer stem cells (CSCs) are characterized by therapeutic resistance (25). The results led us to further inspect the stemness-related pathways of TMZ-resistant cells.

Acquired TMZ resistance-related differentially expressed proteins. Using a relative expression level of 0.5-fold change as the threshold, proteins in the U251 and U251R cell lines were compared. As a result, more than 200 significant differentially expressed proteins were obtained (data not shown), including Ferritin, JAK2, MYC, MDM4, PARP, SOX2 and SRC. Of these, 160 proteins were upregulated and 40 proteins were downregulated.

GOs of the differentially expressed proteins were statistically analyzed. According to the enrichment list, the top 20 GO categories in which differentially expressed genes were identified included: Transmembrane receptor protein tyrosine kinase function, negative regulation of cell apoptosis, positive regulation of cell proliferation, protein phosphorylation and drug response process (Fig. 2A). Similarly, pathways of the

genes corresponding to differentially expressed proteins were also statistically analyzed. The results indicated that up and downregulated genes predominantly participated in 20 significant pathways, including the PI3K-Akt, Ras, MAPK and FoxO signaling pathways, as well as some stemness-related signaling pathways (Fig. 2B).

Acquired TMZ resistance-related molecular networks. According to the expression dataset acquired from the protein microarray assay, TMZ resistance-related molecules and the pathways in which they participated were investigated using the Human Protein Reference Database. On this basis, a signal transfection network was constructed (Fig. 2C). The proteins marked in pink were recognized as stemness- and EMT-related. They were at relatively significant positions, and interacted with downstream and upstream molecules participating in cell differentiation, proliferation, invasion and migration pathways. This molecular profiling, together with the results of the sphere-formation assay, indicated a phenotype transition from differentiation to stemness in TMZ-resistant cells.
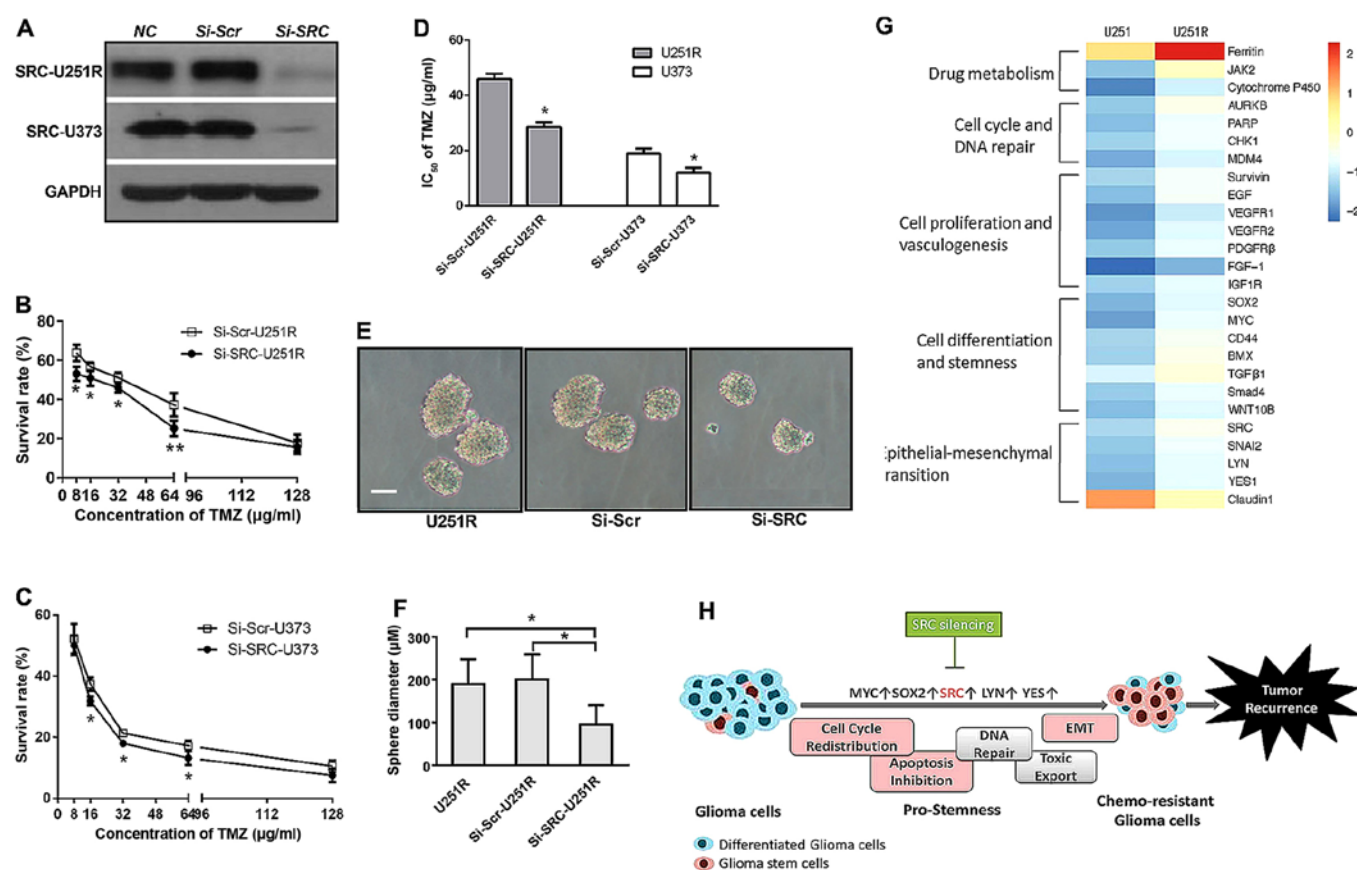
Figure 3. Effect on cell viability, TMZ resistance and self-renewal capacity by SRC knockdown. (A) Stable lentiviral transfection targeting SRC mRNA significantly reduced its expression in both U251R and U373 cells. (B and C) SRC knockdown significantly reduced the survival rate of U251R and U373 cells, compared with their counterparts that were transfected with non-targeting siRNA. (D) Glioblastoma cells with transfected siRNA-directed SRC inhibition exhibited markedly reduced IC_{50} values as compared with the control cells with scramble siRNA transfection. (E and F) SRC silencing markedly suppressed the self-renewal capacity of U251R cells. Si-Scr refers to scramble siRNA; Si-SRC refers to siRNA targeting SRC. (G) Representative molecular signatures of acquired resistance by means of a heat map generated from protein microarray. (H) Schematic illustration of acquired TMZ resistance. TMZ treatment induced a pro-stemness process resulting in chemoresistance, which could be interrupted by SRC silencing. Pink represents the mechanisms investigated in the present study. Data are presented as the mean ± SD of triplicate experiments. *$P<0.05$; **$P<0.01$ between groups. The calibration bar in E=100 μm. TMZ, temozolomide.

TMZ induction upregulates SFK family kinases in chemoresistant U251 cells at both the protein and mRNA levels. It was previously reported that glioma stem cells (GSCs) isolated from GBM patient tissues are more resistant than normal GBM cells to TMZ and radiotherapy (25). Consistent with this, the results of our sphere formation assay strengthened the stemness-chemoresistance link in GBM cells. Further inspection of the molecular signatures revealed a variety of signaling pathways that were related to the stem-like phenotype. Among them, a group of SRC family kinases (SFKs) attracted particular attention. It has previously been reported that SFKs can mediate stem-like signaling and EMT in breast cancer, and in head and neck cancer (37,38). Thus, to demonstrate that SFKs are indeed upregulated in the chemoresistant U251R cell line, we conducted an immunoblotting experiment, the results of which were in accordance with those of the microarray experiment, revealing upregulation of SRC, YES and LYN (Fig. 2D). Furthermore, RT-qPCR analysis confirmed that the mRNA levels of these proteins in TMZ-resistant U251 cells were notably higher compared with those in the TMZ-sensitive cells (relative expression 3.68 for YES, 2.33 for LYN and 2.89 for SRC) (Fig. 2E).

shRNA-mediated SRC knockdown reduces self-renewal activity and enhances TMZ sensitivity. It was previously found that inhibitors of several SFK proteins, including an ATP-competitive dual SFK/ABL inhibitor dasatinib, suppressed the migration of GSCs *in vitro* (37). Consistent with this, we revealed that the characteristics of TMZ-resistant U251R cells were similar to those of GSCs, and a series of SFK members were significantly involved in the signaling to confer TMZ resistance. We then surmised that inhibition of SFKs would affect the TMZ sensitivity of U251R cells, which was hypothetically related to cell stemness. In particular, SRC was not only significantly upregulated, but also located in the central hub of signaling networks. Therefore, we selectively performed transient siRNA transfection against human SRC and successfully inhibited SRC expression in the U251R and U373 cell lines (Fig. 3A). Then, we performed a viability assay and the results revealed that lentivirus-induced SRC inhibition partially reversed the increased survival curve of resistant U251R (Fig. 3B), and decreased the survival rate of the U373 cells (Fig. 3C). Accordingly, the IC_{50} values calculated based on the survival curve were clearly reduced in both cell lines (45.91 vs. 28.58 μg/ml in U251R, and 18.89 vs. 11.98 μg/ml in

U373) (Fig. 3D). Next, we repeated the sphere-formation assay to observe the effect of SRC-knockdown on cell self-renewal. The increase in sphere diameter in U251R cells was abolished after Si-SRC transfection. The average diameter was 186 μm in U251R, 200 μm in scramble siRNA-transfected U251R, and 102 μm in SRC siRNA-transfected U251R cells (Fig. 3E and F). These collective results indicated that enhanced sensitivity to TMZ in U251R and U373 cells is associated with an SRC-mediated stem-like phenotype.

Discussion

The underlying mechanism for acquired TMZ resistance in glioblastoma is considered to be complex (10). In addition to the well-studied mechanisms of adenosine triphosphate binding cassette (ABC) superfamily-associated drug transport to avoid the penetration of chemotherapeutics into the brain (11-13), glioblastoma cells may also initiate DNA damage repair. This could include enhanced base excision repair (BER), nucleotide excision repair (NER), impaired MMR and the more specific single/double-stand break repair pathways to restore DNA integrity (6,14-16). In addition, two equally important cell fate-decisive processes, apoptosis and autophagy, are heavily involved in chemoresistance. The former is usually determined by the balance of Bcl-2 family members, comprised of pro-apoptotic and anti-apoptotic factors (13,17,18), while the latter depends on the stage and extent of lysosomal degradation of chemotherapeutic toxicants (19,20). Moreover, drug metabolism activity may serve as another reinforcement to protect cancer cells from drug-induced cytotoxicity, which is represented by increased expression of glutathione S-transferase (GST) in glioblastoma cells (21). Finally, two cell differentiation-related phenotypes, EMT and cancer stemness (e.g., glioma stem cells), have been shown to give tumors quiescent properties, in order to evade the therapeutic window of most alkylating agents (9,22).

In the present study, we managed to successfully isolate U251R cells with a four-fold IC_{50} value, compared with the parental U251 cell line, by incremental TMZ induction, which imitated clinical TMZ administration. FACS analysis revealed an increased rate of G2/M arrest and a decreased S-phase fragment, as well as a reduced apoptotic rate in U251R cells compared with the parental U251 cells. These results indicated that the acquisition of TMZ chemoresistance could be attributed to cell cycle alterations and apoptosis suppression. G2/M arrest, a distinct phenomenon following TMZ treatment, is widely accepted to be critical for TMZ toxicity caused by O^6-G lesions and the subsequent deleterious MMR response (19). It is argued that the DNA repair response is tightly linked with the G2 checkpoint and G2/M arrest, with the notion that glioblastoma cells can initiate DNA repair pathways when arrested by TMZ treatment so as to prevent cells from apoptotic death (23,24). The current findings of both G2/M arrest and apoptosis inhibition support the view that G2/M arrest induced by TMZ is not just a destructive but also a reconstructive process for glioma cells. Another effect of TMZ on glioblastoma cells was the reduced duplication phase. This has previously been reported in six glioblastoma cell lines treated with 100 μM TMZ for 72 h (19). Similarly, our FACS study found that a reduced rate of S-phase cells

was accompanied by slower proliferation in the U251R cell line (data not shown). Previously, Ye *et al* reported that GSCs are slow-cycling and tumor-initiating cells. Additionally, Chen *et al* indicated that CD133⁺ head-neck cancer stem cells expressed low levels of epithelial differentiation maker CK18 and had a reduced apoptosis rate (25,26). Since DNA repair, apoptosis suppression and slower growth are all characteristics of cell stemness, we performed a sphere-formation assay in the present study, and found that TMZ-treated U251R cells exhibited greater self-renewal capacity compared with TMZ-sensitive cells. Considering previous findings with our current results, we suggest that U251R cells may acquire stem-like attributes, which confer their chemoresistance during TMZ treatment.

Next, we performed a high-throughput comparative protein microarray in order to further investigate the mechanisms for acquired chemoresistance. We first noticed that MGMT, MMR members (MSH2/3/6) and ABC family proteins, which participate in DNA repair and drug detoxification, were not significantly altered between U251 and U251R cell lines. Although the methylation status of the MGMT promoter could not be determined by the antibody-based screening, the results implied that the mechanisms of chemoresistant glioma cells are versatile. In addition, 200 differentially expressed molecules, participating in a variety of biological processes and signaling pathways, were identified.

Ferritin is an intracellular iron storage protein that localizes to the cell nuclei to protect DNA from oxidative damage. It has been reported that ferritin blockade can increase tumor sensitivity to chemotoxins in glioblastoma cells (27). As the most significant upregulated protein in the TMZ-resistant cell line, ferritin is likely to play a critical role in the regulation of TMZ chemoresistance. It is well known that cytochrome P450s (CYPs) are crucial to drug metabolism in addition to GST. Increasing expression of cytochrome P450 in U251R indicated that cytochrome P450 is likely to participate in the process of detoxification by accelerating TMZ metabolism (28). JAK2 and AURKB are two other upregulated molecules able to catalyze phosphorylation of a spectrum of protein substrates. A recent study found that JAK2-mediated Y41 phosphorylation and AURKB-mediated S10 phosphorylation on histone H3 were associated with radio-chemoresistance in patients with GBM. Kinase inhibitors targeting AURKB enhanced both radiation- and TMZ-sensitivity (29,30). In addition, AURKB is essential for G2 checkpoint-related G2/M transition. Therefore, from our cell cycle analyses, it is reasonable to surmise that there is a close relationship between G2/M arrest and the upregulation of AURKB expression. We noticed the upregulated expression of various DNA repair- and apoptosis-associated proteins, including PARP, CHK1, MDM4 and Survivin (31-34), which was concordant with the observed G2/M arrest and apoptosis inhibition in U251R cells. Furthermore, transmembrane receptor tyrosine kinase (i.e., growth factor receptor) has previously been correlated with glioblastoma therapeutic resistance through MDR1 (a major membrane protein transporter) (35,36). According to the protein-chip results, several members of growth factor receptor signaling pathways, including EGF, VEGFR1, VEGFR2, PDGFRβ, FGF-1 and IGF-1R, were upregulated in the U251R cell line.

Most notably, a group of stemness- and EMT-related proteins, including MYC, SRC, SOX2, CD44, BMX, TGFβ1, SMAD4, SNAI2, LYN and YES, were overexpressed in U251R cells, whereas epithelial proteins, such as Claudin1 and E-cadherin, were downregulated. In the past, Auger *et al* identified a stem cell-like genotype in SNB-19 TMZ-resistant variants, while Ye *et al* observed slow-cycling and strong radiation-resistance in patient tumor-derived GSC cultures (25,37). Additionally, EMT is commonly regarded to be closely associated with cell stemness, since EMT transition could lead to misplaced stemness properties, while cancer stem cells are capable of aberrantly communicating with peri-tumor niches for migration like mesenchymal cells (26). Collectively, with the observed stem-like behavior of U251R cells, we propose the hypothesis that glioma cells developing stemness convert to chemoresistant glioma cells (Fig. 3H).

SFKs form a family of non-receptor tyrosine kinases that is intensively involved in glioma tumorigenesis, invasion and migration. There are nine members of the SFK family, of which c-Src (also referred to as SRC), Fyn, Lyn, Yes and Lck were shown to be highly expressed in GBM samples (38,39). Our protein-chip based observations indicated significant upregulation of and a central role for SRC in the networks regulating TMZ-resistance. Accordingly, SRC silencing was able to reverse the acquired survival advantage of U251R cells, while sensitizing U373 cells to TMZ treatment. Empirically, SRC has been linked to cancer cell stemness. Boivin *et al* and Chen *et al* proposed that CD133 is a novel binding partner of SRC. SRC inhibitor PP2 reduced the expression of SOX2-interacting stemness marker Oct4 and could reverse the EMT pattern in head-neck cancer stem cells (26,40). Consistent with this, we observed that SRC silencing in U251R cells inhibited their self-renewal, a prominent biological characteristic of stem-like cells. Moreover, Li *et al* demonstrated that SRC/Akt/mTOR signaling cascades are important for the maintenance of leukemic stemness (41). In fact, our previous study reported a suppressive effect of Akt2 knockdown on TMZ sensitivity both *in vitro* and *in vivo* (13). Therefore, the regulatory axis of SRC/AKT2 remains promising, although the current findings may not establish a direct connection between SRC-mediated stemness interruption and TMZ chemoresistance.

There were several limitations of the present study, which may weaken the clinical relevance of the results. Firstly, the variant U251R cell line was not derived from patients with GBM. Secondly, radiation treatment was not administered along with TMZ to induce the therapy resistance in U251 cells. However, we depicted a network with a wide range of molecules and signaling pathways conferring the acquired TMZ resistance, and proposed a promising therapeutic modality targeting the pro-stemness process that is regulated by SRC.

Acknowledgements

Availability of data and materials

The datasets used during the present study are available from the first author JL upon reasonable request.

Authors' contributions

HH, JL and YC conceived and designed the study. JL, JZ, HF, SL, JL and QX performed the experiments. JL and WW conducted the bioinformatics. JL and GQ wrote the paper. XW, CS and GQ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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