Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Temozolomide combined with PD-1 Antibody therapy for mouse orthotopic glioma model

Bailing Dai [a, *], Na Qi [b], Junchao Li [c], Guilong Zhang [d]

[a] Department of Radiology, The Fifth People's Hospital of Shanghai, Fudan University, Shanghai, 200240, China
[b] Department of Radiology, Shanghai East Hospital, Tongji University, Shanghai, 200123, China
[c] Department of Radiology, Laizhou City People's Hospital, Yantai, 261400, China
[d] Department of Neurosurgery, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, 210009, China

ARTICLE INFO

*Article history:*
Received 5 May 2018
Accepted 10 May 2018
Available online xxx

*Keywords:*
Glioma
Temozolomide
PD-1 Antibody
Chemotherapy
Immunotherapy

ABSTRACT

*Purpose:* Temozolomide (TMZ) is the most frequent adjuvant chemotherapy drug in gliomas. PDL1 expresses on various tumors, including gliomas, and anti-PD-1 antibodies have been approved for treating some tumors by FDA. This study was to evaluate the therapeutical potential of combined TMZ with anti-PD-1 antibody therapy for mouse orthotopic glioma model.

*Methods:* We performed C57BL/6 mouse orthotopic glioma model by stereotactic intracranial implantation of glioma cell line GL261, mice were randomly divided into four groups: (1) control group; (2) TMZ group; (3) anti-PD-1 antibody group; (4) TMZ combined with anti-PD-1 antibody group. Then the volume or size of tumor was assessed by 7.0 T MRI and immunohistochemistry, and the number of CD4 and CD8 infiltrating cells in brain tumor and spleen was evaluated by immunohistochemistry. Western blot was used to evaluate the expression of PDL1. Furthermore, Overall survival of each group mice was also evaluated.

*Results:* Overall survival was significantly improved in combined group compared to other groups ($\chi 2 = 32.043$, $p < 0.01$). The volume or size of tumor was significantly decreased in combined group compared with other groups ($F = 42.771$, $P < 0.01$). And the number of CD4 and CD8 infiltrating cells in brain tumor was also obviously increased in combined group (CD4 $F = 45.67$, $P < 0.01$; CD8 $F = 53.75$, $P < 0.01$).

*Conclusion:* Anti-PD1 antibody combined with TMZ therapy for orthotopic mouse glioma model could significantly improve the survival time of tumor-bear mice. Thus, this study provides the effective preclinical evidence for support clinical chemotherapy combined with immunotherapy for glioma patients.



## 1. Introduction

Malignant gliomas including Glioblastoma Multiforme (GBM) are the most frequently occurred primary malignant tumors (accounts for at least 70% in adult) in the central nervous system [1]. In spite of combining multi-therapies (surgery, chemotherapy or radiotherapy etc.) over decades to optimize the treatments, the median survival of the patients has no significant increase (the 5-year survival rate is below 5%). Nowadays, chemotherapy is still the main adjuvant therapy of gliomas. Temozolomide (TMZ) is the first-line chemotherapy drug approved by the US Federal Drug Administration (FDA), but its efficiency is only about 45%, due to the exist of tumor drug resistance [2].

With the rise of tumor immunotherapy, the tumor immune escape plays a very important role in the progression of tumor, and tumor microenvironment normally protects tumor cells from killed by immune system. The immunosuppressive status of patients with gliomas is related to tumor microenvironment, which can secrete cytokines to inhibit T cell proliferation and differentiation, and affect the number and function of $CD4^+$ and $CD8^+$ cells and dendritic cells [3]. Furthermore, chemotherapy drug resistance is connected with the cytotoxicity induced by lack of adaptive immune response [4]. And the interaction between PD-1 and its ligand PD-L1 is the main mechanism to mediate immunosuppression in tumor microenvironment [5]. And the PD1 receptor

* Corresponding author. Department of Radiology, The Fifth People's Hospital of Shanghai, Fudan University, 801 Heqing road, Minhang District, Shanghai, 200240, China.
*E-mail address:* dbl751206@126.com (B. Dai).

https://doi.org/10.1016/j.bbrc.2018.05.064
0006-291X/

expressed on the activated T lymphocyte binding with the PD-L1 ligand expressed on the tumor cells can reduce the ability of the T cell and result in immune tolerance.

Programmed Death Factor 1 receptor (PD1) as type I transmembrane receptor belongs to the immunoglobulin superfamily, expressed on $CD4^{-}CD8^{-}$ thymocytes, peripheral $CD4^{+}$, $CD8^{+}$, NK T cells, B cells, macrophages and dendritic cells. It has two types of ligands, named as programmed death factor 1 ligand (PD-L1) and PD-L2 [6]. PD-L1 plays a key regulatory role, and highly expressed in various tumors, such as glioma, ovarian cancer, melanoma and lung cancer *etc.* [7]. And patients with tumors overexpressing PD-L1 normally correlates with the size of tumor, the number of lymph node, the grades of tumor and the overall survival of patients [8]. Thus, overexpression of PDL1 in patients with tumors suggests poor prognosis. Similarly, the expression level of PD-L1 in gliomas is also closely related to the grades of gliomas [9,10]. Except expressed on glioma cells, PD-L1 also expressed in the around of tumors, for example the infiltrative lymphocytes or adjacent neurons [11,12], it implies the poor prognosis [13].

However, because of the presence of the blood-brain barrier, the central nervous system is often referred to as immunological privileged site, with immune function defection. Therefore, it will be a promising treatment which through the immune cells selectively though the BBB to kill glioma cells in the CNS [14]. The role of anti-PD-1 monoclonal antibody maintains T cells as activated state by binding to the PD1 receptor on the surface of T cells, so as to exhibit the ability of killing the tumor.

Thus, in this study, we were the first time to assess the therapeutic potency of combined chemotherapeutic drug TMZ with anti-PD1 antibody in mouse orthotopic glioma model. And we found that the combination treatment group achieved significant therapeutic effects compared to control group, such as it significantly reduced the lesion of tumor-bearing mice, and obviously extended the survival time of tumor-bearing mice.

## 2. Material and methods

### 2.1. Glioma cells

Mouse glioma cell line GL261 purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). GL261 cells were cultured in DMEM culture medium (10% fetal bovine serum + 1% penicillin, Gibco), and the plates were placed at 37 °C incubator (5% CO2 + 5% O2).

### 2.2. Mouse orthotopic glioma model built

Eighty male C57BL/6 J mouse (20–22 g, 6–8 weeks) purchased from the Comparative Medical Center of Yangzhou University. Under the supervision of animal experiment welfare ethical review of Southeast University, mouse orthotopic glioma model was constructed as below: $5 \times 10^4$ GL261 glioma cells (5 μl) were injected into the right cerebral cortex of the anesthetized mice, the injection rate was 0.5 μl/min, and the specific sites were 1.5 mm after the fontanelle, 1.5 mm on the right, 1.5 mm from the skull surface. The second day after operation, the mice were randomly divided into four groups (n = 15–22 mice/group): control group, TMZ chemotherapy group (TMZ, Sigma), anti-PD1 monoclonal antibody treatment group (RMP1-14, BioXCell), and combined TMZ with anti-PD1 monoclonal antibody group. The dose of anti-PD1 monoclonal antibody was 200 μg/times (dissolved in sterile saline), intraperitoneal injection (ip), alternate dose, total of three times; the dose of TMZ was 50 μg/kg, (dissolved in 5% DMSO and 5% Solutol-15 sterile saline), ip, for 5 consecutive days; combined treatment group was given the same amount of anti-PD1 antibody and TMZ treatment; and the control group was given the same amount of sterile saline treatment. All animals were euthanatize by $CO_2$ asphyxia with the following symptoms, such as weight loss> 20%, poor physical condition, cachexia, cannot move, drowsiness, ataxia, seizures, bending hump posture and other heavy neurological deficits symptoms.

### 2.3. Magnetic resonance imaging

The tumor size of mice was performed by 7.0 T small animal magnetic resonance scanners (Germany, Brooke). Mice were firstly anesthetized by 1.5–2.5% isoflurane and 0.8 L/min $O_2$, and then placed them in a 72 mm quadrature volume coil. Tumor growth and volume size were assessed by the T2 WI imaging sequence of MRI. Ten days after modeling, mice were checked by MRI, and examined followed by every 4–6 days, until 21 days. The tumor diameter and short diameter of largest layer were measured by the post-processing software of MRI, and then calculated the tumor volume in accordance with the formula ($width^2 \times length \times 0.5$). Finally, mice were euthanized at 1–2 days before expected death, and obtained the survival curve time (Kaplan-Meier curves) of tumor-bear mice by GraphPad 5.0 software and IBM SPSS 20.0 software.

### 2.4. Immunohistochemistry (IHC)

The brains of mice were immobilized with paraformaldehyde, and embedded by paraffin. The slices were incubated by normal goat serum at room temperature, and then incubated with rabbit anti-mouse CD4 antibody and rabbit anti-mouse CD8 primary (Bioss, China; 1: 100, 4 °C overnight); DAB staining, and finally observed in microscope. The criteria of positive results was: the cytoplasm was brown particles. Under the high magnification of microscope (×400), each slice randomly selected eight images and calculated the mean number of positive cells (number/$mm^2$) by using IPP 6.0 software.

### 2.5. Western blot

Total proteins were extracted from glioma tissues with lysis buffer (RIPA), and quantified by a bicinchoninic acid protein assay kit (KeyGen, China). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 20–40 μg of protein from each sample. The electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Millipore) and incubated with diluted primary antibodies against PDL1 (1: 1000; Abcam), β-actin(1:10000,Abcam), followed by incubation with a horseradish peroxidase–conjugated secondary antibody (1: 5000; Santa Cruz). Quantification of western blots was performed according to the manufacturer's protocols.

### 2.6. Statistical analysis

SPSS19.0 statistics software package was used for data analysis. Data are presented as mean ± standard deviation (SD, $x \pm s$) of at least three independent experiments. One-way ANOVA and multiple comparison tests were used to evaluate the volume or size difference between the tumor treatment group and the non-treatment group, with P value < 0.05 considered statistically significant. Kaplan-Meier survival curve was used to analyze the survival time of the model mice thought by Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test.

Please cite this article in press as: B. Dai, et al., Temozolomide combined with PD-1 Antibody therapy for mouse orthotopic glioma model, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.064