Molecular characteristics of single patient-derived glioma stem-like cells from primary and recurrent glioblastoma

Jia Shi, Xuchen Dong, Wei Han, Peng Zhou, Liang Liu, Haiyang Wang, Qianqian Jiang, Haoran Li, Shan Cheng, Suwen Li, Jiaqi Yuan, Zhiyuan Qian and Jun Dong

Glioblastoma has high recurrence, while the sensitivity of recurrent glioblastoma to chemotherapy is lower than that of primary glioblastoma. Moreover, there is no standardized treatment for recurrent glioblastoma. Unfortunately, the biological mechanism of recurrent glioblastoma is still unclear, and there are few related studies. We compared the phenotypes of clinical glioblastoma specimens, in-vitro cultured glioma stem-like cells (GSCs) and patient-derived xenograft tumor (PDX) models to explore the molecular genetic characteristics of primary and recurrent glioblastoma from the same patient. In vitro, SU5-2, GSCs derived from recurrent glioblastoma specimens, had stronger proliferative activity and self-renewal ability. Meanwhile, SU5-2 was more resistant to temozolomide and invasive than SU5-1, which derived from primary glioblastoma specimens. Further analysis of the expression of costimulatory molecules showed that the expression of B7-H1, B7-H2 and B7-H3 of SU5-2 were upregulated. In vivo, Kaplan–Meier survival curve analysis showed that the median survival of the recurrent PDX group was worse. The results of gene detection in vitro, PDX model and clinical samples were consistent.

Our results showed that the GSCs based on glioblastoma specimens and the PDX models could replicate the main molecular genetic characteristics of original tumors, which provided a reliable experimental platform for both tumor translation kinds of research and screening of molecular therapeutic targets. Anti-Cancer Drugs 33: e381–e388

Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: glioblastoma, patient-derived xenograft tumor model, glioma stem cells, recurrence

Introduction

Glioblastoma is the most common and invasive malignant tumor in the central nervous system. Its 5-year survival rate is less than 10% [1]. Though small molecular targeted drugs related to tumor treatment have existed in recent years [2], the inner mechanism of occurrence, progression, and recurrence of glioblastoma is still far from definite and needs further exploration. There is still an urgent need for drug detection platforms that can accurately reflect the cyogenetics and molecular characteristics for glioblastoma in vitro and in vivo. However, the classic glioma cell lines such as U87MG and U251, widely used in tumor mechanism researches, have accumulated molecular mutations unrelated to the genetic characteristics of the original gene during the long-term culture in vitro [3]. The U87MG human glioma cell retained by the American Type Culture Collection has lost the main molecular genetic characteristics of the original glioma [4]. Therefore, it is important to establish primary tumor cells which can accurately reflect the original tumor molecules. Glioma stem-like cells (GSCs) have self-renewal, proliferation, multi-differentiation potential and tumorigenicity, which are related to the resistance of glioblastoma to chemotherapy and radiotherapy [5], leading to the progression and recurrence of glioblastoma [6]. In addition, the technology of the patient-derived xenograft (PDX) model provides a reliable and effective platform for preclinical drug efficacy evaluation. Compared with the tumor model established by cell line, the PDX model reduces the culture procedure in vitro and retains tumor stromal components, namely tumor microenvironment, which is in line with the morphological and molecular biological characteristics of clinical tumor tissue and is a better clinical simulation model.

In this study, a case of glioblastoma with spontaneous recurrence without the interference of radiochemotherapy-induced hyperprogression was studied. We not only established PDX model, but also established GSCs in primary culture to compare the molecular genetic characteristics of the original glioma [4].
characteristics among original glioblastoma specimens, GSCs in vitro and PDX models, so as to lay a foundation for further transformation research.

Methods
Clinical sample
A 32-year-old man was admitted to the hospital with a headache. MRI showed a left-frontal lesion with a strengthening effect (5.1 × 4.0 cm), and the symptoms disappeared after complete tumor resection. The postoperative pathology was glioblastoma. After the operation, the patient refused chemoradiotherapy and routine follow-up. Half a year later, the headache recurred and MRI showed the tumor recurred in situ (4.8 × 4.2 cm). In the second operation, the previous approach was used, and the postoperative pathology was still glioblastoma. The patient refused chemoradiotherapy again and died 9 months later. Tumor specimens were collected during the operations. IDH and PDGFRα mutation was detected by PCR sequencing, MGMT methylation was carried out by methylation-specific PCR, Pten mutation was analyzed by immunohistochemical staining and EGFR amplification was detected by fluorescent in-situ hybridization (EGRF detection meets any of the following is positive: EGFR scattered signal >4 cells >40%; EGFR/CEP7 signal ratio >2.0; EGFR cluster signal >4 cells >10%; EGFR scattered signal >15 cells >10%). The study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University.

Reagents and animals
Dulbecco’s modified eagle medium, fetal bovine serum (FBS), basic fibroblast growth factor and epidermal growth factor (EGF) were purchased from Gibco, Grand Island, New York, USA. Primary antibodies against CD133, β3-tubulin, Nestin, glial fibrillary acidic protein (GFAP), B7-H1 (PD-L1), B7-H2, B7-H3 and Ki67 were obtained from Cell Signaling Technology, Danvers, Massachusetts, USA. Three to four weeks Balb/c male nude mice were purchased from Selleck, Houston, Texas, USA. CD133, β3-tubulin, Nestin, GFAP, B7-H1, B7-H2 and B7-H3 antibodies against B7-H1 (PD-L1) for 30 min after fixation and membrane breaking, then centrifuged and resuspended for flow cytometry (Guava EasyCyte 6HT-2L, Merck Millipore, Darmstadt, Germany). The experiment was repeated three times.

Flow cytometry analysis
Cells were incubated with fluorescent antibody B7-H1 (PD-L1) for 30 min after fixation and membrane breaking, then centrifuged and resuspended for flow cytometry (Guava EasyCyte 6HT-2L, Merck Millipore, Darmstadt, Germany). The experiment was repeated three times.

Cell invasion assay
Matrigel (BD, Franklin Lake, New Jersey, USA) was diluted with a serum-free medium (1:5) to pretreat the transwell chamber (Corning, Corning, New York, USA). The procedure of GSCs invasion was as described [9]. Each group was repeated three times.

Western blot
Total protein was extracted by radio-immunoprecipitation assay (Beyotime, Shanghai, Jiangsu, China). Protein concentration was determined with a standard BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Western blot procedure was performed according to the standard protocol. Primary antibodies against B7-H1 (PD-L1, #13684; CST, USA), B7-H3 (#14058; CST, USA) and B7-H2 (#ab209262; Abcam). Rabbit anti-β-actin was used as normal loading control. An ECL plus kit (Thermo Fisher Scientific) and a ChemiDoc Touch Imaging System (BioRad, Hercules, California, USA) were applied to detect the chemiluminescence signals. Each experiment was repeated three times.

PDX model
Fresh tumor tissue (2 mm³) derived from surgical specimens was transplanted to caudate nucleus of 3–4 weeks Balb/c nude mice with a self-made tissue inoculation needle stereotactic instrument [10]. The overall survival (OS) of mice was recorded and analyzed by Kaplan-Meier survival curves. Intracranial transplanted tumors were dissected, fixed by neutral formalin.

Immunohistochemical staining
Immunohistochemical staining was performed as previously described [8]. Diluted Rabbit anti-human primary
antibody (#ab7260, #ab105389, #ab15580, 1:200) was added and incubated at 4°C overnight after antigen repair and goat serum blocking. Goat anti-rabbit IgG secondary antibody incubated for 1 h. Then, diaminobenzidine staining and hematoxylin re-staining, dehydrated and sealed. Under the microscope, five pictures were taken randomly for each and the positive cells were counted.

**Immunofluorescent staining**
Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 20 min, blocked with 10% goat serum for 1 h. 10% goat serum was used to prepare the primary antibody (#ab105389, #ab222782, #ab7260, 1:100), incubated overnight at 4°C. Diluted fluorescent antibody incubated for 2 h. A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL kit, Roche, Switzerland) assay (TUNEL kit) was applied to detect GSCs apoptosis, and procedures followed the standard protocol. The nuclei were stained with 4,6-diamino-2-phenyl indole. Reviewed and quantified under a fluorescence microscope (Nikon, Kawasaki, Japan).

**Statistical analysis**
Graphpad 7.0 software was used for data analysis. The t-test was used for comparison between the two groups. P < 0.05 was considered statistically significant.

**Results**

**Clinical sample pathological analysis**
MRI showed oval irregular ring enhancement, central necrosis and peritumoral edema in the left frontal lobe (Fig. 1a and f). Both the pathological results were glioblastoma. In H&E morphology, cells of the relapsed specimen had a higher density and more cell atypia. It was easy to find tumor giant cells with higher microvesel density and a wider range of necrosis (Fig. 1b and g). In addition, the expression of GFAP in primary glioblastoma was higher than that in relapsed glioblastoma while Nestin positive cells were more in relapsed glioblastoma (Fig. 1c, d, h, i, k), indicating a higher ratio of tumor stem/initiating cells in recurrent glioblastoma. Ki67, as a sensitive indicator of mitosis, was used to detect cell proliferation. We found that the positive rate of Ki67 in recurrent samples was higher (Fig. 1e, j, k), which meant that the recurrent tumor had stronger proliferative activity. Gene detection analysis showed that IDH, Pten and PDGFRα (Exon 12 and 18) genes were not mutated in primary and recurrent glioma tissues, MGMT in primary gliomas was methylated, EGFR was not amplified, while MGMT

| Samples                  | Methylation of MGMT | IDH mutation | Pten mutation | PDGFRα mutation | EGFR amplification |
|--------------------------|---------------------|--------------|---------------|-----------------|--------------------|
| Primary clinical specimen| +                   | −            | −             | −               | +                  |
| Recurrent clinical specimens| −               | −            | −             | −               | +                  |
| SU5-1                    | +                   | −            | −             | −               | −                  |
| SU5-2                    | −                   | +            | −             | −               | −                  |
| Primary PDX              | +                   | −            | −             | −               | −                  |
| Recurrent PDX            | −                   | −            | −             | −               | +                  |

PDX, patient-derived xenograft tumor.
in relapsed glioblastoma was unmethylated and \textit{EGFR} amplification was positive (Table 1 and Fig. 1l).

**SU5-2 showed stronger self-renew and proliferative activity \textit{in vitro}**

We established GSCs named SU5-1 and SU5-2 by cell culture of primary and recurrent tumor samples. The experimental methods refer to our previous report [7]. Both GSCs lines are characterized by neuropheres and expressed stem/progenitor cell markers Nestin and CD133 (Fig. 2a–c and g–i). Subsequently, GSCs were induced to differentiate with 10% FBS, the cells showed adherent growth, pseudopodia and diverse morphology (Fig. 2d and j). In order to assess the differentiation of SU5-1 and SU5-2, we performed immunofluorescence to verify the expression of GFAP and tubulin, which are markers of glial and neurons lineage, respectively (Fig. 2e, f, k and l).

In order to demonstrate the self-renewal ability and proliferation ability of SU5-1 and SU5-2 \textit{in vitro}, we carried out a neurosphere formation assay and cell viability assay. By quantifying the number of neurospheres under different cell density gradients, we found that SU5-2 had more neurospheres at the same cell concentration (Fig. 2m). Simultaneously, we measured the diameter of the neurospheres of both cells, the average diameter of the neurospheres formed by SU5-2 was larger than that of SU5-1 (Fig. 2n). These results indicated that SU5-2 had stronger self-renewal ability \textit{in vitro}. In the cell viability assay, SU5-2 also showed stronger proliferation activity (Fig. 2o).

**SU5-2 showed stronger chemoresistance and invasiveness \textit{in vitro}**

Temozolomide is the chemotherapy in the standard treatment protocol (STUPP) for glioblastoma [11]. We applied different concentrations of temozolomide to SU5-1 and SU5-2, and found that these two GSCs were resistant to temozolomide, but only inhibited by high concentration of temozolomide (Fig. 3a), which was consistent with previous reports [12]. Comparing the proliferation ability of the two GSCs lines at the concentration of 400, 800, 1600 μM of temozolomide, temozolomide inhibited SU5-1 more than SU5-2 (Fig. 3a). For further verification, TUNEL assay was performed at the concentration of 400μM of temozolomide, and the result revealed that the apoptosis of SU5-1 was more obvious (Fig. 3b).

Similarly, we compared the self-renewal ability of SU5-1 and SU5-2 under temozolomide. With the increasing concentration of temozolomide, the number and diameter of

---

**Fig. 2**

SU5-2 showed stronger self-renew and proliferative activity \textit{in vitro}. (a, g) Under the phase-contrast microscope, SU5-1 and SU5-2 showed spherical growth (Scale bar 400 μM). (b, c, h, i) CD133 and Nestin were positively expressed in SU5-1 and SU5-2, determined by immunofluorescence staining (b, h scale bar 20 μM, c, i scale bar 50 μM). (d, j) Briefly cultured SU5-1 and SU5-2 after differentiation with 10% FCS (Scale bar 400 μM). (e, f, k, l) GFAP and β3-tubulin were positively expressed after differentiation, determined by immunofluorescence staining (Scale bar 50 μM). (m, n) The quantification of numbers and diameter of SU5-1 and SU5-2 neurospheres showing that the neurosphere formation ability of SU5-2 was stronger. *P<0.05, **P<0.01, ***P<0.001. (o) The proliferation activity of SU5-1 and SU5-2 was measured by CCK8 assay. *P<0.05, **P<0.01, ***P<0.001. CCK8, cell counting kit-8; GFAP, glial fibrillary acidic protein.
neurospheres in SU5-1 and SU5-2 decreased, which was more apparent in SU5-1 (Fig. 3c and d). These results implied that SU5-2 from recurrent tumor specimens showed stronger resistance to temozolomide in vitro.

Afatinib and neratinib are dual inhibitors of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 tyrosine kinase, which can be used alone or as adjuvants in chemotherapy, especially for EGFR+ cells [13]. In order to verify the efficacy, we detected the sensitivity of afatinib to SU5-1 and SU5-2 in different concentration gradients. The results showed that afatinib had a stronger inhibitory effect on SU5-2, which was EGFR positive (Fig. 3e). Immediately, we treated SU5-2 with temozolomide combined with different concentrations of afatinib. Cell viability assay showed that low concentration (<3 μM) of afatinib could not enhance the cytotoxicity of temozolomide, while high concentration (>10 μM) of afatinib combined with temozolomide significantly enhanced the inhibition of SU5-2. According to previous studies, the dose of afatinib in combination with other chemotherapeutics was in the range of 0.5–1 μM [14–16]. Considering that a high concentration of afatinib itself had a toxic effect on EGFR+ cells, the enhanced inhibitory effect after combination was more likely to be the superposition of chemotherapy agents, rather than the sensitization effect of afatinib on temozolomide (Fig. 3f).

High invasiveness promotes the progression and recurrence of glioblastoma [9]. Therefore, we explored the invasive properties of SU5-1 and SU5-2 in vitro by transwell assay which showed that the SU5-2 was more invasive than that of SU5-1 (Fig. 3g).

Upregulation of PD-L1 expression in SU5-2
The expression of PD-L1 in glioblastoma was about 40% [17]. Meta-analysis showed the expression of PD-L1 was negatively correlated with the OS of glioblastoma, and high expression of PD-L1 was a biomarker of glioblastoma progression and poor prognosis [1]. In order to determine the expression of PD-L1 in SU5-1 and SU5-2, Western blot and flow cytometry were performed. Results showed that the expression of B7-H1 (PD-L1), B7-H2 and B7-H3 in SU5-2 which derived from recurrent glioblastoma was upregulated than that in SU5-1 (Fig. 4a).
Flow cytometry analysis also confirmed that the positive rate of B7-H1 (PD-L1) was higher in SU5-2 (Fig. 4b and c). These results might suggest that SU5-2 had a stronger inhibitory effect on T cell anti-tumor immunity.

**PDX models of primary and recurrent glioblastoma**

In vivo, the OS was used to evaluate the tumorigenicity and invasiveness of the tumor. We established PDX models of primary and recurrent glioblastoma in order to retain the original tumorigenic characteristics. The median survival of the primary PDX group was 31 days as the recurrent PDX group was 26 days. Kaplan–Meier survival curve analysis showed that the prognosis of recurrent PDX was worse ($P < 0.05$) (Fig. 4d). In transplanted tumors, the GFAP and Nestin expression was consistent with the clinical glioblastoma samples (Fig. 4e, f, h, i and k). Moreover, Ki67 proliferation index was higher in the recurrent PDX, suggesting that recurrence glioblastoma had more proliferative activity (Fig. 4 G, J, K). Genetic analysis showed that the results of *EGFR* amplification, genes mutation of *IDH*, *Pten* and *PDGFRα*, *MGMT* methylation in the primary and recurrent PDX models were consistent with clinical glioblastoma specimens (Table 1).

**Discussion**

The molecular mechanism of glioblastoma occurrence, progression and the research of anti-tumor drugs have always been a hot spot in the field of neurotumor [18]. However, the tool cells commonly used have accumulated a lot of molecular genetic variations that are not related to original tumors. In addition, glioblastoma has a high degree of molecular genetics differences, which makes cell lines express different biomarkers [19]. In particular, the drug-resistant cell lines induced in vitro cannot faithfully retain the molecular characteristics of primary tumor. Therefore, the establishment of primary tumor cell lines with different characteristics will help us to further study the mechanism of glioblastoma recurrence and chemoresistance, providing a reliable platform for basic research, clinical translation and drug screening.

In this study, primary and recurrent glioblastoma specimens of the single patient were primarily cultured separately. During the course of the patient, tumor cells had no molecular genetic changes such as super progression caused by radiotherapy and chemotherapy pressure selection [20]. The natural recurrence of tumor depends on tissue remodeling initiated by tumor stem cells. Consequently, we established primary and recurrent GSCs lines, verified their differences in molecular genetics, and evaluated their stability in original glioblastoma specimens, in vitro and PDX models. The results suggested that the peculiarities of the original glioblastoma were still retained after primary culture in vitro and
transplantation in vivo. SU5-2 cells derived from recurrent glioblastoma had stronger proliferation, self-renewal and invasion capacity.

At present, the efficacy of chemotherapy for glioblastoma is limited [21], which is related to the difficulty of passing through the blood-brain barrier and the heterogeneity of glioblastoma leading to chemotherapy tolerance [22]. In this study, temozolomide, the first choice of chemotherapy for glioblastoma, was applied to primary and recurrent GSCs, respectively. We found that a low concentration of temozolomide had no obvious inhibitory effect on GSCs. Although this patient did not undergo chemotherapy screening, SU5-2 based on the recurrence specimen still showed higher tolerance to temozolomide than SU5-1. PD-1/PD-L1 has been the focus of tumor immunity [23]. Tumor cells overexpressing PD-1/PD-L1 have stronger chemotherapy tolerance [24], suggesting that the PD-1/PD-L1 pathway is not only related to antitumor immunity of tumor T cells [25], but also closely related to chemoresistance. However, the latter is rarely been involved in the study of glioblastoma. In order to explore the problem, we not only analyzed the expression of B7-H1 (PD-L1) in GSCs, but also detected B7-H2 and B7-H3 which also belong to B7 family of costimulatory molecules. The results showed that these proteins were upregulated in SU5-2. In addition to the role of the immune checkpoint, PD-1/PD-L1 may be a target molecule for chemotherapy sensitization and provide ideas for clinical treatment of glioblastoma.

High-frequency mutations are presented in 17% of recurrent glioblastoma samples treated with temozolomide, most of which come from genes encoding DNA mismatch repair proteins. These high-frequency mutations are consistent with the genetic phenotype of recurrent glioblastoma [26]. Glioblastoma in radiotherapy and chemotherapy can be regarded as an evolutionary process of continuous replacement of cell clones. In this study, the primary culture of glioblastoma without chemoradiotherapy was carried out, and the GSCs lines and PDX models were established as well. It was helpful to observe the natural properties of GSCs and compare molecular biological characteristics changes. However, due to the failure to obtain normal tissue samples from patients, it was difficult to compare the molecular differences between primary and recurrent glioblastoma in the follow-up genome-wide analysis, so only limited target molecular changes could be analyzed.

With the advancement of cell culture technology, the primary cell model and corresponding PDX model derived from patients' surgical specimens can faithfully replicate the molecular genetic characteristics of original tumors, which provide a stable, reliable, and sufficient experimental platform for research on tumor-related properties and screening of clinical drug targets.

Conclusion
In this study, we compared the biological differences of the primary and recurrent GSCs from the single glioblastoma patient at both in-vivo and in-vitro levels. Compared with our previous report [27], which described ectopic tumor recurrence after postoperative radio-chemotherapy with no difference in genetic changes between the primary and recurrent GSCs. We showed a case of glioblastoma patient with spontaneous recurrence in situ, the corresponding molecular analysis is free of the interference of radio- or chemotherapy-induced hyperprogression of glioblastoma. Besides, we not only established PDX model to reproduce the tumorigenic characteristics of glioblastoma in vivo but also established primary and recurrent GSCs lines by brief primary cell culture, which could reflect the individualized characteristics and were conducive to the subsequent individualized treatment of glioblastoma.

Acknowledgements
The study was funded by Natural Science Foundation of Jiangsu Province (grant no. BK20201172); Clinical Special Disease Diagnosis and Treatment Technology in Suzhou (grant no. LCZX201807); Key project of Jiangsu Health Commission (ZDB202016); Young Talent Development Plan of Changzhou Health Commission (grant no. 2020-233-CZQM2020013) and Major Science and Technology Project of Changzhou Municipal Health Commission (ZD202005).

J.S. was a major contributor to molecular experiments and article writing. X.C.D. and W.H. performed statistical analysis of the experimental data. P.Z., L.L. and H.Y.W. cultured cells and in-vitro experiments. Q.Q.J. and H.R.L. were involved in a review of the article. S.C. and S.W.L. were patient management and sample collection. J.Q.Y. and Z.Y.Q. were responsible for animal feeding and tumorigenicity experiments. J.D. was responsible for the design and supervision of the experiments. All authors read and approved the final article.

Procedures performed in the study were in accordance with the ethical standards of the institutional research committee.

This study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University.

The datasets are available from the corresponding author on reasonable request.

Conflicts of interest
There are no conflicts of interest.

References
1. Xue S, Song G, Yu J. The prognostic significance of PD-L1 expression in patients with glioma: a meta-analysis. Sci Rep 2017; 7:4231.
2. Kurz SC, Cabrera LP, Hastie D, Huang R, Unadkat P, Rinne M, et al. PD-1 inhibition has only limited clinical benefit in patients with recurrent high-grade glioma. Neurology 2018; 91:e1355–e1359.
3 Allen M, Bjerke M, Edlund H, Nelander S, Westermark B. Origin of the U87MG glioma cell line: good news and bad news. Sci Transl Med 2016; 8:354re3.

4 Reardon S. US science agencies face budget limbo. Nature 2016; 537:149.

5 He J, Liu Y, Lubman DM. Targeting glioblastoma stem cells: cell surface markers. Curr Med Chem 2012; 19:6050–6055.

6 Zhou BB, Zhang H, Danelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 2009; 8:806–823.

7 Sun C, Dai X, Zhao D, Wang H, Rong X, Huang Q, Lan Q. Mesenchymal stem cells promote glioma neovascularization in vivo by fusing with cancer stem cells. BMC Cancer 2010; 11:1240.

8 Bucarelli M, Marconi M, Pacioni S, De Pascalis I, D’Alessandris QG, Martin M, et al. Inhibition of autophagy increases susceptibility of glioblastoma stem cells to temozolomide by igniting ferroptosis. Cell Death Dis 2018; 9:841.

9 Galavotti S, Bartesaghi S, Faccenda D, Shaked-Rabi M, Sanzone S, McEvoy A, et al. The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells. Oncogene 2013; 32:699–712.

10 Fei XF, Zhang QB, Dong J, Diao Y, Wang ZM, Li RJ, et al. Development of clinically relevant orthotopic xenograft mouse model of metastatic lung cancer and glioblastoma through surgical tumor tissues injection with trocar. J Exp Clin Cancer Res 2010; 29:84.

11 Han B, Meng X, Wu P, Li Z, Li S, Zhang Y, et al. ATRX/EZH2 complex epigenetically regulates FADD/PARP1 axis, contributing to TMZ resistance in glioma. Theranostics 2020; 10:3351–3365.

12 Tao Z, Li T, Ma H, Yang Y, Zhang C, Hai L, et al. Autophagy suppresses self-renewal ability and tumorigenicity of glioma-initiating cells and promotes Notch1 degradation. Cell Death Dis 2018; 9:1063.

13 Reardon DA, Nabors LB, Mason WP, Perry JR, Shapiro W, Kavan P, et al. BI 1200 36 Trial Group and the Canadian Brain Tumour Consortium. Phase I/ randomized phase II study of afatinib, an irreversible ErbB family blocker, with or without protracted temozolomide in adults with recurrent glioblastoma. Neuro Oncol 2015; 17:430–439.

14 Guo G, Gong K, Pulyappadambo VT, Panchani N, Pan E, Mukherjee B, et al. Efficacy of EGFR plus TNF inhibition in a preclinical model of temozolomide-resistant glioblastoma. Neuro Oncol 2019; 21:1529–1539.

15 Vengoji R, Macha MA, Nimmakayala RK, Rachagani S, Siddiqui JA, Mallya K, et al. Aflatinib and temozolomide combination inhibits tumorigenesis by targeting EGFRvIII-cMet signaling in glioblastoma cells. J Exp Clin Cancer Res 2019; 38:266.

16 Barbarisi M, Barbarisi A, De Sena G, Armenia E, Aurilio C, Libutti M, et al. Boswellic acid has anti-inflammatory effects and enhances the anticancer activities of temozolomide and afatinib, an irreversible ErbB family blocker, in human glioblastoma cells. Phytother Res 2019; 33:1670–1682.

17 Wang Z, Zhang C, Liu X, Wang Z, Sun L, Li G, et al. Molecular and clinical characterization of PD-L1 expression at transcriptional level via 976 samples of brain glioma. Oncoimmunology 2016; 5:e1196310.

18 Shankar GM, Kirtane AR, Miller JJ, Mazdyasni H, Rogner J, Tai T, et al. Genotype-targeted local therapy of glioma. Proc Natl Acad Sci USA 2018; 115:EB8388–EB8394.

19 Diaz AK, Baker SJ. The genetic signatures of pediatric high-grade glioma: no longer a one-act play. Semin Radiat Oncol 2014; 24:240–247.

20 Lopez GY, Van Zille J, Onodera C, Grenert JP, Yeh I, Bastian BC, et al. The genetic landscape of gliomas arising after therapeutic radiation. Acta Neuropathol 2019; 137:139–150.

21 Koshkin PA, Chistiakov DA, Chekhonin VP. Role of microRNAs in mechanisms of glioblastoma resistance to radio- and chemotherapy. Biochemistry (Moscow) 2013; 78:325–334.

22 Baker GJ, Yadav VN, Motisch S, Koschmann C, Calinescu AA, Mineharu Y, et al. Mechanisms of glioma formation: iterative perivascular glioma growth and invasion leads to tumor progression, VEGF-independent vascularization, and resistance to antiangiogenic therapy. Neoplasias 2014; 16:543–561.

23 Zhang J, Bu X, Wang H, Zhu Y, Geng Y, Nihira NT, et al. Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance. Nature 2018; 553:91–95.

24 Ishibashi M, Tamura H, Sunakawa K, Kondo-Onodera A, Okuyama N, Hamada Y, et al. Myeloma drug resistance induced by binding of myeloma B7-H1 (PD-L1) to PD-1. Cancer Immunol Res 2016; 4:779–788.

25 McClanahan F, Riches JC, Miller S, Day WP, Kotsiou E, Neuberg D, et al. Mechanisms of PD-L1/PD-1-mediated CD8 T-cell dysfunction in the context of aging-related immune defects in the Eμ-TCL1 CLL mouse model. Blood 2015; 126:212–221.

26 Wang J, Cazzato E, Ludewig E, Frattini V, Rosenbloom DI, Zaisins S, et al. Clonal evolution of glioblastoma under therapy. Nat Genet 2016; 48:768–776.

27 Huang Q, Zhang QB, Dong J, Wu YY, Shen YT, Zhao YD, et al. Glioma stem cells are more aggressive in recurrent tumors with malignant progression than in the primary tumor, and both can be maintained long-term in vitro. BMC Cancer 2008; 8:304.