Abstract. – OBJECTIVE: Diabetes mellitus (DM)-mediated impaired glucose metabolism increase in the glioblastoma (GB) risk by inducing hyperglycemia and hyperinsulinemia. An integral membrane transport protein, glucose transporter 3 (GLUT3) facilitates glucose transport into GB tumor cells. We aimed to explore the regulation of GLUT3 in GB tumors of patients who were concurrently diagnosed with DM.

PATIENTS AND METHODS: Formalin-fixed paraffin-embedded (FFPE) tumor samples were collected from 93 GB patients and retrospectively analyzed. Of the total, 15 patients were concurrently diagnosed with DM (GB-DM). The role of GLUT3 in tumor aggressiveness was evaluated by analyzing its correlation with Ki67, P53 expression, MALAT1 expression, and peripheral blood glycated hemoglobin (HbA1c) level. T98G cells were treated with empagliflozin and metformin to modulate GLUT3. The RNA expression of GLUT3, SOX2, and MALAT1 was analyzed by real-time qPCR. The lactate levels of T98G cells were measured by Cobas c502 analyzer. A scratch wound assay was performed to investigate the migration rate of T98G cells.

RESULTS: GLUT3 expression was lower in GB-DM tumors than in GB-only tumors. In GB-DM, the expression of tumoral GLUT3 and peripheral blood glycated hemoglobin (HbA1c) levels were negatively correlated with P53 and Ki67. A decreased GLUT3 shortened the disease-free survival duration in GB-DM patients. Empagliflozin reduced GLUT3, while metformin-induced GLUT3 in T98G cells. The empagliflozin-mediated GLUT3 suppression induced SOX2 and MALAT1 expressions and influenced the migration capacity of T98G cells.

CONCLUSIONS: Our findings suggest that the low GLUT3 expression of the tumors of GB-DM patients may induce the production of adenosine triphosphate (ATP) from cellular energy sources other than glucose metabolism. However, further studies are warranted to confirm these results.

Key Words: Diabetes mellitus, Glioblastoma, HbA1c, Glucose metabolism, GLUT3.

Introduction

Diabetes mellitus (DM) increases the cancer risk and affects prognosis. Evidence suggests several interlinks between DM and cancer. In addition, hyperglycemia and hyperinsulinemia caused by DM have been reported to provoke the transformation into cancer cells. Glioblastoma (GB), the most common malignant primary glioma of the central nervous system, is a cancer type wherein comorbidity such as DM commonly affects the patient’s survival.

The adenosine triphosphate (ATP) production of cells is vital to several essential cellular processes and requires glucose metabolism. In DM, glucose metabolism is commonly impaired. In addition, excessive glucose concentrations due to an imbalance in glucose metabolism could provoke invasiveness and chemoresistance of GB tumors. A family of integral membrane transport proteins, the glucose transporter (GLUT) s, facilitates glucose transport into the cells. GLUT3 is expressed explicitly by neurons and recognized as the neuronal GLUT among these proteins, playing a role in brain glucose homeostasis and function.

In GB, aggressive features of tumors have been linked to the overexpression of
GLUT3 in glioblastoma. In contrast, it has been reported to be downregulated in DM. Therefore, the expression and function of GLUT3 in tumors of GB patients who also suffer from DM remain controversial.

In this study, we have described the regulation of GLUT3 in GB tumors in patients who also have DM and its potential effect on tumor progression. In addition, we uncovered the role of GLUT3 on GB cell aggressiveness by pharmacologically modulating its expression.

Patients and Methods

Patients

This study retrospectively assessed the formalin-fixed paraffin-embedded (FFPE) tumor tissue samples of 93 GB patients (56 male, 37 female). Fifteen of these patients (10 male, 5 female) were also diagnosed with type 2 DM (GB-DM) during the GB treatment. The diagnosis of DM was made in accordance with the standards of the American Diabetes Association for medical care in diabetes. The Ki67 and P53 expressions were sourced from the pathology records to classify the tumor samples based on their aggressiveness. Long non-coding RNA (lncRNA) Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) expression of the samples was determined as described in our previous study.

In this study, we did not include patients who received neoadjuvant chemoradiotherapy, those previously treated for other cancers, or those with a family history of cancer. The 85-month follow-up data of the patients were subjected to survival analyses. This study was approved by the Local Ethics Committee of Bursa Uludag University (approval number 2017-13/98).

Cell Lines and Reagents

T98G, a GB cell line, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells (passage 15) were grown in Dulbecco’s Modified Eagle’s Medium-F12 (DMEM/ F12; HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), L-glutamine (2 mM, BIOCHROME, Rahel-Hirsch, Berlin, Germany), and sodium pyruvate (1 mM, BIOCHROME, Rahel-Hirsch, Berlin, Germany) in a 5% CO2 incubator at 37°C.

Empagliflozin was purchased from Boehringer Ingelheim (500 nM, Biberach, Baden-Württemberg, Germany), while metformin was obtained from Bilim Pharmaceuticals (6 mM, Beyoğlu, Istanbul, Turkey).

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

A commercial RNA Isolation Kit was used to extract the total RNA from FFPE tumor samples (Zymo Research, Orange, CA, USA), and the total RNA of cells was extracted with a trizol reagent (Invitrogen, Carlsbad, CA, USA) as previously described. A DU 730 spectrophotometer measured the RNA concentrations and quality (260 nm:280 nm; Beckman Coulter, Inc., Brea, CA, USA). Total RNA (500 ng) was reverse transcribed to cDNA according to the instructions of ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs; Ipswich, MA, USA). The RNA expression level of GLUT3, SOX2, and MALAT1 was determined according to the protocol of TaqMan™ Gene Expression Assays by using the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The expression of GAPDH was used for normalization, and the relative quantification of GLUT3 RNA was calculated by using the 2−ΔΔCt method.

Lactic Acid Measurement

The continued medium of empagliflozin- and metformin-treated T98G cells were collected, and the lactic acid level was measured on the Cobas c502 instrument by using an enzymatic assay according to the manufacturer’s instruction (Lact2; Roche Diagnostics, IN, USA).

Scratch Wound-Healing Assay

A confluent monolayer of T98G cells was scratched by dragging a 100-μL pipette tip across the thin membrane. The wounded monolayers were cultured for 24 h in empagliflozin and metformin. The image of the injured area of the monolayers was captured at 0 h and 24 h. The size of the wounded area was measured with the ImageJ v1.53s (National Institutes of Health, Bethesda, MD, USA). Each experiment was performed in duplicates.

Statistical Analysis

The difference in the RNA expression of GLUT3 between GB-only and GB-DM was performed using an independent sample t-test. Pearson’s correlation analysis was performed to determine the association of GLUT3 RNA expression between the RNA expression of IncRNA MALAT1, the protein expression of Ki67 and P53, and peripheral blood hemoglobin A1C (HbA1c) levels. A Kaplan-Meier analysis and a log-rank test were performed to identify the effect of GLUT3 expression on the survival of GB-only and GB-DM patients.
One-way analysis of variance with Tukey’s post-hoc analysis was performed to analyze the RNA expression of GLUT3, SOX2, and MALAT1, the lactic acid production, and the scratch wound-healing assays after empagliflozin and metformin treatments of T98G cells. All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 28.0; IBM Corp., Armonk, NY, USA). Statistical significance was defined in a 95% confidence interval, and \( p < 0.05 \) was considered to indicate statistical significance.

**Results**

**Patients’ Characteristics**

The age of patients at the time of GB diagnosis was similar between the GB-only and GB-DM patients (\( t = -1.73; \ p = 0.087 \)). The mean age of these patients was 58.17 ± 1.30 and 63.26 ± 1.74 years, respectively. In 10 GB-only and 4 GB-DM patients, the primary tumors were localized in the parietal region of the brain. In addition, the tumor was detected in the frontal region in 25 GB-only and 5 GB-DM patients; in the temporal region in 23 GB-only and 3 GB-DM patients; in the occipital region in 19 GB-only and one GB-DM patients; the insular region in one GB-only and one GB-DM patients; in the cerebellum in one GB-DM patient.

**GLUT3 Expression was Elevated in Tumors of GB-Only Patients, while it was Weakly Induced in GB-DM**

The RNA expression of GLUT3 was lower in the tumors of GB-DM patients than in those of GB-only patients (\( t = 2.360; \ p = 0.023 \); Figure 1A). In addition, in GB-DM patients, the reduced GLUT3 expression was correlated with elevated HbA1c levels (\( r = -0.886; \ p < 0.001 \); Figure 1B).

**DM-Reduced GLUT3 RNA Promotes GB Tumor Aggressiveness**

In GB-only tumors, the induced GLUT3 RNA expression was not correlated with the MALAT1 lncRNA (\( p = 0.142 \)), Ki67 (\( p = 0.759 \)), or P53 (\( p = 0.246 \)) protein expressions. In addition, no association was detected between the GLUT3 RNA expression and tumor size in GB-only tumors (\( p = 0.948 \)). In contrast, the reduced RNA expression of GLUT3 was correlated with induced MALAT1 lncRNA (\( r = -0.620; \ p = 0.014 \); Figure 2A) and Ki67 (\( r = -0.902; \ p < 0.001 \); Figure 2B) protein expressions of GB-DM tumors.

In addition, the mean disease-free survival of GB-only patients was shortened depending on the high GLUT3 RNA expressions (GB-only-GLUT3\(^{high}\): 11.64 ± 1.44 months; GB-only-GLUT3\(^{low}\): 36.16 ± 15.31 months; Figure 2C). Conversely, low GLUT3 RNA expression lessened the mean disease-free survival in GB-DM patients (GB-DM-GLUT3\(^{high}\): 18.14 ± 4.13 months; GB-DM-GLUT3\(^{low}\): 5.62 ± 1.59 month; Figure 2D).

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**Figure 1.** GLUT3 RNA expression in GB-only and GB-DM patients. A, The comparison of GLUT3 RNA between the tumors of GB-only and GB-DM patients. B, The correlation between GLUT3 RNA and blood HbA1C levels in GB-DM patients. Seven DM-GB patients were on intensive insulin therapy; one was on oral antidiabetic medication. Strict glucose control was achieved (HbA1c <7.5) in two of these patients. In addition, in 5 of those patients, diabetic polyneuropathy and peripheral vascular disease were detected, and blood sugar control was poor (HbA1c >9). The three GB-DM patients were newly diagnosed with DM and had not received antidiabetic therapy before GB diagnosis. GB: Glioblastoma; GB-only: Tumor tissue of patients diagnosed with GB but not DM. GB-DM: Glioblastoma together with diabetes mellitus. Statistical significance was calculated using an independent sample t-test for A and B and Pearson’s correlation analyses for C. \( p\)-values less than 0.05 were considered significant.
Diabetes affects GLUT3 in glioblastoma

Figure 2. DM-reduced GLUT3 induced GB tumor aggressiveness. The correlation of GLUT3 RNA with MALAT1 LncRNA (A) and Ki67 protein expression (B). C-D. The effect of GLUT3 on disease-free survival of GB-only and GB-DM patients. GB-only: Tumor tissue of patients diagnosed with GB but not DM. GB-DM: Glioblastoma together with diabetes mellitus. Empagliflozin, an insulin regulator, metformin, to confirm the role of glucose transport and metabolism in GB cells. Our findings revealed that, while GLUT3 RNA expression was reduced by empagliflozin (p < 0.001), it was induced by...
metformin ($p < 0.001$) in T98G cells when compared to that in the untreated cells (Figure 3A). The RNA expression of a stem-like cancer cell gene $SOX2$ and IncRNA, $MALAT1$, was higher in GLUT3-suppressed cells by empagliflozin than in the untreated cells ($p < 0.001$, Figure 3B-C). In contrast, in T98G cells with metformin-induced GLUT3, the $SOX2$ RNA was reduced ($p < 0.001$), and $MALAT1$ was unaffected when compared to that in untreated cells (Figure 3B-E). In line with this, empagliflozin induced lactic acid production ($p = 0.005$). However, metformin did not affect the level of lactic acid compared to that in the untreated T98G cells. A wound-healing assay revealed the effect of empagliflozin suppression and metformin induction of GLUT3 on the T98G cell migration rate. These findings suggest that the migration rate of empagliflozin-treated cells was almost similar to those in the untreated cells in 24 h ($p > 0.05$), while metformin reduced the migration capacity ($p = 0.049$). These findings suggest that suppressing GLUT3 can induce lactate metabolism and aggressive tumor features, including stem-like cell phenotype and the migration ability of T98G cells.

**Discussion**

GB cells undergo aerobic glycolysis to produce ATP to support their growth, which paves the way for a microenvironment that promotes metastasis due to the conversion of glucose to lactate, as explained by the Warburg effect—a phenomenal transformation of glucose to lactate in an anaerobic microenvironment of tumor cells. Therefore, elevated blood glucose level is recognized as one of the risk factors for GB. GLUT3 supports aerobic glycolysis by glucose uptake in astrocytes and its transport across the

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**Figure 3.** The in-vitro effect of GLUT3 induction and suppression on T98G cells. The RNA expression of (A) GLUT3, (B) $SOX2$ and (C) $MALAT1$ upon empagliflozin (500 nM) and metformin (6 mM) treatments for 24 h. (D) The effect of empagliflozin and metformin on lactic acid production and (E) cell migration rate of T98G cells. All analyses were performed in three technical repeats. $p$-values were calculated using One-Way ANOVA and Tukey’s test. $p$-values lower than 0.05 were considered significant. UNT: Untreated, Emp: empagliflozin, Mtf: Metformin.
GLUT3 has been shown to be highly expressed in high-grade gliomas, suggesting its predominant role in glucose transport in GB tumors. Furthermore, our findings showed an increased RNA expression of GLUT3 in GB tumors relative to that in undiagnosed tissues at their surgical margins. High glucose concentrations have been shown to promote the expression of the aggressive features of GB cells, including enhanced proliferation and colony formation in vitro. This effect was suggested by inducing the function of the G-protein-coupled chemoattractant receptor and epidermal growth factor. Similarly, our data showed reduced lifespan in GB patients with high GLUT3-expressing tumors. Although these tumors had high Ki-67 index and elevated P53 expressions, indicating tumor growth, our findings did not determine a linear correlation between them. A previous study suggested that the role of GLUT3 in aerobic glycolysis could be independent of P53.

In addition, while GLUT3 RNA expression was analyzed in an equal amount of RNA for each sample, the sectioned FFPE tissues’ heterogeneity in tumor cell density may have hindered a possible linear correlation between GLUT3 and Ki67 or P53 in this study.

DM contributes to GB progression by impairing energy metabolism through the expression of changing glucose transporters. The glucose transporters, including GLUT3, were downregulated in DM patients’ blood leukocytes. Supporting this observation, our findings indicated a negative correlation between GLUT3 and HbA1c levels in GB-DM patients. In addition, we detected a lower GLUT3 expression of GB tumors in GB-DM patients, suggesting the adverse effect of DM in brain cells resulting from impaired glucose transport mechanisms. A loss of GLUT3 was meant to lead to a glucose deficit, which may cause the halting of ATP-dependent cellular processes in the brain cells.

In contrast, tumor cells metabolize lactate, glutamine, and fatty acids as alternative nutrients to produce energy in glucose-deprivation conditions and cause lactic acidosis, which, in turn, generates a novel way for cancer survival. To support this hypothesis, we reduced the expression of GLUT3 by using a SGLT2 inhibitor empagliflozin, and induced it via a modulator of glucose uptake, metformin. Our findings indicate that the empagliflozin mediated a reduction of GLUT3 induced the lactic acid production of T98G cells, while metformin did not. These findings suggest that a decreased GLUT3-mediated glucose starvation could result in a similar scenario caused by the Warburg effect through the production of lactic acidosis. A past study showed that insufficient nutrition could promote the Warburg effect by producing reactive oxygen species and inducing the phosphorylation of AMP-activated protein kinase. Lactic acidosis leads to the development of an acidified microenvironment that provides an environment conducive to invasion and metastasis. Indeed, in our study, the loss of GLUT3 in tumors of GB-DM patients was correlated with enhanced tumor Ki67 index and a shortened disease-free life expectancy. In addition, empagliflozin suppression of GLUT3 failed to attenuate the migration rate of T98G cells, although it was reduced by metformin, suggesting that although the tumors of GB-DM patients have a low capacity for cellular glucose uptake, they could maintain their energy metabolism to survive and continue growing.

P38 mitogen-activated protein kinase (P38-MAPK) signaling participates in DM pathogenesis as a result of an excessive caloric intake. Therefore, P38-MAPK signaling inhibitors are used to target the Warburg effect. P38-MAPK has been shown to be regulated by non-coding RNA, for instance, an abnormal expression of a MAPK targeting microRNA (miRNA), miR-362, promotes DM. Moreover, the replacement of miR-362 inhibited cell growth and metastasis in GB. Similar to miRNA, IncRNA plays a role in the maintenance of glucose metabolism by directly or indirectly affecting the Warburg effect.

Glucose has been reported to induce MAPK targeting IncRNA MALAT1 in the serum of DM patients. In addition, high MALAT1 expression has been linked to augmented production of inflammatory cytokines in diabetic retinopathy. Moreover, MALAT1 was demonstrated to regulate cancer glucose metabolism by enhancing glycolysis, aggravating glucose deprivation-induced neuronal endoplasmic reticulum stress, as well as inducing severe metabolic acidosis.

Our previous study emphasized the overexpression of IncRNA MALAT1 in GB tumors of GB-DM patients than in those of GB-only patients. In addition, our current findings indicated a correlation between reduced GLUT3 expression and a high level of MALAT1 in GB-DM patients. Furthermore, the in vitro suppression of GLUT3 by empagliflozin induced the expression of MALAT1. Past studies have suggested that MALAT1 induces GB cell aggressiveness by...
regulating SOX2 to promote the stem-like cancer cell phenotype of GB cells, promoting cancer cell aggressiveness. Supporting this finding, we found that empagliflozin not only induced MALAT1 but also elevated the expression of SOX2. Therefore, our results suggest that the aggressive tumor phenotype of GB-DM patients can be explained by low GLUT3 expression of GB cells, which could maintain the tumor microenvironment to facilitate an aggressive phenotype by the overexpression of MALAT1.

Conclusions

Our preliminary findings revealed that GB and GB-DM patients’ tumors adopt different metabolic mechanisms to provide ATP for tumor growth. While the tumors of GB-only patients provide the required energy through high glucose uptake via GLUT3, GB-DM patients produce ATP from other cellular energy sources through anaerobic metabolism, which increases lactic acid production. Therefore, DM-related metabolic adaptation of GB tumors to glucose shortage caused by low GLUT3 expression could exaggerate aggressive GB tumor phenotype by promoting stem-like cancer cell phenotype with an induced MALAT1 and SOX2 expression. Considering the rarity of GB-DM patients, further studies are warranted to clarify the energy sources mechanism of GB tumors under low-cellular glucose conditions in a large study cohort as well as to investigate the therapeutic targeting potential of GLUT3-mediated glucose shortage to treat DM-induced GB tumors.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability
All data generated or analyzed during this study are included in this published article. The data supporting this study’s findings are available from the corresponding author upon request.

Ethics Approval
This study was approved by the Local Ethics Committee of Bursa Uludag University (approval number 2017/13/98).

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Diabetes affects GLUT3 in glioblastoma


