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# 2-Deoxy-D-glucose increases GFAT1 phosphorylation resulting in endoplasmic reticulum-related apoptosis via disruption of protein *N*-glycosylation in pancreatic cancer cells

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ABSTRACT

The glycolytic inhibitor 2-deoxy-D-glucose (2DG) causes energy starvation, affecting cell viability in a wide range of cancer cell lines. To determine the action of 2DG in pancreatic cancer, we performed proteomic analysis of pancreatic cancer cell line after 2DG treatment. Eighty proteins showed differential expression and among these, proteins involved in phosphohexose metabolism were upregulated. Up-regulation of glutamine: fructose 6-phosphate aminotransferase 1 (GFAT1), which belongs to the hexosamine biosynthesis pathway (HBP) that produces uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) to maintain glycoprotein, was validated by evaluation of mRNA and protein levels. Therefore, we assessed the amounts of total *N*-glycoproteins. Unexpectedly, we found a reduction of total *N*-glycoproteins and phosphorylation of GFAT1 by AMP-activated protein kinase (AMPK). These data may shed light on HBP dysfunction. Furthermore, we found endoplasmic reticulum (ER) stress accompanied by increased expression of ER stress markers, such as glucose response protein 78 (GRP78) and C/EBP-homologous protein (CHOP), in 2DG-treated cells. Moreover, the additive activation of AMPK by metformin (Met) synergistically enhanced the reduction of protein *N*-glycosylation and cell growth inhibition in the presence of 2DG. These results suggest that 2DG reduces *N*-glycosylation of proteins following the increase of phosphorylation of GFAT1 and results in the inhibition of cell growth mediated by ER stress in pancreatic cancer cells.



## 1. Introduction

Glucose metabolism is altered in several diseases, including metabolic diseases, neurodegenerative disorders, diabetes mellitus, inflammation, and cancer. In contrast to normal cells, cancer cells use glycolysis intermediates for other metabolic pathways, such as ATP production, nucleic acid synthesis, and membrane synthesis, in order to adapt to the rapid cell division [1]. It has been found that the metabolic changes in cancer cells are regulated by the mutation status of various oncogenes [2]. However, it is extremely difficult to estimate the metabolic character of each cancer from the mutation status of various oncogenes, because the mutation status of each individual gene in different cancer cell lines or cancer patients is diverse.

Pancreatic ductal adenocarcinoma (PDAC) is a cancer with one of the poorest prognoses in the world. Recently, metabolic aberrations of PDAC have been unveiled using several comprehensive approaches. The metabolic alterations in PDAC cells contributes to their survival in the poor nutrient environment of PDAC [1,3], with changes in the expression of constituents of metabolic processes such as glutamine metabolism [4] and fatty acid metabolism [5].

2-Deoxy-D-glucose (2DG) induces ATP depletion and endoplasmic reticulum (ER) stress, autophagy, cell cycle arrest and ultimately, apoptosis in several cell lines [6]. Given these diverse effects on cell growth, 2DG has been widely used as an enhancer of

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anti-cancer treatments such as the Bcl-2 antagonist ABT-737 [7], irradiation [8] and mitochondrial respiratory inhibitors [9] *in vitro* and in xenograft experiments. However, the detailed mechanism remained unclear. In this study, we confirmed that the anti-cancer mechanism of 2DG may be related to the induction of ER stress-induced apoptosis via disruption of protein *N*-glycosylation through the suppression of glutamine: fructose 6-phosphate aminotransferase 1 (GFAT1), known as the rate-limiting enzyme of the hexosamine biosynthesis pathway (HBP) [10], in PDAC cells. The phosphorylation of GFAT1 at Ser243 b y AMPK is important for its enzyme activity [11] and may be effective for HBP dysfunction. In addition, we found for the first time that metformin (Met) synergistically increased the anti-cancer effect via the enhancement of *N*-glycosylation disorder and enhanced the growth inhibition of pancreatic cancer cells by 2DG treatment.

## 2. Materials and methods

### 2.1. Cell culture

MIAPaCa2 and PANC-1 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and were cultured at 37 °C in RPMI-1640 [#22400, Thermo Fisher Scientific (Thermo), Rockford, IL, USA] supplemented with 10% fetal calf serum in humidified 5% $CO_2$. All lines were tested over the course of ~3 passages.

### 2.2. Cell growth analysis

Cells were seeded into 6-well plates at a concentration of $1.0 \times 10^5$ cells/well and treated with 2DG and/or metformin at various concentrations. After 2–4 days, the total number of cells was counted after propidium iodide (PI) staining using a C-Reader (Digital Bio Technology, Seoul, Korea), an automatic cell counter based on cell imaging.

### 2.3. Label-free proteome analysis of pancreatic cancer cells exposed to 2DG and proteomic data analysis

Label-free proteomics and data analysis were performed according to the protocol described previously [12–14] with some modifications. The detailed proteomic analysis procedure is described in supplementary materials and methods.

### 2.4. Western blotting

Western blotting was performed as described previously [15]. Antibodies to the following proteins were used: GFAT1 [1:1000, #28121, Immuno-Biological Laboratories (IBL), Gunma, Japan], phospho-GFAT1 (Ser243; 1:2000, #28123, IBL), GRP78 (1:1000, #ab21685, Abcam, Cambridge, UK), AMPKα [1:1000, #5832, Cell Signaling Technologies (CST), Danvers, MA, USA], phospho-AMPKα (Thr172; 1:1000, #2535, CST), ACC (1:1000, #3676, CST), phospho-ACC (Ser79; 1:1000, #11818, CST), β-actin (1:10000, #A5316, Sigma-Aldrich, St. Louis, MO, USA).

Protein *N*-linked glycosylation was detected by a lectin binding assay in which concanavalin A (ConA) binds to mannose-containing sugar chain moieties. The protein-transferred membrane was incubated with ConA-Biotin (4 ng/mL, #J1001003, J-Oil Mills, Tokyo, Japan) in TBS-T following three washes and soaked in High Sensitivity NeutrAvidin-HRP (1:50000, #31030, Thermo) for at least 1 h.

### 2.5. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed as described previously [16]. The expression levels were analyzed using TaqMan probes (Thermo) for GFAT1 (#Hs00899865_m1), CHOP (#Hs00358796_g1), and 18 S rRNA (#Hs03928990_g1).

### 2.6. Analysis of apoptotic cell death in cultured cells

Cell death analysis was performed as described previously [17].

### 2.7. Metabolite analysis

Metabolite analysis was performed as described previously with slight modification [18]. The detailed metabolite analysis procedure is described in supplementary materials and methods.

### 2.8. Statistical analysis

Data are expressed as means ± SEM. All statistical analyses were performed using GraphPad, Prism 7.0 (GraphPad, SanDiego, CA, USA). Unpaired *t*-test and one-way ANOVA using a *post-hoc* Dunnett's or Tukey's test were used to analyze the differences between groups. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Proteomic analysis reveals that 2DG induces changes in the expression level of amino-sugar metabolic enzymes

To clarify the effect of 2DG on the cell growth and intracellular ATP concentration of pancreatic cancer cell lines, we analyzed the cell number and intracellular ATP concentration of MIAPaCa2 and PANC-1 cells after 2DG treatment. Cell number was clearly decreased in a 2DG dose- and time-dependent manner, while intracellular ATP concentration was reduced (data not shown).

To explore the molecular mechanisms of 2DG in cell growth inhibition of pancreatic cancer cells, we performed label-free semi-quantitative proteomic analysis of 2DG-treated MIAPaCa2 cells. Among 1342 types of proteins identified, expression of 48 proteins was increased, while 32 proteins decreased. Detailed protein expression data are summarized in Supplementary Tables S1–4. Differentially-expressed proteins in the 2DG-treated or untreated MIAPaCa2 cells were applied to the DAVID database for functional grouping. Furthermore, the up-regulated proteins related to the amino sugar and nucleotide sugar metabolism process were identified using the KEGG database (Fig. 1A). Differentially-expressed proteins associated with critical biological processes such as catabolic metabolism and cell death were also identified (Fig. 1B). Several enzymes involved in the HBP which is responsible for the formation of UDP-GlcNAc from F6P, were identified in both KEGG pathway (Fig. 1A) and biological functions (Fig. 1B) analyses. These enzymes are shown in Fig. 1C. On the other hand, the expression level of hexokinase as a target of 2DG and other glycolysis enzymes did not change (Supplementary Fig. S1).

### 3.2. 2DG affects GFAT1 expression, ER stress and apoptosis

To clarify the effect of 2DG on the translation and transcription of GFAT1, the expression levels of protein and mRNA of GFAT1 in 2DG-treated and untreated MIAPaCa2 cells were analyzed by western blotting and RT-qPCR respectively. 2DG treatment enhanced GFAT1 protein expression in a dose-dependent manner (Fig. 2A), while GFAT1 mRNA was significantly increased in 2DG-