Int J Clin Exp Med 2017;10(12):16057-16063
www.ijcem.com /ISSN:1940-5901/IJCEM0057589

Original Article

The effect of astragalus polysaccharide on the expression of PCNA and GFAP protein in C6 glioma rat model

Zifeng Zhu[1], Qian Xiu[2], Liang Han[3], Zhaohui Li[1], Yu Tian[1]

Departments of [1]Neurosurgery, [2]Otolaryngology, [3]Pathology, China-Japan Union Hospital of Jilin University, Changchun, Jilin, China

Received May 17, 2017; Accepted November 3, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Glioma is characterized as strong aggressive and fast proliferation. Astragalus polysaccharide (APS) can inhibit glioma cell proliferation and improve the immune imbalance. This study observed the impact of APS on glial fibrillary acidic protein (GFAP) and proliferating cell nuclear antigen (PCNA) expression in C6 glioma rat model. SD rats were randomly divided into five groups, including control (group A), model group (group B), APS group (group C), temozolomide (TM) group (group D), and APS + TM group (group E). C6 glioma cells were injected to the rat brain to establish the animal model. TM was administrated for continuous five days by gavage since the fifth day after injection of C6 glioma cells in group D and E. APS was intraperitoneal injected for continuous 15 days in group C and E. Glioma volume and inhibition rate were calculated. TGF-β1 and IL-10 levels were tested by ELISA. PCNA and VEGF expressions were detected by immunohistochemistry. GFAP protein expression was determined by Western blot. Glioma volume, TGF-β1, IL-10, PCNA, and VEGF levels in group B were higher than that in group A. GFAP expression was lower in group B compared with group A ($P < 0.05$). Glioma volume, TGF-β1, IL-10, PCNA, and VEGF levels in group C, D, and E were obviously lower than that in group B, whereas GFAP expression was higher than group A ($P < 0.05$). Group E showed the strongest anti-tumor effect, followed by group D and group C ($P < 0.05$). APS suppressed rat C6 glioma proliferation and enhanced the anti-tumor effect of TM through regulating PCNA, VEGF, and GFAP protein expressions and improving immune function.

Keywords: Astragalus polysaccharide, temozolomide, PCNA, GFAP, glioma

Introduction

Glioma is featured as high invasive and poor prognosis [1, 2]. In recent years, in spite of the promotion of glioma treatment, such as precise positioning radiotherapy, minimally invasive neurosurgery, and chemotherapy drugs targeting cell cycle, the prognosis failed to obtain significant improvement [3, 4]. As an important biological behavior of glioma, high invasiveness leads to high recurrence rate and mortality rate. In clinic, glioma cannot be fully eliminated by surgery, radiotherapy, or chemotherapy. Furthermore, adjuvant radiochemotherapy after surgery may decrease the immune function and affect prognosis. Therefore, improving the immune state is of great significance in the treatment of glioma [5, 6]. It is revealed that the traditional Chinese medicine polysaccharide can enhance the immunity and suppress glioma proliferation. Astragalus polysaccharide (APS) can restrain a variety of tumor cell growth through reducing telomerase activity and inducing cell cycle stagnation. It was confirmed that APS can suppress glioma cell proliferation and improve the rat immune imbalance [7, 8]. However, the specific mechanism of APS in suppressing glioma cell growth has not been fully elucidated. Rat C6 glioma model is widely applied in the basic research of glioma, since its biological characteristic is similar to human [9, 10]. This study detected tumor pathological morphology, serum cytokine levels, GFAP, VEGF, and PCNA protein expressions to explore the potential mechanism of APS on C6 glioma rat from the aspects of immunity, neovascularization, and glioma related protein.

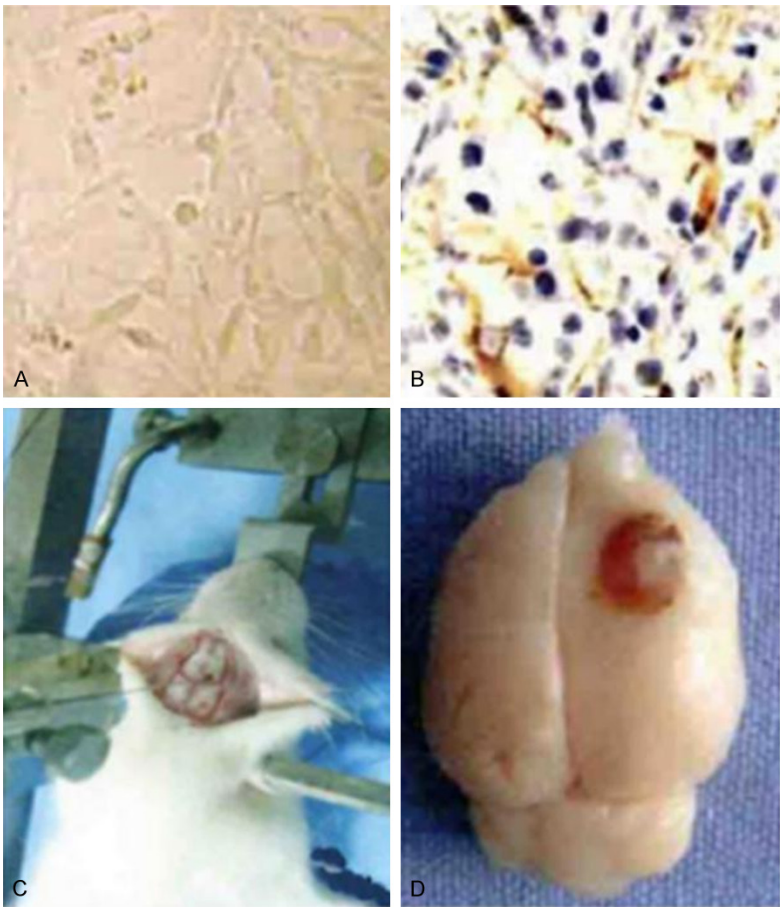

Figure 1. C6 glioma rat model establishment. A. C6 glioma cell; B. GFAP positive in model group (× 400); C. Stereotaxic apparatus injection; D. Glioma general observation.

Materials and methods

SD rats and cell culture

A total of 75 healthy SD rats at six-month old and weighted 200-220 g were provided by the Animal Experiment Center, Chinese Academy of Medical Science (SYXK-2013-0025). The rats were randomly divided into five groups, including control, model group, APS group, temozolomide (TM) group, and APS + TM group with 15 in each group. C6 glioma cells were injected to the rat brain to establish the animal model. APS was intraperitoneal injected for continuous 15 days at 250 mg/kg/d. TM was intragastrically administrated for continuous five days since the fifth day in group D and E at 20 mg/kg/d.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University.

Drugs and reagents

Rat C6 glioma cell line was purchased from Shanghai cellular biochemistry, Chinese academy of medical science. Chloral hydrate, CMC, and paraformaldehyde were got from Suzhou Youran Chemical Co., Ltd. Goat anti rabbit horse radish peroxidase labeled secondary antibody was bought from Beijing Zhonghe Zhengan Biological Technology Co., Ltd. TM was obtained from Jiangsu Disheng Pharmaceutical Co., Ltd. APS was purchased from Tianjin Sanofi-Aventis Pharmaceutical Co., Ltd. Rabbit anti GFAP polyclonal antibody was obtained from CST. TGF-β1 and IL-10 ELISA kits were got from ZSbio. PCNA and VEGF immunohistochemistry kit was bought from Santa Cruz. Microscope was provided by Olympus. Quantity One software was used for image analysis. Stereotaxic apparatus was got from Chengdu Instrument Factory.

Animal model establishment

According to the reference [11], the rat was anesthetized by intraperitoneal injection. The head was fixed on the stereotaxic apparatus. A total of 1 μl C6 glioma cells at 1×10^{10}/L were injected to the right brain caudate nucleus at 2 μl/min. Equal amount of normal saline was injected to the rats in group A.

Tumor volume and inhibition rate calculation

The rat was killed on the 16th day after surgery. Tumor volume was measured and inhibition rate was calculated.

Elisa

A total of 5 ml blood was extracted from the common carotid artery and centrifuged to obtain supernatant. Serum TGF-β1 and IL-10 levels were tested using the ELISA kit according to the manual.

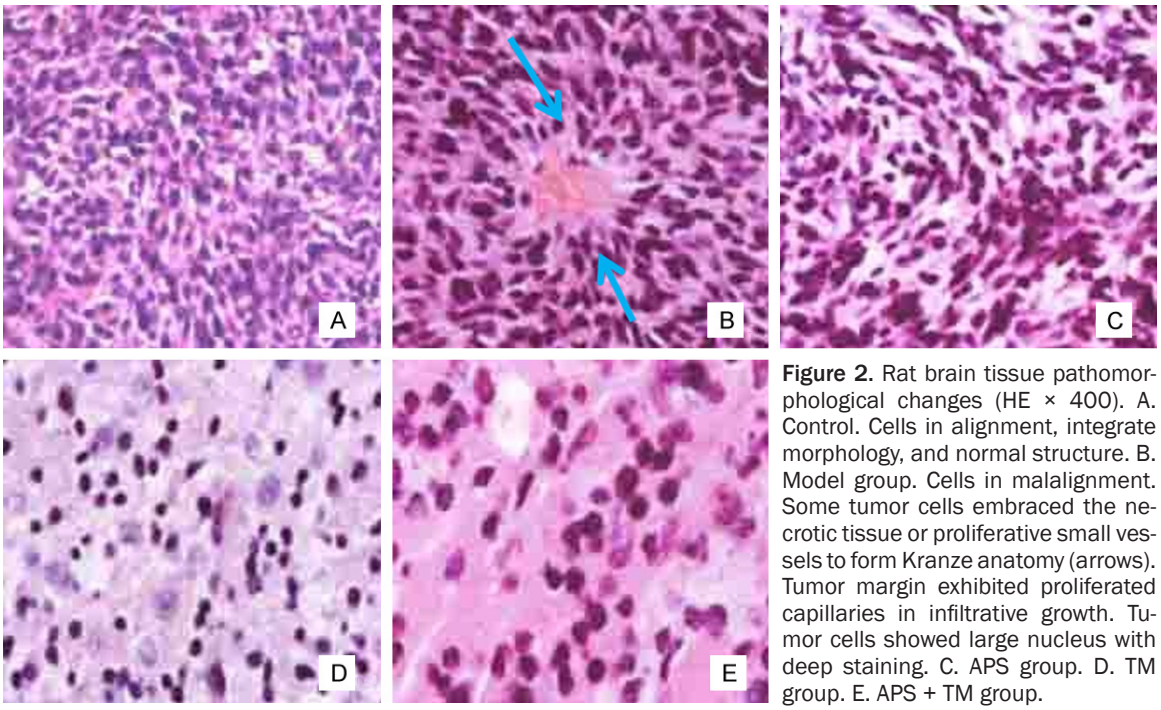

Figure 2. Rat brain tissue pathomorphological changes (HE × 400). A. Control. Cells in alignment, integrate morphology, and normal structure. B. Model group. Cells in malalignment. Some tumor cells embraced the necrotic tissue or proliferative small vessels to form Kranze anatomy (arrows). Tumor margin exhibited proliferated capillaries in infiltrative growth. Tumor cells showed large nucleus with deep staining. C. APS group. D. TM group. E. APS + TM group.

HE staining

The brain was soaked in paraformaldehyde. The brain tissue at 1.4 mm posterior to the bregma was sectioned and embedded. After stained by hematoxylin and eosin, the slice was sealed and observed under the microscope.

Immunohistochemistry

The brain was soaked in paraformaldehyde and sectioned at 5 μm. After dewaxing and antigen retrieval, the slice was treated by H_2O_2. Next, the slice was incubated in primary antibody (PCNA, VEGF, 1:100), and further treated by enzyme labeled anti rat/rabbit polymer. After stained by DAB and treated by dehydration, the slice was sealed and observed under the microscope. Image-pro plus was used for image analysis. Five views were randomly selected to record the positive cell number.

Western blot

The glioma tissue was lysed and centrifuged to obtain the protein. The protein was quantified by BCA method and separated by SDS-PAGE. Next, the protein was transferred to membrane and blocked. Then the membrane was incubated in primary antibody (GFAP, GAPDH, 1:100) and secondary antibody (1:100). At last, the membrane was developed and analyzed by Quantity One software. Absorbance value ratio = GFAP absorbance value/GAPDH absorbance value.

Statistical analysis

All data analyses were performed on SPSS 20.0 software. The measurement data was depicted as mean ± standard deviation ($\bar{x}$ ± S) conforming to normal distribution. The data were compared by one-way ANOVA and LSD test. $P < 0.05$ was considered as statistical significance.

Results

Phenotype and pathological changes of glioma rat

The rat activity, water intake, and appetite decreased after inoculation. The left limb appeared hemiplegia after 9 days. Protopsis and peri-orbital bleeding were observed in the model group on the 10th day. The hair color and activity in the drug groups (group C, D and E) were improved compared with those in model group after 10 days. The rat was killed on the 16th day after surgery. The brain was extracted and the tumor was found. GFAP staining was positive, indicating glioma model was successfully established. HE staining was used

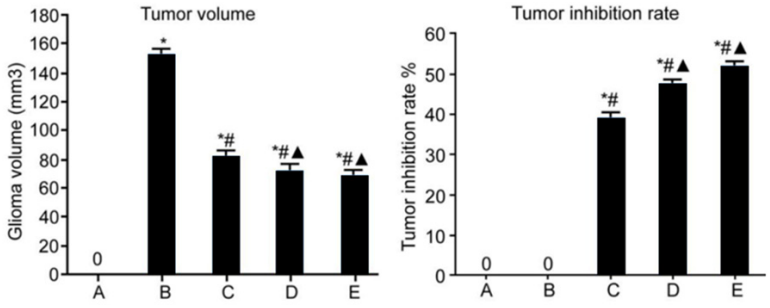

Figure 3. Tumor volume and inhibition rate comparison. A. Control; B. Model group; C. APS group; D. TM group; E. APS + TM group. *P < 0.05, compared with group A. #P < 0.05, compared with group B. △P < 0.05, compared with group C. □P < 0.05, compared with group D.

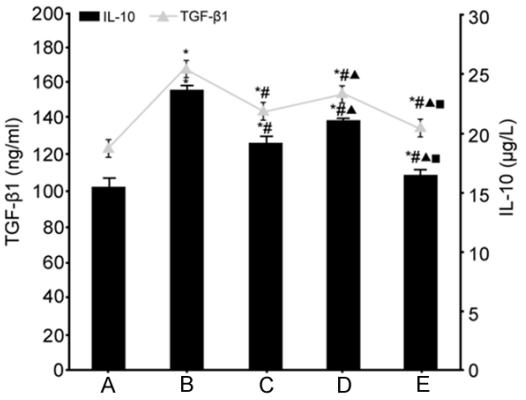

Figure 4. The impact of APS on serum TGF-β1 and IL-10. A. Control; B. Model group; C. APS group; D. TM group; E. APS + TM group. *P < 0.05, compared with group A. #P < 0.05, compared with group B. △P < 0.05, compared with group C. □P < 0.05, compared with group D.

to observe the pathomorphological changes. The cells in the model group exhibited infiltrative growth. The cell number in the drug group was obviously lower than the model group, accompany with necrosis, apoptosis, tumor cell density decrease, inflammatory cell infiltration, and gliocytes proliferation. APS + TM group exhibited significant effect (**Figures 1** and **2**).

Reduced glioma tissue volume after drug treatment

Compared with control, tumor volume in model group significantly increased (P < 0.05). Tumor volume declined in group C, D, and E compared with model group. The inhibition rate was larger than 30%. Group E showed the strongest effect, followed by group D and group C (P < 0.05) (**Figure 3**).

Decreased serum levels of TGF-β1 and IL-10 after APS treatment

Serum TGF-β1 and IL-10 levels were highest in group B, followed by group D, group C, group E, and group A (P < 0.05) (**Figure 4**), suggesting APS could inhibit inflammatory through reduce the secretion of pro-inflammatory cytokines *TGF-β1 and IL-10*

Reduced PCNA and VEGF protein expression after APS administration

As PCNA and VEGF have been demonstrated to be associated with the pathogenesis of several tumors, we also measure the expression of PCNA and VEGF. As seen in **Figures 5**-**7**, the PCNA and VEGF protein expressions in tumor tissue were higher in group B, which was reduced in group D, group C, group E, and group A (P < 0.05) (**Figure 5**).

The impact of APS on GFAP protein expression

GFAP protein expression obviously declined in group B compared with group A (P < 0.05). GFAP protein level was highest in group A, followed by group E, group D, group C, and group B (P < 0.05) (**Figures 6** and **7**).

Discussion

Affected by the blood brain barrier, some drugs cannot enter the central nervous system, which limits the application of chemotherapy drugs. Because of the invasive growth of glioma, surgery cannot achieve the effect of radical removal. Since intracranial tumor is insensitive to radiation, the treatment effect on glioma is poor. Moreover, radiochemotherapy may reduce immune function, thus to affect the clinical treatment effect [12, 13]. APS showed significant inhibitory effect on a variety of tumor cells. It plays anti-tumor effect through enhancing immune function. This study injected C6 glioma cells to the rat to establish the animal model, aiming to investigate the effect of APS on C6 glioma rat. The rat brain was extracted on the 16th day after modeling. The tumor formation was observed and GFAP was positively expressed, suggesting successful model estab-

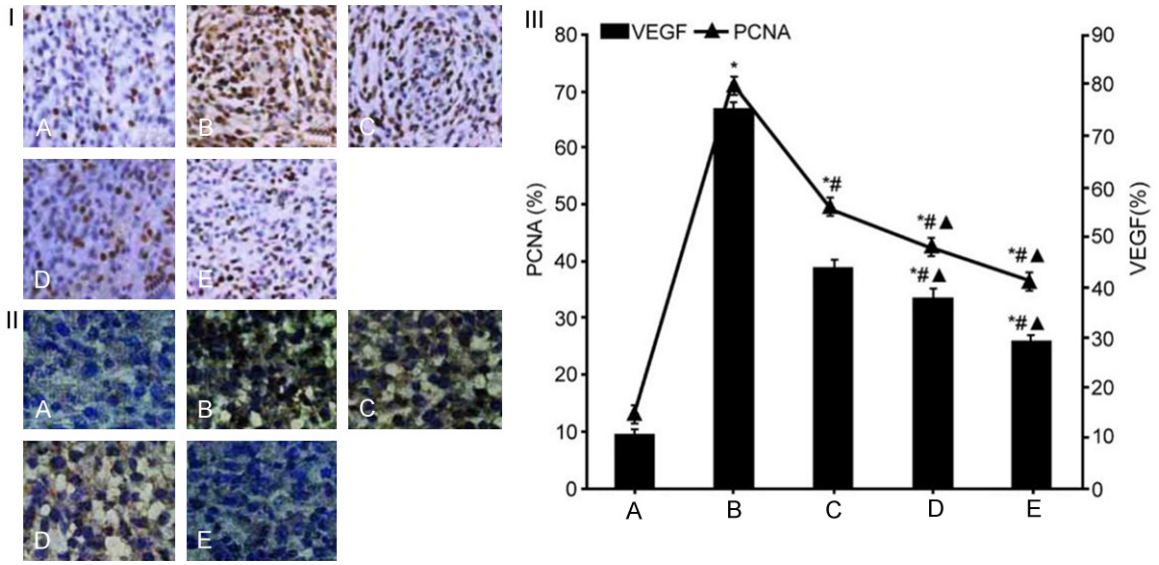

Figure 5. The impact of APS on PCNA (I) and VEGF (II) protein expression (× 400). A. Control; B. Model group; C. APS group; D. TM group; E. APS + TM group. Quantified data was shown in (III). *P < 0.05, compared with group A. #P < 0.05, compared with group B. △P < 0.05, compared with group C. □P < 0.05, compared with group D.

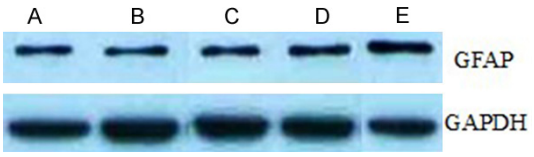

Figure 6. The impact of APS on GFAP protein expression.

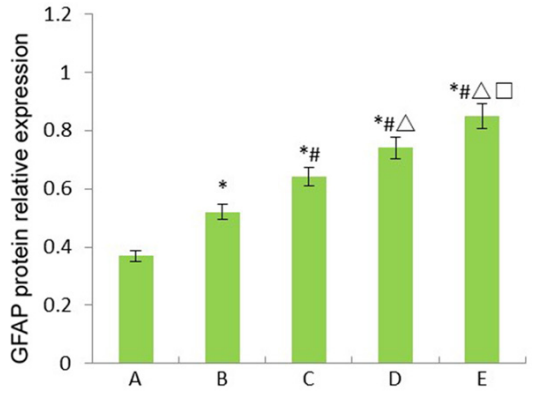

Figure 7. The impact of APS on GFAP protein relative expression level. A. Control; B. Model group; C. APS group; D. TM group; E. APS + TM group. *P < 0.05, compared with group A. #P < 0.05, compared with group B. △P < 0.05, compared with group C. □P < 0.05, compared with group D.

lishment. HE staining was adopted to observe the pathomorphological changes. The cells in the model group exhibited infiltrative growth. The cell number in the drug group was obviously lower than the model group, accompany with necrosis and apoptosis. APS + TM group exhibited significant effect, indicating that APS can suppress C6 glioma growth and enhance the anti-tumor effect of TM.

TGF-β1 can be synthetized and secreted by glioma cells. It can promote tumor cell invasion and local neural microglia differentiation, resulting in immunosuppression microenvironment, which is in favor of tumor growth, VEGF and MMPs expression, and glioma invasion and metastasis [14, 15]. Consistent with the role of TGF-β1 in the promotion of tumor cell invasion and metastasis, we found significantly higher level of TGF-β1 in model group, which were reduced after treatment, further supporting the involvement of TGF-β1 in the pathogenesis of glioma. Regulatory T cell plays its role as inhibitory effector T cell by secreting IL-10. IL-10 leads to the immune tolerance of regulatory T cell on glioma cells via reducing dendritic cell and antigen-presenting cell related molecule expression and decreasing cellular immunity [16, 17]. Our results showed that serum IL-10 level was obviously declined in the drug group, suggesting that APS may play its anti-tumor effect by regulating TGF-β1 and IL-10 expressions and improving immunosuppres-

sive state. However, the molecular mechanism by how APS affects the expression of TGF-β1 and IL-10 remains unclear and requires further investigations.

Tumor invasion and metastasis are related to angiogenesis. Glioma malignancy and prognosis show certain correlation with blood vessel density in the tissue. VEGF plays a critical role in the pathological and physiological angiogenesis. It was showed that VEGF overexpressed in glioma tissue [18, 19]. Consistent with this, in this study, we also found that VEGF level in glioma tissue was markedly higher than the normal brain. VEGF declined in drug group, revealing that APS may suppress glioma angiogenesis and block C6 glioma growth through regulating VEGF expression. PCNA is closely associated with cell cycle and can reflect tumor growth. Cyclin-CDK complex can bind with PCNA to regulate cell cycle. PCNA expression is related to glioma malignancy and prognosis. PCNA positive expression rate increases following glioma pathological upgrading. GFAP mainly distributes in the astrocytes of the central nervous system. GFAP expression is correlated with tumor cell differentiation [20-22]. In our study, PCNA expression was obviously higher in glioma tissue compared with normal control, while GFAP level was markedly lower. PCNA declined, whereas GFAP elevated in drug group, indicating that APS plays its anti-tumor effect might via regulating PCNA and GFAP proteins. However, the exact mechanism by how APS regulates the expression of PCNA and GFAP in glioma was not investigated in the present study, which would be the main study limitation of our study. Further studies on the molecular mechanism are required in the future.

Conclusion

APS suppressed rat C6 glioma proliferation and enhanced the anti-tumor effect of TM through regulating PCNA, VEGF, and GFAP protein expressions and improving immune function.

Acknowledgements

This project supported by the National Science Foundation of China (NO. 30672159) and New Century Excellent Talents in Chinese Universities (NO. NCET-06-0306).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yu Tian, Department of Neurosurgery, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun, Jilin, China. Tel: +86-431-84995981; Fax: +86-431-84995981; E-mail: zhaohuilirfv@163.com

References

[1] Munthe S, Sorensen MD, Thomassen M, Burton M, Kruse TA, Lathia JD, Poulsen FR, Kristensen BW. Migrating glioma cells express stem cell markers and give rise to new tumors upon xenografting. J Neurooncol 2016; 130: 53-62.

[2] Lin L, Wang G, Ming J, Meng X, Han B, Sun B, Cai J, Jiang C. Analysis of expression and prognostic significance of vimentin and the response to temozolomide in glioma patients. Tumour Biol 2016; 37: 15333-15339.

[3] Oliva CR, Markert T, Ross LJ, White EL, Rasmussen L, Zhang W, Everts M, Moellering DR, Bailey SM, Suto MJ, Griguer CE. Identification of small molecule inhibitors of human cytochrome c oxidase that target chemoresistant glioma cells. J Biol Chem 2016; 291: 24188-24199.

[4] Chen B, Liang T, Yang P, Wang H, Liu Y, Yang F, You G. Classifying lower grade glioma cases according to whole genome gene expression. Oncotarget 2016; 7: 74031-74042.

[5] Jiang H, Cui Y, Wang J, Lin S. Impact of epidemiological characteristics of supratentorial gliomas in adults brought about by the 2016 world health organization classification of tumors of the central nervous system. Oncotarget 2017; 8: 20354-20361.

[6] Schiff D. Molecular profiling optimizes the treatment of low-grade glioma. Neuro Oncol 2016; 18: 1593-1594.

[7] Liu H, Zhou L, Shi S, Wang Y, Ni X, Xiao F, Wang S, Li P, Ding K. Oligosaccharide G19 inhibits U-87 MG human glioma cells growth in vitro and in vivo by targeting epidermal growth factor (EGF) and activating p53/p21 signaling. Glycobiology 2014; 24: 748-765.

[8] Yang B, Xiao B, Sun T. Antitumor and immunomodulatory activity of Astragalus membranaceus polysaccharides in H22 tumor-bearing mice. Int J Biol Macromol 2013; 62: 287-290.

[9] Bryukhovetskiy I, Manzhulo I, Mischenko P, Milkina E, Dyuizen I, Bryukhovetskiy A, Khotimchenko Y. Cancer stem cells and microglia in the processes of glioblastoma multiforme invasive growth. Oncol Lett 2016; 12: 1721-1728.

[10] Stojkovic S, Podolski-Renic A, Dinic J, Pavkovic Z, Ayuso JM, Fernandez LJ, Ochoa I, Perez-Garcia VM, Pesic V, Pesic M. Resistance to DNA damaging agents produced invasive pheno-

type of rat glioma cells-characterization of a new in vivo model. Molecules 2016; 21.
[11] Sun C, Yu Y, Wang L, Wu B, Xia L, Feng F, Ling Z, Wang S. Additive antiangiogenesis effect of ginsenoside Rg3 with low-dose metronomic temozolomide on rat glioma cells both in vivo and in vitro. J Exp Clin Cancer Res 2016; 35: 32.
[12] Dunn-Pirio AM, Vlahovic G. Immunotherapy approaches in the treatment of malignant brain tumors. Cancer 2017; 123: 734-750.
[13] Chekhonin IV, Gurina OI, Cherepanov SA, Abakumov MA, Ionova KP, Zhigarev DK, Makarov AV, Chekhonin VP. Pulsed dendritic cells for the therapy of experimental glioma. Bull Exp Biol Med 2016; 161: 792-796.
[14] Busek P, Balaziova E, Matrasova I, Hilser M, Tomas R, Syrucek M, Zemanova Z, Krepela E, Belacek J, Sedo A. Fibroblast activation protein alpha is expressed by transformed and stromal cells and is associated with mesenchymal features in glioblastoma. Tumour Biol 2016; 37: 13961-13971.
[15] Cortese K, Daga A, Monticone M, Tavella S, Stefanelli A, Aiello C, Bisio A, Bellese G, Castagnola P. Carnosic acid induces proteasomal degradation of Cyclin B1, RB and SOX2 along with cell growth arrest and apoptosis in GBM cells. Phytomedicine 2016; 23: 679-685.
[16] Bao Z, Duan C, Gong C, Wang L, Shen C, Wang C, Cui G. Protein phosphatase 1 gamma regulates the proliferation of human glioma via the NF-kappaB pathway. Oncol Rep 2016; 35: 2916-2926.
[17] Bao Z, Wang Y, Yang L, Wang L, Zhu L, Ban N, Fan S, Chen W, Sun J, Shen C, Cui G. Nucleostemin promotes the proliferation of human glioma via Wnt/beta-catenin pathway. Neuropathology 2016; 36: 237-249.
[18] Liu K, Zhang Q, Lan H, Wang L, Mou P, Shao W, Liu D, Yang W, Lin Z, Lin Q, Ji T. GCN5 potentiates glioma proliferation and invasion via STAT3 and AKT signaling pathways. Int J Mol Sci 2015; 16: 21897-21910.
[19] Yang L, Tao T, Wang Y, Bao Z, He X, Cui G. Knocking down the expression of TRA2beta inhibits the proliferation and migration of human glioma cells. Pathol Res Pract 2015; 211: 731-739.
[20] Qu DW, Liu Y, Wang L, Xiong Y, Zhang CL, Gao DS. Glial cell line-derived neurotrophic factor promotes proliferation of neuroglioma cells by up-regulation of cyclins PCNA and Ki-67. Eur Rev Med Pharmacol Sci 2015; 19: 2070-2075.
[21] Ma JW, Zhang Y, Li R, Ye JC, Li HY, Zhang YK, Ma ZL, Li JY, Zhong XY, Yang X. Tetrandrine suppresses human glioma growth by inhibiting cell survival, proliferation and tumour angiogenesis through attenuating STAT3 phosphorylation. Eur J Pharmacol 2015; 764: 228-239.
[22] Gonzalez-Gomez P, Crecente-Campo J, Zahonero C, de la Fuente M, Hernandez-Lain A, Mira H, Sanchez-Gomez P, Garcia-Fuentes M. Controlled release microspheres loaded with BMP7 suppress primary tumors from human glioblastoma. Oncotarget 2015; 6: 10950-10963.