Original article

^{99m}Tc-Tetrofosmin Uptake Correlates with the Sensitivity of Glioblastoma Cell Lines to Temozolomide

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Abstract

^{99m}Tc-tetrofosmin (^{99m}Tc-TF) is a single-photon emission computed tomography tracer that has been used for brain tumor imaging. The aim of the study was to assess if ^{99m}Tc-TF uptake by glioblastoma cells correlates with their response to temozolomide (TMZ). We investigated the correlation of TMZ antitumor effect with the ^{99m}Tc-TF uptake in two glioblastoma cell lines. The U251MG cell line is sensitive to TMZ, whereas T98G is resistant. Viability and proliferation of the cells were examined by trypan blue exclusion assay and xCELLigence system. Cell cycle was analyzed with flow cytometry. The radioactivity in the cellular lysate was measured with a gamma scintillation counter. TMZ induced G_2/M cell cycle arrest in U251MG cells, whereas there was no effect on cell cycle in T98G cells. Lower ^{99m}Tc-TF uptake was observed in U251MG cells that were exposed to TMZ compared to control (P = 0.0159). No significant difference in respect to ^{99m}Tc-TF uptake was found in T98G cells when exposed to TMZ compared to control (P = 0.8). With ^{99m}Tc-TF, it was possible to distinguish between TMZ-sensitive and resistant glioblastoma cells within 6 h of treatment initiation. Thus, ^{99m}Tc-TF uptake may consist a novel approach to assess an early response of glioblastoma to chemotherapy and deserves further investigation.

Keywords: ^{99m}Tc-tetrofosmin, glioma, temozolomide

Introduction

Glioblastoma is the most frequent and most malignant primary brain tumor. Despite intensive clinical and basic research and several novel therapeutic methods, the median survival remains short in the range of about 15 months.[1] Current treatment involves surgical excision followed by radiotherapy with concurrent and adjuvant chemotherapy.[1] The methylating agent temozolomide (TMZ) is the first-line chemotherapy used for glioma treatment and the combination with radiotherapy demonstrated in significantly prolonged survival.[2] The methylation status of the methylguanine methyltransferase gene, *MGMT*, is an established predictor of benefit of TMZ. *MGMT* gene encodes a DNA repair protein. Promoter methylation of the *MGMT* gene results in epigenetic silencing and is associated with impaired DNA-repair activity.[3] Apart from *MGMT*, glioblastomas might be intrinsically resistant to TMZ, or TMZ resistance can be developed during treatment.

^{99m}Tc-tetrofosmin (^{99m}Tc-TF) is a single-photon emission computed tomography (SPECT) tracer that has also been utilized among others SPECT tracers for brain

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tumor imaging.[4,5] ^{99m}Tc-TF is a monovalent lipophilic cation and enters viable cells by passive transport, which is also aided by the negative electric potential of the cell membrane. This tracer can be located within the cytosol, with only a small fraction incorporating into the mitochondria.[4] This agent has been found useful *in vivo* for the assessment of glioma aggressiveness and grade and for the differentiation of glioma recurrence from treatment-induced necrosis.[6,7] In this study, we examined whether ^{99m}Tc-TF can be used to identify drug resistance factors in glioblastoma. As proof of concept, we employed two glioblastoma cell lines, a resistant and a sensitive to TMZ treatment, and we investigated whether differences exist in ^{99m}Tc-TF uptake between the two cell lines.

Materials and Methods

Cell lines and treatment conditions

The human glioma cell lines U251MG were obtained from Dr. W. K. Alfred Yung (Department of Neuro-Oncology, M. D. Anderson Cancer Center, Houston, TX) and T98G were purchased from (American Type Culture Collection; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Grand Island, NY) that was supplemented with 1% penicillin/streptomycin (Gibco, BRL) and 10% fetal bovine serum cells were grown at 37°C in a 5% CO_2 atmosphere as has been described in detail elsewhere.[8]

Cell adhesion assay

We used the xCELLigence Real-time Cell Analyzer (Roche Diagnostics, GmbH, Mannheim, Germany) for the measurements. This system monitors the status of cells, namely cell number and adhesion, by performing calculations of the electrical impedance across microelectrodes at the bottom of the culture plates. These plates contain wells similar to that of 96-well plates (E-Plates, Roche Diagnostics). The analyzer automatically calculates the frequency-dependent electrical impedance and provides the cell index (CI). As more cells attach on E-Plates, the impedance value is higher, leading to a larger CI number.[9] The system provides the calculation of normalized CI as the quotient of CI at each time point to CI at a reference time point. Cell cultures were treated 1 day after dispersion of cells in the wells of E-plates and monitored for 72 h. Values of normalized CI are presented as mean of two different measurements.

Viability assay

Cultures of human glioma cells were treated with TMZ (Schering-Plough) at various concentrations (100, 250 and 500 mM). Cell viability was evaluated by trypan blue exclusion assay. Each assay was carried out at least 3 times and is represented as the mean value of different experiments. Cell cultures were evaluated every day by light microscopic observation and viability tests were performed when the cytotoxic effect was prominent. Cell proliferation was also continuously monitored for 72 h after treatment every 30 min using the xCELLigence system, via calculation of the CI.

Flow cytometric analysis of DNA cell cycle

Cells were treated with TMZ at concentration of 500 μM. Untreated cells were used as negative control. All samples were run 3 times of at least three independent experiments. Flow cytometric analysis of propidium iodide (PI) was done at day 3. For the DNA cell cycle, cells were trypsinized then centrifuged and washed with buffer phosphate buffered saline (PBS) and finally incubated with PI-working solution (50 μg/mL PI and 20 mg/mL RNase A and 0.1% Triton X-100) for 20 min at 37°C in the dark. The PI fluorescence of 10,000 individual nuclei was measured using a flow cytometer (FACScalibur, Becton Dickinson San Jose, California, USA). The fractions of the cells in G_0/G_1, S, and G_2/M phases were analyzed by the use of Cell Quest software program (BD Biosciences) and were determined for each histogram as the mean peak fluorescence intensity.

Radioactive tracer experiments

^{99m}Tc-TF (Myoview, GE Healthcare, UK) was prepared according to the manufacturer instructions. The radiochemical purity of the radiotracer was <95%.

Cell kinetic studies

About 5×10^5 cells were platted to each 10 cm plate in diameter. At the 4th day, 200 μCi (7.4 10^6 Bq) (200 μl) of ^{99m}Tc-TF was added to the medium. The cells were leaved for an incubation period of 30 min and then the medium was discarded. The cells were then rapidly washed 3 times with PBS at 4°C. Thereafter, the cells were treated with 0.5 mL of trypsin. When the cells had been detached from the bottom of the well, 1 mL of DMEM was added to block the proteolytic action. Cell clumps were removed by at least 10-fold repeating pipetting of the trypsin/DMEM mixture. The cells were then harvested and centrifugated at 3000 rpm for 10 min. After centrifugation, the supernatant was discarded, and we counted the radioactivity in the remaining pellet 10 times with a gamma scintillation counter (Wizard 2, Perkin Elmer, USA). All experiments were performed in triplicate and repeated 3 times.

Statistical analysis

Unless otherwise stated, data are expressed as mean ± standard deviation. The significance of

differences between experimental conditions was determined using Mann–Whitney test. Differences were considered statistically significant at $P < 0.05$.

Results

Sensitivity to temozolomide of glioblastoma cells

To investigate the sensitivity to TMZ of U251MG and T98G cell lines, we utilized xCELLigence system. 2×10^3 U251MG and T98G cells were seeded and 20 h later, the cells were exposed to escalating concentrations of TMZ (0, 100, 250, and 500 μM). U251MG cells were sensitive to TMZ, and the effect was most prominent at a concentration of 500 μM. T98G cells were resistant even at a concentration of 500 μM. The CI of U251MG cells started to diminish after 24 h of treatment at a concentration of 500 μM of TMZ, whereas in T98G, the CI was similar to the control even at a concentration of 500 μM of TMZ [Figure 1]. After 72 h of treatment, there was a significant decrease in the CI of U251MG cells that were treated with 500 μM of TMZ compared to control ($P < 0.001$), whereas the difference was not significant for T98G cells ($P = 0.38$). These results were also verified using trypan blue exclusion test (data not shown).

Effect of temozolomide on cell cycle

To investigate the effect of TMZ in U251MG and T98G cell cycle, both cell lines were exposed to a concentration of 500 μM TMZ for 72 h. In U251MG cell line, TMZ induced cycle arrest at G_2/M phase. In T98G cells, TMZ had virtual no effect on cell cycle [Figure 2].

^{99m}Tc-tetrofosmin predicts response to temozolomide

We studied the effect in ^{99m}Tc-TF uptake when the U251MG and T98G cell lines, we exposed to TMZ. 2×10^5 U251MG and T98G cells were seeded, and 4 days later the cells were exposed to TMZ in the concentration of 500 μM. Six hours later, we calculated the ^{99m}Tc-TF uptake in treated and control cell lines. A significant lower ^{99m}Tc-TF uptake in U251MG cells that were exposed to TMZ was found relative to control ($P = 0.0159$). On the contrary, no statistically significant difference was found in T98G cells ($P = 0.8$) [Figure 3].

Discussion

In this study, we found that ^{99m}Tc-TF uptake differs in glioma cells that were exposed to TMZ. In T98G cells

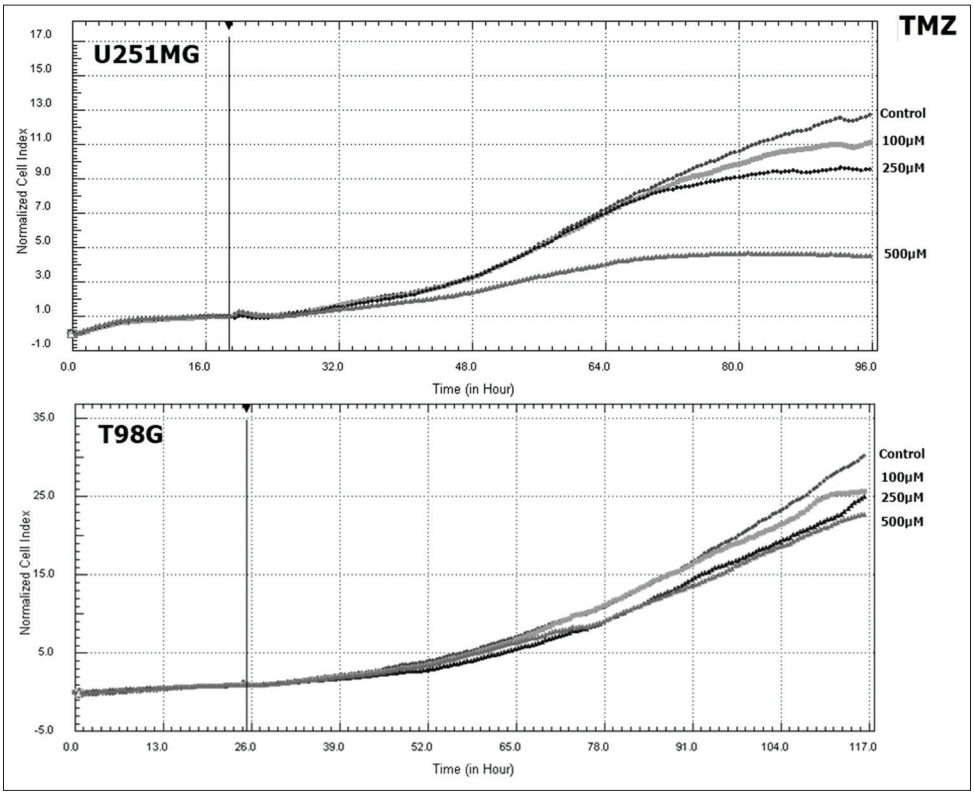

Figure 1: Normalized cell index curves of U251MG and T98G cell lines as generated by xCELLigence real-time cell analysis. On the E-Plate devices 2×10^3 of U251MG and T98G cells per well were seeded. Twenty to twenty-four hours after seeding the cells were treated with escalating concentrations (100, 250 and 500 μM) of temozolomide. Responses were monitored every 30 min by automated cycling between the real-time cell analysis HT station and the incubator. For U251MG cells, temozolomide produced a dose-dependent decrease in proliferation. For T98G cells, temozolomide had no effect in proliferation even at a concentration of 500 μM

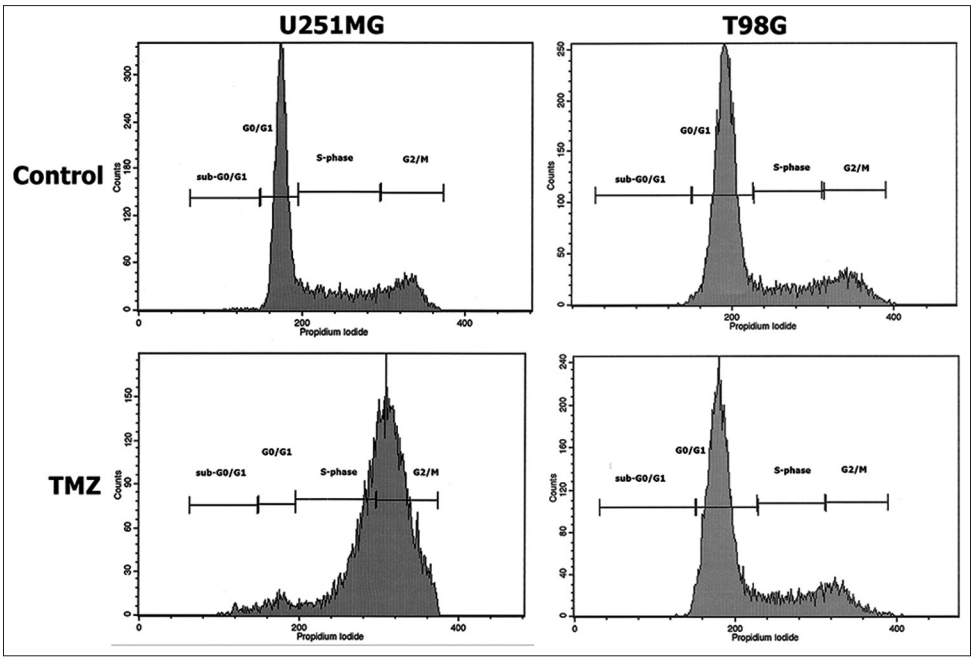

Figure 2: Cell cycle analysis by flow cytometry of U251MG and T98G cell lines. 10^4 U251MG and T98G cells were seeded in a 24-well-plate and 24 h later they were exposed to 500 μM temozolomide. After treatment for 72 h cells were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry. As control, untreated cells were used. A total of 10,000 cells were counted for each cell line. temozolomide induced G_2/M (M3) cell cycle arrest in U251MG cells, whereas no effect on cell cycle was observed in T98G cells

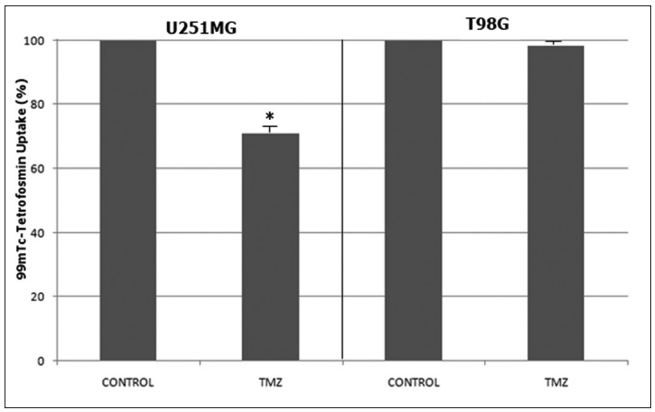

Figure 3: ^{99m}Tc-tetrofosmin uptake following treatment. ^{99m}Tc-tetrofosmin uptake measured in U251MG and T98G cells following treatment with temozolomide (500 μM) for 6 h. Significant differences are indicated with asterisk when $P < 0.05$

which are resistant to TMZ, no statistically significant difference was found in the ^{99m}Tc-TF uptake after exposure to TMZ, whereas in U251MG cells which are sensitive to TMZ, a significant lower ^{99m}Tc-TF uptake was found due to TMZ exposure. To the best of our knowledge, no previous study investigated if ^{99m}Tc-TF uptake could predict sensitivity to TMZ in glioblastoma cell lines.

TMZ is the first-line chemotherapy for glioblastoma since it prolongs survival and delays progression without major adverse events and without having an impact on quality of life. Even in elderly, TMZ is comparable to radiotherapy regarding overall survival and progression-free survival.[10] This agent methylates several nucleophilic sites, mainly at guanine-N7, adenine-N3, and guanine-O6. The latter is responsible for the anticancer activity and results in a continuous cycle of DNA base mismatch repair. These can lead to strand breaks and eventually cell death.[11] O6-methylguanine can be removed by *MGMT* gene, thus if the tumor expresses this protein, the effect of TMZ is limited. Furthermore, in the absence of DNA mismatch repair, *MGMT* inhibition does not increases sensitivity to TMZ.[12] TMZ induces cell cycle arrest in G_2/M phase and apoptosis.[13] This was verified in this study.

^{99m}Tc-TF is a SPECT tracer and has been proven a promising agent for brain tumor imaging.[5] In gliomas, ^{99m}Tc-TF uptake has been correlated with tumor grade and aggressiveness as assessed by Ki-67 index.[6,7] A correlation of ^{99m}Tc-TF uptake with patients' prognosis was also found. Contrary to other tracers such as ^{99m}Tc-MIBI, ^{99m}Tc-TF showed higher uptake in glioma cell lines and was influenced to a smaller degree from the gliomas multidrug resistance phenotype.[14] Finally, ^{99m}Tc-TF proved to be more lipophilic than MIBI, thus could enter easier in glioma cells.[15]

In this study, two glioma cells lines were exposed to TMZ. In the U251MG cell line, there is no MGMT protein expression and is TMZ-sensitive, whereas in T98G cell line, there is MGMT expression and is TMZ-resistant.[16]

Based on the results of xCELLigence and trypan blue exclusion test, TMZ at a concentration of 500 μM exerts its action and induces cell death at least after 24 h in U251MG cell line, whereas no effect was observed in T98G cells. By calculating ^{99m}Tc-TF relative uptake after exposure to TMZ for 6 h lower ^{99m}Tc-TF uptake in U251MG cells was found than in the unexposed cells. No difference was observed in T98G cells. Thus, ^{99m}Tc-TF uptake might be an early indicator of tumor response to TMZ treatment. Considering the glioblastomas rapid growth rate, assessment of TMZ efficacy early in the course of treatment is important. Nowadays, additional therapeutic options are currently available for glioma treatment such as anti-VEGF treatment, thus early identification of patients with TMZ resistant tumors might result in timely decision for alternative treatment modalities in the nonresponding patients. Another interesting question is whether ^{99m}Tc-TF uptake might be an adjunct for establishing the minimum effective dose for treatment.

The positron emission tomography (PET) tracer 3′-deoxy-3′-(^{18}F)-fluorothymidine (^{18}F-FLT) has been recently reported to be useful for the early evaluation of the response of glioblastoma multiforme to TMZ chemotherapy using a xenograft mouse model of human glioblastoma.[17] In another study, ^{18}F-FLT micro-PET was found to be a sensitive predictor of TMZ and bevacizumab treatment efficacy in U87MG and U251 experimental human glioma models. Measurements of cerebral blood volume by micro-magnetic resonance imaging (MRI) were less sensitive than FLT. (^{18}F)-fluorodeoxyglucose micro-PET was predictive in the U87MG model but not in the U251 model.[18] Nevertheless, PET is not so widely available, whereas SPECT has the advantage of wider availability and lower cost.

Apart from TMZ, ^{99m}Tc-TF might be an early indicator of response to treatment with other chemotherapeutic agents. Thus, it might be useful for the preclinical development of anticancer agents testing of *in vitro* effects. Since diminishment of tumor volume by CT or MRI has been most often used for the assessment of response to treatment and tumor shrinkage may be an effect that requires considerable time, metabolic imaging by ^{99m}Tc-TF may detect early a response to chemotherapeutic regimens.

An important limitation of this study pertains to the absence of a glioma xenograft model for testing whether response to TMZ could be monitored early, in the course of therapy by noninvasive ^{99m}Tc-TF SPECT. In conclusion, given that ^{99m}Tc-TF has been found useful for imaging brain tumors, assessment the response to treatment might be a novel application that deserves further investigation.

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Conflicts of interest

There are no conflicts of interest.

References

1. Liu Y, Shete S, Etzel CJ, Scheurer M, Alexiou G, Armstrong G, *et al.* Polymorphisms of LIG4, BTBD2, HMGA2, and RTEL1 genes involved in the double-strand break repair pathway predict glioblastoma survival. J Clin Oncol 2010;28:2467-74.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-96.
3. Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS, Futscher BW. Methylation of discrete regions of the O6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. Mol Cell Biol 1997;17:5612-9.
4. Fukumoto M. Single-photon agents for tumor imaging: 201Tl, 99mTc-MIBI, and 99mTc-tetrofosmin. Ann Nucl Med 2004;18:79-95.
5. Birbilis T, Boussios N, Papatheodossiou L, Matis G, Zissimopoulos A. Is there a role for spect with (99m) Tc-tetrofosmin in the diagnostic work up of a brain tumor? Maedica (Buchar) 2013;8:347-50.
6. Alexiou GA, Fotopoulos AD, Papadopoulos A, Kyritsis AP, Polyzoidis KS, Tsiouris S. Evaluation of brain tumor recurrence by (99m) Tc-tetrofosmin SPECT: A prospective pilot study. Ann Nucl Med 2007;21:293-8.
7. Fotopoulos AD, Kyritsis AP, Tsiouris S, Al-Boucharali J, Papadopoulos A, Voulgaris S, *et al.* Characterization of intracranial space-occupying lesions by 99mTc-Tetrofosmin SPECT. J Neurooncol 2011;101:83-9.
8. Alexiou GA, Tsamis KI, Vartholomatos E, Peponi E, Tzima E, Tasiou I, *et al.* Combination treatment of TRAIL, DFMO and radiation for malignant glioma cells. J Neurooncol 2015;123:217-24.
9. Ke N, Wang X, Xu X, Abassi YA. The xCELLigence system for real-time and label-free monitoring of cell viability. Methods Mol Biol 2011;740:33-43.
10. Hart MG, Garside R, Rogers G, Stein K, Grant R. Temozolomide for high grade glioma. Cochrane Database Syst Rev 2013;4:CD007415.
11. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C. Temozolomide: A review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev 1997;23:35-61.
12. Alvino E, Castiglia D, Caporali S, Pepponi R, Caporaso P, Lacal PM, *et al.* A single cycle of treatment with temozolomide, alone or combined with O(6)-benzylguanine, induces strong chemoresistance in melanoma cell clones *in vitro*: Role of O(6)-methylguanine-DNA methyltransferase and the mismatch repair system. Int J Oncol 2006;29:785-97.
13. Hirose Y, Berger MS, Pieper RO. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. Cancer Res 2001;61:1957-63.
14. Alexiou GA, Xourgia X, Vartholomatos E, Tsiouris S, Kalef-Ezra JA, Fotopoulos AD, *et al.* Comparison of (99m) Tc-tetrofosmin and (99m) Tc-sestamibi uptake in glioma cell lines: The role of p-glycoprotein expression. Int J Mol Imaging 2014;2014:471032.

15. Perek N, Koumanov F, Denoyer D, Boudard D, Dubois F. Modulation of the multidrug resistance of glioma by glutathione levels depletion – Interaction with Tc-99M-Sestamibi and Tc-99M-Tetrofosmin. Cancer Biother Radiopharm 2002;17:291-302.
16. Van Nifterik KA, Van den Berg J, Slotman BJ, Lafleur MV, Sminia P, Stalpers LJ. Valproic acid sensitizes human glioma cells for temozolomide and γ-radiation. J Neurooncol 2012;107:61-7.
17. Viel T, Schelhaas S, Wagner S, Wachsmuth L, Schwegmann K, Kuhlmann M, *et al*. Early assessment of the efficacy of temozolomide chemotherapy in experimental glioblastoma using [18F] FLT-PET imaging. PLoS One 2013;8:e67911.
18. Corroyer-Dulmont A, Pérès EA, Petit E, Guillamo JS, Varoqueaux N, Roussel S, *et al*. Detection of glioblastoma response to temozolomide combined with bevacizumab based on μMRI and μPET imaging reveals [18F]-fluoro-L-thymidine as an early and robust predictive marker for treatment efficacy. Neuro Oncol 2013;15:41-56.