Original Article
Bitterness in sugar: O-GlcNAcylation aggravates pre-B acute lymphocytic leukemia through glycolysis via the PI3K/Akt/c-Myc pathway

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Abstract: Abnormal cellular energetics has emerged as a hallmark of cancer cells. Deregulating aerobic glycolysis can alter multiple metabolic and signaling pathways in cancer cells, and trigger unlimited growth and proliferation. Accumulating evidence suggests that elevated levels of protein modification with β-*N*-acetylglucosamine (O-GlcNAcylation) along with dysregulation of O-GlcNAc transferase (OGT) and/or O-GlcNAcase (OGA) levels may act as a nutrient sensor in cancer cells. However, the underlying mechanism of O-GlcNAcylation and the relationship between O-GlcNAcylation and glycolysis are largely unknown in pre-B acute lymphocytic leukemia (pre-B-ALL). In this study, CD19^{+} bone marrow mononuclear cells (BM-MNCs) from untreated pre-B-ALL patients displayed increased O-GlcNAcylation levels, upregulated OGT, and downregulated OGA. Patients with higher lactate dehydrogenase (LDH) levels exhibited higher O-GlcNAcylation levels with OGT upregulation and overactivation of the PI3K/Akt/c-Myc pathway. The extracellular acidification rate (ECAR) and the mRNA expression of key enzymes in glycolysis were determined to assess glycolysis activation. Our results revealed the existence of abnormal glycolysis in the CD19^{+} BM-MNCs of pre-B-ALL patients. The knockdown of OGT decreased the ECAR and downregulated glycolysis-related enzymes in Nalm-6 cells via the PI3K/Akt/c-Myc pathway. The suppression of OGT slowed the rate of proliferation and induced apoptosis in Nalm-6 cells. The glycolysis inhibitor 2-deoxy-D-glucose induced cytotoxicity of Nalm-6 cells, which was potentiated by OGT-siRNA. These findings suggested that O-GlcNAcylation could be a hallmark of pre-B-ALL, which has considerable therapeutic potential in clinical practice.

Keywords: O-GlcNAcylation, pre-B acute lymphocytic leukemia, O-GlcNAc transferase, glycolysis, PI3K/Akt/c-Myc pathway

Introduction

As an essential posttranslational modification (PTM) process for proteins, O-GlcNAcylation can alter the structures of proteins to improve the precision of their regulation [1]. Almost all subcellular compartments contain O-GlcNAcylation-related proteins; the process occurs in nuclear membranes and nuclei, particularly on nuclear pore complex proteins [2]. This modification is a dynamic and reversible process, which adds the monosaccharide β-*N*-acetylglucosamine (O-GlcNAc) to the serine and threonine residues of intracellular proteins (O-GlcNAcylation). In cells, 2%-5% of glucose enters via the hexosamine biosynthesis pathway (HBP). UDP-GlcNAc is an end product of the HBP [3] and a donor substrate in O-GlcNAcyla-

tion cycling. In contrast to other PTMs that are mediated by a series of enzymes to guarantee site specificity, O-GlcNAcylation is controlled by 2 key enzymes. O-GlcNAc transferase (OGT) appends UDP-GlcNAc to serine and threonine residues. The deglycosylating enzyme O-GlcNAcase (OGA) can cleave the modification [4]. Protein O-GlcNAcylation is involved in a series of complex biological processes, such as gene expression, transduction, protein degradation, immune protection, and cell proliferation [5].

Eighty years ago, Otto Warburg found that, in contrast to normal cells, tumor cells support their energy requirements through high levels of anaerobic glycolysis, even in the presence of sufficient oxygen [6]. The amount of ATP generated by glycolysis is considerably lower than

Table 1. Basic clinical characteristics of the patient

	Pre-B-ALL	Healthy donor	P
Age (months), median (range)	36 (14-69)	35.5 (18-60)	0.689
Gender, male/female	10/14	6/4	0.457

that from oxidative phosphorylation, but macromolecules can be provided to satisfy the increased energy requirement [7]. In addition to solid tumors, leukemia cells undergo overactive anaerobic glycolysis to produce energy for survival [8]. Tumor cells consume and utilize considerably more glucose than normal cells [9]. Metabolic disorder has been considered an important hallmark of malignancy [10]. O-GlcNAcylation is sensitive to intracellular glucose levels and, thus, is used as a nutrient sensor and metabolic regulator [11]. Numerous proteins associated with tumorigenesis are also O-GlcNAcylated [12]. O-GlcNAcylation, OGT, and OGA levels are altered in the tumorigenesis and progression of various cancers, such as bladder [13], prostate [14], breast [15], human laryngeal [16], and colorectal cancer [17]. Therefore, we hypothesized that the O-GlcNAcylation level of proteins can be used as a tumor marker.

The PI3K/Akt pathway can regulate the uptake and utilization of glucose [18]. Aberrant PI3K/Akt pathway activity plays an important role in hematologic malignancies. This pathway may also serve as a pro-survival factor in leukemic stem cells and contribute to disease progression. Inhibitors of this pathway elicit therapeutic effects on leukemia [19]. The PI3K/Akt pathway also participates in cell survival and metabolic processes [20]. A well-known oncogene in leukemia, c-Myc, regulates tumor glycolytic activity [21]. It is also a key downstream molecule in the PI3K/Akt pathway [22]. However, its functions in the glycolytic progress of acute leukemia cells remain unclear.

Intracellular O-GlcNAcylation is associated with the pathogenesis of chronic lymphocytic leukemia [23], but the relationship between O-GlcNAcylation and glycolysis in human chronic or acute leukemia remains unknown. In this study, we found that O-GlcNAcylation was higher in CD19+ bone marrow mononuclear cells (BM-MNCs) from pre-B acute lymphocytic leukemia (pre-B-ALL) patients compared with cells from healthy donors, while OGT was upregulated and OGA was downregulated. O-GlcNAcy-

lation correlated with lactate dehydrogenase (LDH) levels in pre-B-ALL patient cells. The suppression of OGT-mediated O-GlcNAcylation affected glycolysis via the PI3K/Akt/c-Myc pathway. The knockdown of OGT induced cytotoxicity to Nalm-6 cells and increased their sensitivity to the glycolysis inhibitor 2-deoxy-D-glucose (2-DG). Our results suggested that OGT-mediated O-GlcNAcylation could be a tumor marker and a novel therapeutic target for pre-B-ALL.

Materials and methods

Ethical statement

This study was carried out in conformity with ethical standards and was approved by Institutional Review Board of the Qilu Hospital Authority.

Isolation of human pre-B-ALL cells

BM samples were obtained from 24 pre-B-ALL patients at the time of initial diagnosis prior to chemotherapy and 10 healthy donors at Qilu Hospital of Shandong University from September 2015 to September 2016, with informed consent from the children's legal guardians (clinical characteristics in **Table 1**). The experiments are in accord with the ethical standards established by the institutionThe specimens were collected in a sterile manner into preservative-free heparin. BM-MNCs and CD19+ BM-MNCs were isolated, respectively, with a lymphocyte separation medium (Haoyang, Tianjin, China) and through positive selection with CD19 MicroBeads (Miltenyi), according to the manufacturers' instructions. BM-MNCs were then stored in a frozen stock solution containing 10% dimethyl sulfoxide and 90% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) for subsequent experiments. The LDH levels in patient plasma samples were collected from their laboratory tests upon hospital admission. Plasma LDH greater than 400 units/L were considered high (n = 15) and plasma LDH levels lower than 400 units/L were denoted as low (n = 9) [24].

Cell culture and reagents

The human pre-B-ALL cell line Nalm-6 was provided by Prof. Daoxin Ma (Qilu Hospital, Jinan, Shandong, China). Nalm-6 cells were cultured

Table 2. Primer sequences of genes in glycolysis

Gene ID	Primer sequence (5'-3')	Length
HK2	Forward primer: TTCCCCTGCCACCAGACTAA	20
	Reverse primer: TCAAAGTCCCCTCTCCTCTGG	20
Glut1	Forward primer: GTCTGGCATCAACGCTGTCT	20
	Reverse primer: GTTGACGATACCGGAGCCAA	20
HIF-α	Forward primer: ATTTTGGCAGCAACGACACA	21
	Reverse primer: CGTTTCAGCGGTGGGTAATG	20
LDHA	Forward primer: CCAGTGTGCCTGTATGGAGT	20
	Reverse primer: CGTAAAGACCCTCTCAACCACC	22
PFK1	Forward primer: CAGGCTCCCTCCATCCTCA	19
	Reverse primer: CTGCCTCCTAGCGACTCTTC	20

in RPMI-1640 (Gibco) containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibico) at 37°C in 5% CO_2 and a normal level of oxygen. LY294002 and 2-DG were purchased from Sigma-Aldrich, and recombinant human insulin-like growth factor 1 (IGF-1) was purchased from PeproTech.

Western blot analysis

Protein extracts from isolated CD19^{+} BM-MNCs (prior to and following different in vitro treatments) were prepared in RIPA lysis buffer (Beyotime, Shanghai, China). Then, proteins (60 μg) were separated on 10% acrylamide gels and transferred to polyvinylidene fluoride (PV-DF) membranes (Millipore). The following commercially available antibodies and dilutions were used for western blotting: anti-O-GlcNAc (RL2) (ab2739, Abcam; 1:1000), anti-OGT (ab-96718, Abcam; 1:1000), anti-MGEA5 (OGA) (ab124807, Abcam; 1:1000), anti-PI3K (ab-86714, Abcam; 1:1000), anti-AKT (ab81283, Abcam; 1:1000), anti-AKT (phosphoS473) (ab-81283, Abcam; 1:1000), anti-c-Myc (ab32072, Abcam; 1:1000), anti-c-Myc (phosphoT58) (ab-28842, Abcam; 1:1000), and anti-β-actin (60-008-1-Ig, Proteintech; 1:2000). After incubation with primary antibodies overnight at 4°C, the PVDF membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (SA00001-1, Proteintech, 1:10000) or -rabbit (SA00001-2, Proteintech, 1:10000) secondary antibody for 1 h at 25°C. Blots were developed using the ECL chemiluminescence detection kit (Millipore) and analyzed with Image Studio™ DiGits Version 4.0. All target protein signal intensities were normalized to β-actin signals.

Real-time polymerase chain reaction

Total RNA was extracted from cell lysates with TRIzol® reagent (Invitrogen Life Technologies), following the manufacturer's protocol. We prepared cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The mRNA levels were quantified by quantitative real-time (qRT)-polymerase chain reaction (PCR) with 1×SYBR® Green PCR Master Mix (TOYOBO) in an Applied Biosystems® 7500 PCR system (Applied Biosystems). The relative expression of each target gene was normalized to β-actin using gene-specific primers (primer sequences in **Table 2**).

siRNA transfection

Nalm-6 cells were transfected with siRNA targeting OGT (OGT-siRNA, GenePharma) or negative control (NC) siRNA (GenePharma) using Lipofectamine® 2000 (Invitrogen). The sequences of the siRNAs were: OGT-siRNA, sense, 5'-GCUUGCAAUUCAUCACUUUTT-3', and anti-sense, 5'-AAAGUGAUGAAUUGCAAGCTT-3'; NC-siRNA, sense, 5'-UUCUCCGAACGUGUCACGUT-T-3', and anti-sense, 5'-ACGUGACACGUUCGG-AGAATT-3'.

Extracellular flux analysis

We determined the bioenergetics of Nalm-6 cells using the XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA). Cells ($5×10^4$) were cultured in specialized cell culture plates coated by Cell-Tak (BD Biosciences). The extracellular acidification rate (ECAR) was detected using the XF Glycolysis Stress Test kit (Seahorse Bioscience) strictly in accordance with the user's guide.

Flow cytometry

The purity of sorted CD19^{+} BM-MNCs was detected by flow cytometry with APC-conjugated mouse anti-human CD19 antibody (555415, BD Biosciences) and APC-conjugated mouse IgG1, κ-isotype control (555751, BD Biosciences). To identify apoptotic cells after OGT-siRNA or 2-DG (0, 2.5, and 5 mmol/L) treatment, Nalm-6 cells were stained with a FITC Annexin V Apoptosis Detection Kit (BD Biosci-
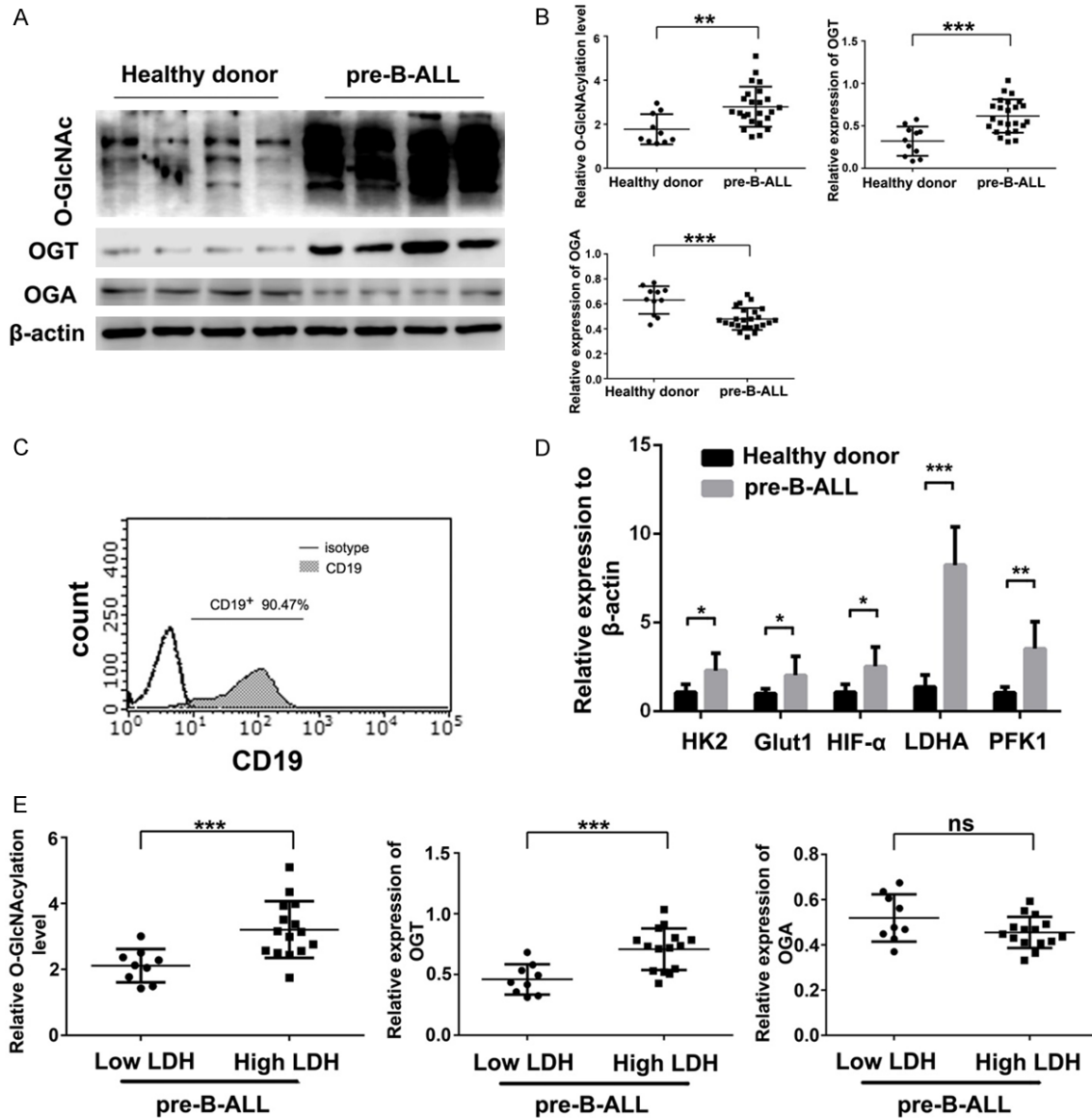
Figure 1. Pre-B-ALL cells exhibit elevated O-GlcNAcylation and overactive glycolysis. A, B. Western blot and statistical analyses of O-GlcNAcylation, and OGT and OGA expression from CD19+ BM-MNCs from 10 healthy donors and 24 pre-B-ALL patients. β-actin was used as a loading control. C. The separation purity after positive selection with CD19 MicroBeads compared to an isotype control. D. Levels of glycolysis-related enzyme mRNA quantified by qRT–PCR and normalized to β-actin. Differences were exhibited as fold changes compared with the healthy donor group. E. Statistical analysis of O-GlcNAcylation, OGT, and OGA isolated from CD19+ BM-MNCs from the low LDH group (n = 9) and the high LDH group (n = 15) by western blotting. *P < 0.05; **P < 0.01; ***P < 0.001. The results are expressed as mean ± SD.

ences). Flow cytometry was performed with a Guava® easyCyte 6HT-2L (Millipore), and the results were analyzed with guavaSoft™ 3.1.1 software (Millipore).

Cell counting kit-8 assay

We used the Cell Counting Kit-8 (CCK-8) assay (Dojindo) to measure the OGT-siRNA and

2-DG-induced inhibition of Nalm-6 proliferation. OGT-siRNA or 2-DG-treated cells (2×10⁴) were cultured in a 96-well plate. After treatment, 10 µL of CCK-8 reagent was added per well. After 2 h of continuous culture in an incubator, the absorbance values of the plates at 450 nm were detected using a spectrophotometer (Bio-Rad Laboratories) and recorded for analysis. The times of treatment and the con-

centrations of reagents are shown in the relevant figures and/or the corresponding figure legends.

Statistical analysis

All data were expressed as mean ± SD. Unpaired parametric (2-tailed Student's t-test) or nonparametric (Mann-Whitney U test) tests were used to compare between 2 independent groups. One-way ANOVA analysis was used when comparing differences among the means of 3 or more independent groups. All experiments were repeated independently 3 times. Prism 6 software (San Diego) and SPSS 18.0 (IBM) were used for statistical analyses, and differences where P < 0.05 were considered statistically significant.

Results

O-GlcNAcylation and glycolysis are increased in pre-B-ALL cells compared with normal B cells

Abnormal O-GlcNAcylation levels and expression of the key enzymes OGT and OGA occur in numerous malignant diseases. However, no clear evidence has emerged in pre-B-ALL. Protein extracts from the CD19+ BM-MNCs of 24 pre-B-ALL patients and 10 healthy controls were immunoblotted with RL2 antibody, which recognizes O-GlcNAc moieties. In addition to the O-GlcNAcylation level, we also evaluated changes in the protein expression of OGT and OGA. We found that O-GlcNAcylation was markedly increased (*P* < 0.01) in CD19+ BM-MNCs from pre-B-ALL patients compared with those from healthy donors; in addition, the expression of OGT was upregulated (*P* < 0.001), while OGA was downregulated in pre-B-ALL cells (*P* < 0.001) (**Figure 1A** and **1B**). The purity after CD19 MicroBead separation was up to 90.47% (**Figure 1C**).

O-GlcNAcylation is sensitive to metabolism, especially glycometabolism. Thus, we examined if pre-B-ALL CD19+ BM-MNCs had aberrant glycolysis. For this purpose, we measured several essential components of the glycolytic cycle, including hexokinase 2 (*HK2*), glucose transporter 1 (Glut1), hypoxia inducible factor 1 alpha (*HIF1A*), lactate dehydrogenase A (*LDHA*), and phosphofructokinase-1 (*PFK1*) via qRT-PCR. In comparison with the healthy cells, we found that pre-B-ALL cells had upregulated

mRNA levels of all these factors, especially 5.951-fold and 3.366-fold increases in *LDHA* and PFK1, respectively (**Figure 1D**).

LDH is an important enzyme in glycolysis [25]. To further understand the relationship between O-GlcNAcylation and glycolysis, we compared the O-GlcNAcylation levels in groups of pre-B-ALL patients with different LDH levels. We found that patients with higher LDH levels exhibited considerably higher O-GlcNAcylation levels (*P* < 0.001) and OGT expression (*P* < 0.001), compared with the lower LDH patients; however, they did not have significantly different OGA levels (**Figure 1E**). These findings indicated a positive correlation between O-GlcNAcylation and plasma LDH levels in pre-B-ALL patients. These findings suggested that O-GlcNAcylation may be involved in the regulation of glycolysis.

The glycolytic pathway is regulated by the PI3K/Akt/c-Myc pathway in pre-B-ALL cells

To date, several reports have showed that c-Myc is a downstream signaling molecule in the PI3K/Akt pathway [26, 27], but its function is unclear in pre-B-ALL cells. We employed Nalm-6 cells as a model for pre-B-ALL; we treated them with various concentrations of the PI3K/Akt pathway inhibitor LY294002 for 48 h, at which point phosphorylation of Akt (*P* < 0.001) and c-Myc (*P* < 0.001) were markedly decreased (**Figure 2A** and **2B**). Therefore, the PI3K-Akt pathway may regulate the expression of c-Myc in pre-B-ALL.

To investigate the relationship between the PI3K/AKT/c-Myc pathway and glycolysis, we investigated if the PI3K/Akt/c-Myc pathway is more active in the high LDH pre-B-ALL CD19+ BM-MNCs than in cells from the low LDH group. As expected, we found that the protein expression of PI3K (*P* < 0.05) and the phosphorylation of Akt (*P* < 0.05) and c-Myc (*P* < 0.05) were markedly increased in the high LDH pre-B-ALL group (**Figure 2C** and **2D**). To further substantiate that glycolysis can be regulated by the PI3K/Akt/c-Myc pathway, we cultivated Nalm-6 cells with LY294002 and measured the production of lactic acid via the ECAR to evaluate the impact on glycolysis. As expected, treatment with LY294002 resulted in an evident decrease in the baseline ECAR (*P* < 0.01), glycolytic capacity (*P* < 0.001), and glycolytic reserve (*P* < 0.01) (**Figure 2E** and **2F**). Taken together, these
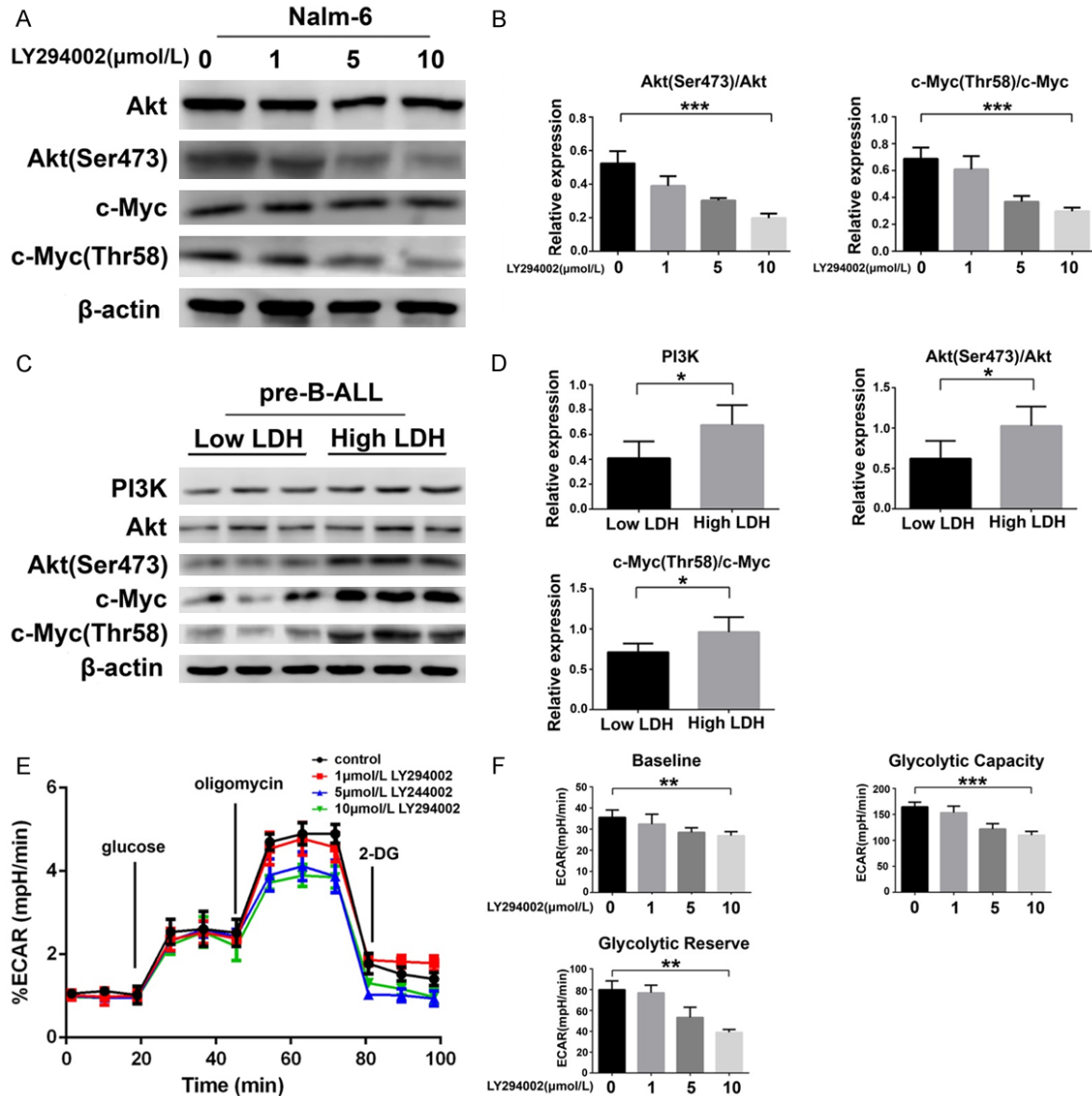
Figure 2. Glycolysis is regulated by the PI3K/Akt/c-Myc pathway in pre-B-ALL cells. A, B. Western blot and statistical analyses of the phosphorylation of Akt (Ser473) and c-Myc (Thr58) in Nalm-6 cells treated with LY294002 (0, 1, 5, and 10 μmol/L). C, D. Western blotting of proteins in the PI3K/Akt/c-Myc signaling pathway, and statistical analysis of PI3K expression, and the phosphorylation of Akt (Ser473) and c-Myc (Thr58) in CD19^{+} BM-MNCs from low LDH and high LDH pre-B-ALL patients. E, F. ECAR in Nalm-6 cells treated with LY294002 (0, 1, 5, and 10 μmol/L) for 48 h. The relative ECAR (% ECAR) is shown as a percentage of the baseline measurement (set as 100%). Statistical analyses of the baseline ECAR, glycolytic capacity, and glycolytic reserve are also shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. The results are expressed as mean ± SD.

results indicate that the PI3K/Akt/c-Myc pathway is essential for glycolysis in pre-B-ALL cells.

Suppression of OGT-mediated O-GlcNAcylation affects glycolysis via the PI3K/Akt/c-Myc pathway

To investigate if the change in O-GlcNAcylation is related to the inhibition of glycolysis in pre-B-

ALL cells, we downregulated O-GlcNAcylation with OGT-siRNA and harvested the cells 48 h after transfection. To confirm the interference efficiency, we measured the protein expression of OGT by western blot, which indicated high efficiency by the OGT-siRNA (**Figure 3A**).

Given that we had successfully constructed a low O-GlcNAcylation cell model, we next exam-
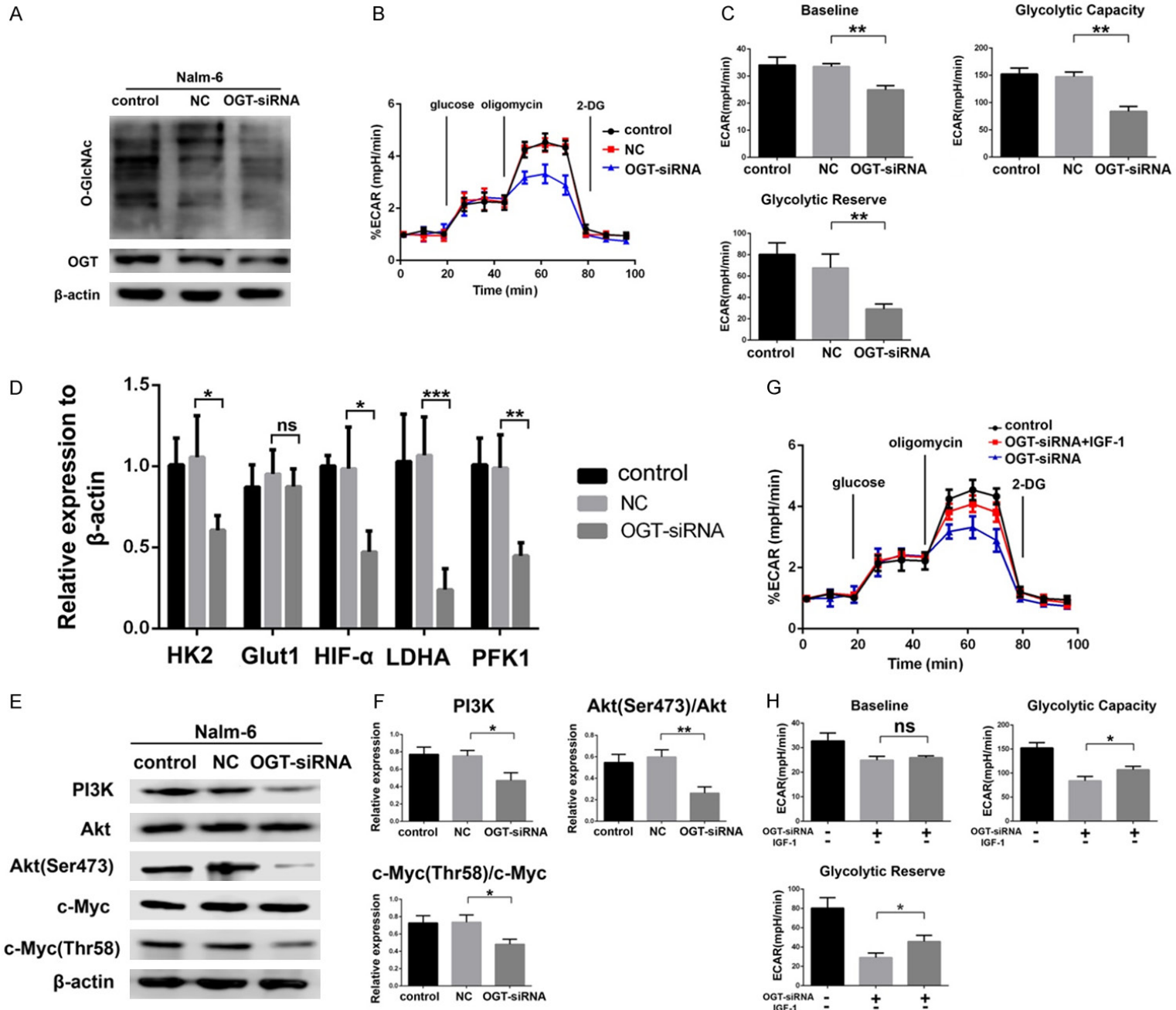
Figure 3. OGT-mediated O-GlcNAcylation could regulate glycolysis via the PI3K/Akt/c-Myc pathway. (A) Cell lysates from Nalm-6 cells transfected with double-distilled H_2O (control), negative control siRNA (NC), or OGT-siRNA by Lipofectamine® 2000 for 48 h were collected for western blot and statistical analysis of O-GlcNAcylation and OGT expression. (B) ECAR was measured in Nalm-6 cells transfected with control, NC, or OGT-siRNA. The relative ECAR (% ECAR) is shown as a percentage of the baseline measurement (set as 100%). Statistical analyses of baseline ECAR, glycolytic capacity, and glycolytic reserve shown in (C). (D) Levels of glycolysis-related enzyme mRNA quantified by qRT–PCR and normalized to β-actin in Nalm-6 cells transfected with control, NC, or OGT-siRNA for 48 h. (E, F) Western blot of proteins in the PI3K/Akt/c-Myc signaling pathway and statistical analyses of the phosphorylation of PI3K, Akt (Ser473), and c-Myc (Thr58) in Nalm-6 cells transfected with control, NC, or OGT-siRNA for 48 h. (G, H) The ECAR was measured in the OGT-siRNA group and the group that received co-treatment with OGT-siRNA and IGF-1 (an activator of PI3K). $*P < 0.05$; $**P < 0.01$. The results are expressed as mean ± SD.

ined if the suppression of OGT affected glycolysis. Nalm-6 cells transfected with OGT-siRNA exhibited a decrease in their baseline ECAR ($P < 0.01$), glycolytic capacity ($P < 0.01$), and glycolytic reserve ($P < 0.01$) (**Figure 3B** and **3C**). To confirm that the knockdown of OGT regulated glycolytic metabolism, we measured specific glycolysis-related molecules using qRT-PCR, which revealed that the mRNA levels of HK2, HIF-α, LDHA, and PFK1 were markedly deceased compared to the levels in the nontransfected cells (**Figure 3D**). PI3K, Akt, and c-Myc are O-GlcNAcylated; thus, it is likely that OGT regulates the PI3K/Akt/c-Myc pathway in pre-B-ALL cells. As expected, the knockdown of OGT decreased the expression of PI3K, as well as the phosphorylation of Akt and c-Myc (**Figure 3E** and **3F**).

To further demonstrate the function of the PI3K/Akt/c-Myc pathway in O-GlcNAcylation-induced alterations to glycolysis, we measured the ECAR in Nalm-6 cells treated with IGF-1 (an activator of PI3K) after OGT-siRNA knockdown. IGF-1 rescued the reduction in the ECAR caused by OGT knockdown (**Figure 3G** and **3H**). Overall, our data suggested that the suppression of OGT-mediated O-GlcNAcylation affects glycolysis via the PI3K/Akt/c-Myc pathway.

Downregulation of OGT induces cell apoptosis and potentiates the cytotoxicity of 2-DG in Nalm-6 cells

Given that OGT is a critical regulator of glycolysis, we examined if changes in glycolysis influenced O-GlcNAcylation and OGT. 2-DG is a known glycolysis inhibitor that targets HK2 [28]. HK2 is reported to be antineoplastic via targeting metabolic pathways in cancer cells [29, 30], but its function in pre-B-ALL cells is unclear. After the treatment of Nalm-6 cells with different concentrations of 2-DG for 72 h, western blotting revealed that O-GlcNAcylation

and OGT expression were reduced (**Figure 4A** and **4B**). This finding indicated that OGT-mediated O-GlcNAcylation is suppressed when glycolysis is inhibited.

To elucidate the function of OGT in 2-DG-induced cytotoxicity to Nalm-6 cells, we performed CCK-8 assays and flow cytometric analyses of annexin V/propidium iodide staining to assess cell proliferation and apoptosis. The CCK-8 assay indicated that treatment with OGT-siRNA alone induced the suppression of Nalm-6 proliferation at 72 and 96 h ($P < 0.01$ for OGT-siRNA versus NC, **Figure 4C**). However, decreased Nalm-6 cell proliferation was observed after treatment with 2.5 mM 2-DG for 48 h ($P < 0.001$ for 2-DG versus control, **Figure 4D**). Significantly higher cytotoxicity was detected in Nalm-6 cells treated with both OGT-siRNA and 2-DG than in cells treated with 2-DG alone at 48 h ($P < 0.01$ for 2-DG+OGT-SiRNA versus 2-DG). By contrast, a negligible effect was detected when these cells were treated with OGT-siRNA alone at 48 h (**Figure 4C** and **4D**). On the basis of the CCK-8 assay results, we next used flow cytometric analysis to assess cell apoptosis. We found that the knockdown of OGT for 72 h induced an approximately 2-fold increase in cell apoptosis over the NC group (21.72 ± 1.903% versus 11.07 ± 1.069%, **Figure 4E** and **4F**). Consistent with the CCK-8 assay results, treatment with 2-DG caused cell apoptosis (34.66 ± 2.566% versus 11.07 ± 1.069%), which was potentiated by OGT-siRNA (52.40 ± 3.037% versus 34.66 ± 2.566%, **Figure 4E** and **4F**) in Nalm-6 cells.

Discussion

Pre-B-ALL is a hematopoietic malignancy defined as the malignant proliferation of early B lymphoid precursors that turns into clonal expansion [31]. Although pre-B-ALL is sensitive to chemotherapy, 20% to 30% of pre-B-ALL
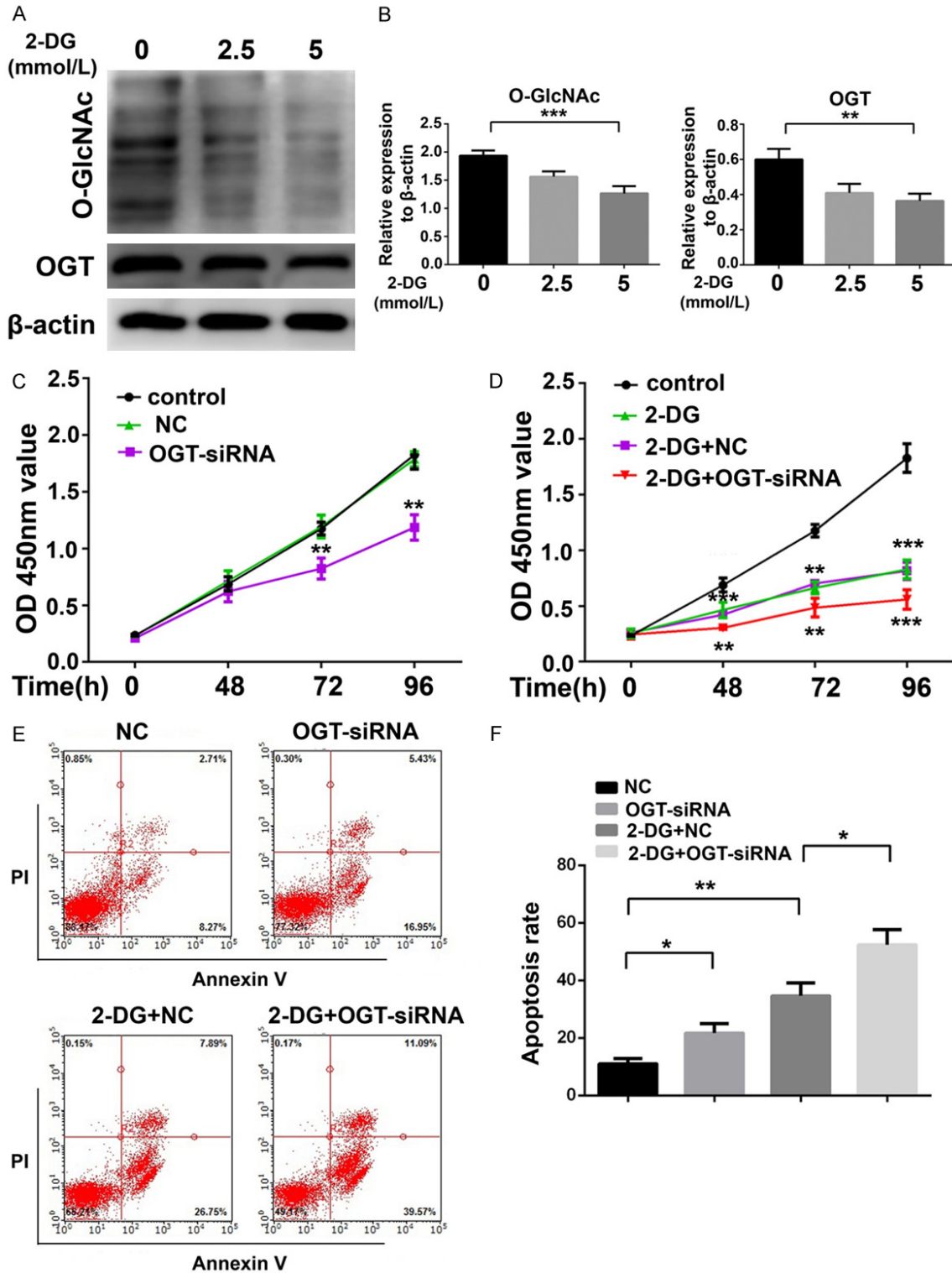
Figure 4. Suppression of OGT induces cell apoptosis and potentiates the cytotoxicity of 2-DG in Nalm-6 cells. A, B. Western blot and statistical analyses of O-GlcNAc and OGT in Nalm-6 cells treated with 2-DG (0, 2.5, and 5 mmol/L) for 48 h. C. Proliferation of Nalm-6 cells transfected with OGT-siRNA and NC-siRNA measured by CCK-8 assay. D. Proliferation of Nalm-6 cells transfected with OGT-siRNA or co-treated with OGT-siRNA and 2-DG (2.5 mmol/L) measured by CCK-8 assay. E, F. Cell apoptosis and statistical analyses of Nalm-6 cells transfected with OGT-siRNA, or co-treatment with OGT-siRNA and 2-DG (2.5 mmol/L) for 72 h. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The results are expressed as mean ± SD.

patients cannot achieve complete remission and eventually relapse because of drug resistance and immune escape [32]. Moreover, although the pathogenesis of pre-B-ALL at the genetic level is known, the role of O-GlcNA-cylation of proteins in pre-B-ALL is not fully understood.

The results of this study demonstrated for the first time that the level of O-GlcNAcylation in CD19^{+} pre-B-ALL BM-MNCs was significantly higher than in healthy cells, and was accompanied by the upregulation of OGT and the downregulation of OGA. Furthermore, higher O-GlcNAcylation levels and overactivation of the PI3K/Akt/c-Myc pathway were detected in pre-B-ALL cells from patients with higher LDH levels. Knockdown of OGT downregulated the key enzymes in glycolysis and induced a significant decrease in the ECAR; however, IGF-1, an activator of PI3K, rescued the inhibition. In addition, knockdown of OGT slowed proliferation and caused apoptosis in Nalm-6 cells. Combination treatment with 2-DG and OGT-siRNA led to more serious cytotoxicity to Nalm-6 cells. These results demonstrated the ability of O-GlcNAcylation to aggravate pre-B-ALL through regulation of glycolysis via the PI3K/Akt/c-Myc pathway.

Increasing evidence suggests that O-GlcNAcylation may be strongly related to the pathogenesis and progression of certain cancers [33], such as prostate [34], breast [35], colorectal [36], liver [37], and pancreatic cancers [38]. At present, aberrant O-GlcNAcylation and its 2 key enzymes are regarded as biomarkers of certain cancers. An earlier report suggested that the gene expression of OGA decreases by about 56% when screening from 40 candidate genes in breast cancer [39]. More recently, O-GlcNAcylation levels were found to be associated with poor prognosis in prostate cancer patients, and siRNA-mediated OGT knockdown enhanced prostate cancer cell proliferation and invasion [40]. In our study, we found that O-GlcNAcylation is significantly enhanced in CD19^{+} pre-B-ALL BM-MNC protein extracts, along with increased OGT and decreased OGA expression. Furthermore, the knockdown of OGT expression via siRNA resulted in not only decreased cell proliferation, but also increased cell apoptosis in Nalm-6 cells. Therefore, increased O-GlcNAcylation and aberrant expression of the O-GlcNAc cycling enzymes could

be regarded as a biomarker for the oncogenesis and tumor progression of pre-B-ALL. Moreover, OGT targeting may be a novel treatment for pre-B-ALL.

Despite increasing evidence for the critical role of O-GlcNAcylation in various cancers, the molecular mechanisms of the PTM in leukemia are unclear. Here, we detected a positive correlation between O-GlcNAcylation levels and plasma LDH from clinical cases. LDH, which includes the subunits LDHA and LDHB, is critical in aerobic glycolysis [41]. Recently, LDHA was established to play an essential role in the maintenance and progression of tumors [42]. Furthermore, cancer cells rely on aerobic glycolysis to generate energy to maintain cell growth [18]. Therefore, we investigated if O-GlcNAcylation correlated with glycolysis in pre-B-ALL. We first found that glycolysis is overactive in pre-B-ALL cells. Then, we utilized siRNA to downregulate the expression of OGT and found decreased mRNA expression of glycolytic-related enzymes). Among the glycolytic enzymes examined, LDHA and PFK1 were markedly reduced, as was previously reported with regard to PFK1 modification by O-GlcNAc at Ser-529 [43]. These data provide evidence that the molecular mechanism of O-GlcNAcylation-induced cytotoxicity may be the inhibition of glycolysis.

The PI3K/Akt pathway is aberrantly active in a large number of malignancies, including leukemia. Consistent with previous research [26, 27], our study confirmed that c-Myc is a downstream molecule in the PI3K/Akt pathway and that constitutively activated PI3K/Akt/c-Myc existed in the cells of pre-B-ALL patients with high LDH. Akt and c-Myc undergo both phosphorylation and O-GlcNAcylation [44]. However, the relationship between OGT and PI3K/Akt/c-Myc in glycolysis remains unclear. In this study, we silenced the expression of OGT via OGT-siRNA and found that PI3K and the phosphorylation of Akt and c-Myc were all decreased in Nalm-6 cells. The findings indicate that OGT may participate in regulating the activation of the PI3K pathway in pre-B-ALL. To evaluate the change in glycolysis, we directly measured lactic acid production via an XF instrument to determine ECAR values. Glycolytic capacity is the maximum potential ECAR level when mitochondrial ATP production is inhibited by oligomycin. A decreased ECAR caused by blocking

glycolysis with 2-DG is defined as the glycolytic reserve. When Nalm-6 cells were treated with a PI3K inhibitor or OGT-siRNA, their baseline ECAR level, glycolytic capacity, and reserve deceased to varying degrees. In addition, co-treatment of Nalm-6 cells with OGT-siRNA and IGF-1 (an activator of PI3K) increased their ECAR compared with that of the OGT knockdown group. These results reveal that the PI3K/Akt/c-Myc pathway plays a key role in the changes in glycolysis caused by the knockdown of OGT.

Numerous cancer cells satisfy more of their energy requirements by glycolysis than by mitochondrial oxidative phosphorylation [45]. Although glycolysis generates less ATP, the rate of production can be 100 times faster to meet energy demands [46]. Various glycolytic enzymes, such as GLUT1 [47], hexokinase [48], and LDHA [49], have been targeted for therapy in tumors. Here, we demonstrated that the HK2 inhibitor 2-DG could induce cytotoxicity to Nalm-6 cells. Nalm-6 cells treated with 2-DG displayed significant upregulation of OGT protein expression and higher O-GlcNAcylation levels. Moreover, we found that OGT knockdown could potentiate the cytotoxicity caused by 2-DG. Our findings suggest that a combination therapy of glycolytic inhibitors with an O-GlcNAcylation inhibitor may be a powerful and effective treatment for human leukemia.

Therefore, our study is the first to demonstrate, using clinical cases, that O-GlcNAcylation and its key enzymes could be regarded as pre-B-ALL biomarkers. This study is also the first to examine the key role of OGT in glycolysis mediated by the PI3K/Akt/c-Myc signaling pathway. OGT targeting could be applied therapeutically in the future. A combination of glycolytic inhibitors with O-GlcNAcylation inhibitors may be an effective therapy for clinical use in pre-B-ALL.

Acknowledgements

Disclosure of conflict of interest

None.

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