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Brain Urea as a Potential Biomarker of Neoplasm Progression

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Abstract

Metabolic reprogramming is a key feature driving oncogenesis in cancers. Recent studies have revealed that protein metabolism is largely altered in gliomas facilitating its malignant growth. Urea is the end product of nitrogen metabolism which is mainly produced by arginase. The interdependence of arginase and other biochemical mechanisms triggered scientific research interest. This research aimed to investigate the relationships between the urea as the main parameter of protein metabolism and glioma progression. It was also the most pronounced relationship between urea and the level of the nuclear protein Ki-67 as a marker of proliferative activity and O-6-methylguanine-DNA methyltransferase (MGMT), which performs DNA repair. Postoperative material from 20 patients with gliomas of different grades of anaplasia was analyzed.

Keywords

Glioma, Peritumoral Zone, Urea, Gliomal Molecular Genetic Markers, Ki-67, MGMT

1. Introduction

Urea is well known as the end product of nitrogen metabolism which is formed by arginase, a manganese-containing enzyme that catalyzes the conversion of L-arginine to urea and L-ornithine. The hydrolytic function of arginase was known from the identification of the Krebs-Henseleit urea cycle, but the interdependence of arginase and other biochemical mechanisms triggered scientific interest. Despite the early findings showing that arginase was mostly expressed in the mammalian liver [1], and to a lesser extent in the kidney [2], this enzyme was also identified in organs where the urea cycle is not present [3] [4]. With the

isolation of arginase from different tissues and comparing the physicochemical properties, it became evident that different isoforms exist. Most plants, bacteria, yeasts, and invertebrates have only one arginase isoform, arginase 2 (ARG 2), and it is located in the mitochondria. The majority of animals that metabolize excess nitrogen as urea also express arginase 1 (ARG 1), and it is localized in the cytosol. In some vertebrates, A1 is expressed in the liver, red blood cells, and specific immune cell populations, whereas ARG 2 is highly expressed in the kidney and is also expressed in some other tissues, including the brain and retina.

Primary brain tumors are hallmarked for their destructive activity on the microenvironment. Gliomas are the most common primary tumors of the central nervous system and brain: 14.3% of all tumors and 49.1% of malignant tumors [5]. Survival results show that grade 1 tumors have the highest survival rate. Grade 4 tumors have the worst results: only 6.8% of patients live for 5 years after diagnosis [5]. In gliomas, in addition to the tumor zone itself, there is also a perifocal (peritumoral) area, which is associated with up to 90% of relapses [6]. The mosaic character of metabolic changes in glioma tissue is noteworthy, which is associated with the heterogeneity of kinetic and metabolic polymorphism and the variety of lesions of the hemato-tumor barrier. At the same time, it is necessary to take into account the fact that proliferation within the tumor occurs asynchronously. There are also differences in the metabolism of normal and tumor cells. The border region always plays a dual role, containing elements that limit the tumor process on the one hand, and on the other, being a substrate for further tumor progression. [7] [8]. This is due to the fact that the peritumoral region has a specific cellular composition (immune cells, various proteins, inflammatory mediators, metalloproteinases, pro- and antioxidants, oxygen content, acidity, etc.), molecular and biochemical features, which distinguishes it from both tumor and normal brain tissue. Besides, these parameters tend to change at different stages of the tumor process. This phenomenon can be explained by the cross-exchange of cells with adjacent zones and changes in biochemical parameters against the background of tumor metabolism. Processes that contribute to the progression of gliomas occur in this zone. Thus, the study of the peritumoral zone is very important for determining the optimal boundaries of resection during surgical treatment, evaluating drug resistance, and predicting the likelihood of recurrence and further progression of the tumor [9] [10] [11].

In recent years, the understanding of the regulation of tumor metabolism has significantly improved. Accumulating evidence shows that tumor cells reprogram their metabolism to meet high energy demands, and coordinate markedly elevated biosynthetic processes and energy production, which in turn promote rapid growth and division of tumor cells [12] [13]. Numerous point mutations and copy number variations have been shown to drive glioma cells' metabolic state, affecting tumor growth and patient outcomes. Among the most common, IDH mutations, EGFR amplification, mutation and MGMT promoter mutation have emerged as key patterns associated with upregulated metabolism of proteins, lipids and carbohydrates [14].

Protein dysregulation and neuronal death may lead to greater protein breakdown, and so to increased urea production. Widespread elevations in brain urea have, in recent years, been reported not only in tumors, but in certain types of age-related dementia [15].

This study aims to find relationships between urea content as one of the main protein metabolism parameters and gliomal progression. We have investigated the spatial distribution of molecular changes associated with glioma progression using the analysis of the specific concentration of urea relative to brain total protein in order to determine molecular markers for early diagnosis of tumor growth. The relationship between urea and molecular genetic markers of gliomas has also been analyzed. The metabolic alterations and their connection with gliomal molecular markers are the driving force in the investigation of the mechanisms of the complex relationship between molecular aberrations, metabolism profile, and tumor behavior. The balance between high protein metabolism, which could be estimated by urea and gliomal markers, influences on the tumor metabolism, enhancing malignant processes such as cell proliferation and invasion.

2. Materials and Methods

2.1. Material

Tumor tissues (area 1), peritumoral zones (area 2), adjacent non-cancerous tissues (area 3) and blood, were collected as postoperative material at the Federal State Budgetary Educational Institution of Higher Education "Privolzhsky Research Medical University" of the Ministry of Health of the Russian Federation with informed consent before antitumor therapy from 20 patients aged 39 - 61 years with gliomas of varying degrees of anaplasia. The histological diagnosis was established according to the WHO classification of CNS tumors [16]. All the patients were divided into two groups: the low level of anaplasia (1 - 2 Grade) and the high level (3 - 4 Grade). The control group consisted of brain tissue and blood from 6 individuals (4 men, 2 women) who died as a result of trauma. The study was approved by the Ethics Committee of PRMU. Exclusion criteria: under 18 years old; presence of gross somatic pathology; gliomas with multifocal growth.

2.2. Method

Preparation of tissue homogenates for biochemical research. Preparation of tissue homogenate for biochemical studies was carried out in a refrigerating room at a temperature of 0˚C. The postoperative material was washed in 0.32 M sucrose solution, pH = 7.4, and cleaned from the shells. The tissue was then homogenized at a speed of 200 rpm. in a homogenizer (glass-Teflon) in a 10-fold volume of the isolation medium containing 0.32 M sucrose, 10 mM tris-HCl and 1 mM EDTA, pH = 7.4. Biochemical studies were performed in tumor and brain tissue homogenates. Fasting blood was collected from the cubital vein of the patients in a volume of 10 ml into a tube with citrate as an anticoagulant.

Analysis of urea concentration. The urea concentration was determined by the urease-salicylate method using the Urea-Novo kit from Vector-Best, Russia. The

results were recalculated per 1 g of protein, which was determined by the Lowry method using a set of reagents from Sintacon Company LLC, Russia.

Measurement of Tumor Markers Postoperative material was fixed in 10% formalin solution and was processed according to the standard procedure. The following antibody clones were used: Anti-MGMT (clone EP337), item AC-0307RUO (Epitomics, USA), Ki-67 antibodies (clone SP6) (Thermo Scientific, USA). The levels of MGMT, Ki-67 markers were determined by the proportion of positive nuclear staining. The values obtained were expressed as the proportion of stained cells in 10 fields of view at x400 magnification [17] [18]. When investigating the presence of MGMT promoter methylation, nuclear staining in less than 15% of cells was considered positive [19].

Analysis of protein-protein interaction. To identify the relationship between the above-mentioned immunohistochemical markers and the enzymes of urea metabolism, the protein-protein interactions were analyzed using the databases of STRING (STRING: functional protein association networks (string-db.org)), BioGrid (BioGRID | Database of Protein, Chemical, and Genetic Interactions (thebiogrid.org)), Signor (SIGNOR 2.0 (uniroma2.it)) and KEGG PATHWAY (https://www.kegg.jp/). A search for interactions was carried out between the markers of tumor growth of gliomas MGMT, Ki-67 and the urea metabolism enzymes: Arginine decarboxylase (ADC), Arginase2 (ARG2), Ornithine decarboxylase 1, and Agmatinase.

Statistical Analysis. Statistical data processing was carried out using the AnalystSoft Inc., Statplus package, version 6 (https://www.analystsoft.com/ru/). The selection of the main characteristics and statistical criteria for their comparison was carried out after studying the distribution of the characteristic and its comparison with the Gaussian distribution according to the Kolmogorov-Smirnov/ Lillifors, Shapiro-Wilk criteria. Since the data distribution was different from normal, the results were presented as medians, quartiles, and nonparametric comparison methods were used. The reliability of the differences obtained was assessed using nonparametric tests (Mann-Whitney U test). For all statistical tests, p values < 0.05 were considered statistically significant. The values of the specific concentration of urea in all 3 areas of the brain tissue relative to the control group were analyzed. For the MGMT marker, the rank biserial correlation coefficient was used because the results obtained were presented on a nominal dichotomous scale (yes/no), and the specific urea concentration was measured on an ordinal scale. To analyze the relationship between the Ki-67 marker and urea content, Spearman's rank correlation coefficient was used for nonparametric data with the calculation of the correlation coefficient and its level of significance, since Ki-67 has a numerical expression.

3. Results and Discussion

Different distribution of relative content of urea to protein was found in all three zones: adjacent non-cancerous brain tissue (3), peritumoral (2) and tumor area itself (1) (**Table 1**).

Table 1. The value of relative content of urea in the tumor tissues and blood plasma of patients with gliomas.

Relative content of urea in different tumor areas, mM/g protein	Intact, n = 6	1 - 2 grade, n = 7 (Median; quartiles)	Mann-Whitney U-test	3-4 grade, n = 13 (Median; quartiles)	Mann-Whitney U-test
Adjacent non-cancerous tissues (3)	0.241 (0.211; 0.281)	1.998 * (1.718; 2.372	0.0495	1.959 * (0.975; 10.229)	0.010
Peritumoral zone (2)	0.241 (0.211; 0.281)	1.621* (1.381; 5.013)	0.0495	1.959 * (0.975; 10.229)	0.010
Tumor (1)	0.241 (0.211; 0.281)	1.920 * (1.421; 3.260)	0.020	1.260 * (0.340784314; 2.648)	0.051
Blood plasma	0.085 (0.08; 0.099)	0.092 (0.066; 0.110)	0.664	0.105 (0.089; 0.129)	0.065

Legend: Statistically significant differences are reported * $p < 0.05$; n - number of observations.

At all stages of anaplasia, this ratio undergoes a significant increase in all three zones relative to control healthy tissue. The value of urea/protein was the highest in tumor (1), exceeding 10 times to the control group at early stages and in 5 times at III-IV Grade. The progression of the tumor from early stages to the final Grade changed this coefficient in 20%. In peritumoral area (2) this parameter increased at 6.7 and 8 times respectively Grade I-II and Grade III-IV. No differences in urea/protein ratio were found in adjacent non-cancerous tissues at all the stages.

It is known that urea can be formed not only as a result of hydrolysis of arginine under the action of the enzyme arginase (**Figure 1**). It was proved that glioma cells are sensitive to arginine [20]. L-arginine metabolism in normal and tumor cells is different. Arginine is an amino acid critically involved in many cellular processes, including synthesizing nitric oxide and polyamines and is a direct activator of mTOR, a nutrient-sensitive kinase. It is actively involved in carcinogenesis. However, it is also considered an essential or semi-essential amino acid due to the intrinsic ability of normal cells to synthesize arginine from citrulline and aspartate through ASS (argininosuccinate synthase) and ASL (argininosuccinate lyase).

High values of the specific concentration of urea at grade II-IV, regardless of the tumor zone relative to the control healthy tissue, are a sign of a reactive change in the metabolism of brain tissue in the presence of a malignant neoplasm.

High values in the peritumoral zone of the tumor are due to active protein metabolism, as well as good blood supply to this zone. Intensive blood supply is caused by pathological angiogenesis, which develops as a result of the active production of vascular endothelial growth factor [22] and a number of others. Local hypoxia resulting from the mismatch of the vascular wall with the oxygen needs of tumor cells triggers transcription factor (HIF)-1α, which promotes tumor invasion and transformation of the peritumoral zone into a tumor one. In addition, studies show that the accumulation of lactic acid, actively produced by tumor cells, promotes the stimulation of the expression of high levels of VEGF and arginase I, which support tumor growth, metastasis and angiogenesis, as well as inhibit antitumor immunity [23]. The metabolism of arginine undergoes

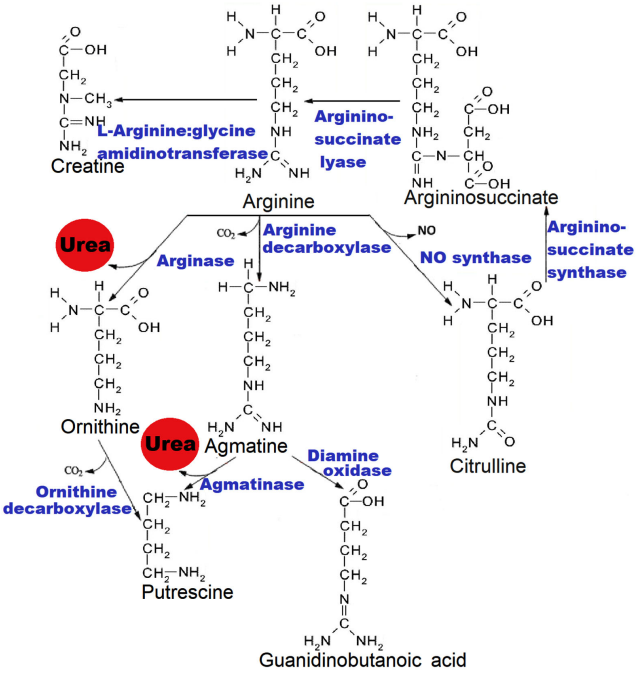

Figure 1. Metabolic pathways for urea formation. (Modified by Piletz *et al*., 2013 [21]).

remodeling, increasing the activity of arginase 2 [24] and the production of urea respectively. Urea, which is a carrier of nitrogen of amino acids formed during the enhanced breakdown of proteins in the process of carcinogenesis, is formed not only by the action of arginase, but also by the action of the enzyme agmatinase. Endogenous agmatine, formed as a result of the decarboxylation of arginine, is induced in response to hypoxia and suppresses the synthesis of NO. Nitric oxide has a dual role: it participates in the initiation and progression of cancer, but also limits the proliferation and invasion of cancer and promotes antitumor immune response [25]. Besides, the other product of metabolism of arginine—ornithine/putrescine—is used for the active production of polyamines [26], which regulate cell proliferation (**Figure 1**).

To date, it is no longer possible to predict the behavior of tumors without taking into account immunohistochemical and molecular genetic aspects. WHO classification of tumors of the central nervous system (2016) became the first stage in the formation of a new approach, and WHO classification consolidated this direction (2021) [16]. To establish the existing relationships between immunohistochemical markers of gliomas and the studied of urea metabolism enzymes, a bioinformatic analysis was carried out using databases on the interactions of molecular biological objects with an average confidence of 0.400. Protein-protein interactions were only considered if the Combined Score between nodes was ≥0.6. Direct biologically significant interactions between Ki-67 and Arginase2 were identified. An indirect effect of MGMT on the functional state of Arginine decarboxylase, Arginase2, and Ornithine decarboxylase 1 has also been established. The determination of the mitotic index Ki-67 is a fairly routine me-

thod in the diagnosis of many oncological processes. It is a labile non-histone nuclear protein that is actively expressed in the G1, S, G2, M phases of the cell life cycle [27]. Its quantitative assessment in glial neoplasms helps to assess the overall potential of tumor progression, identify areas with higher proliferative activity, and sometimes it can serve as a determinant factor in distinguishing gliomas of varying degrees of malignancy [28]. Another predictive marker is considered to be the methylation of the MGMT promoter. Enzyme o-6-methylguanine-DNA methyltransferase is known to be involved in DNA repair [29]. Methylation is one of the mechanisms for regulating gene expression in the body by blocking the attachment of RNA polymerase to the promoter [30], this leads to a decrease in the ability of tumor cells to repair damaged DNA sites after the action of chemotherapy drugs with an alkylating agent [31].

The correlations between urea concentration and the markers of tumor growth are represented in **Table 2**. The greatest number of significant relationships was found for Ki-67 and for MGMT. A direct dependence of the urea level in the non-cancerous brain tissue adjacent to the tumor and the peritumoral zone on the value of the mitotic index Ki67 (coefficients 0.444 and 0.404, respectively) and the inverse dependence (Spearman's coefficient - 0.478) of the urea content on the methylation of the MGMT promoter was observed.

The data on the specific concentration of urea were divided into groups depending on the immunohistochemical profile according to the corresponding marker. At a low level of the mitotic index Ki-67 (less than 10%), a significantly lower specific concentration of urea is observed in non-cancerous brain tissue adjacent to the tumor (**Figure 2**).

In the group without methylation of the MGMT promoter, a significantly higher specific concentration of urea was detected in non-cancerous brain tissue adjacent to the tumor (**Figure 3**).

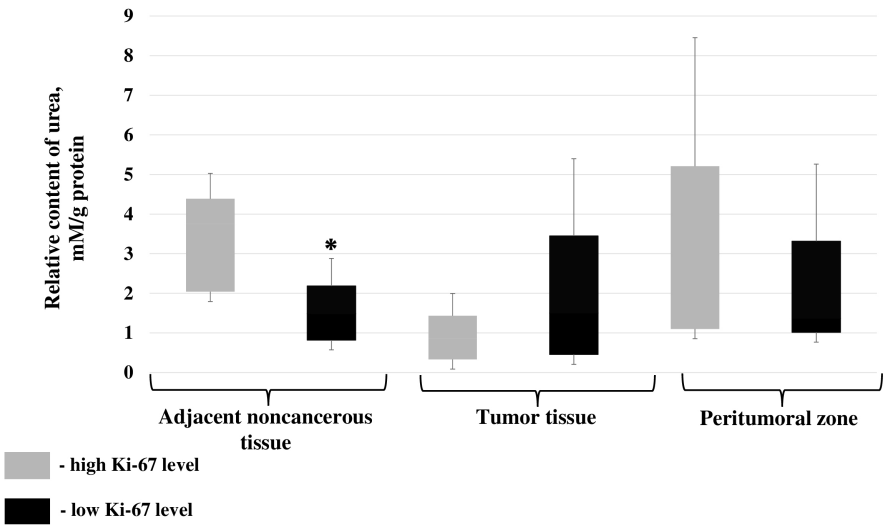

Figure 2. Medians and interquartile ranges of relative content of urea in gliomas as function of the value of the Ki-67 mitotic index. Legend: *- statistically significant differences in comparison with the high Ki-67 level.

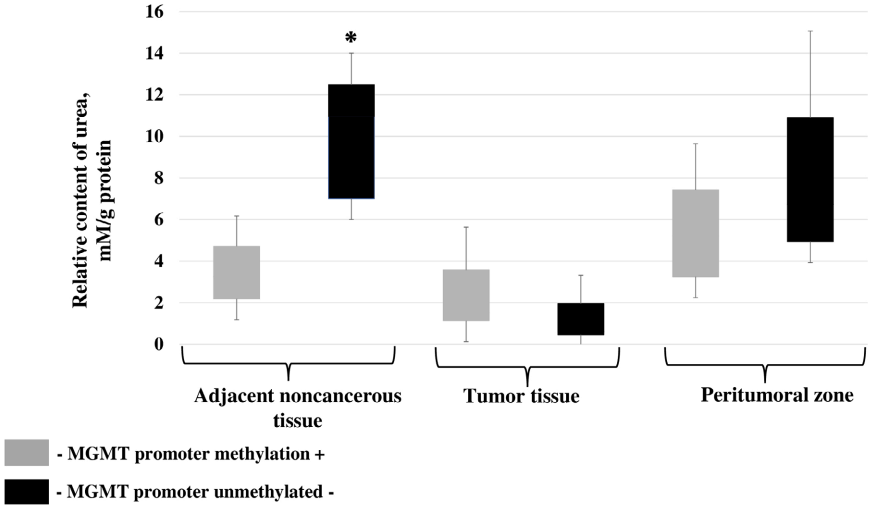

Figure 3. Medians and interquartile ranges of relative content of urea in glioma tissue depending on the promoter of MGMT gene which carries out the DNA repair. Legend: *-statistically significant differences in comparison with the MGMT promoter methylation.

Table 2. Correlation between immunohistochemical markers of gliomas and the relative content of urea in brain tumors in 20 individuals (Spearman's coefficient).

	Relative content of urea		
	Adjacent noncancerous tissue	Peritumoral zone	Tumor tissue
Ki-67	0.444* (p = 0.04)	0.404* (p = 0.05)	−0.272 (p = 0.520)
MGMT	−0.478* (p = 0.05)	−0.179 (p = 0.650)	0.161 (p = 0.620)

Legend: Statistically significant differences are reported * $p < 0.05$.

The mitochondrial isoform Arginase 2 promotes the conversion of arginine into ornithine and urea, besides arginine is decarboxylated into agmatine with further conversion into urea and putrescine. Ornithine is converted into putrescine under the action of ornithine decarboxylase 1, and putrescine, in turn, into polyamines—spermidine and spermine—substances that promote not only active cell proliferation, but also DNA replication (**Figure 1**). High competition for nutrients disrupts antitumor immunity and suppresses the physiological cytotoxic function of T cells, which are also sensitive to arginine, while both systemic and local amino acid deficiency is observed. The microenvironment can have an impact on the depletion of reserves. Myeloid suppressor cells secrete the cytosolic enzyme ARG1 into the extracellular matrix, which cleaves arginine, thereby becoming involved in the process of suppressing T lymphocytes [32]. Pathological angiogenesis is an absolute requirement for tumor growth, activated mainly due to local hypoxia and active production of vascular endothelial growth factor and the main growth factor obtained from fibroblasts both in the tumor cells themselves and in glial cells. Nitric oxide contributes to an increase in blood flow and vascular permeability due to its direct muscle relaxant effect and angiogenesis by enhancing these angiogenic factors, and also affects the proliferation of

vascular cells such as endothelial and smooth muscle cells [33]. Mutation or loss of the tumor suppressor gene p53 leads to resistance to NO-mediated cell death, providing a selective advantage for the growth of abnormal cells [34]. Arginine decarboxylation product agmatine inhibits the production of nitric oxide by reducing the activity of NO synthase 2 (NOS-2) in macrophages and astroglial cells by reducing the level of NOS-2 protein. Increased calcium levels in glioma tumor cells inhibit ADC1 [26], which provide a substrate for arginase and NO synthase, thereby contributing to an increase in NO levels, the formation of polyamines and depletion of agmatine. In oncogenesis with the TP53 mutation, active ornithine decarboxylase 1 can indirectly affect the expression of the mutant protein and impaired apoptosis, which is manifested by the development of neoplasms and increased resistance of tumor cells to chemotherapy.

The next point of our investigation was to check the specific concentration of urea relatively protein content in blood to find whether the correlation between brain and liver urea has had. No changes in this parameter were found, only the tendency of rise at the last stages of tumor. Located in the endothelial cells of the capillaries of the brain, the blood-brain barrier (BBB) has specific properties of strict control. However, these specific properties of BBB can be changed in pathology [35]. In brain tumors, the physical and metabolic properties of the blood-brain barrier are modified, which is renamed the blood-brain tumor barrier (BBTB) [36]. As glioma growth progresses, the permeability of the blood-brain tumor barrier increases [37], this may be the reason of blood urea content increases at Grade III-IV of the tumor.

4. Conclusion

The insights into gliomas metabolism have revealed significant changes in the upregulation of catabolism and biosynthesis of proteins leading to enhanced growth of tumors. The leading role in these changes belongs to the peritumoral zone. The border region plays a dual role, containing elements that limit the tumor process on the one hand, and on the other, being a substrate for further tumor progression. It has been shown that relative concentration of urea as the end product of nitrogen metabolism can be used as the simple biomarker of the metabolic activity of this perifocal area and thus, determines the further neoplasm progression. Amino acid (arginine) pathways may contain druggable targets for gliomas. Drugs that deplete arginine and, thus, urea, may be effective against brain tumors, and should be studied in conjunction with chemotherapy, so arginine deprivation is becoming a novel and promising clinical strategy for metabolism-based cancer therapy [38] [39]. O(6)-methylguanine-DNA methyltransferase (MGMT) status may help to choose between chemotherapy and radiotherapy[management of gliomas [40]. Yet, the questions remain: Do different cells within the tumor have different metabolic strategies or preferred metabolic substrates? Do gliomas with different oncogenic driver mutations (e.g., in p53, IDH) have different metabolic strategies or preferred metabolic substrates?

Further understanding of the metabolic alterations in gliomas will definitely shed light on the investigation not only tumor itself but which is more effective for prevention of anaplasia progression the state of peritumoral and adjacent tissues, those pathways, which could be targeted pharmacologically to slow growth and invasion of glioma. It should be expected that many new fields or breakthroughs will appear in the future.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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