A Supplemented High-Fat Low-Carbohydrate Diet for the Treatment of Glioblastoma

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Abstract

Purpose: Dysregulated energetics coupled with uncontrolled proliferation has become a hallmark of cancer, leading to increased interest in metabolic therapies. Glioblastoma (GB) is highly malignant, very metabolically active, and typically resistant to current therapies. Dietary treatment options based on glucose deprivation have been explored using a restrictive ketogenic diet (KD), with positive anticancer reports. However, negative side effects and a lack of palatability make the KD difficult to implement in an adult population. Hence, we developed a less stringent, supplemented high-fat low-carbohydrate (sHFLC) diet that mimics the metabolic and antitumor effects of the KD, maintains a stable nutritional profile, and presents an alternative clinical option for diverse patient populations.

Experimental Design: The dietary paradigm was tested in vitro and in vivo, utilizing multiple patient-derived glioblastoma lines.

Introduction

Glioblastoma (GB) is the most common high-grade glioma in adults, with extremely poor prognosis. Tentacle-like projections and pseudopalisading necrosis integrate into normal brain tissue making complete surgical resection difficult. Extreme cellular heterogeneity, extensive genetic aberrations, and inadequate early detection make effective long-term control of GB challenging. Current standard of care (SOC) is limited to surgery, radiation, and chemotherapy (temozolomide/TMZ), with median survival of 9 to 12 months and 5-year survival less than 5% (1).

Glucose metabolism and the Warburg Effect have gained traction as a potential tumor weakness and exploitable treatment area. Where normal cells utilize glucose for high-yield energy production in the mitochondria (1:36ATP), tumor cells demand higher levels of glucose for diminished energy production, via lactate in the cytosol (1:4ATP) and nucleotide synthesis in the pentose phosphate pathway. This metabolic characteristic, termed the Warburg Effect, is an essential byproduct of rapid cellular proliferation and promoted during tumorigenesis by oncogenic metabolic reprogramming. Hence tumor cells acquire the ability to sustain proliferative signaling mechanisms, which subsequently promotes malignant glycolysis (2). The PI3K/Akt/mTOR pathway plays a central role in human cancers by way of constitutive activity, increased growth factor expression and glocolytic activation, resulting in feed-forward loops of glucose consumption, proliferation and survival (3). PTEN, a key regulator of the PI3K/Akt/mTOR pathway, is frequently inactivated in GB.

Research into dysregulated cellular metabolism has given rise to the notion that dietary therapies for cancer patients may have significant clinical utility. GB has been proposed to be a promising candidate for dietary intervention due to its substantial reliance and utilization of glucose (4, 5). At the forefront of dietary anticancer therapy is the ketogenic diet (KD), which is a high-fat, low-carbohydrate, low-protein diet, used for decades to treat refractory epileptic seizures. Extreme carbohydrate restriction mimics a fasting state, resulting in reduction of blood glucose and induction of ketone bodies (e.g., β-hydroxybutyrate/BHB;
Translational Relevance

The emerging role of metabolism in many types of cancers provides a novel target for new therapeutics. A possibility is dietary therapies that are designed to take advantage of the unique energetic needs of tumor cells. One such option is the use of a very high-fat and low-carbohydrate diet (90%:5%, respectively) called the Ketogenic Diet (KD). This diet has been used to treat epilepsy for nearly 90 years, and recently its application as a cancer therapeutic has been explored. Although preclinical studies demonstrate its efficacy and clinical case reports support its feasibility, the KD is difficult to implement due to its stringent nature. Here, we report the development of an sHFLC, which mimics the main physiologic effects of the KD, namely reduced glucose and increased ketones. In addition, the sHFLC diet is able to reduce glioblastoma (GB) tumor cell proliferation and extend lifespan in GB animal models.

ref. 6). Ketone bodies are suitable energy replacements for normal cells with functional mitochondria (7), but have been shown to be unsuitable for tumor cells (8,9), as tumor cell mitochondrial functions are dysregulated (10). Existing preclinical data support the KD (11–13) and a calorie-restricted KD (RKD; refs. 14, 15) in the treatment of brain cancer by diminishing tumor growth and increasing animal survival. Clinical reports (5), case reports (16,17), and pilot trials (18–20) have demonstrated that the KD is safe, has low toxicity, and is applicable to cancer patients.

These publications note that there are obstacles in its use as a cancer treatment due to undesirability for long-term use, with some reporting mild-to-severe side effects (21). It has been proposed that more endurable dietary regimens should be developed and tested, as not all patients may tolerate the same dietary restrictions (22). Therefore, we sought to develop a less restrictive KD-like diet that would exhibit the same physiologic phenotype and antitumor efficacy. By supplementing a high-fat, low-carbohydrate (sHFLC) diet, moderate protein diet with specialized medium-chain triglycerides [MCT; 60%(30%):30%:10%::Fat:MCT:Protein:Carb], we hypothesize that a more balanced diet can be implemented, resulting in diminished tumor progression. MCTs were specifically chosen based on carbon chain lengths (C8:C10::97%:3%), which allow them to rapidly diffuse from the gastrointestinal tract into the hepatic portal system and travel directly to the liver where they are converted into ketone bodies (6). We believe it is possible to provide a more nutritionally complete, flexible, and palatable anticancer diet with the sHFLC, which could target a diverse patient population and increase patient compliance.

Materials and Methods

Cells

Patient glioblastoma tumors were dissociated and cultured as previously published (23). All lines tested and authenticated via STR-profiling following ICLAC protocols. Culture media are serum free, supplemented with EGF at 20 ng/mL. Glucose restricted media were tested with a glucometer and supplemented with glucose at plating and every-other-day for the 7-day passage cycle. BHB was administered in the media at plating day only and measured at day 1 and passaging day 7 using the OneTouch meter.

Images

After sphere formation, cells were fixed with PFA, stained with DAPI, and imaged using Leica DM 2000 2.5× fluorescent microscope. Images were quantified using Macnification.

Animals

All experiments were approved by IACUC. NOD/SCID animals were housed under standard animal husbandry procedures. Transplants: Animals were transplanted per previously published protocols (24). For subcutaneous xenografts, dissociated gliomaspheres were transplanted (1×10⁶). Endpoint criteria of 15×15 mm (1766mm³) were used. For intracranial xenografts, animals were stereotactically injected with dissociated gliomaspheres (2×10⁶) into the striatum (2 ML, and 2.5 DV). Endpoint criteria were >20% body weight loss, BCS ≤2, and neurologic deficits.

Diet creation and delivery

The ketogenic diet was readymade Bioserv #F3666. The sHFLC and control diets were created using ingredients from Purina Test Diet. The sHFLC diet final calorie% was 10:30:30 (30) carbohydrates:protein:fat (MCTs: 3.2:2.8:1 fats:protein:carb). The sHFLC diet provided 5.66 kcal/g gross energy where fat, carbohydrates, protein, and fiber comprised 404 g/kg, 125 g/kg, 355 g/kg, 73.12 g/kg respectively. The control diet calorie% was 55:20:25 carbohydrates:protein:fat (1:1.8:4.9 fats:protein:carb). The control diet delivered 4.67 kcal/g gross energy where fat, carbohydrates, protein, and fiber comprised 115 g/kg, 570 g/kg, 208 g/kg, 72 g/kg, respectively. Food (ad libitum) was changed daily. Upon SC tumor palpation, (1×1mm) animals were randomized and assigned respective diets. Intracranial animals were randomized post-transplant and assigned respective diets 5 days after final transplant.

Tumor occupancy

Quantification of tumor occupancy was determined using ImageJ. Three images for each brain (4 control, 3 sHFLC, 3 KD) were taken from rostral, medial, and caudal positions of the tumor.

IHC

Tissue was formaldehyde fixed, cryopreserved, or embedded in paraffin. Sections were blocked and incubated in primary antibody overnight at 4°C. Fluorescent secondary antibody was applied for 1 hour at RT. Slides were coverslipped-using VectaShield with DAPI. Biotinylated secondary antibody was applied for 2 hours at RT followed by 1 hour Vectastain Elite ABC kit and DAB peroxidase kit. Slides were coverslipped with Cytoseal.

Western blot analysis

Proteins were extracted from subcutaneous tumors and quantified using Qubit. Equal amounts of protein were loaded into each well (20–40 µg). Gels were run for 30 minutes at 200 V and then transferred using a xCell-II blot module (1 hour, 35A, RT). Proteins of interest were detected using chemoluminescence. Membranes were exposed (Kodak), stripped, and probed for actin.

1For detailed experimental procedures please see supplemental methods file.
Flow cytometry

Cells from culture and ex-vivo cells from SC tumors were run on a BD Biosciences LSRII flow cytometer. Briefly, 0.5 x 10⁶ cells were blocked, permeabilized, and incubated with primary antibodies overnight at 4°C. Secondary antibody was applied for 1 hour at RT. Flow analysis was performed using FlowJo software.

RNA isolation and RT² PCR Human mTOR Profiler

Total RNA was isolated from SC tumors using RNeasy Plus Mini RNA isolation kit. RT² first strands and RT² SYBR Green ROX qPCR Mastermix were utilized. RT-PCR was performed using Qiagen (PAHS-098ZE-4), on ABI 7900HT. Housekeeping genes were determined using NormFinder.

Results

Restricted glucose slows proliferation of patient-derived gliomaspheres

Using four different previously published GB patient-derived cell lines (L0, L1, L2, and S3; refs. 24–26), we examined the effects of glucose restriction in-vitro. For this publication, L0 will be used as the primary cell line due to its robust proliferative activity, and L2 will be used as the secondary line since it closely resembles the proliferative and metabolic activity of L0. Cells were grown under three glucose conditions: normal (glucose) media (NG: 500±mg/dL), physiologic (glucose) media (PG: 90–110 mg/dL), and physiologic-low (glucose) media (LG: 65–80 mg/dL). Figure 1A shows altering glucose concentrations in the media significantly reduced L0 cell proliferation over time. The cell lines revealed a significant reduction in mean expansion under PG and LG when compared with NG (Supplementary Fig. S1A–S1C), as well as a significant difference between PG and LG media (Fig. 1B), as confirmed by a MTT Assay (Fig. 1C). To assess the reduction in fold expansion, L0 was analyzed for proliferation (Ki-67-Fig. 1D; MCM2-Fig. 1E) and apoptosis markers (active-caspase-3-Fig. 1F). Lowering glucose concentrations resulted in a significant reduction in Ki-67 and MCM2 expression, in both PG and LG, as well as a significant increase in active caspase-3 between NG and LG. Therefore, alterations in glucose availability, to levels equivalent to a low glucose state, are sufficient to slow the proliferation of gliomaspheres while concomitantly increasing apoptosis.

The clonogenic frequency and proliferative rate of individual clones (24) were assessed in 96-well culture plates under all conditions (Fig. 1G). There was a statistically significant reduction in both the clonogenic frequency (Fig. 1H) and the mean proliferative rate of each clone (Fig. 1I), in all lines (Supplementary Fig. S1D—S1I). Quantification of the self-renewing stem cell symmetrical division rate (k_Li; ref. 24) demonstrated a significant decrease in cancer stem cell expansion under reduced glucose conditions (Fig. 1J). The combination of diminished stem cell division rates, cellular fold expansion, and proliferation markers indicates that lowering glucose affects not only the putative stem cell population, but also the non-stem cell population.

Ketone treatment in vitro further suppresses proliferation of patient-derived GB cells

To determine ketone effects in vitro and model the sHFLC dietary phenotype (low glucose and high ketones), a dose response experiment was performed. BHB treatment in all glucose conditions resulted in a dose-dependent reduction in fold expansion beginning at 4 mmol/L (Fig. 2A). BHB EC₅₀ were calculated for each glucose condition (Fig. 2A). On the basis of the BHB EC₅₀ of L0 from PG, as well as the knowledge that this is an achievable, efficacious ketosis plasma concentration for patients on a high-fat diet (7), all in vitro experiments were performed with 4 mmol/L BHB (KTX). The MTT assay also showed reduction in enzymatic activity post BHB treatment beginning at 1 mmol/L, but without dose dependence (Supplementary Fig. S2A—S2C). To confirm the reduction in fold expansion, we performed 50 consecutive single passages with 3-glucose concentrations with KTX, and we still observed a significant reduction in fold expansion (Fig. 2B) and MTT enzyme activity (Fig. 2C) in all lines (Supplementary Fig. S2D—S2E). We assessed proliferation and apoptosis in L0 KTX and found an enhanced reduction in Ki-67 (Fig. 2D) and MCM2 (Fig. 2E) expression, but no increase in active caspase-3 activity (Fig. 2F) post KTX. Clonogenic frequency, proliferation rate of individual clones, and tumor stem cell division rate were each determined under restricted glucose, and in conjunction with KTX (Fig. 2G). A significant reduction was observed in clonogenic frequency (Fig. 2H) and mean proliferation rate of each clone (Fig. 2I) in all glucose media when exposed to KTX, in all lines (Supplementary Fig. S2G—S2L). Ki₆ quantification demonstrated a significant reduction in stem cell division rate in all KTX groups compared with glucose controls, with a significant reduction in the low glucose and KTX combination (Fig. 2J) in all lines (Supplementary Fig. S2H, S2J, and S2L). Together, this evidence supports the potential anti-tumorigenic effect of the sHFLC dietary phenotype by diminishing the proliferative potential of the putative stem cell and non-stem cell populations.

The sHFLC diet is safe and nutritionally complete

Dietary breakdown for all xenograft experiments is shown in Supplementary Fig. Table 1. Detailed descriptions of diet creation and dietary timelines are provided in the Supplemental Methods. Animals placed on the sHFLC and KD had a significant reduction in blood glucose, above hypoglycemic levels, compared to controls, with no difference between the sHFLC and KD (Fig. 3A and Supplementary Fig. S3A). Blood ketones were significantly increased in mice maintained on the sHFLC and KD to a safe level, with a statistical difference between the groups (Fig. 3B and Supplementary Fig. S3B). To compare blood glucose and ketone levels among different anticancer dietary therapies, a simple glucose ketone index (GKI) is used. The GKI is a single number, and can be used both clinically and preclinically, to identify a therapeutic zone (27). On the basis of this formulation, our average calculated GKIs are 24.4 ± 7.14, 3.1 ± 1.07, and 1.94 ± 0.67 for the control, sHFLC and KD, respectively. These values are comparable with other published preclinical studies reporting a therapeutic effect using a RKD (range of 1.8–5.7; ref. 27). To compare nutritional differences and health status of mice on sHFLC and KD, body weight and blood analyses were performed. Patients on RKD report losing approximately 20% of their body weight (28), whereas patients on KD report initial body weight fat-loss followed by stabilization (29). Surprisingly, subcutaneous-L0 mice fed the sHFLC diet had a significant increase in body weight compared with the KD and control (Fig. 3C). In other xenograft experiments, sHFLC-fed mice had a significant increase in body weight compared with KD-fed mice only (Supplementary Fig. S3C—S3E). This may be due to increased caloric intake of a nutritionally complete, palatable diet, as animals are fed ad libitum. Nutritional insufficiencies from a high-fat diet can place...
strain on the body's vital organs such as the liver, kidneys, or heart (28). Analysis of serum albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine transaminase (ALT), and iron levels in tumor-bearing mice fed the sHFLC diet for over 1 month (Fig. 3D–H) showed normal levels in all enzymes tested. KD-fed animals had reduced serum albumin levels (Fig. 3D) and increased serum ALP levels (Fig. 3E), which has been demonstrated in long-term KD-fed mice (30). Analysis of cholesterol,
triglycerides, HDL, LDL, and non-esterified fatty acids (NEFA) were also performed (Fig. S1-M), as high cholesterol levels are a concern with a high-fat diet (31). Reports of mice fed the Bioserv-KD describe increases in hepatic triglycerides and fat accumulation, together with a reduction in body weight, due to choline deficiency combined with low-protein and high-fat content (32). In line with these findings, animals fed the Bioserv-KD showed a significant increase in cholesterol (Fig. 3I), triglycerides (Fig. 3J), HDL (Fig. 3K), and LDL levels (Fig. 3L). sHFLC-fed animals showed statistically significant high levels of HDL only (Fig. 3K). Together, these results indicate that the sHFLC diet is nutritionally safe.

The sHFLC diet slows tumor progression, increases survival, and reduces tumor burden in subcutaneous and orthotopic xenograft models

In our preclinical subcutaneous xenograft model, L0 GB cells were grown until palpation (1 × 1 mm), at which time dietary treatments were introduced. Overall tumor volume for the sHFLC and KD-fed mice were similar, showing a significant reduction in tumor progression compared with controls (Fig. 4A and Supplementary Fig. S4A). In addition, compared with control, both treatment groups demonstrated a significant delay in time to reach a tumor volume of 50 mm³; under which tumor progression is not considered to have occurred (Fig. 4B). Both treatment groups also demonstrated a significant delay in time to reach 1,766 mm³ (15 × 15 mm: endpoint; Fig. 4C). These results indicate increased progression-free survival and time to reach endpoint in both experimental groups. Similar results were obtained with L2, with respect to body weight, tumor progression, and delayed progression to endpoint (Supplementary Fig. S3C and S4B and S4C). Overall, the sHFLC diet demonstrated similar antitumor efficacy as compared with the KD, in a subcutaneous xenograft model of tumor progression.

We assessed the efficacy of the sHFLC diet on tumor burden and host survival using histologic techniques in our intracranial xenograft model. Brains from tumor-bearing animals were stained with H&E and human-specific Nestin and tumor burden was confirmed in all three groups (Fig. 4D and Supplementary Fig. S4D–S4F). Animals fed the sHFLC and KD demonstrated a 3-fold reduction in tumor occupancy compared with controls (Fig. 4E).

Brains from mice fed the control and sHFLC diets, when stained with active caspase-3 and Ki-67, showed no change in apoptosis, but did show a reduction in proliferation (Supplementary Fig. S4G).

It has been proposed that a potential combinatorial treatment of metformin with carbohydrate restriction could result in enhanced antitumor efficacy (33). Mice fed the KD and sHFLC alone demonstrated a significant increase in survival compared with the control-fed mice (Fig. 4E and Supplementary Fig. S4H), statistically equal to metformin alone. In both xenograft models, metformin alone was able to reduce blood glucose, reduce tumor progression, and increase survival, yet the combination sHFLC diet and metformin showed no additive or synergistic effects (Fig. 4E; Supplementary Fig. S4I–S4K). These data show a lack of combinatorial efficacy, potentially stemming from an overlapping mechanism of action. Metformin is a well-characterized activator of AMP-activated protein kinase (AMPK), an enzyme responsible for cellular energy homeostasis and negative regulation of the mTOR pathway. It is possible that the energy restriction via the sHFLC is also activating AMPK and, in turn, inactivating the mTOR pathway. These data indicate that the sHFLC diet is capable of increasing animal survival while minimizing tumor burden, is as effective as metformin, and may mechanistically overlap in AMPK-mediated inactivation of the mTOR pathway.

sHFLC nutritional intervention correlates with downregulation of the mTOR pathway in implanted GB cells

To determine the potential mechanistic effects of the sHFLC diet, we analyzed 86 key genes in the mTOR pathway, through rTPCR, using an mTOR Profiler Kit. Four control subcutaneous tumors and four sHFLC-fed subcutaneous tumors were analyzed. Overall, there was a global downregulation of mTORC1/ mTORC2 genes in sHFLC-fed tumors compared with control (Fig. 5A), with an inverse relationship observed in negative pathway regulators. All significantly down-or-upregulated genes are shown in Fig. 5B and grouped as functional sets. Comparison of regulatory genes in functional groups indicates that the most affected clusters are the positive mTORC1 regulators and the translational effectors downstream of mTORC1/2. Inhibition of

Figure 2.
Mimicked sHFLC in vitro (low glucose and ketone treatment) further reduces the proliferation of patient-derived gliomaspheres. A, dose response curve of L0 patient-derived gliomaspheres treated with increasing concentrations of ketones (β-hydroxybutyrate, BHβ), expressed via fold expansion. Assay performed in normal glucose-containing media, physiologic media, and low glucose media to determine EC50 value and optimal ketone treatment in vitro. The β-hydroxybutyrate (BHβ) EC50 for NG = 15.937 mM/L, PG = 4.065 mM/L, and LG = 2.021 mM/L. B, fold expansion of consecutive single-passage patient-derived L0 and L2 cells treated with 4 mM ketones (KTX) in combination with normal (NG) and altered glucose media (physiological-PG and low-LG). Fold expansion calculated by dividing final density by plating density. N = 50+ passages. C, MTT enzymatic assay of patient-derived L0 and L2 treated with 4 mM ketones (KTX) in combination with normal (NG) and altered glucose media (physiological-PG and low-LG). Dual wavelength subtraction 750 – 595 CD. Expressed as a percentage of control gliomaspheres grown in normal media (NG). N = 3 assays/12 wells per assay. D, quantification of percent positive Ki-67 L0 cells grown over a 7-day passage cycle in normal (NG) and altered glucose media (physiological-PG and low-LG), exposed to 4 mM/L β-hydroxybutyrate treatment (KTX) measured by flow cytometry (n = 10). E, quantification of percent positive MCM2 L0 cells grown over a 7-day passage cycle in normal (NG) and altered glucose media (physiological-PG and low-LG), exposed to 4 mM/L β-hydroxybutyrate treatment (KTX) measured by flow cytometry (n = 10). F, quantification of percent positive active caspase-3 L0 cells grown over a 7-day passage cycle in normal (NG) and altered glucose media (physiological-PG and low-LG), exposed to 4 mM/L β-hydroxybutyrate treatment (KTX) measured by flow cytometry (n = 10). G, DAPI-stained 96-well images of patient-derived GBM cell line O grown in normal glucose-containing media, physiologic media, and low glucose media with and without 4mM/L ketone treatment. Gliomaspheres plated 250 cells per well and grown for 7 to 10 days, fixed and stained with 4% PFA/0.1% DAPI and imaged using a Leica DMI 4000 B microscope. White box surrounding enlarged gliomasphere in bottom right corner. H, quantification of 96-well DAPI-stained images using Maphancing for sphere forming frequency, expressed as a percentage of the control (normal glucose grown cells) n = 20 wells. I, quantification of 96-well DAPI stained images using Maphancing for average sphere diameter (um). n = 200+ cells. J, K, K, Mathematical modeling of stem cell divisions, Kn = LN(N0)/t, whereby f = fold expansion, division of cell count at end of passage/ cell count at start of passage, and t = time (days in culture). Statistics performed using a one-way ANOVA with Bonferroni post hoc comparative test. *P < 0.01; **P < 0.001; ***P < 0.0001 when compared with NG control. #P < 0.01; ##P < 0.001; ###P < 0.0001 when compared with physiologic control (PG). *P < 0.01; **P < 0.001; ***P < 0.0001 when compared with low control (LG).
both mTORC1/mTORC2 has been shown to be a potent anti-cancer therapy (34).

To investigate further the negatively affected mTOR effector genes, we assessed ribosomal S6, its activator p70S6K and mTOR release of 4EBP1. In addition, AMPK activation and phosphorylation of mTOR were assessed, as metformin primarily targets AMPK. Flow analysis of multiple ex vivo single-cell–dissociated sHFLC-fed tumors confirmed a statistically significant reduction in the phosphorylation of ribosomal S6 (Fig. 6A), the release of the translational repressor 4EBP1 (Fig. 6B) and a significant increase in AMPK activation (Fig. 6C), when compared with control. The proliferation marker MCM2 was also diminished in all treatments groups (Fig. 6D). Control-fed tumors served as positive mTOR controls with constitutive activity levels (PTEN negative; ref. 26), and metformin-fed tumors served as negative mTOR controls (AMPK activator; ref. 35. This was confirmed using IHC analysis of subcutaneous tumors and xenograft mouse brains (Fig. 6E and F), as well as through Western blot analysis of subcutaneous tumors (Fig. 6G; targeting the S6 regulator p70S6K, p-mTOR, AMPK, and multiple epitopes of 4EBP1). The phosphorylation of 4EBP1, and subsequent release of eIF4E, results in initiation of translation, and modulation of this release directly affects tumorigenicity (36). Therefore, we conclude that the sHFLC diet can potentially diminish cellular proliferation.
through AMPK-mediated mTOR inactivation of 4EBP1 and through protein synthesis via ribosomal S6.

Discussion

We report a dietary intervention that produces low circulating glucose while elevating ketones and results in a substantial reduction in GB cellular proliferation. Maintaining in-vitro glucose at levels representing physiologic (90–110 mg/dL) or physiologic-low but not hypoglycemic (65–80 mg/dL), reduces the clonogenic frequency of gliomaspheres, potentially affecting the cancer stem cell population. The addition of BHB also reduces GB proliferation and when combined with reduced glucose levels, results in further reduction in proliferation. Here, we demonstrate that a high-fat, low-carbohydrate diet supplemented with MCT oil (sHFLC) is able to slow tumor progression and increase survival. In vivo, the sHFLC diet was similar to the ketogenic diet (KD) in antitumor efficacy, but showed nutritional advantages in body weight, organ enzyme levels, and lipid profile. Finally, we demonstrate that the sHFLC diet affects the mTOR signaling pathway by reducing expression of upstream regulators and translational downstream effectors.

We utilized patient-derived GB stem cell lines that have been demonstrated to not only recapitulate the genotype and phenotype...
of the primary tumor (37), but also to be driven by a cancer stem cell population (38). They are grown in serum-free conditions, which serves as a clinically relevant model for studying GB in vitro (39). Standard tissue culture media are supersaturated with glucose to provide an excess of nutrients for continuous growth. However, available glucose is variable and highly dependent on nutritional

Figure 5.
The shFLC diet diminishes mTORC1/2 signaling. A, clustergram of 78 selected mTOR-associated genes from Qiagen RTProfiler with 86 key mTOR genes. Color gradient of clustergram shows consistent downregulation of the mTOR pathway. Clustergram has four different animal tumors from the control and shFLC-fed groups. Tumors analyzed were subcutaneously transplanted patient-derived line 0 gliomaspheres. B, table of significant P values of selected mTORC1/2 substrate genes grouped in functional sets. Housekeeping genes were determined using NormFinder and normalized using the Qiagen analysis software. Statistics performed using a Student t test.
Figure 6. shFLC potential mechanism of action. A–D, assessment of mTOR substrate activation via flow cytometry. Graphically represented as a percent of positive staining based on control. Mean fluorescent intensity for each treatment group was averaged across multiple animal samples and normalized to be expressed as a percent compared with the normal fed control mice = 100%. E, immunofluorescent-stained subcutaneous xenograft tumor sections (5 μm) cryopreserved and embedded in OCT. Green = antibody/Blue = DAPI. All images were taken with a 10× lens on a Leica DM 4000 microscope. F, immunofluorescent-stained mouse brain sections from intracranial xenograft (10 μm) cryopreserved and embedded in OCT. Green = antibody/Blue = DAPI. All images were taken with a 10× lens on a Leica DM 4000 microscope. G, Western blot analysis for mTOR substrates using Metformin treatment as a positive control as well as mTOR siRNA. Quantification with ImageJ to the right. *P < 0.01, **P < 0.001; ***P < 0.0001 compared with control; #P < 0.01, ##P < 0.001; ###P < 0.0001 compared with KD. All statistics performed using one-way ANOVA with Bonferroni post hoc test.
status or disease state in vivo. Excess glucose, as seen in GB patients with persistent hyperglycemia, leads to poor patient survival (40). It has also been suggested that diets with a high glycemic index may increase the risk of tumorigenesis (41), and low-carbohydrate, high-protein diets that limit circulating glucose can delay cancer development and progression (42). In vitro, glucose withdrawal and treatment of GB cells with the non-metabolizing glucose analog 2-deoxy-glucose (2DG) results in up to 95% growth inhibition (43). These studies demonstrate the dependence of cancer cells on glucose to promote survival and cellular growth. To assess whether 2DG results in up to 95% growth inhibition, in cell populations that also differ in proliferation and symmetrical stem cell divisions, suggesting that elevated ketones affect the putative cancer stem cell population (24). This effect was seen in all patient-derived cell lines, which include the classical (L0, L1, L2) and proneural (S3) subtypes of GB. GB subtypes represent the variation in mutational status that may affect some aspect of metabolism and glycolysis. The proneural subtype exhibits isocitrate dehydrogenase 1 (IDH1) mutations, which alter the cell's ability to regulate redox; NAD(P)H levels and metabolize several macromolecules (52), potentially making this subtype more susceptible to metabolic interventions.

The combination of reduced glucose and increased ketone bodies has shown an enhanced antitumor effect. The KD mimics these biologic effects and has been proposed as a treatment for GB and other cancers (5). This was first explored in two pediatric patients with advanced-stage astrocytomas, following SOC treatments, and was able to produce long-term (>5 years) tumor maintenance, employing a KD supplemented with MCT oil (16). Since this landmark report, numerous groups have investigated the antitumor efficacy of a KD and an RKD in several types of cancer. RKD in experimental mouse models of glioma has been shown to be antitumorigenic, antiangiogenic, and prosurvival (14), while also being anti-invasive, anti-inflammatory, and proapoptotic by targeting signaling pathways related to glucose and glutamine metabolism (34). In animal models, feeding with the KD ad libitum has been reported to increase survival and reduce tumor growth (11). Other preclinical animal models such as gastric cancer (53), colon cancer (54), and metastatic cancer (13) have used the KD, reporting similar antitumorigenic effects. Several case reports describe the applicability (20) and combinatorial safety of KD and RKD with current SOC therapies (17), with some patients experiencing stable disease on the diet and others experiencing inability to maintain (19). In 2014, the first phase one clinical trial assessing the safety of the KD on recurrent glioblastoma determined that the diet is safe and feasible, warranting other trials to explore the combinatorial potential of the KD with cytotoxic treatments (18). The KD in the context of cancer is promising, however patient populations are diverse, as are their dietary needs (21), creating a demand for alternative dietary interventions for tumor management (22).

We designed the sHFLC diet for long-term sustainable maintenance of GB and for increased flexibility and palatability. We utilized NOD/SCID xenografts in this study for the advantages in studying measurable outcomes of human tumor responses to therapy. It should be noted, however, that NOD/SCID mice display the effects of both type I and type II diabetes, are immunocompromised and have an altered metabolic state. Our preclinical models of tumor progression and survival support the feasibility of the sHFLC diet, as we can effectively slow the progression of GB and increase animal survival with diet alone (Fig. 4), using two patient-derived GB lines. Notably, sHFLC-fed animals, compared with the Bioserv-KD, had superior nutritional status demonstrated by increased body weight and improved blood analyte and lipid profiles (Fig. 3). The Bioserv-KD we utilized contains large amounts of lard-based fat, and low levels of protein and choline, which increases hepatic triglycerides and
cholesterol while simultaneously reducing body weight (32). Furthermore, the 1:6 carbohydrate:fat KD utilized in this study is higher in fat content than the KD typically administered to patients (1:4). Patients on a 1:4 or 1:3 KD can better manage fat sources to attenuate issues such as weight loss and increased cholesterol, which have been demonstrated here with animals on a 1:6 KD and attenuated with animals on the shFLC. Our GKI values were comparable to previous publications, but were more indicative of an RKD than an ad libitum KD. However, when comparing GKI values to human data, patients receiving a KD supplemented with MCTs (also fed ad libitum) exhibit values closer to the ones we report. We hypothesize that the utilization of MCTs, which inherently increase ketone body production in vivo, may skew the GKI towards a more favorable value. MCT supplementation has also been shown to inhibit proteolytic and lipolytic factors of cachexia and increase body weight in cachectic cancer patients (55), which is not typically a problem for brain tumor patients but may still be useful for patients with other cancer types susceptible to metabolic intervention.

Pharmacologic intervention to temper circulating glucose has been explored, with the assumption that a combination of KD and metformin could work together as a comprehensive anticancer therapy (33). We proposed to enhance our dietary paradigm with the addition of Metformin to the shFLC diet (Supplementary Table S1). Metformin alone significantly reduced tumor volume and enhanced survival in two preclinical glioma models, indicating metformin as a promising anticancer therapy. When metformin was combined with the shFLC diet, there were no additive or synergistic effects (Supplementary Fig. S4), indicating possible overlapping mechanisms of action. Metformin has multiple proposed antitumor mechanisms, but it is well characterized to activate AMPK, a negative regulator of the mTOR pathway (33). The anticonvulsant mechanism of the KD is still unclear, however, one proposed mechanism is also inhibition of the mTOR pathway (56, 57). Taking into account the proposed KD mechanism, the known metformin mechanism and the lack of combinatorial antitumor efficacy, we posit that a potential mechanism of action for the shFLC diet is through diminished mTOR signaling (Figs. 5 and 6). A large body of literature exists on the mTOR pathway, its substrates and how its hypo/hyper-activation results in various disease states (35). The mTOR pathway is one of the largest and most utilized pathways in cellular signaling, with two complexes (mTORC1/mTORC2) that have demonstrated a role in tumorigenesis. Recently, it’s been shown that inhibition of both mTORC1/mTORC2 signaling results in dramatically reduced cell viability in glioma cell lines, as well as inhibition of tumor growth in vivo (34). In our assessment of the shFLC diet’s effects on the mTOR pathway, we found significant reduction in both mTORC1/mTORC2 signaling (Fig. 5). In our preclinical models, shFLC-fed tumors showed significant reduction in downstream mTOR effector signaling, whereby reduction in phosphorylation of 4EBP1 and ribosomal S6 inhibits both translation and protein synthesis needed for cellular replication (36) (Fig. 6). Taken together, these findings indicate that inhibition of the mTORC1/2 pathway can be achieved through dietary intervention, resulting in a potent anti-cancer treatment.

Our work demonstrates that there is a distinct relationship between metabolism and proliferation that can be exploited by changing the energy sources in the body. Further research into the biochemical reactions of metabolic intermediates may shed more light on how ketone bodies are differentially utilized by tumor cells, as the role of mitochondria in tumor propagation and carcinogenesis is multifaceted and incompletely understood. Nevertheless, we effectively show that a combination of low glucose and high ketones results in negative proliferative effects on gliomaspheres, which can be translated in vivo with the shFLC diet. This diet reduces overall tumor burden and increases survival, equivalent to a strict 1:6 KD, and has a complete nutritional profile. Hence we propose that dietary therapy, such as the shFLC diet, could be utilized in the management of GB.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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