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Tissue Expressions of Regulatory Enzymes of the Krebs Cycle in Low- and High-grade Gliomas

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ABSTRACT

AIM: To compare tissue levels of the regulatory enzymes related to the Krebs cycle between low, and high-grade supratentorial gliomas.

MATERIAL and METHODS: Forty patients who underwent surgery for supratentorial gliomas (19 with low-grade and 21 with high-grade gliomas) were evaluated. The regulatory enzymes directly involved in the Krebs cycle, namely pyruvate dehydrogenase, citrate synthase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase, and two enzymes that indirectly regulate the Krebs cycle, namely glutamate dehydrogenase and glutaminase, were quantitatively studied in tumor tissues using ELISA. The results were compared between the two groups.

RESULTS: The levels of all enzymes were higher in the high-grade glioma group but only pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase levels showed statistical significance. Moreover, all enzymes showed higher tissue levels in grade-II compared to grade-I gliomas, but only two enzymes, glutamate dehydrogenase and glutaminase, reached significantly higher levels. In the high-grade glioma group, all enzymes again showed higher tissue levels in grade-IV gliomas than in grade-III gliomas, but none showed statistical significance.

CONCLUSION: Regulatory enzymes of the Krebs cycle are increased in high-grade gliomas compared to low-grade gliomas. Glutaminolysis enzymes, namely glutamate dehydrogenase and glutaminase, which are required for resupplying the Krebs cycle, are also increased in order to meet the high energy demand in high-grade gliomas.

KEYWORDS: Glioma, High-grade glioma, Krebs cycle, Low-grade glioma, Surgery

INTRODUCTION

Gliomas are the most common central nervous system tumors, accounting for more than 50% of all primary brain tumors (10). They are broadly categorized according to their cells of origin and four classes are generally used in clinical practice. Grades I and II are low-grade gliomas (LGGs), whereas grades III and IV are high-grade gliomas (HGGs). Grades II through IV are diffuse gliomas that make total surgical removal impossible in the majority of cases, and survival outcomes are largely dependent on grade, with grade IV having the worst overall survival despite every effort (10). Thus, it seems that understanding the molecular events behind the pathogenesis of gliomas is of utmost importance, and better treatment strategies are urgently required because of the short survival rate, especially for grade IV gliomas. Median survival time has been reported as approximately 15

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months after standard treatment including surgical resection, followed by radiation and chemotherapy with temozolomide in grade IV gliomas (5).

Studies focusing on the molecular events will explore the mainstay of future glioma treatment, and enzymes of the Krebs cycle (citric acid cycle or tricarboxylic acid cycle) have become the focus of several studies. IDH-1 and IDH-2 mutations have been discovered in gliomas, and patients with such gliomas have been reported to have better survival rates than those with wild-type gliomas (15). A large number of studies of mutations of the Krebs cycle enzymes and have shown that the Krebs cycle may play a very important role in glioma biology (6,7).

Considering that individual Krebs cycle enzymes are regulated, studies of the main regulatory enzymes may lead to better experimental and/or clinical studies in the future and thus development of more sophisticated treatment strategies, which are needed for treatment in addition to surgical glioma removal. The aim of this prospective study was to measure tissue expression of Krebs cycle regulatory enzymes in both LGG and HGG and to identify any differences between the two groups. To the best of our knowledge, this is the first study to compare tissue levels of all of the Krebs cycle regulatory enzymes in LGG and HGG.

MATERIAL and METHODS

This study was approved by the Local Ethics Committee of Cerrahpasa School of Medicine, Istanbul University-Cerrahpasa, and does not contain any data exposing any patient's identity. Surgery was performed after all patients signed a written informed consent form (Date: 2 April 2019, No: 83045809-604.01.02).

This prospective study included a series of patients who underwent surgery for supratentorial gliomas between April 2019 and April 2021 in our clinic. All patients were operated on by the same surgeon using a similar technique and were followed-up regularly after surgery. The inclusion criteria were strictly applied, and patients were included who were 1) at least 18 years of age, 2) had no cancer related to other organ system, and 3) had histologically proven glioma. Patients who had recurrent tumors or underwent surgery by another team at a different neurosurgical center were excluded.

Age, sex, tumor lateralization and localization, and histopathological diagnosis were noted, and all patients underwent magnetic resonance imaging (MRI) before and within 72 hours of surgery. During surgery, tumor tissues were obtained and stored at -80°C until the Krebs cycle regulatory enzymes were assayed. In this study, pyruvate dehydrogenase (PDH)-(lipoamide) kinase isozyme-1, mitochondrial; citrate synthase (CS); glutamate dehydrogenase (GDH); α-ketoglutarate dehydrogenase (α-KDH); glutaminase (GLS) kidney isoform, mitochondrial; and isocitrate dehydrogenase (IDH) were evaluated in tumor tissues in both low- and high-grade gliomas.

Enzyme Assay

All tissue samples stored at -80°C were homogenized in phosphate-buffered saline (0.01 M, pH 7.4). The homogenate was centrifuged at 2700 × g for 10 minutes. Proteins in the obtained supernatant were measured. Proteins were measured using a colorimetric method in a biochemistry auto-analyzer (Roche Diagnostic/Cobas 8000-c702). The sandwich-ELISA principle (human ELISA kits from MyBioSource) was used to measure mitochondrial PDH (lipoamide) kinase isozyme-1 (catalog no. MBS764901), CS (catalog no. MBS2516040), GDH (catalog no. MBS763282), α-KDH (lipoamide) (catalog no. MBS2602828), mitochondrial GLS kidney isoform (catalog no. MBS2602981), and IDH (catalog no. MBS266748). The results are provided as nanograms per milligram protein.

Statistical Analysis

We used a commercially available statistics software package (SPSS version 22.0; SPSS, Inc., Chicago, IL, USA]) for all statistical analyses. The mean ± SD was calculated for each parameter. The nonparametric χ^2 and Mann-Whitney U tests were used as appropriate for comparisons. Correlation analysis was performed using Pearson' correlation analysis. Differences were considered statistically significant for $p<0.05$.

RESULTS

Forty patients (19 women and 21 men) with a mean age of 43.37 ± 16.22 years were included. MRI before surgery demonstrated tumors on the right side in 17 patients (42.5%) and on the left side in 23 patients (57.5%). The frontal lobe (n=14; 35%) was the most common location of the gliomas, followed by multilobar (n=10; 25%), temporal (n=9; 22.5%), occipital (n=3; 7.5%), and parietal (n=2; 5%) lobes, and two patients (5%) had glioma on the thalamus. The LGG and HGG groups included 19 and 21 patients, respectively, with no statistically significant difference in mean age (p=0.35) or sex (p=0.53) between the groups (Table I). Furthermore, there was no significant difference regarding lateralization of the tumors between the groups (p=0.55). Histopathologic diagnosis showed grade I in 6, grade II in 13, grade III in 11, and grade IV in 10 patients. In the LGG group, diffuse astrocytoma (grade II) was the most common, found in eight patients. Other diagnoses included diffuse oligodendroglioma (grade II) in four, pilocytic astrocytoma (grade I) in two, angiocentric glioma (grade I) in two, and low-grade astrocytoma (grade I), low-grade glial (grade I), and diffuse non-mutant glial tumor (grade II) in one patient each. In the HGG group, diagnoses included anaplastic astrocytoma (grade III) in seven, non-mutant grade IV in five, wild-type grade IV in four, anaplastic oligodendroglioma (grade III) in three, anaplastic pleomorphic xantho-astrocytoma (grade III) in one, and small cell grade IV in one patient.

Regarding the tissue expression of the regulatory enzymes (Table II) in both groups, the mean levels of all enzymes were higher in the HGG group than in the LGG group, with PDH (p=0.0001), CS (p=0.001), and IDH (p=0.007) showing significantly higher levels. The tissue levels of all enzymes were higher in grade II than in grade I gliomas, but the difference was significant for only two enzymes: GDH (p=0.01) and GLS (p=0.01). As in the LGGs, the tissue levels of all enzymes

Table I: Statistical Summary of Demographic Variables in 40 Patients Operated on Supratentorial Gliomas

Parameters	LGG (n=19)	HGG (n=21)	p-value
Mean age (years)	40.3 ± 18.8	46.0 ± 13.3	0.35
Gender (F/M)	10/9	9/12	0.53
Lateralization (R/L)	9/10	8/13	0.55

***F:** Female; **HGG:** High-grade glioma; **L:** Left; **LGG:** Low-grade glioma; **M:** Male; **R:** Right. Values are given as mean ± standard deviation.*

Table II: Summary of Statistical Analysis Regarding the Six Kreb's Cycle Enzymes Between the Two Groups

Variables (ng/mg)	LGG (n=19)	HGG (n=21)	p-value
Pyruvate DH	6.16 ± 5.67	17.99 ± 15.39	**0.0001**
Citrate synthase	294.98 ± 91.27	436.88 ± 136.89	**0.001**
Glutamate DH	8.03 ± 6.07	10.28 ± 11.12	0.86
α-Ketoglutarate DH	2.08 ± 0.66	2.21 ± 1.26	0.61
Glutaminase	5.11 ± 3.61	5.90 ± 4.58	0.52
Isocitrate DH	3.06 ± 1.39	4.77 ± 2.26	**0.007**

***DH:** Dehydrogenase; High-grade glioma; **LGG:** Low-grade glioma. Values are provided as mean ± standard deviation.*

were higher in grade IV gliomas than in grade III gliomas, the differences were not significant (p>0.05). In the whole group, tissue levels of PDH, CS, and IDH tended to increase from grade I to grade IV.

Correlation analysis showed positive correlations between PDH and CS (p=0.003), PDH and α-KDH (p=0.001), PDH and IDH (p=0.00001), CS and α-KDH (p=0.01), and CS and IDH (p=0.04). As expected, GLS and GDH were positively correlated (p=0.02), and α-KDH was positively correlated with both GLS (p=0.01) and IDH (p=0.02).

DISCUSSION

The Krebs cycle includes a series of enzymatic reactions in mitochondria and is the main pathway for ATP production linked to oxidative phosphorylation, with 10 ATP molecules formed per cycle (1). This cycle plays a pivotal role in metabolism and is the final common pathway for the oxidation of carbohydrates, lipids, and proteins (1). The involvement of the Krebs cycle in cancer metabolism, including glioma, is the result of adaptation of cancer cells to adverse conditions such as hypoxia (4). Neoplasms reprogram their metabolism, which leads cancer cells to be addicted to glucose and glutamine in order to meet the demands of their high proliferative activities (7,8). The acidic environment in cancer cells comes from the production of lactate because fast-growing cancer cells exhibit high rates of glycolysis despite the presence of oxygen, known as the Warburg effect or aerobic glycolysis (13). Although glycolysis proceeds at a high rate, it cannot compensate for high ATP demand; thus, fast-growing tumor cells such as HGGs, also generate ATP via the Krebs cycle and oxidative phosphorylation (4,9).

Recent studies have demonstrated that targeting some intermediates or enzymes of the Krebs cycle may alter glioma metabolism and be a therapeutic option. Inhibition of GLS, which hydrolyzes glutamine to glutamate, which is subsequently converted to an intermediate molecule of the Krebs cycle, α-ketoglutarate (α-KG), slows growth of glioma cells with mutant IDH-1 (12). Mutant IDH-1 then converts α-KG into an oncometabolite, D-2-hydroxyglutate (2-HG) (2). Increased levels of 2-HG lead to both increased oxidative stress and an increased rate of glycolysis. The high rate of glycolysis causes formation of pyruvate, which is reduced to lactate, thus forming an acidic environment, which is common in cancers including gliomas (1,2).

The current literature includes studies mainly focusing on IDH-1 and IDH-2 mutations and glutamine addiction in gliomas (3,8,12). Because there has been no study reporting tissue levels of the Krebs cycle regulatory enzymes in gliomas in the English literature, it is very difficult to compare our results with the current literature. We analyzed the tissue expression of regulatory enzymes of the Krebs cycle in LGG and HGG. The main sites for regulation in the Krebs cycle are non-equilibrium reactions catalyzed by CS, which is responsible for the initial reaction to form citrate by forming a carbon-carbon bond between acetyl-CoA and oxaloacetate. The second regulatory enzyme is IDH, which dehydrogenates isocitrate to α-KG. The importance IDH mutations in glioma formation is well known, and its effect on glioma prognosis has been discussed extensively in the literature. The third regulatory enzyme is α-KDH, which catalyzes the oxidative decarboxylation of α-KG to form succinyl-CoA. The last regulatory enzyme is PDH, which forms acetyl-CoA from pyruvate (1). Although GLS and GDH are not directly involved in the Krebs cycle, they indirectly regulate the Krebs cycle by forming an important intermediate,

Table III: Statistical Comparisons Within Each Group

Variables (ng/mg)	LGG (n = 19)		HGG (n = 21)		p-value*	p-value**
	G-I/6	G-II/13	G-III/11	G-IV/10		
Pyruvate DH	5.16 ± 5.36	8.34 ± 6.20	12.66 ± 7.53	22.83 ± 19.20	0.05	0.26
Citrate synthase	281.1 ± 98.59	324.9±71.28	420.3 ±122.1	451.9 ± 153.4	0.53	0.72
Glutamate DH	6.18 ± 4.16	12.0 ± 7.96	6.93 ± 4.64	13.9 4± 14.91	**0.01**	0.57
α-Ketoglutarate DH	1.92 ± 0.56	2.42 ±0.79	1.84 ± 0.66	2.54 ± 1.59	0.07	0.57
Glutaminase	3.56 ± 1.89	8.47 ± 4.31	3.95 ± 1.66	8.04 ± 5.81	**0.01**	0.07
Isocitrate DH	3.03 ± 1.55	3.11 ± 1.11	3.74 ± 1.70	5.71 ± 2.37	0.59	0.09

DH: *Dehydrogenase;* ***G:*** *Grade;* ***HG:*** *High-grade glioma;* ***LGG:*** *Low-grade glioma.* ***P*:*** *Comparisons within LGGs;* ***P**:*** *Comparisons within HGGs. Values are provided as mean ± standard deviation.*

α-KG (1). Glutamine and glutamate are important anaplerotic substrates, and glutamine is hydrolyzed to produce glutamate by the action of GLS (1). Glutamate is subsequently converted to α-KG by GDH. Recent studies have demonstrated the importance of glutamate addiction in glioma, and targeting GLS led to slower rates of growth for glioma cells with mutant IDH-1 (7,8,12).

Our results are in line with the current idea that fast-growing tumors like gliomas have to meet their energy demand by reprogramming their metabolism. In this context, cancer cells need more energy because glycolysis cannot compensate for their high ATP requirement. Thus, the Krebs cycle comes into play for providing more ATP to gliomas with a high proliferative index. Our results showed that four regulatory enzymes that directly regulate the Krebs cycle (PDH, CS, α-KDH, and IDH) had higher levels in HGGs than in LGGs, but no significant differences were found between grade III and grade IV gliomas. These results are expected because grade III gliomas also have a high proliferative index. We also evaluated tissue expression of two enzymes that indirectly regulate the Krebs cycle, namely GLS and GDH (1). Results related to these two enzymes are also in line with the theory that to provide sufficient energy to fast-growing gliomas, the Krebs cycle intermediates must be resupplied, and fast-growing tumors become glutamine-addicted (8). It is well-known that glutamine is a nonessential amino acid, but in pathological conditions like cancer, it becomes essential (11,14). Glutamate and glutamine are important anaplerotic substances because by the actions of GLS and GDH, they yield α-KG, an important intermediate of the Krebs cycle (1). The Krebs cycle is maintained by these two enzymes, and the glutamine-derived α-KG remains a major way to supply the Krebs cycle (1, 8). Our correlation analysis supports the above data that GLS is correlated with both GDH and α-KDH.

Limitations

The authors of the present study are aware of the limitations. The first and most important limitation is the number of patients. Lower number of patients in our study is mainly due to the COVID-19 pandemic, and we think that number should be increased in future studies in order to provide more results that are inclusive. The second limitation is the lack of normal brain tissues serving as a control group, but it should be appreciated that obtaining normal brain tissues in glioma, especially in extensive gliomas, may not be ethical and could result in unwanted neurological deficits. Finally, we did not obtain the levels of the enzyme products together with the enzymes, which would add important data to the current literature.

CONCLUSION

Regulatory enzymes of the Krebs cycle are increased in HGGs compared to LGGs, and glutaminolysis enzymes, which are required for resupplying the Krebs cycle, are also increased in order to meet the high energy demand in HGGs because cancer cells become glutamine-addicted.

AUTHORSHIP CONTRIBUTION

Study conception and design: BT, TT
Data collection: BT
Analysis and interpretation of results: BT, BO, MI, HS, BBI, TT
Draft manuscript preparation: BT, RK, MEA, TK
Critical revision of the article: BT, TT
All authors (BT, RK, MEA, BO, MI, HS, BBI, TK, TT) reviewed the results and approved the final version of the manuscript.

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