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Original Article

Glioma cells with Tim-3 expression induce resistance to chemotherapy drug

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Abstract: Purpose: T cell immunoglobulin-3 (Tim-3) as a negative regulator of anti-tumor immunity has been widely understood. However, the mechanism that Tim-3 depresses anti-tumor are unclear in drug-resistance glioma cells. The present research corroborates Tim-3 expression in drug-resistance glioma cells. Materials and methods: Two glioma sublines (U87 and U251) which had been continually cultured were retested for the study. Cell proliferation activity, cell viability in early and late stage, the expression of the Tim-3 RNA, were detected by CCK-8 and real-time PCR experiments. Enhancement of sensitivity of glioma cells to chemotherapeutic agent was tested after inhibiting Tim-3 expression by ADV-antisense Tim-3. Results: U87 and U251 glioma cells were killed by various doses of temozolomide (TMZ). The CCK-8 experiment showed that the low, middle and high dose of TMZ could advance the cell apoptosis. With the increase of TMZ concentration, the ratio of apoptosis cells accordingly augmented. Expression of Tim-3 was higher in the high dose groups than the lower and middle, but BAT3 decreased. The difference between the two groups was statistically significant ($P< 0.05$). The killing effect increased after interfering Tim-3 expression by ADV-antisense Tim-3. Tim-3 expression was associated with drug resistant glioma cells. Conclusion: Tim-3 expression in the drug-resistant glioma cells leads to resistance to therapeutic reagents. Significantly, down-regulating the expression of Tim-3 improved the potential of TMZ treatment. Tim-3 expression may play an antiapoptotic functional role in drug-resistance glioma cells by protecting cancer cells.

Keywords: Glioma, cell lines, Tim-3, drug resistance

Introduction

Glioblastoma is the most malignant and devastating primary brain tumour in adults. Current treatment remains insufficient as these tumors are inclined to relapse despite extensive resection followed by radiochemotherapy. Several independent factors can explain drug resistance that results in final therapy failure. Determining the cause of drug resistance and applying additional treatment strategies to prolong survival of malignant glioma patients are required. This study elaborates relevant mechanisms related to glioma resistance toward chemotherapy. Investigating resistance mechanisms to different drug modalities are prevalent in the tumor [1]. Therefore, the development of novel strategies to enhance the efficacy of TMZ would be effective.

Tim-3 can specifically discriminate Th1 cells and Th2 cells in both mice and humans [2].

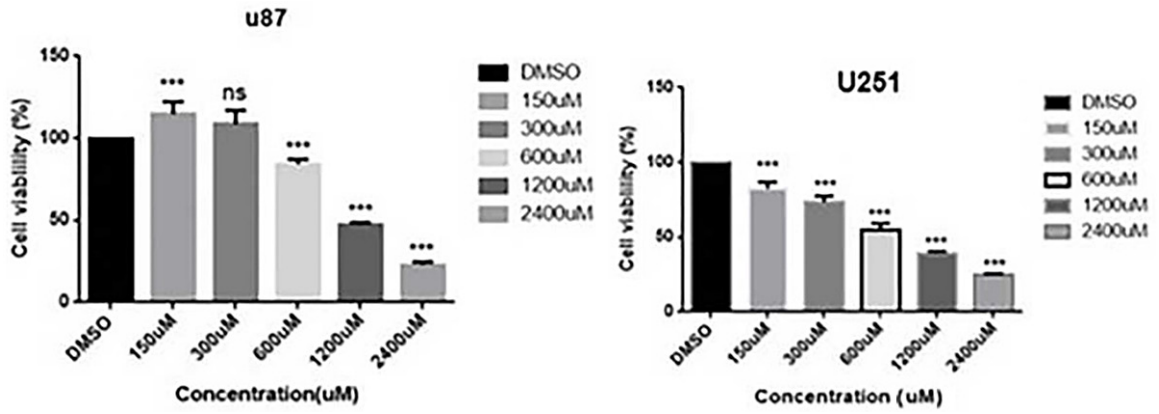

Figure 1. TMZ killing ability to glioma cell lines is associated with the concentration.

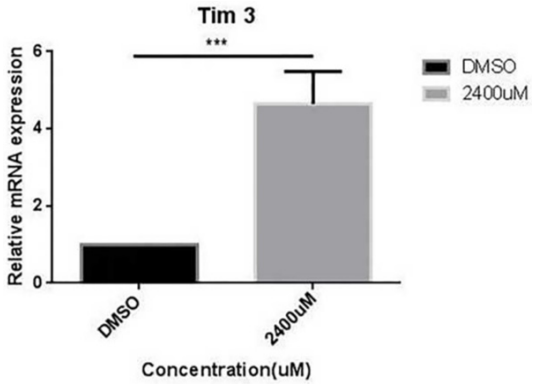

Figure 2. The level of Tim-3 expression in drug resistant cell lines (2400 μM) was significantly high than the control.

Evidence supporting Tim-3 expression in tumor cells has been diffusely reported in recent years [3]. In this study, by using real-time PCR, we found that Tim-3 is expressed on drug resistant glioma cells. The expression level of Tim-3 shows significant correlation with the ratio of drug-resistant cells. Furthermore, knockdown of Tim-3 gene sensitized glioma cells to TMZ treatment by synergistically enhancing apoptosis. The finding of Tim-3 expression in glioma cells opens new perspectives on the function of Tim-3 and suggests that Tim-3 is a potential treatment target for patients with malignant glioma.

Materials and methods

Agents

TMZ were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO), and stored at -20°C. The stock concentration was 200 mmol/L. TMZ were diluted in serum-free medium at indicated concentrations for treatment.

Cell culture

U87 and U251 cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (Invitrogen, Carlsbad, CA) supplied with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37°C with 5% CO_2. When the cells covered the bottom of culture plate 80%-90%, began adding various concentrations of TMZ, cultured for 24, 48, and 72 hours.

Cell viability assay

Cell viability of U87 and U251 after TMZ incubation was assessed using Cell Counting Kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Briefly, U87 and U251 cells were seeded in 96-well flat-bottomed plates at a density of 3×10^4 in 180 μL of conditioned medium per well. After exposure to increasing concentration of TMZ (DMSO or 150-2400 μmol/L) for 72 hours at 37°C in a humidified 5% CO_2 atmosphere, 10 μl CCK-8 solution was added to each well. The absorbance was quantified at 450 nm with a spectrophotometer. Each experiment was performed in triplicate, and each assay was repeated three times.

Reverse transcriptase-polymerase chain reaction detection of tim-3

Briefly, total RNA was extracted from the two glioma cell lines. A 5 sense primer (5-CTGCTGCTACTACTTACAAGGTC-3) and a 3 anti-

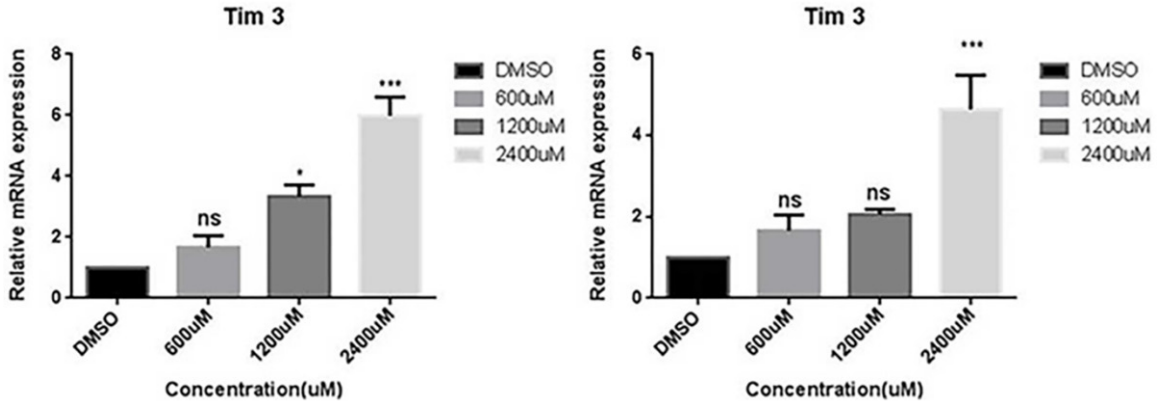

Figure 3. The level of Tim-3 expression was based on the TMZ concentration.

Table 1. The IC_{50} of TMZ to glioma cell lines

Cell line	TMZ IC_{50} (μmol/l)
U87	1200 ± 15
U251	1400 ± 38

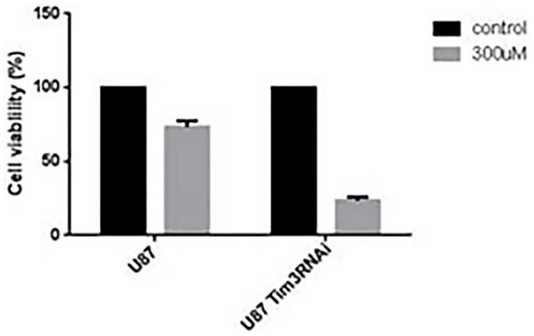

Figure 4. Interfering with the Tim-3 gene enhanced sensitivity of cell lines to TMZ.

sense primer (5-GCAGGGCAGATAGGCATTCT-3) were used to amplify Tim-3 transcripts. A 5-sense primer (5-CTCACGAAACTGGAATAAG-C-3) and a 3- antisense primer (5-AAGCCACA-CGTACTAAAGGT-3) were used to amplify a b-actin internal control. Total RNA extracted from PBL was used as positive control. The primers used for RT-PCR detection of Tim-3 were designed to span introns to avoid false positive amplifications resulting from DNA amplifications. Additionally, total RNA product was used without reverse transcription as templates for PCR as negative control to be sure free of genomic DNA contamination.

Statistical analysis

All statistical analyses were carried out using the Graph-Pad Prism 5.0 software program. The data are expressed as mean ± standard error. Student's t-test was used to determine any statistically significant differences. *P* values are two sided, with P<0.05 considered to indicate a statistically difference.

Results

U87 and U251 glioma cells were resistant to TMZ

Tim-3 played a crucial role in the resistance of glioma cells to TMZ. Interference of Tim-3 expression reversed this effect. By using viability assay, U87 and U251 glioma cells were found to partially survive well even after incubation with a high concentration of TMZ. The results are shown in **Figures 1** and **3**. The IC_{50} for TMZ in U87 and U251 glioma cell lines was calculated to be 1200 μmol/L and 1400 μmol/L (**Table 1**). Therefore, U87 and U251 glioma cell lines presented a remarkable insensitivity to TMZ. Tim-3 expression level went up in drug resistance glioma cell lines than the control group (**Figure 2**). After knockdown of Tim-3 gene via virus, the viable cell rates of the U87 and U251 were 19.45 ± 1.45 and 17.68 ± 1.38% compared to 42.78 ± 1.26% for the TMZ only group (P<0.05, **Figure 2**), following treatment with 1200 μmol/L TMZ. The injection of TMZ significantly induced the death of the glioma cell line, but did not markedly affect the growth of the resistant cell lines, when compared to the control. This result confirmed the lack of response to TMZ, and showed further that this drug resistance was maintained.

Tim-3 inhibits TMZ-induced apoptosis in U87 and U251 cell lines

Knockdown of Tim-3 gene sensitizes cancer cell lines to the chemotherapeutic agent via the

enhancement of apoptosis. TMZ alone elicited a moderate increase in the fraction of hypodiploid cells, while knockdown of Tim-3 gene observably increased cell death. Quantitative analysis demonstrated that the cell lines increased from 42.78 ± 1.26% in TMZ-treated cell lines to 81.55 ± 19.2% in cells with combined treatment (TMZ + interfering Tim-3 expression). The findings suggest induction of apoptosis by knocking down Tim-3 expression in glioma cells. To further confirm the induction of apoptosis, activation of cells by CCK-8 after 72 h TMZ treatment was performed. The assay demonstrated that TMZ incubation after knocking down Tim-3 expression resulted in a percentage of apoptotic cells significantly greater than that seen in glioma cells treated without interfering Tim-3 expression.

Interfering with expression of Tim-3 enhances sensitivity of cell lines to TMZ

Following 1200 IU/ml TMZ treatment to cells by interfering with the Tim-3 gene, the viable cell rates of the U87 and U251 cells were 19.45 ± 1.45 and 17.68 ± 1.38%, respectively, compared to 81.55 ± 19.2% and 76.45 ± 11.7% for the TMZ only. The result revealed that interfering with the Tim-3 gene remarkably enhanced the sensitivity of glioma cell lines to TMZ (**Figure 4**).

Discussion

High grade gliomas remain one of the most intractable and lethal solid tumors. The median survival time of patients is roughly 15 months, despite surgical resection, radiotherapy, and chemotherapy [1, 2, 4]. TMZ can effectively prolong overall survival and has become the most important chemotherapeutic agent for patients with high grade glioma [5]. Unfortunately, the antitumor activity of TMZ is not always durable due to intrinsic or acquired drug resistance. Therefore, identification of the cause of drug tolerance to TMZ is highly desired.

Tim-3 which can affect therapy of cancer in the tumor microenvironment, is a surface molecule that can specifically distinguish Th1 cells from Th2 cells in both mice and humans [2]. Interestingly, we observed that drug-resistant glioma cell lines had Tim-3 expression, and are presently in the process of determining the proximity of this role. The coincidental finding of Tim-3 expression being resistant to chemotherapeutic-induced apoptosis led to our efforts to determine whether Tim-3 expression serves a functional role in triggering drug resistance in glioma. To investigate this potential mechanism, drug resistance glioma cells were explored. These cells normally do not have a major population positive for Tim-3. In the current study, we found Tim-3 expression in the drug resistance cell lines, and interfering with Tim-3 expression could enhance sensitivity of cell lines to TMZ *in vitro*. TMZ significantly inhibited cell growth and induced apoptosis in glioma cell lines, so the vast majority of glioma cells are sensitive to TMZ.

The results of the present study demonstrate that expression of Tim-3 elevated resistance of glioma cells to TMZ strongly and suggested the necessity for implementation of therapeutic regimens that consider Tim-3 as a potential target. As a matter of fact, one study has implied that anti-Tim-3 monoclonal antibodies tagged with a therapeutic drug could destroy cancer cells. This approach may have had two consequences, one being the anticipated specific delivery of the therapeutic agent and second the unexpected enhancement of the efficacy of agents by interfering with Tim-3 expression [6].

In support of cell signaling, drug resistant cell lines were shown to have a low BAT3 expression compared with common glioma cell lines. These cells with Tim-3 expression were shown to be more reluctant to undergo apoptosis with less BAT3 expression. BAT3 was bound to Tim-3 in the presence of preventing initiation of apoptosis. These data support that knockdown of Tim-3-directed regulation promotes decreased drug resistance and inhibits apoptosis. Recently, aberrant Tim-3 expression was reported in melanoma cells, contributing to the low adhesion ability of tumor cells and promoting the survival of the tumor [3]. Zhuang et al. also showed a significant role of Tim-3 expression in tumor cells, which serves as an independent prognostic factor for patients with non-small cell lung cancers [7]. Here, for the first time, the role of Tim-3 expression was demonstrated in glioma drug resistant cell lines. In addition, we observed a significant correlation between Tim-3 expression in drug resistant cell lines.

Recent research has supported an important effect of Tim-3 in T cell exhaustion in cancer [4, 8]. Blockade of Tim-3 pathways is more effective in controlling tumor growth than targeting either pathway solely, suggesting these two pathways work synergistically in establishing T cell exhaustion. Tim-3 gene knockdown has not been reported in the glioma cell lines. A functional role for Tim-3 on cytotoxic resistance has not yet been established in this interfering glioma cell lines. The mechanisms for regulating Tim-3 expression in glioma cells are not yet fully understood. Wiener et al. studied that Tim-3 as a negative regulator of T helper 1 -cell responses, resulted in local immunosuppression [2, 3].

Moreover, recent studies have affirmed that Tim-3 overexpression was associated with shorter overall survival of epithelial cancers involving lung cancer and cervical cancer [7, 8]. More importantly than all of these findings, Cao et al. found that down-regulating the expression of Tim-3 in Hela cells inhibited both the migration and invasion [9]. These results certified that Tim-3 not only suppress tumor, but also directly boost cancer progress.

The experiments reported here display that it is possible to cut down drug-resistance using knocking down Tim-3 expression, which affords some protection for drug resistant in tumor cell lines, although the protection is not as complete as that against the original cells. Further experimentation is required along these lines to unravel the maze of variables possible in drug-resistance procedures. It is possible that if immunization could precede chemotherapy, even though protection was not complete, chemotherapy might be more effective and the outgrowth of resistant tumor cells might be prevented. This type of experimentation might also be carried out for other drug resistant tumor cell lines. Consequently, the data provide evidence that cancer cells with Tim-3 expression would be resistant to chemotherapy.

Tim-3 expression in tumor cell lines was detected and was found to correlate with drug-resistance. Tim-3 expression may play an anti-apoptotic functional role in drug-resistance glioma cells. Down-regulating the expression of Tim-3 improved the ability of TMZ treatment to glioma cell lines.

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Disclosure of conflict of interest

None.

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