Experimental study of selective MGMT peptides mimicking TMZ drug resistance in glioma

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ABSTRACT

Background: Glioblastoma (GBM) is a very frequent primary tumour in the cerebrospinal nervous system. Temozolomide (TMZ) is the first-line treatment for patients with GBM. However, some of GBM patients do not respond to TMZ. O6-methylguanine-DNA-methyltransferase (MGMT) remains a major cause. In a previous study, we detected antibodies against MGMT peptides in patients with glioma, and five highly responsive autoantibodies against MGMT-02, anti-MGMT-04, anti-MGMT-07, anti-MGMT-10, and anti-MGMT-18 were identified that could be used to dynamically assess chemotherapy-resistant TMZ. Therefore, targeting MGMT peptides may be a potential therapeutic approach for GBM to fight TMZ resistance.

Methods: First, MGMT-02 and MGMT-04 polypeptides with cell-penetrating peptides were designed and connected to FITC tracer for immunofluorescence localisation. CCK-8 and colony formation assay were performed to evaluate cell proliferation ability. Western blot and immunofluorescence analysis were used to detect the expression of apoptosis-related protein. Flow cytometry was used to detect the proportion of apoptosis in cells.

Results: TMZ-resistant effect of MGMT-02/04 peptides was assessed in intracranial xenograft nude mouse model. We also found reduced apoptosis of cells treated with MGMT-02 and MGMT-04 peptides and TMZ compared with those treated separately with TMZ in vivo and in vitro experiences.

Conclusion: The results of this study indicate that MGMT-02 and MGMT-04 peptides have a role in glioma resistance and that MGMT peptides may serve as a precise target for TMZ-resistant GBM.

1. Introduction

Glioblastoma, often referred to as glioblastoma multiforme (GBM) is the most common and aggressive malignant brain tumour [1]. The typical medical care for GBM involves surgical excision followed by radiation therapy and treatment with the DNA alkylating agent, temozolomide (TMZ) [2]. TMZ is the only “standard” treatment available to patients, because of the fact that TMZ is the only drug available that has been demonstrated to improve the survival of GBM patients [3]. However, the acquisition of TMZ resistance inevitably results in treatment failure and a poor prognosis of GBM patients [4]. The O6-methylguanine-DNA methyltransferase (MGMT) protein has been shown to be an important mechanism underlying treatment resistance [5].

As a “DNA suicide repair enzyme” [6], MGMT eliminates deadly O6-methylguanine (O6-MG) DNA damages produced by TMZ by repairing damaged guanine nucleotides by transferring methyl at guanine O6 to cysteine residues [6,7]. Methylation of the MGMT promoter is believed to inhibit MGMT expression, resulting in TMZ resistance [8]. MGMT expression is largely, but not exclusively, controlled by promoter methylation [9]. The measurement of the methylation of the MGMT promoter is generally predictive of response and overall survival in patients with GBM [10]. Due to the important role of MGMT in TMZ resistance, we wanted to enhance the chemotherapy effect of TMZ by targeting MGMT. In a previous study, Wu et al. (2018) detected autoantibodies against MGMT peptides in the serum of patients and investigated whether we could use these autoantibodies as a novel biomarker for the clinical management of gliomas. We divided MGMT into more than 20 polypeptides by peptide microarray technology and screened the corresponding highly responsive MGMT polypeptides using MGMT autoantibodies. We obtained five highly responsive peptide sequences, namely “MGMT-02, MGMT-04, MGMT-07, MGMT-10, and MGMT-18”.

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Today, thanks to precision medicine, many solid tumours in advanced phases respond well to medications that target the particular genetic changes that fuel tumor growth. Therefore, we propose that these five MGMT peptides may be the direct cause of TMZ resistance in GBM. This study aims to lay a theoretical foundation for designing corresponding monoclonal antibodies based on these five peptides in subsequent experiments to establish new precision treatment targets for gliomas. This will provide a new idea for adjuvant TMZ chemotherapy regimens. Among these five high-response peptide sequences, MGMT-02 and MGMT-04 had the highest responses. Therefore, in this study, we chose peptides MGMT-02 and MGMT-04 to simulate the resistance of MGMT to TMZ to verify the hypothesis that targeting these peptides can potentially reduce resistance to TMZ.

2. Material and methods

2.1. Cell culture

U251 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and SHG140 cells were obtained from primary glioma cells [12]. The cells were grown in Dulbecco’s Modified Eagle Medium/F12 (Corning, NY, USA) with a 10% foetal bovine serum (Gibco, MA, USA) supplement in a 37 °C, 5% CO2 and humidified incubator.

2.2. Construction and location of cell-penetrating peptide

MGMT-02 and MGMT-04 peptides with NTP were obtained from GenScript Biotech Corporation (NJ, USA). The construction and preparation of peptides with NTP is performed as described [13]. To confirm that the peptides entered the nucleus, we performed immunofluorescence analysis. The cells were incubated on slides and treated with peptides. After 24 h, 4% paraformaldehyde was used to fix the cells. Staining with 4’,6-diamidino-2-phenylindole was done on the nuclei (Sigma-Aldrich, MO, USA). Then, we used confocal laser scanning microscopy to observe the cells (Olympus, Tokyo, Japan).

2.3. Cell viability assay

TMZ, MGMT-02 with NTP, and MGMT-04 with NTP were added to 96-well plates containing cell suspensions at a total volume of 100 μL. After induction for 24, 48, and 72 h, 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Japan) was added, and the cells were incubated at 37 °C for 2 h in a constant temperature incubator. Cell viability was evaluated by measuring the difference in optical density values at 450 nm using an enzymatic standard.

2.4. Colony formation assay

The cells were cultured in six-well plates at a density of 1000 cells/well for 2 weeks, and the culture medium was changed every 3 d. Two weeks later, the medium was removed and the wells were washed twice with phosphate-buffered saline (PBS). Colonies were then fixed in 4% formaldehyde for 20 min, washed twice with PBS, and stained with 0.25% crystal violet for 20 min at room temperature. Finally, the plates were washed with distilled water for 15 s and left to dry. The colonies were counted and visualised.

2.5. Western blotting and immunofluorescence analyses

After TMZ and MGMT-02/04 with NTP treatment, the cells were collected in lysis buffer, incubated on ice for 30 min, and centrifuged at 12,000×g at 4 °C for 10 min. The supernatant was collected, and the protein concentration was determined. Equal amounts of protein samples were subjected to 8%-12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated in 5% skim milk with a secondary antibody at room temperature for 1 h using for exposure. Protein bands are quantified, expressed as the ratio of target protein/β-actin, and then graphed as values [14,15].

For immunofluorescence staining, the cells were grown on chamber slides, fixed with 4% paraformaldehyde, and treated with primary antibodies. The secondary antibodies used were Texas fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse or red-conjugated goat anti-rabbit (Molecular Probes, OR, USA).

2.6. Apoptosis analysis by annexin V-FITC/PI

Apoptosis was detected using an annexin V-FITC/PI staining kit (BD Biosciences, NJ, USA). Briefly, 1 × 106 cells were collected and resuspended in 100 μL of binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added, and the cells were incubated for 15 min in the dark at room temperature. The samples were tested using a BD FACS Celse (BD Biosciences). FlowJo software (version 10.3; Tree Star Inc., OR, USA) was then used to analyze the percentages of viable and apoptotic cells [16,17].

2.7. Calcein-AM/propidium iodide (PI) staining

To investigate whether MGMT peptides affect the treatment in vivo, we use SHG140 cells to establish a nude mouse model. Female BALB/c nude mice (5 weeks, 15–17 g) were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). A total of 5 × 106 SHG140 cells with luciferase-encoding lentivirus (GeneChem, Shanghai, China) were injected into the mice (n = 6 per group) as described [19]. SHG140 cells injected into the mouse brain were divided into control and MGMT-02 peptide-treated groups. Next, the two groups of animals with SHG140 cells injection were divided into TMZ-and PBS-treated groups. TMZ was dissolved in 2% dimethyl sulfoxide, 30% PEG-300, and 68% ddH2O. Seven days after implantation, the mice were treated with an equal dose of TMZ (60 mg/kg/d) or PBS every other day for 28 dd during the survival period as described [20]. Intracranial tumour size was assessed, and radiance values were recorded on the 7th, 14th, and 28th day using “IVIS Spectral Real-Time Imaging System (Blandford, USA).” The brains removed from live mice were fixed in 4% paraformaldehyde, paraffin-embedded, and stained with haematoxylin and eosin. Both international animal welfare guidelines and national laws were followed.

2.8. Intracranial xenograft nude mouse model

The Fisher’s exact test was used to determine whether or not a correlation existed. The findings are shown as the average of a minimum of 3 separate trials. Every test had two tails. GraphPad Prism (GraphPad Software Inc, CA, USA) was used to conduct the statistical analysis.

3. Results

3.1. Construction and localisation of MGMT-02 and MGMT-04

We propose that MGMT peptides may be precise targets of MGMT-
mediated TMZ resistance. The most toxic and mutagenic DNA modification produced by TMZ is the addition methyl groups at O6 sites on guanines (O6-MeG), which can be quickly repaired by MGMT [21]. Since MGMT-mediated DNA repair mainly occurs in the nucleus, we used a nucleus-penetrating peptide, RIFHFRIGC (also known as nuclear transport peptide [NTP]), which is composed of 10 amino acids [13] to deliver MGMT peptides to the nucleus. Based on this, we constructed two fusion proteins, MGMT-02-NTP and MGMT-04-NTP, with FITC conjugated on the protein for tracing (Fig. 1B). First, we performed Western blot analysis to examine the expression of MGMT in various human glioma cells. The primary cell lines SHG140 and U251 with positive and negative MGMT expression, respectively, were selected as experimental objects (Fig. 1A). We verified that MGMT-02 and MGMT-04 could enter the nucleus through their fluorescent localisation (Fig. 1C).

3.2. Effect of MGMT peptides on cell proliferation

To examine whether MGMT-02 and MGMT-04 peptides affect glioma proliferation, cell viability and growth were evaluated after treatment with different concentrations of MGMT peptides. The results showed that MGMT-02 and MGMT-04 had no effect on cell viability or growth (Fig. 1D and E). Then we examined the activities of the peptides with TMZ treatment. The effect of TMZ on GBM cell inhibition was assessed using CCK-8. Treatment with higher concentrations of TMZ for longer durations resulted in increased cell inhibition (Fig. 1F). Finally, we chose a 24 h as the experimental time point and obtained the half-maximal inhibitory concentration values.

To determine the appropriate dosing concentrations of MGMT-02 and MGMT-04 peptides in this experiment, we treated the cells with TMZ and different concentrations of peptides. We then observed the concentration of peptides that began to produce a TMZ-resistant effect (Fig. 1G).

3.3. MGMT peptides reduce apoptosis caused by TMZ in glioma cells

Subject to the observations, we concentrated on the effects of the MGMT peptides on TMZ. We investigated the effects of MGMT-02 or MGMT-04 in combination with TMZ on apoptosis. Annexin V-FITC/PI staining was performed to assess whether MGMT-02 or MGMT-04 decreased apoptosis in GBM cells. As shown in Fig. 2A and B, compared with the control groups, MGMT-02 or MGMT-04 peptide treatment resulted in an almost consistent percentage of apoptotic cells, TMZ treatment resulted in a higher percentage of apoptotic cells, and MGMT-02/04 + TMZ treatment resulted in a decreased percentage of apoptotic cells. Cell calcine-AM/PI staining experiments, in which calcine-AM stained live cells and PI stained dead cells, were used to determine live cells. Similarly, compared with the TMZ treatment group, the MGMT-02/04 + TMZ treatment group had a reduced number of dead cells (Fig. 2C).

Next, we examined apoptosis-related proteins via western blotting. The level of cleaved PARP and Bax increased and the level of Bcl2 decreased upon TMZ treatment. With MGMT-02/04 treatment, the level of cleaved-PARP and Bax decreased and the level of Bcl2 increased (Fig. 3A and B). We also performed Bcl2 and cleaved PARP immuno-fluorescence staining to investigate apoptosis (Fig. 3C) and found no significant changes in the levels of Bcl2 and cleaved PARP between the MGMT-02/04 treatment and control group. At the same time, the expression of cleaved PARP increased significantly while that of Bcl2 decreased significantly in the TMZ treatment group. However, similar to the previous results, the expression of cleaved PARP decreased and that of Bcl2 increased in the MGMT-02/04 + TMZ treatment group compared with the TMZ treatment group. Taken together, the results suggest that MGMT-02 and MGMT-04 peptides increase resistance to TMZ in vitro.

3.4. MGMT peptides mimic TMZ resistance in vivo

To investigate whether MGMT peptides reduce TMZ sensitivity in glioma in vivo, we selected MGMT-02 peptides with the highest response for this experiment. SHG140 cells and MGMT-02 treated SHG140 cells were transplanted into 5-week-old female BALB/c mice. Starting on day 7 after successful implantation, PBS or TMZ (60 mg/kg/day) was injected intraperitoneally every other day for 28 d. Subsequently, we observed tumour growth weekly via bioluminescence imaging and used the tumour size and radiographic values as indicators to assess the treatment effect. We found that the growth rate of intracranially transplanted tumours with MGMT-02-treated SHG140 cells showed no noticeable change compared with the control group. At the same time, the therapeutic effect of TMZ was worse in tumours planted with MGMT-02-treated SHG140 cells than in those planted with normal SHG140 cells (Fig. 4A and B). Similar results were observed for survival time (Fig. 4C). In addition, haematoxylin and eosin staining of brain sections from nude mice showed that tumours became significantly smaller after treatment with TMZ, but TMZ efficacy was reduced in tumours planted with MGMT-02-treated SHG140 cells (Fig. 4D). These results are similar to those of the in vitro experiments, confirming the TMZ-resistant effects of the MGMT peptides.

4. Discussion

Currently, TMZ is the only chemotherapeutic agent that can prolong the overall survival of patients with GBM [22]. However, not all patients benefit from TMZ, and current data suggest better outcomes in patients with methylated promoters and no MGMT expression than in those with unmethylated promoters [23]. The mechanism by which TMZ induces apoptosis is through methylation of the location of guanine O6, causing DNA double strands to break and the cell cycle to stagnate [24,25]. The MGMT protein removes the damaging alkyl group from the guanine O6 position and repairs DNA, after which the alkylated protein is degraded [26]. During this process, the MGMT proteins must be continuously supplemented. MGMT expression levels are directly related to the methylation state of the MGMT promoter, which is observed in approximately 45% of patients presenting with GBM and is associated with increased sensitivity to TMZ and prolonged survival [27]. Many studies have also been conducted on the anti-TMZ resistance effects of MGMT, such as O6-benzylguanine and interferon β, but no significant survival benefit has been observed [28,29]. In addition, Gupta SK et al. found that veliparib, an inhibitor of polymerisation of ADP-ribose polymerase, significantly improves TMZ’s effectiveness in tumours with a hypermethylated MGMT promoter but not the unmethylated [30]. Therefore, drug resistance brought on by using TMZ to treat GBM is a significant problem. However, with a deeper comprehension of the development of gliomas, targeted therapies are becoming a breakthrough and a promising treatment strategy. So far, the FDA (United States Food and Drug Administration) has approved more than 100 antibodies. Bevacizumab (FDA approved) is an injection of a humanised monoclonal antibody that binds to and inhibits vascular endothelial growth factor, which is a growth factor that promotes the growth of blood vessels and the growth of tumours [31]. Pembrolizumab is a humanised antibody used to treat advanced hepatocellular carcinoma [32]. However, for GBM, targeted therapy faces a dilemma. Most drugs for targeted therapy, such as cilengitide, cediranin, and nimotuzumab, have failed in phase I and II clinical trials [33–35]. Therefore, this study was based on MGMT peptides, with the aim of establishing a precise target for GBM.

Due to the hope of constraining the drug resistance function of MGMT through smaller fragments, MGMT has been cut into 20 peptides through peptide microarray technology in previous research by Wu et all. (2019). After comparing these 20 peptides with the antibodies in the patient’s serum, it was found that these 20 peptides had different responses. MGMT-02 and MGMT-04 peptides had the highest response.
Fig. 1. Construction and localisation of MGMT-02/04 and their effect on cell proliferation. (A) Western blot of MGMT in glioma cells. (B) Construction of cell-penetrating MGMT-02 and MGMT-04 peptides. (C) Immunofluorescent localisation of MGMT-02 and MGMT-04 peptides in glioma cells. Cells staining was done with MGMT-02/04 (green) and 4',6-diamidino-2-phenylindole (DAPI; blue). Images were captured using a laser confocal microscope. Scale bar, 50 μM. (D) SHG140 and U251 cells were treated with MGMT-02/04 for 24 h. By using the Cell Counting Kit-8 (CCK-8) assay, cell viability was assessed. (E) Colony formation density of MGMT-02/04-treated assessed via colony formation assay. (F) SHG140 and U251 cells were treated with temozolomide (TMZ) for 24, 48, and 72 h. Cell inhibition was determined via CCK-8 assay. (G) Statistics are expressed as the average ± standard deviation of the mean (SEM). *P < 0.05, **P < 0.01, and ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Therefore, these two peptides were selected for this experiment. Since the DNA repair effect of MGMT-02 and MGMT-04 peptides occurs in the nucleus, it is not sufficient to design only peptides. Based on previous studies, the cell-penetrating peptide RIFHFRIGC known as NTP is composed of 10 amino acids and allows the peptide to enter the nucleus to exert its activity [13]. Therefore, in this study, we designed MGMT-02 and MGMT-04 peptides with NTP and traced them with FITC modifications. Subsequently, we verified through fluorescence localisation that these peptides could enter the nucleus, thus further mimicking the role of MGMT proteins.
In summary, this study verified that MGMT-02 and MGMT-04 peptides can imitate the drug-resistant effect of MGMT on TMZ in human glioma cells. The resistance of peptides to TMZ was observed using various apoptotic indicators in this experiment, which proves that MGMT may be a promising target. The five MGMT peptides screened in previous experiments were likely to be the direct cause of GBM resistance to TMZ. We designed and prepared monoclonal antibodies targeting the active site of MGMT in combination with TMZ for the five MGMT peptides. In future experiments, we will verify whether these monoclonal antibodies, combined with TMZ, can enhance the tumour lethality of TMZ by preventing drug resistance in tumour cells.

Author's contributions

YZ and YW designed the experiments and revised the manuscript. GC and KZ analyzed the data and revised the manuscript. YW and HW performed the experiments and wrote the manuscript. HW, WY, GC and ZK analyzed and interpreted the data. HW and YW confirmed the
authenticity of all the raw data. All authors read and approved the final manuscript.

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Declaration of competing interest

All authors disclosed no relevant relationships.

Data availability

No data was used for the research described in the article.

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