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Inhibition of Glioblastoma Tumorsphere by Combination of 2-Deoxyglucose and Metformin

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Inhibition of Glioblastoma Tumorsphere by Combination of 2-Deoxyglucose and Metformin

Directed by Professor Sun Ho Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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June 2015

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Eui Hyun Kim

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ABSTRACT

Inhibition of Glioblastoma Tumorsphere by Combination of 2-Deoxyglucose and Metformin

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(Directed by Professor Sun Ho Kim)

Objective: The treatment failure of glioblastoma is thought to be the presence of refractory cancer cells. It has been suggested that deprivation of tumor bioenergetics by inhibition of multiple energy pathways could be an effective new therapeutic approach for various human tumors. However, the effectiveness of this idea has not been evaluated in glioblastoma tumorsphere (GBM TS) model. We hypothesized that the dual inhibition of glycolysis and oxidative phosphorylation could suppress GBM TS effectively.

Methods: We evaluated the effect of 2-deoxyglucose (2DG) or metformin each alone, and their combination in GBM TS model. The viability of GBM TS was tested in different conditions, and protein expression related to AMP-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) pathway were examined. The influence of combination treatment on cytotoxicity, stemness and invasion properties of GBM TSs was evaluated with sphere formation assay and 3-demensional culture system. Glucose metabolism was assessed with ^{18}fluorodeoxyglucose positron emission tomography (PET) scan. Transcriptome assay was performed

with gene set enrichment analysis. Lastly, in vivo efficacy tests were performed with mouse orthotopic xenograft model in different conditions.

Results: Viability of GBM TS was not decreased by any single or combination treatments of 2DG and metformin. Of note, mTOR-signaling proteins were down-regulated in AMPK-independent manner. Sphere formation was significantly suppressed with 2DG and metformin combination treatment and the proteins related to stemness were down-regulated. Invasion capacity of GBM TS was inhibited by combination treatment in the 3D invasion model assay. PET scan showed ^{18}fluorodeoxyglucose uptake was decreased in the GBM TS treated with the combination of 2DG and metformin. Transcriptome gene set enrichment analysis showed subset of genes related to extracellular matrix and adhesion were negatively correlated when compared before and after combination treatment. Combination of 2DG and metformin prolonged survival of tumor bearing mice, and the tissue examination revealed decreased invasion of GBM TS.

Conclusion: The combination of 2DG and metformin did not show significant cytotoxicity for GBM TS. However, 2DG and metformin effectively decreased the stemness and invasion capacity of GBM TS, and showed potential survival benefit in mouse orthotopic xenograft models. We believe this dual inhibition of cellular bioenergetics would be worth expedited clinical evaluation in the treatment of glioblastoma patients by targeting GBM TS.

Key words: 2-deoxyglucose, glioblastoma, metformin, stemness, tumorsphere

Inhibition of Glioblastoma Tumorsphere by Combination of 2-Deoxyglucose and Metformin

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I. INTRODUCTION

The outcome of a glioblastoma (GBM) is still dismal even with any combination of current best treatment modalities.[1-4] One of the reasons for treatment failure is thought to be the presence of refractory cancer cells.[5,6]

It is well known that cancer cells take advantages from preferential glycolysis for central energy metabolism (Warburg effect)[7-9], but mitochondrial respiration is still a major energy production pathway even under anaerobic condition. In particular, the treatment refractory cells characterized by stem cell-like property are more dependent on oxidative phosphorylation.[10-14] Many studies have shown that inhibition of either glycolysis or oxidative phosphorylation was effective to decrease proliferation, invasion of cancer cells.[15-19] However, with the idea that simultaneous inhibition of multiple metabolic pathways would be more effective for cancer treatment, there have been a few researches which showed deprivation of tumor bioenergetics by inhibition of multiple energy pathways could be an effective new therapeutic approach for various human tumors.[20-22]

However, the effectiveness of this idea has not been evaluated in a GBM

tumorsphere (TS) model.[23,24] We hypothesized that the dual inhibition of glycolysis and oxidative phosphorylation could suppress GBM TSs effectively with synergetic fashion. In this study, we evaluated the effect of the combination of 2-deoxygluocose (2DG) and metformin on GBM TS.

II. MATERIALS AND METHODS

1. Single cell isolation from glioma patients

The specimens from patients with gliomas were freshly obtained from the operation room. Approval was given from the institutional review of Severance Hospital, Yonsei University College of Medicine (4-2012-0212). Informed consent was provided according to the Declaration of Helsinki. Neuropathologists diagnosed each surgical specimen according to WHO classification.[25] For isolation of TSs from GBM specimens, we followed previously published methods.[26,27] We performed the cell isolation procedure within 2 hr after tumor removal using a mechanical dissociation method. Surgical specimens were minced and dissociated with a scalpel in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12; Mediatech, Manassas, VA, USA) and then passed through a series of 100-μm nylon mesh cell strainers (BD Falcon, Franklin Lakes, NJ, USA). Cell suspensions were washed twice in DMEM/F-12 and cultured in complete media (DMEM/F-12) with B27 (1×; Invitrogen, San Diego, CA, USA) plus 20 ng/ml of basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO, USA), 20 ng/ml of epidermal growth factor (EGF; Sigma), and 50 U/ml penicillin/50 mg/ml streptomycin (Gibco, Invitrogen Korea, Seoul, Korea). Two different TSs, GSC11 and TS13-20, were used in this study.

2. Lentiviral vector transfection and expression

GFP-GBM TS for cell counting were generated by growing GBM TS in

complete medium and then applying green fluorescent protein (GFP)-expressing lentiviral supernatants. Polybrene (Sigma) was added to a final concentration of 8 μg/ml and incubated with cells for 18 hr. After infection, the cells were placed in fresh growth medium and cultured in a standard manner. Cells were treated with 1 mg/ml puromycin (Life Technologies Korea, Seoul, Korea) to eliminate uninfected cells and generated stable GFP- GBM TS.

3. Cell viability assay

The effect of 2DG and metformin combination on the survival of GBM TS was determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay.[28] 5 x10^3 GBM TS cells were seeded in 96-well plates and incubated at 37°C for 24 hr and treated with 2DG, metformin and their combination for 5 days. MTS reagent (20μl/well) was added, incubated at 37℃ for 4 hr, and the absorbance was measured at 490nm. Each experiment was repeated three times in triplicate and the results were expressed as % viable cells over control.

4. Protein extraction and Western blot assay

Cells were collected and lysed in lysis buffer (50 mM Tris–Cl, pH 8.0, 150mM Nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, phosphatase inhibitor cocktail solution, protease inhibitor cocktail solution) After centrifugation (for 15 min at 14,000 rpm), supernatants were collected and used as whole cell extracts for Western blot analysis.

For Western blot analysis of proteins, samples were electrophoresed at 120 V in SDS–polyacrylamide gels. After transfer to nitrocellulose membranes, membranes were blocked 1hr in PBS/Tween solution containing 5% non-fat dried milk, probed for 3 hr with primary antibody (1:1000 dilution) and for 1 hr with secondary antibody (1:3000 dilution) as specified in the figures. Visualization was achieved using enhanced chemiluminescence according to

the manufacturer's instructions (Amersham Biosciences).

Western blot analysis was conducted for adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR)-related proteins, stemness markers such as nestin, CD133, Oct3/4, podoplanin, Sox-2, Notch1 and Notch2, and epithelial mesenchymal transition (EMT)-related markers including snail, Zeb1, β-catenin and N-cadherin.

5. GBM TS formation assay

The GSC11 and TS1 cells were cultured as TS in medium consisting of DMEM/F-12 with 2 % 1×B27, 20 ng/ml 0.02 % bFGF, 20 ng/ml of 0.02 % EGF, and 1 % 50 U/ml penicillin/50 mg/ml streptomycin (100×, Gibco, Invitrogen Korea, Seoul, South Korea). The cells were cultured in different conditions for 3 weeks. Cell cultures were observed with an inverted phase-contrast microscope (I×71 Inverted Microscope; Olympus, Tokyo, Japan) to determine morphology and the size of the GBM TSs. Photographs of cells were obtained with a digital camera (DP70 Digital Microscope Camera; Olympus), using DP Controller software (Olympus).

6. Three-dimensional invasion assay model

The GFP stable GSC11 cells (G-GSC11s) grown in spheroid were cultured in collagen I matrices using polydimethylsiloxane (PDMS)-based micro-wells (diameter and depth of microwells: 6mm and 500μm). The microwells were treated with 1% poly (ethyleneimine) (Sigma-Aldrich, St. Louis, MO, USA) solution for 10 min followed by 0.1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and washed with PBS for overnight to make PDMS wells adherent to collagen. The 4mg/ml collagen I matrices are prepared from high-concentration rat tail collagen I (BD Bioscience, CA, USA) using the recommended manual provided by the manufacturer. Briefly, the amounts of

10x phosphate buffered saline (PBS), 1N NaOH, sterile dH_2O and collagen I were mixed to create gels based on the desired final concentration. The solution is well mixed and kept at 4℃ before use. To encapsulate G-GSC11 spheroid, 10ul of collagen I solution (4mg/ml) were pipetted into the micro-well, single G-GSC11 spheroid is placed onto collagen I matrices from culture plate, and 10ul of collagen I solution (4mg/ml) was dropped onto G-GSC11 spheroid. The platform was incubated at 37℃ and 5% CO2 for 30 min. The cell viability was characterized by staining G-GSC11 spheroid with 8μM Ethidium homodimer-1 (Invitrogen Korea, Seoul, South Korea) for 30 min at 37℃ before implantation in collagen matrix. After full gelation, a superlayer of culture medium consisting of DMEM/F-12 with 2 % 1×B27, 20 ng/ml 0.02 % bFGF, 20 ng/ml of 0.02 % EGF, and 1 % antibiotic–antimycotic solution (100×, Gibco, Invitrogen Korea, Seoul, South Korea) is then added. To observe drug effects, drugs are mixed with medium considering final concentration of each drug. Images were taken using an inverted confocal laser scanning microscope (Nikon Ti-E, Tokyo, Japan) to observe dynamic morphology of G-GSC11 spheroids. To quantitate the invasion assay, the maximal area covered by migrating edges of cells was used as a parameter for defining invasiveness (invaded area at certain time/spheroid area at initial time×100). Data are analyzed through image analysis software ImageJ (NIH, Bethesda, Maryland, USA).

7. ^{18}F-fluorodeoxyglucose PET

GBM cell lines were plated on 12-well plates with 3 x10^5 cells per well for 24 hr and treated with 2DG, metformin and their combination for 3 days. The medium was changed to a glucose-free DMEM medium (Gibco) containing approximately 0.5 uCi of ^{18}F-FDG, followed by incubation for 15 min. The cells were washed with phosphate-buffered saline 3 times and 0.1 mL of lysis buffer

was added to each well. The lysed cells were then harvested to measure the amount of radioactivity by gamma-counter (Wallac 148 Wizard 3; Perkin Elmer). The radioactivity measured was normalized to protein content.

8. Gene expression microarray and class comparison

Total RNA was extracted from tissue using a Qiagen miRNA kit according to the manufacturer's protocol. Expression profiles were obtained using Illumina HumanHT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA). Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0)). Data were transformed by logarithm and normalized by quantile method. Genes involving adhesion junction, adhesion molecules (CAMs), ECM-receptor interaction, focal adhesion, regulation of actin cytoskeleton); SNARE interactions in vesicular transport); TGF-beta signaling pathway) defined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway gene sets were filtered and analyzed. All analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. We identified genes that were differentially expressed among the two classes using a random-variance t-test. Genes were considered statistically significant if their p value was less than 0.001. We also performed a global test of whether the expression profiles differed between the classes by permuting the labels of which arrays corresponded to which classes. For each permutation, the p values were re-computed and the number of genes significant at the 0.001 level was noted. The proportion of the permutations that gave at least as many significant genes as with the actual data was the significance level of the global test.

9. Orthotopic xenograft animal model

Male athymic nude mice (Central Lab Animal Inc., Seoul, Korea), aged 4 to 8 weeks, were used in this study. Mice were housed in micro-isolator cages

under sterile conditions and observed for at least 1 week before study initiation to ensure proper health. Lighting, temperature, and humidity were controlled centrally. All experimental procedures were approved by Yonsei University College of Medicine Institutional Animal Care and Use Committee. Mice were anesthetized with a solution of Zoletil® (30 mg/kg; Virbac Korea, Seoul, South Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, South Korea) delivered intraperitoneally. GB TSs were implanted into the right frontal lobe of nude mice using a guide-screw system within the skull, as described previously.[29-33] Mice received 5×10^5 GBM TS cells via a Hamilton syringe (Dongwoo Science Co., Seoul, South Korea) inserted to a depth of 4.5 mm. GBM TS cells were injected into three mice simultaneously using a multiple micro-infusion syringe pump (Harvard Apparatus, Holliston, MA, USA) at a speed of 0.5 μl/min. Then, 500mg/kg of 2DG, 500mg/kg of metformin was administrated to mice intraperitoneally every other day. The body weight of mice was checked every other day. If the weight decreased more than 15 % compared to the original body weight, mice were euthanized according to protocol. When mice died, we carefully removed mouse brains and observed gliomagenesis with H/E staining, and tumor cell invasion with Zeb1 staining. Invading cells were defined as the Zeb1-positive cells outside gross tumor boundary demarcated by H/E staining, and they were counted.

10. Statistical analysis

Data are expressed as means ± standard deviations. Survival of GBM TS cells-implanted mice was evaluated using the Kaplan Meier method. All statistical analyses and graphing were performed using SPSS version 18.0KO software (SPSS Korea, Seoul, Korea).

III. RESULTS

1. 2DG, Metformin and their combination do not inhibit proliferation of GBM TS

GBM TSs were treated by 2DG and metformin for 3 days, and the viability of each GBM TS was evaluated by MTT assay. Any of GBM TS treated by 2DG (4mM), metformin (5mM) and their combination did not show significantly decreased proliferation when compared with the control (Fig. 1A). Interestingly, metformin, which is known to be an AMPK-activator, did not show any increase in the AMPK expression. When GBM TS was treated with metformin together with 2DG, AMPK expression was not increased either. However, the combination of 2G and metformin effectively inhibited mTOR and its related proteins such as Raptor (Fig. 1B).

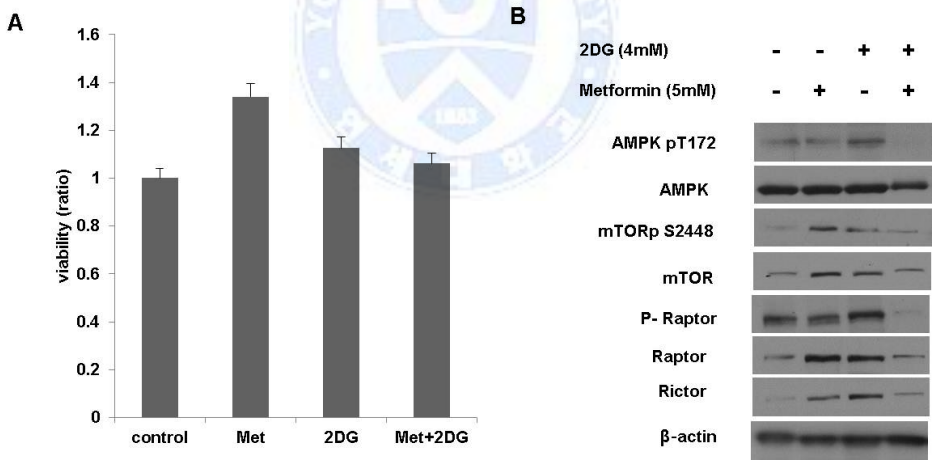
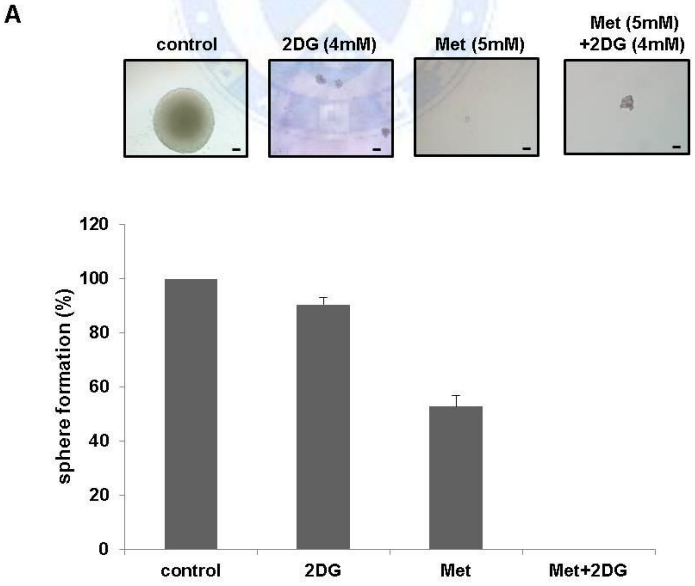


Figure 1. Anti-proliferative effect of 2-Deoxyglucose (2DG) and metformin (Met), (A) 2DG, metformin and their combination did not inhibit proliferation of glioblastoma (GBM) tumorsphere (TS), (B) Increased expression of AMP-activated protein kinase (AMPK) was observed neither in single treatment of metformin nor in combination treatment. Of note, the combination of 2G and metformin effectively inhibited mammalian target of rapamycin (mTOR) and its related proteins.

2. Combination of 2DG and metformin decreases stemness of GBM TS

Sphere formation assay was performed in different conditions. The size of the GBM TS treated with 2DG (4mM) was about the same as the control. Metformin alone showed moderate inhibition of the sphere formation. When GBM TS was cultured in the condition of 2DG and metformin together, this combination treatment showed strong anti-stemness effect as no sphere was observed at all (Fig. 2A). Compared with sphere formation in the condition of metformin (5mM) alone, the combination of 2DG(4mM) and metformin (5mM) showed synergetic anti-stemness effect on GBM TS. Also, the protein expression of stemness-related genes such as nestin, podoplanin, Sox-2, Notch1 and Notch2 were down-regulated by this combination of 2-DG and metformin (Fig. 2B).



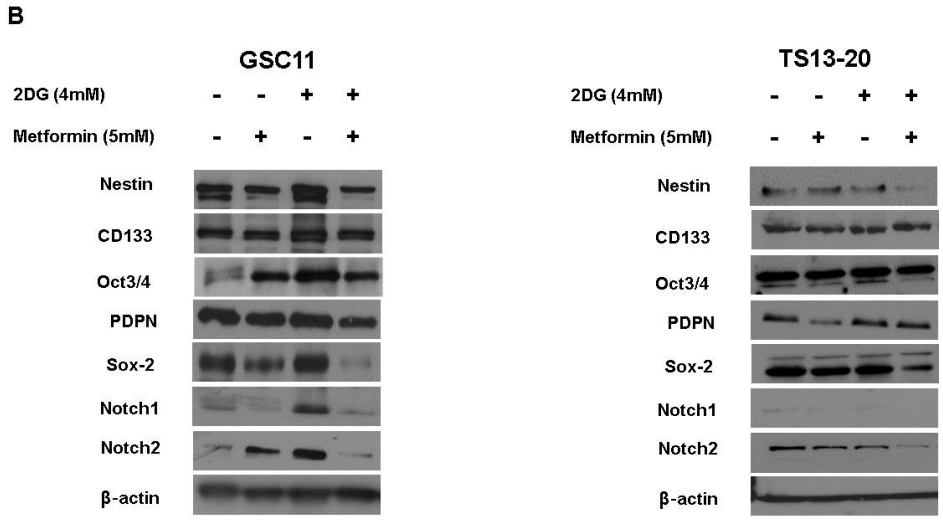


Figure 2. Combination of 2-Deoxyglucose (2DG) and metformin (Met) decreases stemness of glioblastoma tumorsphere, (A) Sphere formation was tested in different conditions. Although metformin single treatment showed moderate inhibition of sphere formation, no sphere formation was observed in the treatment of the combination of 2DG and metformin. (B) Downregulation of stemness-related genes in the combination treatment was confirmed on western blot assay.

3. Combination of 2DG and metformin inhibits invasiveness of GBM TS

For 3D invasion assay, we implanted one type of GBM TS as G-GSC11 in a collagen type I matrix. The implanted GBM TS migrate radially into the collagen matrix, which is physiologically relevant to in vivo tumor behaviors. To observe the distinct effects of drugs on cells starting invasion and invaded cells, two drug treating time points were employed, from implantation point of GBM TS in collagen matrix (Fig. 3A) and 12hr post implantation of GBM TS in collagen matrix (Fig. 3B).

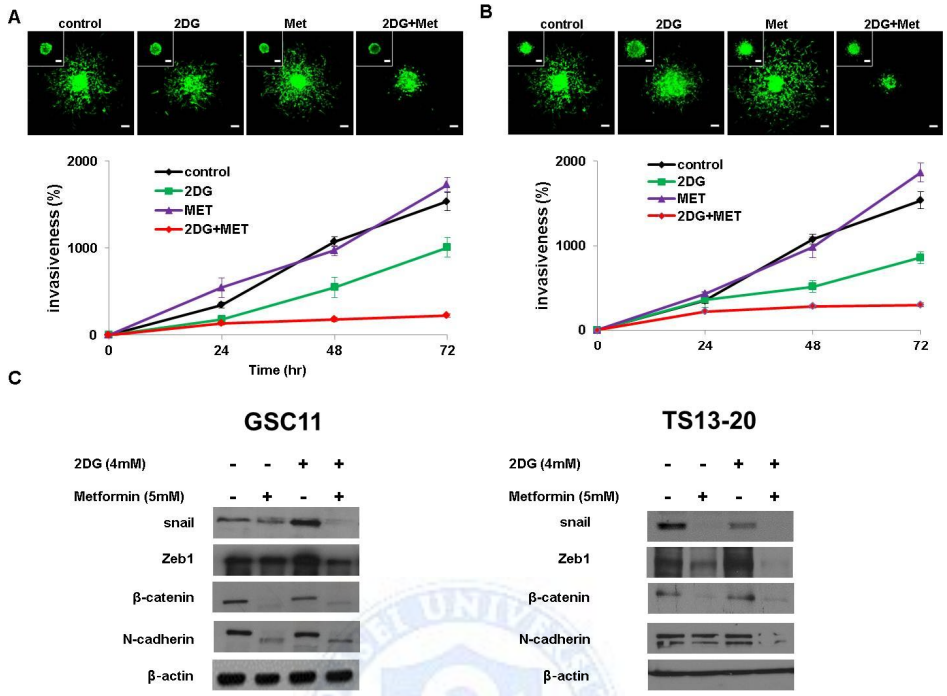


Figure 3. Combination of 2-Deoxyglucose (2DG) and metformin (Met) regulates invasiveness in glioblastoma (GBM) tumorsphere (TS), (A) GBM TS invasion in collagen I matrices, in the presence of various drugs from implantation (images: after 72hr, inset: drug treat point, scale bar: 100μm) and (B) GBS TS invasion in collagen I matrices, in the presence of various drugs from 12hr post implantation (images: after 72hr, inset: drug treat point) were inhibited by combination treatment of 2DG and metformin. Metformin alone did not show inhibition of invasion property of GBM TS. (C) Decreased expression of epithelial mesenchymal transition-related genes was observed on western blot.

After 72 hr, over 80% of GBM TS invasion inhibited with combination of 2DG (4mM) and Metformin (5mM) compared to absence of drugs, both in two drug treating time points. Single treatment of 2DG (4mM) also inhibited invasion about 34% (drug treat from implantation) and 44% (drug treat from 12 hr post implantation).On western blot, the expression of EMT-related markers such as Zeb1, β–catenin, N-cadherin were markedly decreased (Fig 3C). Single treatment of Metformin (5mM) did not inhibit the invasion of GBM TS at all

whereas 2DG alone seemed to decrease the invasiveness of GBM TS, however, this finding was not consistent with the results on western blot.

4. Cellular metabolism; ^{18}F-FDG uptake

The F18-FDG uptake was markedly decreased in GBM TS treated with combination of 2DG and metformin although decreased glucose metabolism was also observed in GBM TS treated with either 2DG or metformin alone (Fig. 4.).

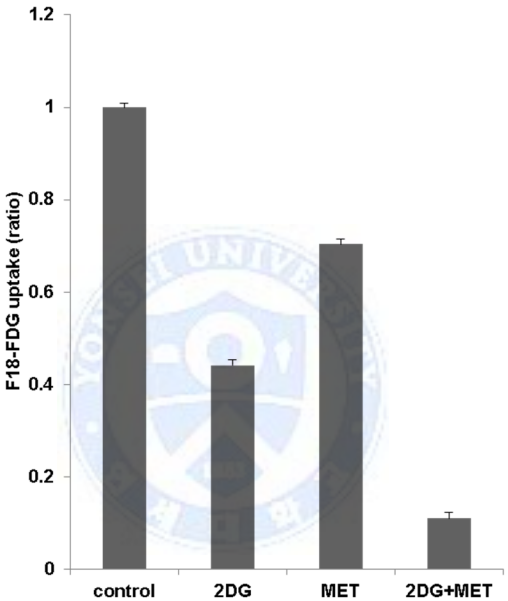


Figure 4. ^{18}F-fluorodeoxyglucose uptake was measured with positron emission tomography, the F^{18}-FDG uptake was markedly decreased in GBM (GSC-11) treated with combination of 2DG and metformin (Met).

5. Gene expression microarray and class comparison

We performed gene expression microarray and genes involving certain pathway were compared between control and 2DG+metformin treated group. (See Fig. 5 and Table 1) In control group of cancer stem cells, genes regulating cellular migration and several EMT related and TGF-beta pathway genes potentially involving EMT pathway were upregulated whereas cellular adhesion molecules were upregulated in treated group.

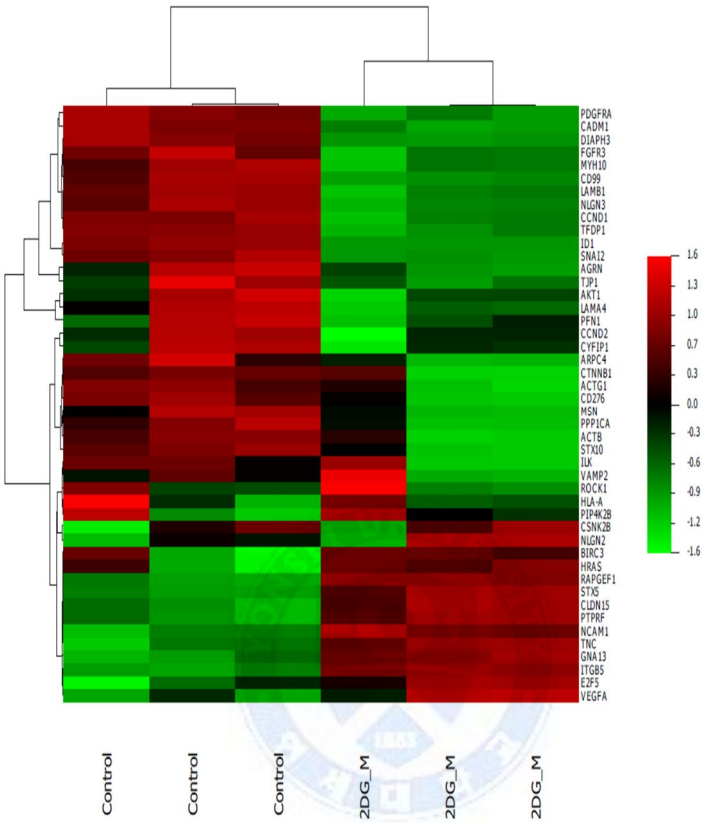


Figure 5. Gene expression microarray and class comparison, Heatmap of gene expression of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway selected gene of control and 2-deoxyglucose/metformin-treated groups. Genes involved in epithelial mesenchymal transition process were downregulated in the treated group.

Table 1. Gene ontology groups of genes with significantly changed expression in combination treatment of 2-deoxyglucose and metformin

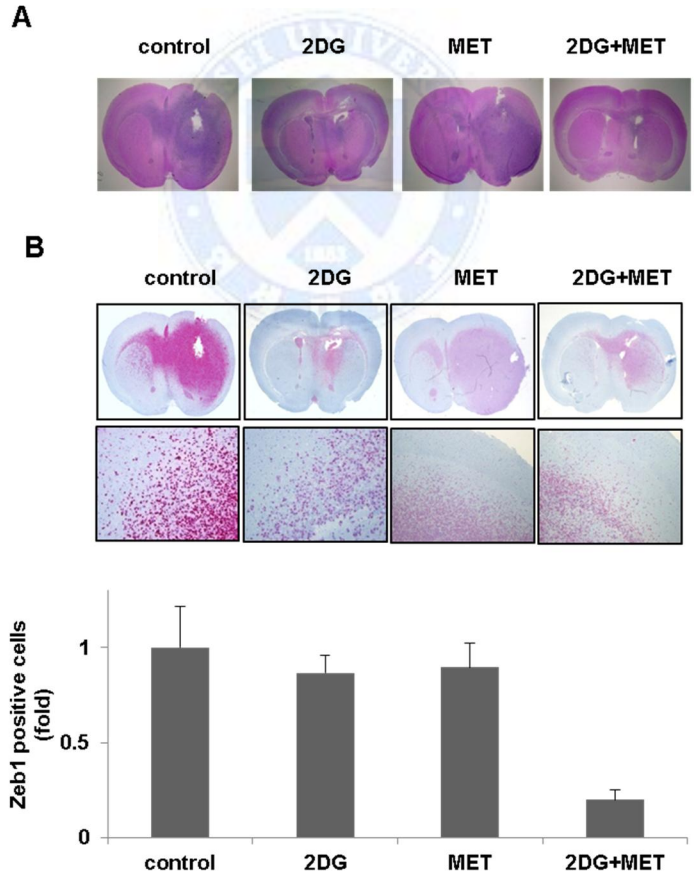
Symbol	Name	Defined Gene List	Parametric p-value	FDR	Fold-change
Genes upregulated in treated group					
TNC	tenascin C	ECM-receptor interaction, Focal adhesion	0.0000001	0.0000001	8.6
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	Integrin Signaling Pathway, Signaling of Hepatocyte Growth Factor Receptor, Focal adhesion, Insulin signaling pathway, Neurotrophin signaling pathway, Renal cell carcinoma	0.0000025	0.0000214	2.58
GNA13	guanine nucleotide binding protein (G protein), alpha 13	PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase, Thrombin signaling and protease-activated receptors, Long-term depression, Regulation of actin cytoskeleton, Vascular smooth muscle contraction	0.0000176	0.0000789	2.53
NCAM1	neural cell adhesion molecule 1	Synaptic Proteins at the Synaptic Junction, Cell adhesion molecules (CAMs), Prion diseases	0.0000403	0.000143	2.2
STX5	syntaxin 5	SNARE interactions in vesicular transport	0.000041	0.000145	2.14
PTPRF	protein tyrosine phosphatase, receptor type, F	Adherens junction, Cell adhesion molecules (CAMs), Insulin signaling pathway	0.0000544	0.000179	2.42
CLDN15	claudin 15	Cell adhesion molecules (CAMs), Hepatitis C, Leukocyte transendothelial migration, Tight junction	0.000104	0.000294	2.42
Genes upregulated in control group					
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	TGF-beta signaling pathway	0.0000001	0.0000001	0.097
DIAPH3	diaphanous-related formin 3	Regulation of actin cytoskeleton	0.0000018	0.0000182	0.38

TFDP1	transcription factor Dp-1	Cell Cycle: G1/S Check Point , Cyclin E Destruction Pathway, Cyclins and Cell Cycle Regulation, E2F1 Destruction Pathway, Influence of Ras and Rho proteins on G1 to S Transition, Regulation of p27 Phosphorylation during Cell Cycle Progression, Cell cycle, TGF-beta signaling pathway	0.0000022	0.0000203	0.34
CADM1	cell adhesion molecule 1	Cell adhesion molecules (CAMs)	0.0000023	0.0000206	0.26
SNAI2	snail family zinc finger 2	Downregulated of MTA-3 in ER-negative Breast Tumors, Adherens junction	0.0000034	0.0000257	0.3
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	CBL mediated ligand-induced downregulation of EGF receptors, Erk1/Erk2 Mapk Signaling pathway, PDGF Signaling Pathway, Phospholipids as signalling intermediaries, Rac 1 cell motility signaling pathway, Role of PI3K subunit p85 in regulation of Actin Organization and Cell Migration, Calcium signaling pathway, Cytokine-cytokine receptor interaction, Endocytosis, Focal adhesion, Gap junction, Glioma, MAPK signaling pathway, Melanoma, Pathways in cancer, Prostate cancer, Regulation of actin cytoskeleton	0.0000036	0.0000263	0.29

CCND1	cyclin D1	BTG family proteins and cell cycle regulation, CARM1 and Regulation of the Estrogen Receptor, Cell Cycle: G1/S Check Point , Cyclins and Cell Cycle Regulation, Inactivation of Gsk3 by AKT causes accumulation of b-catenin in Alveolar Macrophages, Influence of Ras and Rho proteins on G1 to S Transition, p53 Signaling Pathway, WNT Signaling Pathway, Acute myeloid leukemia, Bladder cancer, Cell cycle, Chronic myeloid leukemia, Colorectal cancer, Endometrial cancer, Focal adhesion, Glioma, Jak-STAT signaling pathway, Melanoma, Non-small cell lung cancer, p53 signaling pathway, Pancreatic cancer, Pathways in cancer, Prostate cancer, Small cell lung cancer, Thyroid cancer, Viral myocarditis, Wnt signaling pathway	0.00002	0.000086	0.44
LAMB1	laminin, beta 1	Prion Pathway, Amoebiasis, ECM-receptor interaction, Focal adhesion, Pathways in cancer, Small cell lung cancer, Toxoplasmosis	0.0000413	0.000146	0.45
CD99	CD99 molecule	Cell adhesion molecules (CAMs), Leukocyte transendothelial migration	0.000046	0.000159	0.5
MYH10	myosin, heavy chain 10, non-muscle	Regulation of actin cytoskeleton, Tight junction, Viral myocarditis	0.0001869	0.000479	0.47
FGFR3	fibroblast growth factor receptor 3	Bladder cancer, Endocytosis, MAPK signaling pathway, Pathways in cancer, Regulation of actin cytoskeleton	0.0001944	0.000494	0.52

6. Effects of 2DG and metformin in orthotopic xenograft model

When the mice died after different treatments, their brains were removed and examined with H & E stain. The size of the tumor was much smaller in the mice treated with 2DG and metformin together than in the control group, however, the inhibitory effect of combination treatment was not superior to that of 2DG (Fig. 6A). The number of Zeb1-positive cells located outside the gross tumor boundary was counted, which demonstrated the combination treatment markedly inhibited invasion of GBM TS cells compared with the control and any of single treatments (Fig. 6B). Kaplan-Meier survival analysis showed increased survival of the mice treated with combination of 2DG and metformin compared with the control (P = .008) (Fig. 6C).



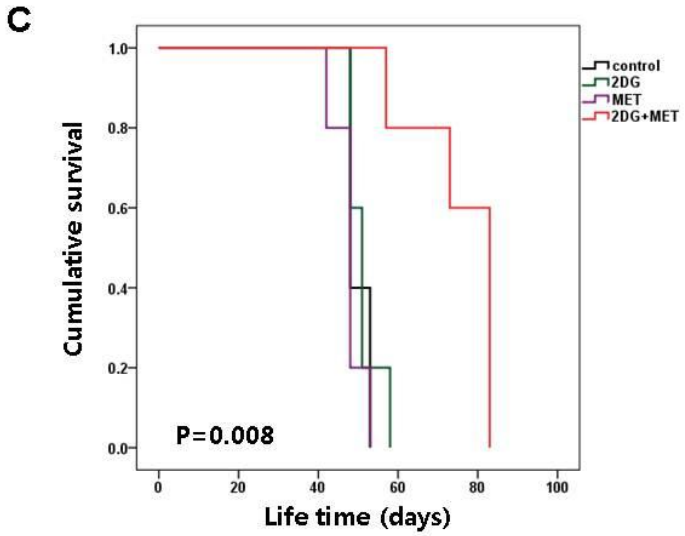


Figure 6. Effect of the combination of 2-deoxyglucose (2DG) and metformin (Met) in an orthotopic xenograft model. (A) The size of the tumor was much smaller in the mice treated with combination of 2DG and metformin than the control and any other treatment groups. (B) Zeb1 immunohistochemistry (IHC) revealed that the combination treatment markedly inhibited invasion of glioblastoma tumorsphere cells compared with the control. (C) Kaplan-Meier survival curve showed increased survival of the mice treated with combination of 2DG and metformin when compared with the control (P = .008). (A) H & E 12 x (B) Zeb1-IHC 12 x

IV. DISCUSSION

Modulation of cancer metabolism is one of emerging approaches for cancer treatment. This is based on the idea that the inhibition of cellular bioenergetics would possibly override the oncogenic signaling pathway.

As cancer cells are more dependent on glycolysis (Warburg effect)[7-9], there have been various attempts to block glycolysis for cancer cell inhibition. In most of these studies, 2DG, a competitive inhibitor of glucose, was used for glycolysis inhibition.[16,22] Various drugs targeting mitochondrial oxidative phosphorylation in TCA cycle, such as AICAR, oxaloacetate, oligomycin and metformin, were extensively tested.[34,35] Especially, the anti-cancer mechanism of biguanide, the most popular anti-diabetic drug, has been now well understood

by numerous researches. It is known that the anti-cancer mechanism of biguanide is mediated by activation of AMPK and consequent inhibition of mTOR pathway.[36-39]

Many studies have shown that inhibition of either glycolysis or oxidative phosphorylation was effective to decrease proliferation, invasion of cancer cells. However, even if glycolysis is inhibited by 2DG, the tricarboxylic acid (TCA) cycle is still working even under hypoxic condition.[40] Although many experiments above showed results supporting therapeutic potential of single pathway inhibition, it is apparent that cancer cells can utilize these two pathways, and one could be an alternative when the other pathway is blocked. Cheong et al. suggested that dual inhibition of glycolysis and oxidative phosphorylation would results in more serious cellular energetic deprivation showing better anti-cancer effect.[20] 2DG is an glucose-mimetic agent, which competes with normal glucose; the effect of 2-DG is like emptying the fuel. On the other hand, oligomycin and metformin directly inhibits cellular energy production in mitochondria. In many studies, this combination of 2DG and metformin showed stronger anti-cancer effect than single treatment.[20-22] For a glioblastoma which is one of the deadliest cancers, Kennedy et al. evaluated the efficacy of dual inhibition of glycolysis and oxidative phosphorylation based on a GBM cell line model.[23] They used oligomycin to suppress oxidative phosphorylation, and 2DG as a glycolysis inhibitor. They observed GBM cell lines which were sensitive to oligomycin did not undergo AMPK activation and adaptive glycolytic switch. On the other hand, when oligomycin-resistant GBM cell lines were treated with 2DG simultaneously, they observed strong anti-proliferation effect.

Cancer stem cell theory postulates that a small population of tumor-initiating cells is responsible for the development, progression and recurrence of cancers.[41,42] Especially, it is believed that tumor-initiating cell with stem properties is responsible for treatment failure.[5] The metabolic

characteristics of a cancer stem cell have not been clearly elucidated. There are many reports that cancer stem cells prefer glycolysis.[10-13] However, other researches support "reverse Warburg effect" that cancer stem cell more depends on oxidative phosphorylation based on the selective toxicity of metformin on a cancer stem cell or a tumor-initiating cell.[17-19,43-45] Viale et al. demonstrated that surviving cancer cells which are responsible for tumor relapse have features of cancer stem cells and depend on oxidative phosphorylation for survival.[14]

In our study, rather than specific GBM cell lines, we chose GBM TS as we believe new therapeutic approach should target or, at least, include tumor-initiating cells (cancer stem cells). First, we checked the cytotoxicity of 4mM of 2DG and 5mM of metformin on GBM TS. Very unlike our expectation that the combination of 2DG and metformin would show strong anti-cancer effect, neither combination nor the single treatment of 2DG and metformin did not inhibit proliferation of GBM TS. Interestingly, cell viability was rather increased with metformin treatment compared with the control. This finding was also consistent with upregulation of mTOR and its related proteins on western blot. Considering AMPK was also activated with metformin treatment as expected[38], we think that metformin-induced mTOR activation could be AMPK-independent in GBM. Liu et al. revealed that metformin suppressed GBM proliferation through PRAS40-mediated mTOR inhibition. They found AMPK activation is not required for anti-proliferative action of metformin.[46] We also noted AMPK-independent regulation of mTOR, however, we failed to show anti-proliferative effect of low dose metformin (5mM) based on a GBM TS model. Würth et al. reported metformin inhibited the viability of GBM tumor-initiating cells concentration-dependently, however, this anti-proliferative effect was not apparent at low dose of metformin either in their experiment.[19] We found that combination treatment of 2DG and metformin inhibited mTOR and its related proteins such as Raptor in an AMPK-independent manner, but it did not lead to anti-proliferative action on GBM TS.

Instead of anti-proliferative actions, the combination treatment resulted in strong inhibition of stemness. No sphere was formed under dural treatment of 4mM of 2DG and 5mM of metformin. This finding was also supported by western blot as expression of stem cell markers such as Sox2, Notch2 were markedly decreased in a combination treatment group. Metformin alone also seemed to decrease sphere formation of GBM TS in a dose dependent manner (Fig. 7), however, downregulation of stem cell markers were not observed.

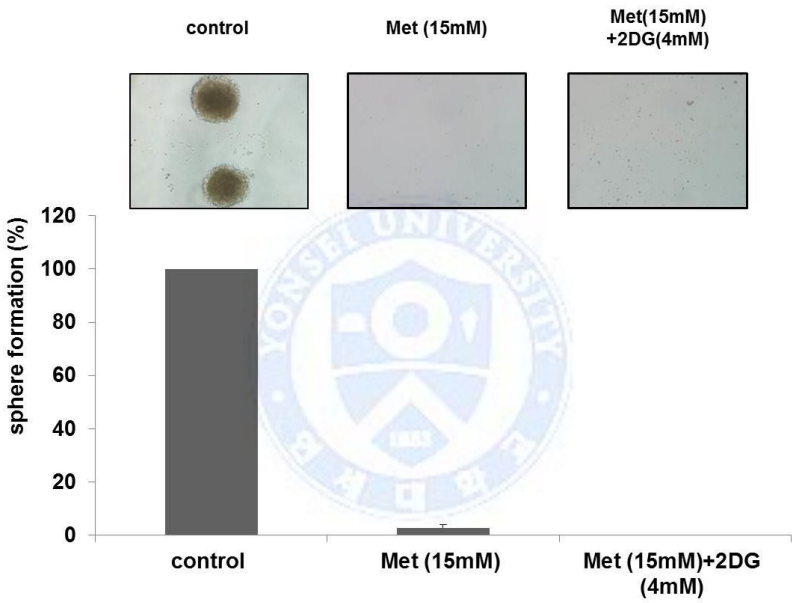


Figure 7. Sphere formation assay with high dose of metformin (Met), Tumorsphere formation was test in different conditions with higher dose of metformin (15mM). Single treatment of metformin showed strongly inhibited sphere formation of GBM TS when compared with combination with 2-Deoxyglucose (2DG).

There are many reports suggesting that tumor invasion is mediated by the cancer stem cell population.[47-49] In order to elucidate whether modulation of cancer metabolism could inhibit invasion properties of GBM TS, we employed an in vivo-like tumor model to observe three-dimensional invasion of GBM TS. To design physiologically relevant platform to in-vivo environment, type I

collagen which is the most abundant matrix in human body was used. After implantation spheroids in collagen matrix, the GSC11 cells in cell-matrix boundary radially invaded into matrix and collagen matrix may mediate their changes in behaviors.[50] In two drug treating time points, combination of 2DG (4mM) and Metformin (5mM) dramatically inhibited the invasion for 3 days while did not induce cell death. The dead cells were exclusively observed at the core of spheroids due to hypoxia (Fig. 8), which means 2DG and metformin treatment does not affect cell viability but purely inhibits invasion of GBM TS.

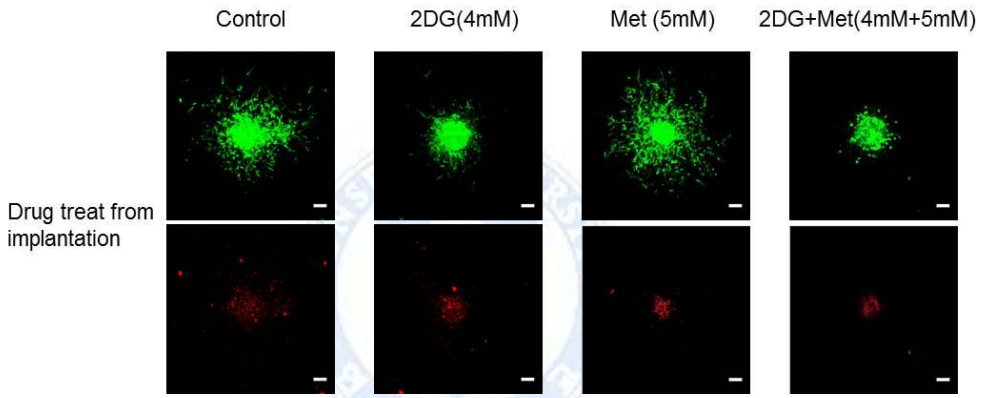


Fig. 8. Viability of glioblastoma (GBM) tumorsphere (TS) in collagen matrix with drugs, Red fluorescence indicating dead cells is mainly localized in the core of the spheroids. The extent and amount of red fluorescence of each treatment group was about the same, and even similar to that of the control GBM TS. (images: after 72hr, scale bar: 100μm, upper row: green fluorescent protein-GBM TS, lower row: dead cell stained GBM TS)
2DG, 2-deoxyglucose; MET, metformin

As for the GBM TS treated with metformin alone, we found that invasion of GBM TS was not inhibited when treated with low concentration of metformin (5mM). But, with high concentration of metformin (15mM), it showed inhibitory effect on invasion of GBM TS (Fig. 9) as previously shown by Bao et al.[51]

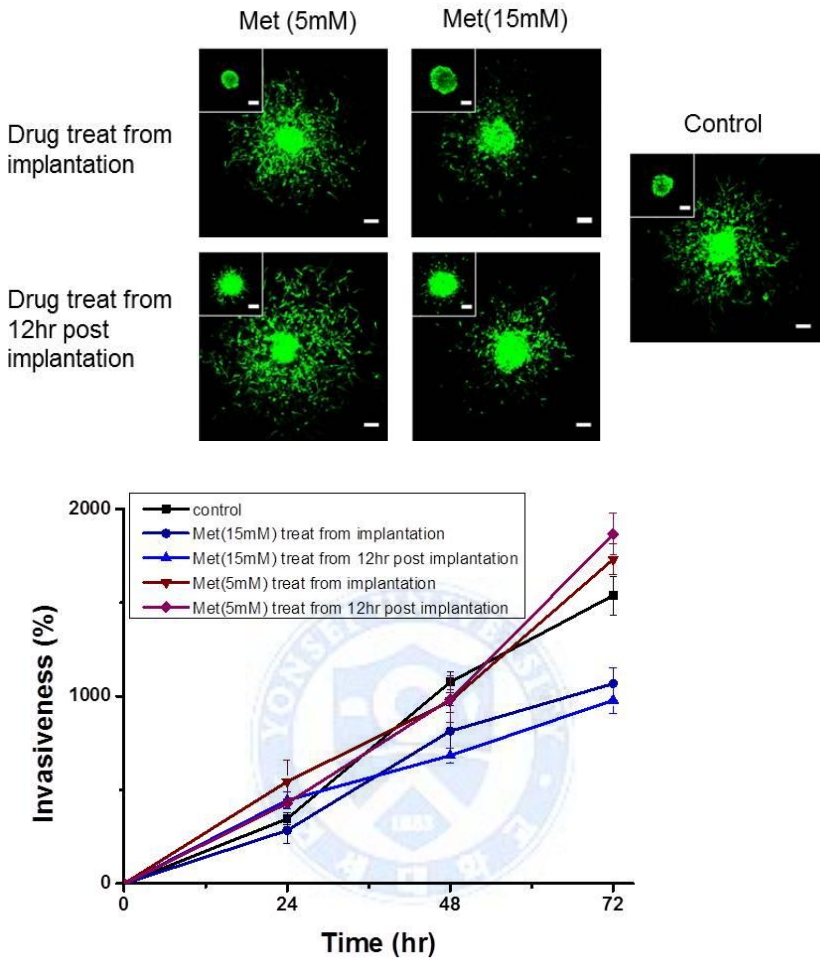


Fig. 9. Anti-invasion effect of metformin single treatment with the comparison of high concentration and low concentration of metformin, Effect of metformin on invasion of glioblastoma tumorsphere depends on the concentration. Invasion of GBM TS was not inhibited in the condition of 5mM of metformin whereas 15mM of metformin showed inhibitory effect on invasion of GBM TS.

In order to clarify whether the anti-stemness and anti-invasion effect of 2DG and metformin is really mediated by altered metabolic status, we checked ^{18}F-FDG uptake from GBM TS with micro-PET. Indeed, the ^{18}F-FDG uptake was markedly decreased in GBM TS treated with combination of 2DG and

metformin.

We conducted trancriptome assay with the comparison between before and after the combination treatment on GBM TS, and found genes related to ECM receptor, adhesion and cytoskeleton, were negatively correlated on GSEA.

2DG and metformin are transported across the blood-brain barrier (BBB) by transport proteins such as GLUT-1 and OCT1, respectively. We noted a possibility that this combination would also show anti-cancer effect for glioblastomas. Indeed, the combination of 2DG and metformin was proved to be effective in an in vivo model as well. In the experiment with an orthotopic xenograft model, the tumor growth was inhibited in the combination treatment group when compared with the control. However, rather than focusing on the tumor size, Zeb1 staining revealed that the combination treatment of 2DG and metformin strongly inhibited GBM TS invasion. Moreover, these strong anti-cancer actions also led to survival benefit.

In our experiment, we used 500mg/kg of metformin administrated by intraperitoneal injection every other day, which was the maximal dose the mice tolerated. Although it is obvious metformin crosses BBB, the CSF concentration of metformin is only 10% of serum concentration. In order to increase the concentration of metformin in CSF, administration of higher dose of metformin is inevitable. 500mg/kg of metformin is apparently quite high dose to be used for human, however, the maximal safe dose of metformin was determined based on daily long-term use for diabetes control, and not for anti-cancer action. Pollak also noted a possibility of short-term use of metformin at higher doses.[38] Also, because 500mg of metformin alone failed to show anti-cancer effect in in vivo setting, we can lower the required dose of metformin for anti-cancer effect by combining with 2DG. Other biguanides such as penformin could be another way to decrease toxicity as its CSF concentration is much higher than that of metformin.[38]

Targeting multiple bioenergetic pathways in combination with

conventional standard therapeutics could be a new therapeutic approach expecting synergetic anti-cancer effect. With the same idea, there has been already increasing number of researches in the literature.[52-54]

V. Conclusion

In summary, the combination of 2DG and metformin did not show cytotoxicity for GBM TS. But, 2DG and metformin effectively decreased the stemness and invasion capacity of GBM TS, and showed potential survival benefit in mouse orthotopic xenograft model experiment. We believe this dual inhibition of bioenergetic pathways could be helpful in the treatment of GBM patients by targeting GBM TS.

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ABSTRACT (IN KOREAN)

2-Deoxyglucose와 metformin 병합요법에 의한 교모세포종 종양구의 억제

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김 의 현

목적: 교모세포종 (glioblastoma)의 완치가 어렵고 결국 재발하는 이유는, 여러 가지 치료법에도 생존할 수 있는 종양세포가 존재하기 때문이다. 종양세포 내에 존재하는 여러 가지 에너지 발생 경로를 차단함으로써 다양한 종양을 효과적으로 억제할 수 있다는 것이 보고되고 있다. 하지만, 이러한 효과가 교모세포종의 종양구 (tumorsphere)에 대하여 증명된 적은 없다. 저자들은 해당과정 (glycolysis)와 산화적인산화 과정 (oxidative phosphorylation)을 동시에 차단함으로 교모세포종 종양구를 억제할 수 있을 것이라는 가정 하에 본 연구를 진행하였다.

재료 및 방법: 저자들은 해당과정을 억제하는 2-deoxyglucose (2DG)와 산화적인산화 과정을 억제하는 metformin 및 그 병합용법에 대하여 교모세포종 종양구 억제에 대한 효과를 확인하였다. 먼저 세포 독성 및 AMP-activated protein kinase (AMPK)와 mammalian target of rapamycin (mTOR) 경로에 관련된 단백질에 대한 발현을 확인하였다. 또한, 병합요법이 교모세포종 종양구의 줄기세포능 및 침윤에 대해 미치는 영향을 종양구 형성 실험과 3D 배양 장치를 사용하여 확인하였다. 또한 ^{18}fluorodeoxyglucose를 이용한 양전자 단층촬영 (positron emission tomography)를 통하여 포도당 대사의 변화를 관찰하였다. 그리고, 전사체 (transcriptome) 분석을 통해 병합용법 전후의 유전자의 변화를 확인하였다. 마지막으로 동물실험을 통한 생체 내 실험을 통하여 종양 형성 능력 및

침윤 능력, 그리고 생존에 대한 분석을 시행하였다.

결과: 교모세포종 종양구에 대한 세포독성은 2DG와 metformin, 그리고 이 둘의 병항요법에도 억제되지 않았다. 주목할 것은, 일반적으로 알려진 mTOR 경로의 억제가 AMPK와의 관련성 없이 관찰되었다는 점이다. 종양구의 형성 능력은 병합요법에서 유의하게 감소하는 것으로 나타났으며, 줄기세포능과 관련된 단백질들의 발현도 유의하게 감소되었다. 종양구의 침윤 능력 역시 병합요법에 의해 유의하게 억제됨을 확인하였다. 양전자 단층촬영을 통하여 병합요법에서 포도당과 관련된 대사 과정이 유의하게 저하되어 있음을 확인하였다. 전사체 분석을 통하여 병합요법 후에 종양에서 세포외 기질 (extracellular matrix)과 세포부착 (adhesion)에 관련된 유전자들의 전사가 억제됨을 확인하였다. 마지막으로, 동물실험을 통하여 2DG와 metformin의 병합요법이 교모세포종의 종양구 침윤 능력을 억제하고, 생존기간을 증가시킴을 확인하였다.

결론: 2DG와 metformin의 병합요법은 교모세포종 종양구에 대하여 종양사멸 효과는 보이지 않았으나, 종양구의 줄기세포능과 침윤 능력을 효과적으로 억세하며, 동물 실험에서 생존기간의 증가까지 보일 수 있음이 확인되었다. 저자들은 주된 세포내 에너지 생성 경로인 해당과정과 산화적인산화 과정을 동시에 차단하는 방법이 교모세포종에 있어 새로운 치료적 접근법이 될 수 있다는 가능성을 확인하였다.

핵심: 2-deoxyglucose, 교모세포종, metformin, 줄기세포능, 종양구