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Original Articles

# Inhibition of GSH synthesis potentiates temozolomide-induced bystander effect in glioblastoma

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the most aggressive human tumors with poor prognosis. Current standard treatment includes chemotherapy using DNA alkylating agent temozolomide (TMZ) concomitant with surgical resection and/or irradiation. However, GBM patients exhibit various levels of the elevated expression of DNA repair enzyme, due to MGMT causing resistance to TMZ. Determination of the MGMT-positive population of primary tumor is important to evaluate the therapeutic efficacy of TMZ. Here we generated TMZ-resistant GBM cells by introducing MGMT into TMZ-sensitive GBM cell line KMG4, and established a model to assess the TMZ-induced bystander effect on TMZ-resistant cells. By mixing TMZ-resistant and -sensitive cells, GBM tumors with MGMT positivity as 50%, 10%, and 1% were generated *in vivo*. We could not observe any bystander effect of TMZ-induced cell death in tumor with 50% MGMT positivity. Although the bystander effect was observed within 20 days in the case of tumor with 1% MGMT positivity, final tumor size at day 28 was the same as control without sensitive cells. This bystander effect was observed *in vitro* using conditioned medium of TMZ-damaged GBM cells, and PCR array analysis indicated that the conditioned medium stimulated stress and toxicity pathway and upregulated anti-oxidants genes expression such as catalase and SOD2 in TMZ-resistant cells. In addition, the reduction of the activity of anti-stress mechanism by using inhibitor of GSH synthesis potentiated TMZ-induced bystander effect. These results suggest that GSH inhibitor might be one of the candidates for combination therapy with TMZ for TMZ-resistant GBM patients.

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## 1. Introduction

Gliomas are the most common primary tumor of the central nervous system, accounting for approximately 30%, and are classified into four clinical grades from I to IV. The most aggressive and lethal of the tumors is glioblastoma multiforme (GBM) [1]. The prognosis for a GBM patient is dismal, and the patients have a median survival of only 14.6 months, mainly because conventional post-surgical chemotherapeutic agents and irradiation exhibit limited effects [2,3].

Temozolomide (TMZ) is an alkylating agent used for malignant glioma including GBM [4]. TMZ induces DNA methylation of guanine at the $O^6$ position and $O^6$-methylguanine incorrectly pairs with thymine, which triggers the mismatch repair (MMR) system leading to double strand break of the genome that results in the arrest of the cell cycle and induction of apoptosis [5,6]. $O^6$-methylguanine methyltransferase (MGMT) removes methylation from the $O^6$ position of guanine and induces TMZ resistance [7]. Thus, the patients with silenced MGMT expression through methylation of the MGMT promoter were reported to have an improved 2-year survival with TMZ treatment together with irradiation [8]. In addition, immunohistochemical study demonstrated that GBM patients exhibit various levels of the elevated expression of MGMT, probably causing various degrees of resistance to TMZ [9]. Thus, determination of the MGMT-positive population of primary tumor is important to evaluate the therapeutic efficacy of TMZ. However, it is controversial whether one can set the cutoff value of MGMT positivity in GBM for evaluation of the application of TMZ treatment.

Despite the expectations that pseudosubstrates of MGMT such as $O^6$-benzylguanine would suppress resistance by depleting MGMT [10–12], clinical trials did not show significant restoration of TMZ sensitivity in patients with TMZ-resistant GBM [13]. Therefore, other therapeutic agents which suppress MGMT expression and sensitize the efficacy of TMZ are highly desired [14].

The so-called "bystander effect" was initially borrowed from the field of gene therapy, where it usually referred to the killing of several types of tumor cells by targeting only one type of cell

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within a mixed population [15]. Radiation and chemotherapy-induced bystander effect has been extensively studied [16,17]. While the bystander effect of Paclitaxel or Mitomycin C in breast cancer and liver cancer, respectively, has been reported, TMZ-induced bystander effect has not been evaluated yet. Although the precise mechanisms bystander effects derived by radiation or chemotherapy are unknown, soluble signaling factors [18,19] and cellular communication such as via gap junctions may be involved [20].

Considering that tumors are highly heterogeneous with respect to their sensitivity towards chemotherapeutic agents, identification of reagents which can enhance the bystander effect is highly desired. In this study, we established a heterogeneous tumor model with differential MGMT positivity by mixing TMZ-resistant and -sensitive cells, so that we could estimate the TMZ-induced bystander effect *in vivo*. Furthermore, using this model we evaluated whether a combination of antioxidant inhibitors and TMZ can act synergistically to enhance bystander TMZ-resistant cell killing.

## 2. Materials and methods

### 2.1. Cells

The human GBM cell line KMG4 was kindly provided by Dr. Kazuo Tabuchi (Saga University, Japan). KMG4 was cultured in Dulbecco's modified minimal essential medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS). Cell line authentication was not carried out by the authors within the last 6 months.

### 2.2. Reagent

3-Amino-1,2,4-triazole (3-AT, an inhibitor of catalase), 2-methoxyestradiol (2-ME, an inhibitor of Mn-SOD), diethyldithiocarbamate (DDC, an inhibitor of Cu, Zn-SOD), and L-buthionine sulfoximine (BSO, an inhibitor of GSH synthesis) were all purchased from Sigma–Aldrich (St. Louis, MO). Temozolomide (TMZ) was purchased from LC Laboratories (Woburn, MA, USA). All reagents were used following the manufacturer's instructions.

### 2.3. Preparation of retrovirus and establishment of stable cell line

For retrovirus production, the pcx4 vector system was used [21,22]. The complete sequences of pcx4pur (puromycin) is available from the GenBank database (AB086386). Full-length cDNAs for human MGMT was subcloned into pcx4pur. Retroviruses were obtained by using 293T cells as packaging cells, and were infected to KMG4 and selected with puromycin (2 μg/ml).

### 2.4. Xenograft

For xenograft preparation, the indicated number of cells was injected s.c. into 6- to 8-week-old female athymic nude mice (BALB/cAJcl-*nu*/*nu*) purchased from Clea Japan (Tokyo, Japan). For evaluation of TMZ treatment, TMZ (50 mg/kg) or control DMSO was administered i.p. days 3–8. The tumor volume ($mm^3$) was calculated by using the following formula: (length × $width^2$)/2. All animal procedures were carried out according to the protocol approved by the institutional Animal Care and Use Committee at Hokkaido University Graduate School of Medicine.

### 2.5. Immunoblotting

Immunoblotting was performed by the method described elsewhere. Cells were lysed with buffer containing 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM PMFS, 1 mmol/L $Na_3VO_4$. Proteins were subjected to SDS-PAGE, and separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore, Billerica, MA, USA). Filters were probed with antibodies obtained from the following sources: anti-MGMT (MT3.1) MAb and anti-Actin MAb (Chemicon, Temecula, CA, USA). Bound antibodies were detected with peroxidase-labeled goat antibody to mouse IgG or goat antibody to rabbit IgG, and visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Freiburg, Germany).

### 2.6. Conditioned medium preparation

$1 \times 10^5$ of KMG4 were cultured in DMEM supplemented with 10% FBS and treated with TMZ (100 μM) or control DMSO. After 5 days the cell-conditioned medium was collected.

### 2.7. PCR array analysis

The expression profile of 84 Stress and Toxicity pathway-related genes was determined using a 96-well format human Stress and Toxicity pathway $RT^2$ Profiler PCR array (SABiosciences, USA) according to the manufacturer's instructions. The array also included 6 housekeeping genes and 3 RNA as internal controls. qPCR were run on an ABI 7900HT qPCR instrument equipped with SDS 2.3 software, using $RT^2$ SYBR Green/ROX qPCR master mix (Applied Biosystems, UK). Data analysis was done by the $2^{-\Delta\Delta Ct}$ method on the manufacturer's Web portal http://www.SABiosciences.com/pcrarraydataanalysis.php (Applied Biosystems, UK).

### 2.8. Immunohistochemical analysis

Formalin-fixed paraffin-embedded xenografts were sectioned and stained using anti-MGMT (MT3.1) (Chemicon, Temecula, CA, USA) antibody. Percentage of KMG4–MGMT cells was calculated by the number of positive cells per 100 tumor cells in different areas under 200× magnification in 10 fields and the mean was calculated.

## 3. Results

### 3.1. Establishment of TMZ-resistant KMG4 GBM cells by MGMT overexpression

To confirm that MGMT is involved in TMZ resistance, we introduced MGMT by using retroviral infection system into the glioblastoma cell line KMG4 without endogenous MGMT (Fig. 1A). Although KMG4 is TMZ-sensitive as expected [14], KMG4 cells expressing MGMT (KMG4–MGMT) exhibited strong resistance against TMZ *in vitro* (Fig. 1A). *In vivo* growth rates of tumors of KMG4 or KMG4–MGMT cells subcutaneously injected into the nude mice were found to be almost equal, and intraperitoneal injection of TMZ completely regressed the tumor of KMG4 1 month after the administration (Fig. 1B). However, TMZ exhibited no effect on the tumor growth of KMG4–MGMT *in vivo* (Fig. 1B). To examine the degree of TMZ resistance within the GBM tumors with different MGMT positivity, mixture of TMZ-sensitive and -resistant cells were subcutaneously injected into nude mice and generated GBM with MGMT positivity as 50%, 10%, and 1%. Three weeks after injection, tumors of the same size were formed with MGMT positivity as 50%, 10%, and 1% as GBM models of different MGMT positivity (Fig. 1C).

### 3.2. Evaluation of TMZ-induced bystander effect in vivo

To evaluate the efficacy of TMZ onto the GBM with differential MGMT positivity, we treated these nude mice with TMZ as intraperitoneal injections. To assess the TMZ-induced bystander effect on TMZ-resistant cells, we compared the xenograft composed of a certain number of KMG4–MGMT cells with that composed of the same number of KMG4–MGMT mixed with the same number of KMG4 (50% MGMT positivity) (Fig. 2A) or 10 times number of KMG4 (10% MGMT positivity) (Fig. 2B) or 100 times number of KMG4 (1% MGMT positivity) (Fig. 2C). In the case of GBM with 50% MGMT positivity, no effect was observed with TMZ treatment (Fig. 2A). In contrast, TMZ-dependent bystander effect was observed in the early stage within 20 days in case of GBM with 10% or 1% MGMT positivity (Fig. 2B and C). However, the growth difference was not observed after day 23 and final tumor volume at day 29 was not significantly changed even in the case of GBM with 1% MGMT positivity. These results suggest that TMZ-induced bystander effect on growth suppression persisted only for a limited duration. The positivity of MGMT after TMZ at day 29 was 100% in any tumor (Fig. 2D). Considering the tumor of KMG4 cells treated with TMZ completely regressed at day 29 shown in Fig. 1B and MGMT positivity was not changed after injection into the subcutaneous of nude mice without TMZ treatment shown in Fig. 1C, 100%

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