Glucose transporter 2-transported glucosamine inhibits glycolysis in cancer cell lines through competition with glucose for hexokinase II

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Abstract. Antiproliferative effects of glucosamine, a glucose derivative with a similar structure to glucose, have been discovered, but the molecular mechanisms are not yet fully understood. Since glucosamine and glucose not only have similar structures but also are catalyzed by the same enzyme, hexokinase (HK), the present study delved into determining whether the antiproliferative effect of glucosamine involved the inhibition of glycolysis by competition with glucose. Whole-genome screening analysis showed that a number of the gene pathways controlled by glucosamine were directly and indirectly involved in glycolysis. *In vitro* experiments revealed that as more glucose was added, the antiproliferative effect of glucosamine decreased. Also, it was found that glucosamine was transported into cells mainly through glucose transporter (GLUT) 2 which was responsible for the antiproliferative effects of glucosamine. In addition, the present study found that cancer cell lines with low expression level of HKII show high sensitivity to glucosamine and a HK inhibitor, 3-bromopyruvate, enhanced the antiproliferative effect of glucosamine. Under hypoxic conditions, activated hypoxia-inducible factor 1α (HIF-1α) inducing glucose uptake and glycolysis hampered glucosamine-induced cell death and *HIF1A* knockdown or HK inhibitors restored the antiproliferative effects of glucosamine. These findings demonstrated that glucosamine is an efficient glycolysis inhibitor and that GLUT2 and HKII play important roles as biomarkers for determining sensitivity to glucosamine. Moreover, the results suggested that the antiproliferative effect of glucosamine may be more efficient when administered in combination with other glycolytic agents or inhibitors targeting HIF-1α.

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Introduction

Glucosamine is a natural amino sugar that is used as one of the building blocks of the body, such as in cartilage and tendon. Therefore, it has been used to treat patients with painful knee osteoarthritis and in complementary and alternative medicine as an osteoarthritis cure (1,2). In addition, a number of studies have reported that glucosamine inhibits cell proliferation and induces cell cycle arrest and apoptosis in various types of cancer (3,4). Other studies have described target proteins of glucosamine, such as transglutaminase 2, p70S6K, hypoxia-inducible factor 1α (HIF-1α), cyclooxygenase-2 (COX-2) and the insulin-like growth factor 1 receptor (IGF-1R)/Akt pathway (5-9). These studies propose that glucosamine has potential value as an antiproliferative drug.

Facilitative glucose transporters (GLUTs) are integral membrane proteins that have 12 transmembrane domains and a binding site for hexose substrates such as glucose, fructose and glucosamine (10). A total of 13 GLUT isoforms with highly conserved amino acid sequences and 12 hydrophobic α-helical domains have been identified and characterized in mammalian cells. Each GLUT isoform has a different expression level in various tissues and organs and plays a specific role in the energy-independent uptake of hexoses. For instance, GLUT1, which is highly expressed in all tissues, is in charge of basal glucose transport; GLUT2, which is abundant in liver, pancreatic islet and retinal cells, has a higher affinity for glucosamine than glucose (11).

Cancer cells undergo metabolic reprogramming to support their rapid growth and proliferation. One hallmark of this reprogramming is the Warburg effect, which describes the preference of cancer cells for aerobic glycolysis, even in the presence of sufficient oxygen (12). This phenomenon allows cancer cells to generate ATP quickly while producing metabolic intermediates essential for biosynthesis. A number

of previous studies have also demonstrated that GLUTs are overexpressed in various types of cancer, facilitating increased glucose uptake to sustain the heightened glycolytic flux. This overexpression of GLUT proteins has been closely associated with aggressive tumor behavior, including metastasis and poor prognosis (13,14).

Once hexoses are transported into the cytoplasm, they are catalyzed by hexokinases (HKs), which carry out the first and rate-limiting step of the hexose metabolic pathway. In addition to glycolysis, HK also performs the irreversible step in the hexosamine pathway that converts hexose to hexose-6-phosphate, using an ATP molecule to start the process (15). In humans and other mammals, four HK isoforms have identified; HKI, II, III and IV (glucokinase). HKI, II and III have a high affinity for glucose and a molecular mass of ~100 kDa, whereas HKIV has a molecular mass of ~50 kDa and can only phosphorylate glucose, giving it a higher K_m than the other HKs (16). During cancer progression, among those HKs, HKII, which has low affinity for glucose and is only slightly expressed in normal tissues, is markedly upregulated (17), suggesting that HKII plays an important role in malignancy.

HIF-1, a heterodimer consisting of α and β subunits, is stabilized under hypoxic conditions and plays a pivotal role in the glycolytic phenotype in human cancers (18). It has been reported that HIF-1α acts as a transcription factor and increases expressions of GLUTs and HKs and thus, tumor cells which are in hypoxic conditions activate HIF-1α to facilitate glucose uptake and glycolysis to satisfy their energy demands (19). Furthermore, since the metabolic reprogramming induced by HIF-1α helps cancer cells to grow, cancer cells turn on HIF-1α even with enough oxygens, a phenomenon called pseudohypoxia (20). These features make cancer cells more resistant to glycolysis inhibitors, such as 2-deoxy-D-glucose (2-DG), by increasing the number of glycolytic enzymes (21).

Malignant cancer cells have an elevated and accelerated glucose metabolism due to increased requirements for glucose, which is essential as an energy source (17). In addition, dysfunction of the Krebs cycle caused by mitochondrial mutations leads to increased dependence on glycolysis by cancer cells for ATP production (22). Therefore, it is not surprising that the increased aerobic glycolysis in cancer is closely related to overexpression of GLUTs and HKII in various types of cancer. A number of researchers have used glucose analogs such as fluorodeoxyglucose and 2-DG and HKII inhibitors such as 3-bromopyruvate (3-BrPA) to study interference of glucose uptake and glycolysis (15,23). As stated for the aforementioned molecules, these results indicate that glucosamine also may be a glycolysis inhibitor.

Based on these preliminary studies, the present study sought to determine whether the antiproliferative activity of glucosamine occurs because it is a glycolysis inhibitor in various cancer cells and whether GLUTs, HK isoforms and HIF-1α affect the sensitivity to glucosamine of cancer cells. It showed a new mechanism of glucosamine to specifically inhibit the glycolytic pathway to suppress cancer progression.

Materials and methods

Cell lines and agents. Human liver cancer cell lines (HepG2 and Hep3B), non-small cell lung cancer cell lines (A549, H1299 and H460) and breast cancer cell lines (MCF-7, MDA-MB-231 and T47D) were purchased from the American Type Culture Collection and cultured in RPMI 1640 (PAA Laboratories GmbH) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH) and 100 U/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. The authenticity of all eight cell lines used in the present study was validated by short tandem repeat (STR) DNA profiling, which was performed using PowerPlex 18D system by commercial service (Cosmo Genetech).

D-(+)-glucose, D-(+)-glucosamine hydrochloride, cytochalasin B and phloretin were purchased from MilliporeSigma, while 2-DG and 3-BrPA were purchased from Tokyo Chemical Industry. In the competition experiment between glucose and glucosamine, glucose-free RPMI 1640 medium (MilliporeSigma) was used and cells were pre-incubated for 24 h with the indicated doses of glucose prior to being treated with glucosamine.

Semiquantitative RT-PCR. Total RNA was isolated from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and chloroform extraction. First-strand cDNA was synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Semiquantitative PCR was performed in a thermal cycler (MyCycler; Bio-Rad Laboratories, Inc.) using gene-specific primer sets and sequences of primers are listed in the supplementary Table SI. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 25-45 cycles of denaturation at 95˚C, annealing at 42-60˚C for 30 sec (depending on the target gene), and elongation at 72˚C for 1 min, with a final extension at 72˚C for 5 min. The reactions were stopped during the exponential phase to ensure accurate comparisons. *ACTB* was used as a reference gene. PCR products were electrophoresed on 1.2-1.5% agarose gel and visualized using ethidium bromide.

siRNA transfection. A549 and HepG2 cells ($2.5x10^5$ cells/well) were seeded in 6-well plates and transfected with double-stranded small interfering RNA (siRNA). Target-specific siRNAs were designed for knockdown of *GLUT1*, *GLUT2* and *HIF1A* were from Bioneer Corporation and MBiotech, respectively. A scrambled siRNA (siSCR) was synthesized and validated by Bioneer Corporation. The sense and anti-sense sequences of siRNAs are listed in the Table SII. Following 25 pmole of siRNA transfection for 24 h at 37˚C using Lipofectamine® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, subsequent experiments were conducted. Knockdown of *GLUT1*, *GLUT2 and HIF1A* expression were validated by semiquantitative RT-PCR. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 25-45 cycles of denaturation at 95˚C, annealing at 42-60˚C for 30 sec (depending on the target gene), and elongation at 72˚C for 1 min, with a final extension at 72˚C for 5 min. The reactions were stopped during the exponential phase to ensure accurate comparisons. *ACTB* was used as a reference gene. PCR products were

electrophoresed on 1.2-1.5% agarose gel and visualized using ethidium bromide.

Western blotting. Whole cell lysates were prepared using RIPA-B lysis buffer containing 150 mM NaCl, 1 mM EDTA (pH 8.0), 20 mM Tris-Cl (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM Na_3VO_4, 5 mM NaF, 10% glycerol and protease inhibitor cocktail (Roche Diagnostics GmbH). Protein concentration was determined using BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equivalent amounts of proteins (10-20 μg per lane, depending on the target protein) were separated by electrophoresis on 6, 8, 10 or 12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Pall Corporation). Membranes were blocked with 5% skimmed milk at room temperature for 1 h. The membranes were then incubated overnight at 4˚C with primary antibodies against phosphorylated (p-)Akt (1:1,000, cat. no. 9271, Cell Signaling Technology, Inc.), Akt (1:1,000, cat. no. 4691, Cell Signaling Technology, Inc.), a/β-tubulin (1:1,000, cat. no. 2148, Cell Signaling Technology, Inc.), IGF-1R (1:1,000; cat. no. sc-713, Santa Cruz Biotechnology, Inc.) and COX-2 (1:1,000; cat. no. sc-1745, Santa Cruz Biotechnology, Inc.). After washing with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat. no. AbC-5001 for anti-mouse antibody and cat. no. AbC-5003 for anti-rabbit antibody, Abclon) for 1 h at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence (AbSignal; Abclon).

MTT assay. Cells ($3.0x10^3$ cells/well) were seeded into 96-well plates, incubated overnight at 37˚C and treated with glucosamine or other agents. Control groups were given dimethyl sulfoxide (DMSO; 0.1% final concentration) or phosphate-buffered saline (PBS) vehicle. Cell viability was measured by MTT assay. An insoluble purple formazan precipitate was solubilized in DMSO and the absorbance was determined by microplate spectrometer at 562 nm.

Cell cycle analysis. A549 and HepG2 cells ($1.5x10^5$) were plated onto 60x15 mm dishes for 1 day and then cells were treated with glucose (1, 10, 20, or 50 mM) and glucosamine (1, 2, or 5 mM) at 37˚C. Glucose was administered for 48 h and glucosamine was co-administered with glucose during the last 24 h of that period. After treatment, cells were washed once with PBS, trypsinized and harvested. All the harvested cells were fixed in 70% ethanol for 1 h at 4˚C. After centrifugation for 5 min. at 1,000 x g and 4˚C, cell pellets were washed twice with PBS and then stained with PI containing RNase A (40 μg/ml) for 30 min at 4˚C in the dark. Total DNA contents were detected by BD FACSCalibur flow cytometer (BD Biosciences), and analyzed using FlowJo 10.10.0 (FlowJo LLC).

Apoptosis analysis. A549 and HepG2 cells ($1.5x10^5$) were plated onto 60x15 mm dishes for 1 day and after treated with glucosamine for 48 h, cells were harvested and washed twice with PBS on ice. Then, cells were stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen; BD Biosciences). Cells were resuspended in 1x binding buffer containing 5 μl of fluorescein isothiocyanate (FITC)-Annexin V and 5 μl of PI. After staining, cells were detected by BD FACSCalibur flow cytometer (BD Biosciences) at 488 and 633 nm, and analyzed using FlowJo 10.10.0 (FlowJo LLC). The apoptotic rate was calculated as the percentage of early apoptotic cells and the percentage of late apoptotic cells. All procedures were carried out according to the manufacturer's instructions.

Measurement of glucosamine consumption rate. Cells ($2.5x10^5$ cells/well) were seeded in 6-well plates and incubated under RPMI 1640 medium containing the indicated concentrations of glucosamine. Glucosamine in harvested culture medium was reacted using D-Glucosamine Assay Kits (K-GAMINE; Megazyme) and optical density was measured with a spectrophotometer at 340 nm. All procedures were carried out according to the manufacturer's instructions.

Measurement of lactate production. The cellular glycolytic efficiency could be estimated by measuring the cellular lactate generation rate. Cells ($2.5x10^5$ cells/well) in the exponential growth phase were plated in 6-well plates for 1 day and cells were washed and treated with indicated concentrations of glucosamine for an additional 24 h. Aliquots of the culture medium were withdrawn and used for analysis of secreted lactic acid using the YSI 2300D STAT Plus Glucose and Lactate Analyzer (YSI Inc.).

Whole genome gene expression analysis. A549 cells were treated with DMSO or 5 mM of glucosamine for 24 h at 37˚C and RNA was isolated. From each sample, 500 ng of total RNA was biotinylated and amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cRNA yield was measured using RiboGreen RNA quantitation kit (Invitrogen; Thermo Fisher Scientific, Inc. and 750 ng of the cRNA sample was hybridized on a human HT-12 expression bead chip (Illumina, Inc.) profiling 48,804 transcripts per sample. The chips were stained with streptavidin and scanned using an Illumina BeadArray Reader (Illumina, Inc.). BeadStudio v3 (Illumina, Inc.) was used to quantile-normalize the data.

Gene ontology (GO) analysis was performed on genes that were increased or decreased by more than 2-fold in the glucosamine-treated sample compared with the control. The analysis was conducted using the gseGO function in the clusterProfiler package (ver. 4.14.3) (24) in R software (ver. 4.4.1, R Core Team, 2024) (25), based on the Gene Set Enrichment Analysis approach. GO terms which have adjust P-value <0.01 were considered markedly regulated GO. Up- or downregulated GO terms were featured by R software.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed on differentially expressed genes using the enrichKEGG function from the clusterProfiler package in R software. The result of glycolysis pathway (hsa00010) was visualized using the Pathview package (Release 3.20) (26).

Statistical analysis. All statistical analyses were conducted using GraphPad Prism 8.0 (Dotmatics) and Microsoft Excel

(Microsoft Corporation). For comparisons involving more than three groups, one-way ANOVA was performed, followed by Tukey's post hoc test. For comparisons between two groups, the Student's t-test was employed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Glucosamine regulates some gene sets related to glycolysis. In 1953, J.H. Quastel and A. Cantero demonstrated, for the first time, that D-glucosamine inhibits the tumor growth in a xenograft mouse model (27). After their findings, a number of studies have sought to discover the mechanism and related molecules in this antiproliferative effect. A number of studies have demonstrated that glucosamine decreases the ATP level in cancer cells and inhibits oncogenes, such as IGF-1R, HIF-1α and COX-2 (8,9,21). However, the mechanism has not been described in detail. Therefore, the present study used a microarray to screen genes that are regulated directly or affected indirectly by glucosamine. Genes induced by glucosamine more than two folds were categorized mainly into pro-apoptosis and cell death (Fig. 1A), while genes downregulated by glucosamine more than two folds mostly belonged to cell cycle and DNA damage responses (Fig. 1B). Next, the present study examined whether the genes involved in carbohydrate metabolism, biomolecules biosynthesis and glycosylation, which pathways are highly associated with glycolysis (28-30), are regulated by glucosamine. Notably, as shown in Table SIII, several genes upregulated by glucosamine more than twofold were involved in these pathways. Also, pathway enrichment of differential genes was used and then the KEGG pathway analysis results viewed to see whether the differential genes are enriched in glycolysis. As a result, differential genes are represented in the glycolysis pathway and their expression is primarily downregulated by glucosamine (Fig. 1C). With all these findings, glucosamine's similar structure to glucose and its reduction of ATP levels in cancer cells (4), as demonstrated by previous studies, it was assumed that glycolysis is the main target pathway of glucosamine.

Competitive relationship between glucose and glucosamine constrains the inhibitory effects of glucosamine in cancer cells. Other than its NH_2 group, glucosamine has a structure very similar to that of glucose. In fact, several studies have demonstrated that glucosamine competes with glucose in carbohydrate metabolism (31-33). Based on this, whether its antiproliferative effect is linked to this competitive relationship was investigated. A recent study has shown that glucosamine exhibits antiproliferative effects on a liver cancer cell line (34), while our previous research demonstrated similar effects on lung cancer cell lines (9). Therefore, the present study initially conducted experiments on HepG2 liver cancer cell line and A549 lung cancer cell line. Under low level of glucose, glucosamine inhibited proliferation of A549 and HepG2 cancer cells, which anti-proliferative effects of glucosamine were reversed by increasing glucose concentration (Fig. 2A). Glucose-treated cells also showed a dose-dependent recovery of the G0/G1 arrest induced by glucosamine (Fig. 2B). Since previous studies showed that glucosamine inhibits the IGF-1R/Akt pathway (9,35), the present study next investigated the effect of glucose on these target molecules. As expected, IGF-1R and p-Akt expression levels that had been reduced by glucosamine were restored as concentrations of glucose increased (Fig. 2C). To determine whether the competitive effect of glucose and glucosamine occurs during glycolysis in cancer cells, the level of lactate, which is produced from pyruvate during glycolysis was investigated. Glucosamine markedly suppressed lactate production, but this suppression was nearly reversed with a high dose of glucose (Fig. 2D). These findings suggested that the antiproliferative effect of glucosamine was closely related to competition between glucose and glucosamine, which occurs during glycolysis.

Antiproliferative effect of glucosamine is related to basal expression level of GLUT2 and basal activity of glucosamine consumption. Glucosamine enters the cell mostly via GLUT2, along with other isoforms of GLUT (11). Although several studies have examined the kinetics of glucosamine transport, there is no direct evidence on how glucosamine, particularly its antiproliferative effects, influences these kinetics. Thus, it was hypothesized that cancer cells with higher expression levels of GLUT2 were more sensitive to the antiproliferative effect of glucosamine. First, the expression level of *GLUT1* and *GLUT2* in cancer cell lines originating from various types of cancer including liver cancer, lung cancer and breast cancer was screened. For this purpose, two liver cancer cell lines (HepG2 and Hep3B), three lung cancer cell lines (A549, H1299 and H460) and three breast cancer cell lines (MCF-7, MDA-MB-231 and T47D) were used. Among them, liver cancer cell lines showed higher levels of *GLUT2* compared with the others, while most of the cell lines showed similar levels of *GLUT1* (Fig. 3A). Then, to assess the correlation between glucosamine uptake and *GLUT2* expression levels, a glucosamine consumption assay was performed and the results showed that liver cancer cell lines, which showed highly expressed *GLUT2*, consumed glucosamine more actively than others (Fig. 3B). Next, whether the expression level of *GLUT2* and the rate of glucosamine uptake are related to the antiproliferative effect of glucosamine was investigated. As a result, liver cancer cell lines, which showed higher expressions of *GLUT2* and actively consumed glucosamine, were more sensitive to glucosamine than others (Fig. 3C). Along with these results, HepG2 and Hep3B cells showed greater decreases in IGF-1R and p-Akt expression levels in the presence of glucosamine than A549 cells, which were the most sensitive among the remaining cell lines (Fig. 3D). Taken together, these results implied that the antiproliferative effect of glucosamine has a strong relationship with the expression level of GLUT2 and the uptake rate of glucosamine.

Antiproliferative effect of glucosamine is dependent on the absorption process of glucosamine into cancer cell lines through GLUT2, not GLUT1. Then it was explored whether cancer cells uptake glucosamine through GLUT2, not GLUT1, and whether the uptake of glucosamine is an essential step for its antiproliferative effects. A549 and HepG2 cells were treated with cytochalasin B, a GLUT1-specific inhibitor, or phloretin, a GLUT2-specific inhibitor, in combination with glucosamine, respectively. Cytochalasin B and phloretin concentrations were taken from previously

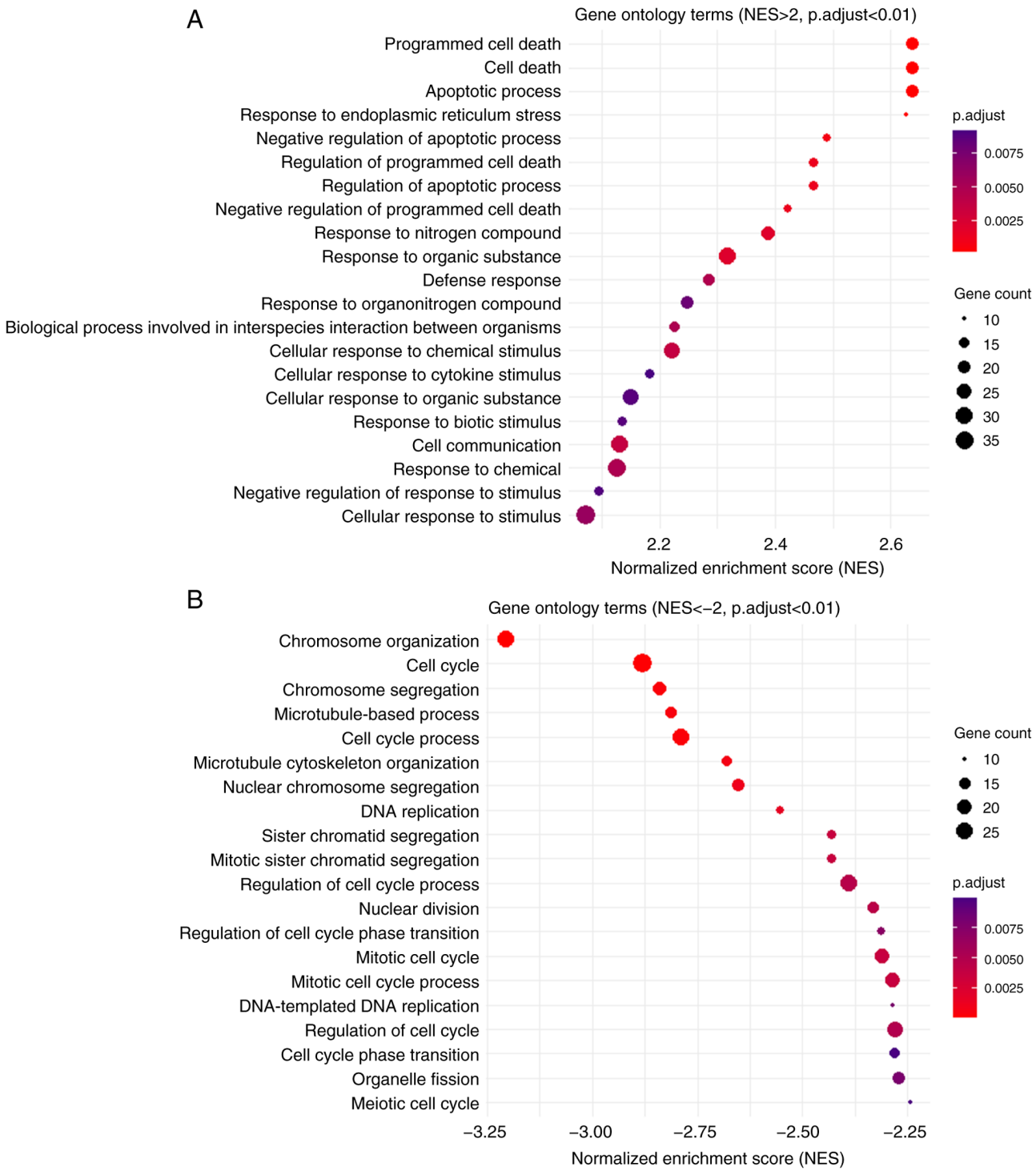

Figure 1. Continued.

reported studies (36,37). As a result, phloretin recovered glucosamine-induced downregulation of IGF-1R and p-Akt expressions in both cancer cell lines, while cytochalasin B showed no recovery effects in A549 cells and partial recovery effects in HepG2 cells (Fig. 4A). In addition, glucosamine shifted the molecular mass of COX-2 as previously reported (38), which were markedly reversed by phloretin, but not by cytochalasin B in A549 cells (Fig. S1). Following these changes in molecular features, phloretin-mediated GLUT2 inhibition sufficiently rescued A549 and HepG2 cells from glucosamine-induced cell death, while the effects of cytochalasin B were not significant (Fig. 4B). Along with the results using inhibitors, GLUT2-knockdowned HepG2 cells by transfection of si*GLUT2* showed recovery of IGF-1R and p-Akt expression levels decreased by glucosamine, while there were no changes in those expressions in GLUT1-knockdowned HepG2 cells (Fig. 4C). The present study also investigated whether treatment with phloretin or si*GLUT2*, which have an inhibitory effect on glucosamine transportation, could decrease consumption of glucosamine

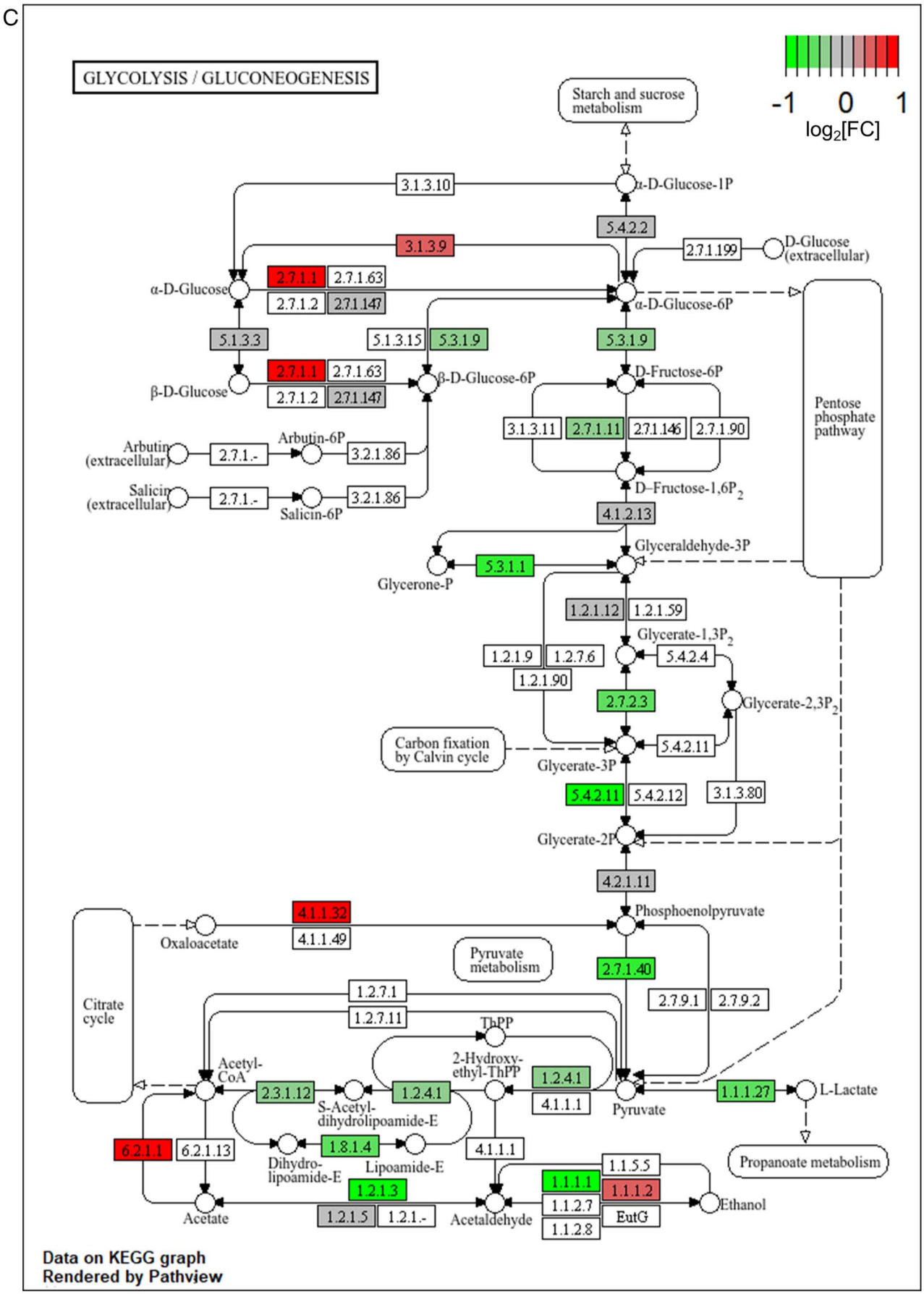

Figure 1. Whole genome gene expression analysis revealed that glucosamine regulates apoptosis and cell cycle. A549 cells were treated with 5 mM of glucosamine for 24 h and Whole genome gene expression analysis was performed. (A) Upregulated and (B) downregulated GO terms in the glucosamine treated sample compared with the control. GO terms possessing adjust P-value <0.01 were listed. (C) KEGG pathway enrichment analysis was performed and the result of the glycolysis pathway (hsa00010) was visualized with Pathview. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

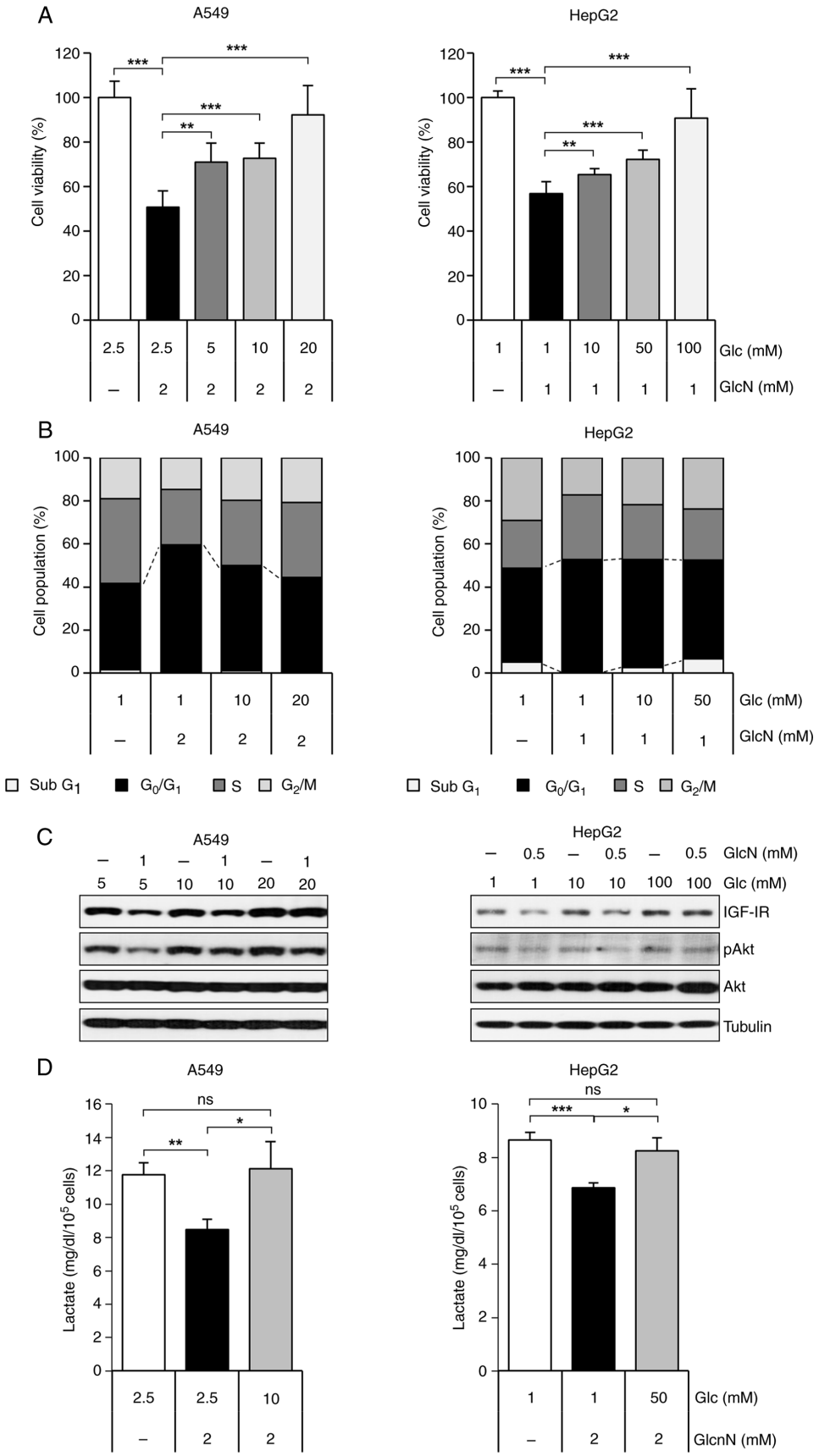

Figure 2. GlcN-induced anticancer effect occurs through competition with Glc. (A) Cell viability of A549 and HepG2 cells after treatment with Glc and GlcN for 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (B) Cell population by cell cycle of A549 and HepG2 cells after treated with Glc and GlcN. Cells were treated with Glc for 24 h, followed by additional 24 h treatment with GlcN. Cell cycle analysis was performed after propidium-iodide staining. (C) Western blot analysis of IGF-1R, p-Akt and Akt expression levels in A549 and HepG2 cells. Cells were treated with indicated concentrations of Glc and GlcN for 24 h. (D) Lactate production in A549 and HepG2 cell lines after treatment with Glc and GlcN for 24 h. Results are presented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ns, no significance; Glc, glucose; GlcN, glucosamine; p-, phosphorylated; IGF-1R, insulin-like growth factor 1 receptor.

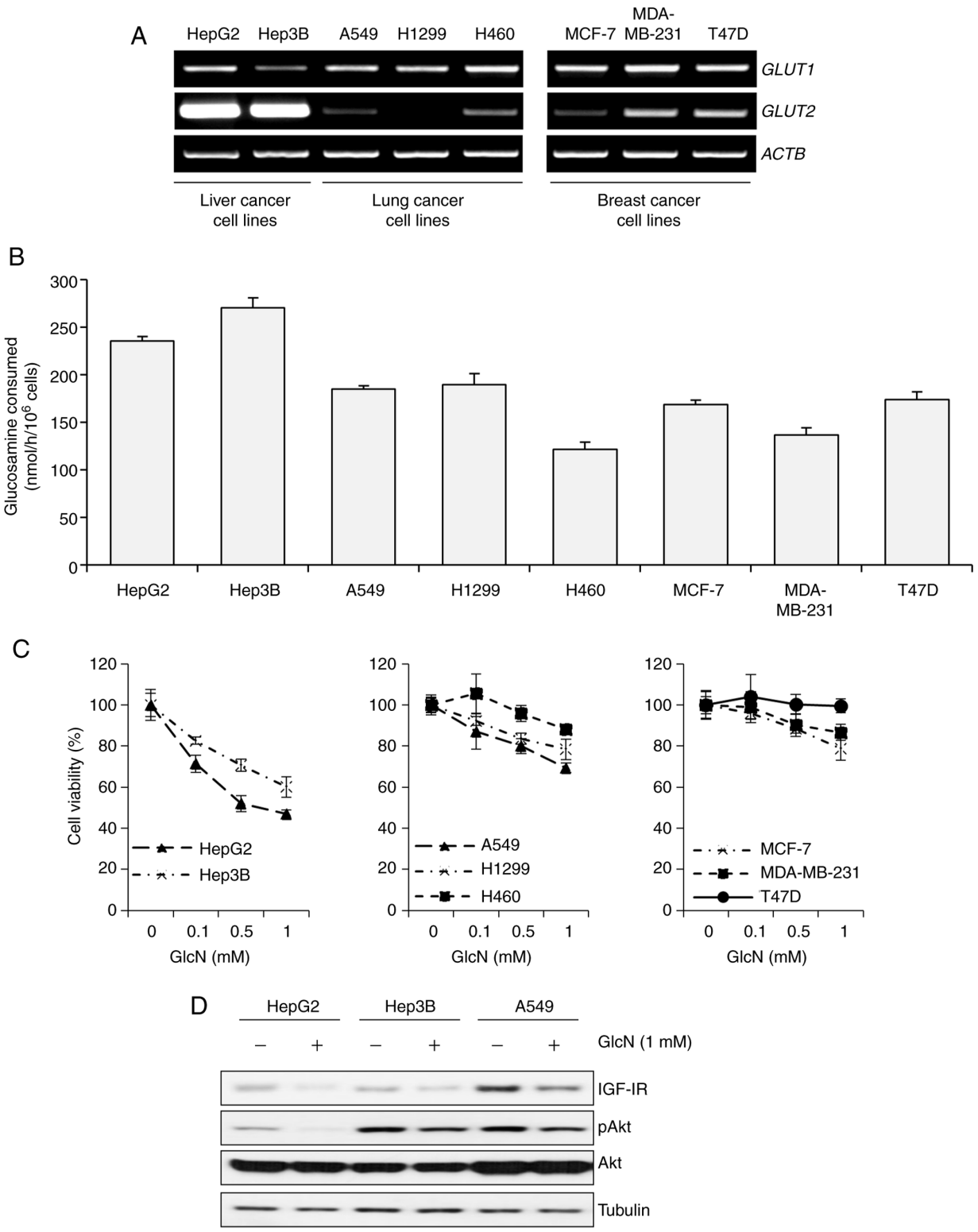

Figure 3. GLUT2 basal level is associated with GlcN sensitivity in liver, lung and breast cancer cell lines. (A) Semiquantitative PCR of *GLUT1* and *GLUT2* in various cancer cell lines. Total mRNA from the following cancer cell lines was isolated and used for analysis: HepG2, Hep3B, A549, H1299, H460, MCF-7, MDA-MB-231 and T47D. *ACTB* was included as a loading control. (B) GlcN consumption rates in the eight cell lines. Each cell line was incubated in Glc-free RPMI 1640 containing both 1 mM Glc and 5 mM GlcN for 6 h. Results are presented as mean ± standard deviation. (C) Cell viability of the eight cell lines after treated with GlcN for 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (D) Western blot analysis of IGF-1R, p-Akt and Akt expression levels in HepG2, Hep3B and A549 cells after 1mM of glucosamine treatment for 12 h. GLUT, glucose transporter; GlcN, glucosamine; IGF-1R, insulin-like growth factor 1 receptor.

in both cancer cell lines and it was found that glucosamine consumption rate was reduced when GLUT2 was inhibited by phloretin and si*GLUT2* (Fig. 4D). These findings demonstrate that the absorption of glucosamine via GLUT2 is the basic and necessary process for the antiproliferative effect of glucosamine.

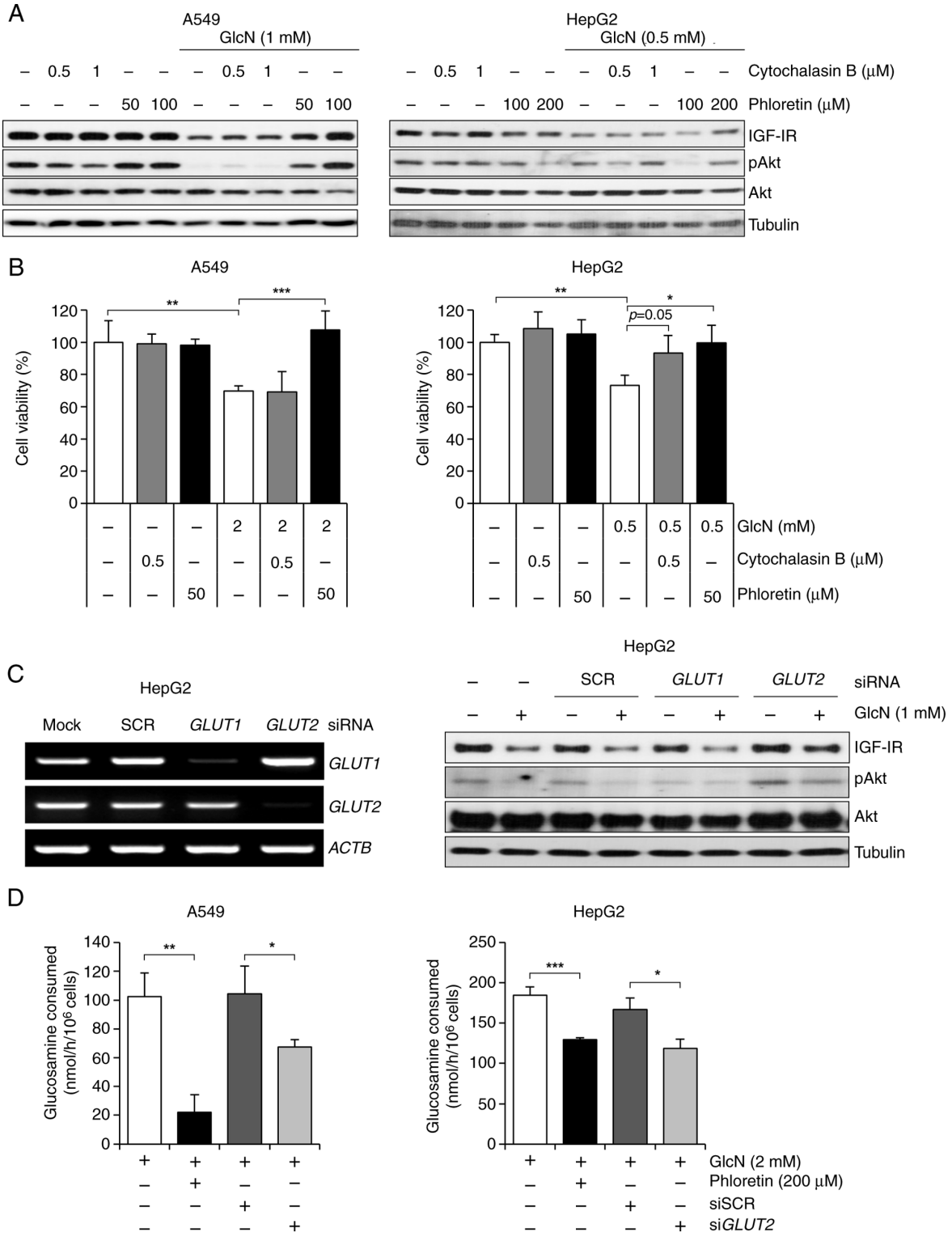

Figure 4. GlcN entrance into cancer cells through GLUT2 leads to its anticancer activities. (A) Western blot analysis of IGF-1R, p-Akt and Akt expression levels in A549 and HepG2 cells treated with cytochalasin B or phloretin for 24 h. Cytochalasin B was used as a GLUT1-specific inhibitor, while phloretin was used as a GLUT2-specific inhibitor. (B) Cell viability of A549 and HepG2 cells after treatment with glucosamine, cytochalasin B and phloretin for 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (C) Left: Semiquantitative PCR of *GLUT1* and *GLUT2* in HepG2 cells after transfection with siSCR, si*GLUT1*, or si*GLUT2*. *ACTB* served as a loading control. Right: Western blot analysis of IGF-1R, p-Akt and Akt expression levels in GLUT1 or GLUT2 knockdown HepG2 cells after 1 mM of GlcN treatment for 24 h. (D) GlcN consumption rates in A549 and HepG2 cells. GLUT2 was inhibited by phloretin or si*GLUT2* and then GlcN treatment for 6 h. Results are presented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. GlcN, glucosamine; GLUT, glucose transporter; IGF-1R, insulin-like growth factor 1 receptor; si, small interfering.

Inhibition of glycolysis by glucosamine is greater in cancer cells with lower HKII levels. Although the other six cell lines expressed lower levels of GLUT2 compared with HepG2 and Hep3B cells, there were differences in the antiproliferative effect of glucosamine among those six cell lines (Fig. 3B and C), suggesting that cellular sensitivity to glucosamine is influenced

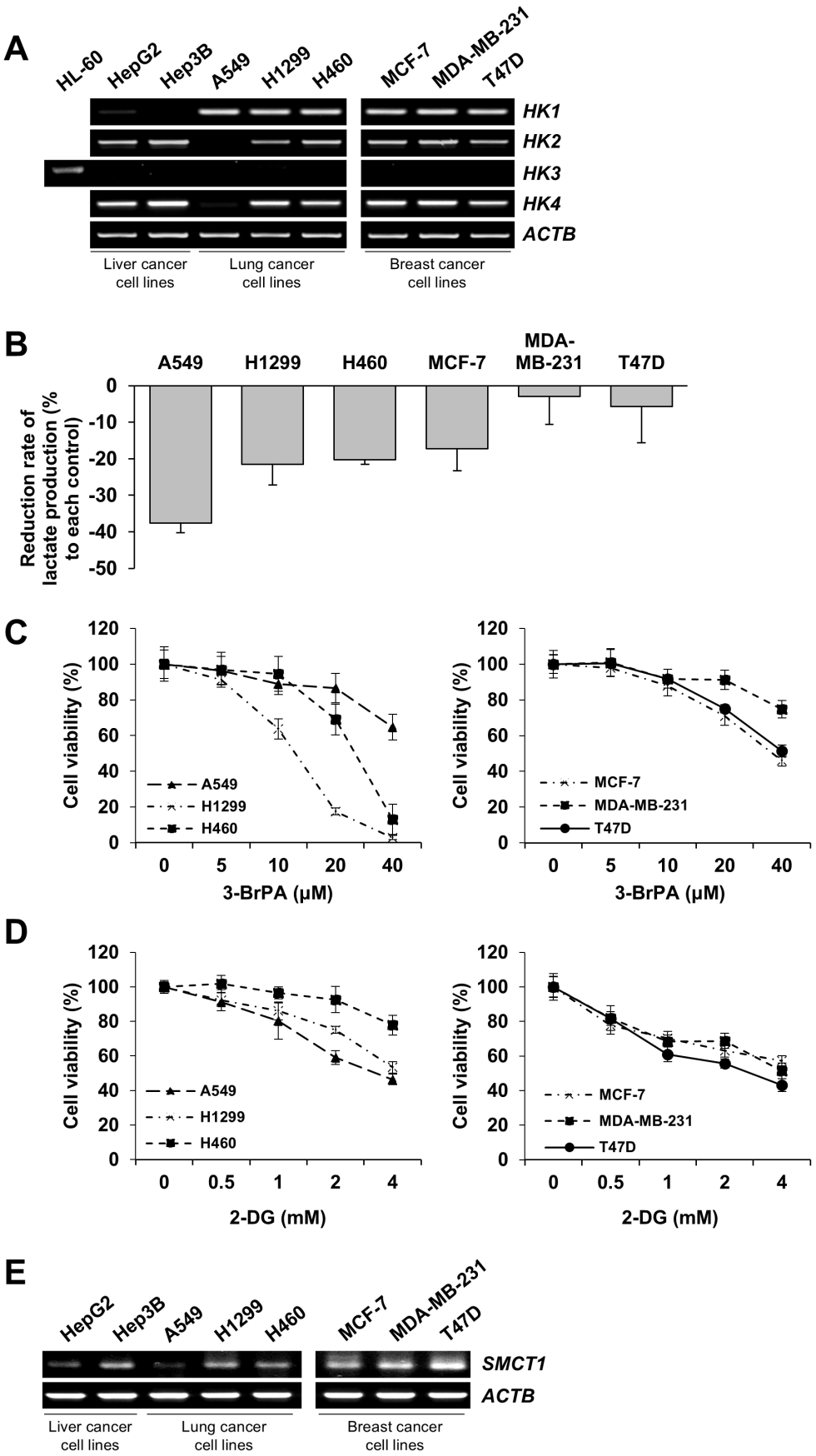

Figure 5. Basal levels of HKs are associated with GlcN sensitivity in human lung and breast cancer cell lines. (A) Semiquantitative PCR of HK isoforms in a variety of cancer cell lines. In the case of *HK3*, HL-60 which have high basal levels of HKIII, were used as a positive control. *ACTB* served as a loading control. (B) Reduction rate of lactate production by GlcN in the indicated cancer cell lines. Cells were treated with PBS or 2 mM or GlcN for 24 h and lactate levels in cultured medium were quantified. Results are presented as mean ± standard deviation. Cell viability of lung and breast cancer cells treated with (C) 3-BrPA or (D) 2-DG for 72 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (E) Semiquantitative PCR of *SMCT1* in the eight cell lines. *ACTB* served as a loading control. HK, hexokinase; GlcN, glucosamine.

by not only uptake through GLUT2 but also by post-uptake processes. Glucosamine can be phosphorylated by yeast hexokinase (39,40). In addition, yeast hexokinase is similar to rat hexokinase, human hexokinase N-terminal and C-terminal in respect of its sequence and structure amino acids, especially in the active site (41). Thus, it could be hypothesized that glucosamine serves as a substrate for hexokinase in mammalian cells including those of human origin (42-44). Thus, the present study examined the basic expression levels of HK isoforms (*HK1-4*) in the studied eight cell lines. Most cell lines showed similar levels of *HK1* expression and rarely expressed *HK3*. Notably, the six cell lines showed lower *GLUT2* expression and reduced glucosamine consumption rates compared with HepG2 and Hep3B cells, A549 cells exhibited the lowest *HK2* expression level compared with the other five cell lines (Fig. 5A). Next, since HK is the first enzyme to be activated during glycolysis and this activation is a rate-limiting step that regulates the speed of glycolysis (45), the differences in glycolytic rates and the extent of glycolysis inhibition by glucosamine was assessed. Notably, the rate of lactate production was most markedly reduced by glucosamine in A549 cells which were the most sensitive to glucosamine after liver cancer cell lines. It was observed that 3-BrPA or 2-DG, which are used as HKII inhibitors (46,47), sufficiently inhibited the proliferation of those six cell lines (Fig. 5C and D). In case of A549 cells, the anti-proliferative effect of 3-BrPA was relatively weak compared with other lung cancer cell lines, H1299 and H460, and this was probably due to the lower level of sodium-coupled monocarboxylate transporter 1 (*SMCT1*), which is responsible for the transportation of 3-BrPA into cells (48), than other cancer cell lines tested (Fig. 5E). Taken together, these results showed that glycolysis inhibition by glucosamine is negatively related to the level of HKs, especially HKII.

Glucosamine and 3-BrPA enhance the antiproliferative activity of one another. 3-BrPA, 2-DG and glucosamine, which are all glycolysis inhibitors, take different pathways to enter cells and work along different mechanisms. For example, 2-DG enters cells via GLUT1 and GLUT4 like glucose and works as a competitor of glucose, which eventually lowers the efficiency of glycolysis (23), whereas glucosamine is transported into cells efficiently via GLUT2 rather than GLUT1 and competes with glucose for HK. By contrast, 3-BrPA is taken up into cells by SMCT1 and directly suppresses the activity of HKII (47,48). Therefore, it was examined whether the antiproliferative effect of glucosamine is elevated when co-treated with 2-DG or 3-BrPA. Co-treatment of 3-BrPA with glucosamine increased the anti-proliferative effects of glucosamine in both A549 and HepG2 cells (Fig. 6A), whereas 2-DG showed a slight combinational effect and no combinational effect in A549 cells and HepG2 cells, respectively (Fig. 6B). Along with these results, glucosamine-induced decreased in IGF-1R and p-Akt expression level were tended to be enhanced by co-treatment of 3-BrPA (Fig. 6C), but not by co-treatment of 2-DG (Fig. 6D). These results suggested that the mechanism of glucosamine inhibiting HK is similar to that of 2-DG and different from that of 3-BrPA and that 3-BrPA may be a suitable HK inhibitor for co-administration with glucosamine.

HIF-1α/HKII signaling is responsible for hypoxia-induced glucosamine resistance. Glycolysis is mainly regulated by the quantity of glucose taken up into cells and the activity of HK, a rate-limiting step of the glycolysis pathway. Therefore, the expression level of GLUTs and HKs is an important factor in the glycolysis process. HIF-1α, one of the upstream transcriptional factors of GLUTs and HKs, plays an important role in controlling glycolysis (19). Based on this knowledge, it was first investigated whether the expression of GLUTs and HKs changes under hypoxic conditions in A549 and HepG2 cells and whether this is regulated by HIF-1α. As expected, the expression of *GLUT1*, *HK1*, *HK2* and *HK4* markedly increased under hypoxic conditions and it was confirmed that knocking down HIF-1 α markedly reduced their expression again (Fig. 7A). The expression of *GLUT2* was constant in the examined conditions and the expression of *HK1* was induced by hypoxic stress in HepG2 cells, but not in A549 cells. Next, the effects of hypoxia on the antiproliferative effects of glucosamine was examined. Notably, glucosamine reduced HIF-1α expression under hypoxic conditions in cancer cells (Fig. S2) and it was found that glucosamine-induced apoptosis was diminished under hypoxic conditions and this hypoxia-induced glucosamine resistance were restored by HIF-1α knockdown (Fig. 7B). Furthermore, cell cycle analysis found that the subG1 hypodiploid population, which was increased by glucosamine, was reduced by hypoxic stress and this hypoxic stress-induced change was not observed in HIF-1α depleted cells (Fig. 7C). Along with these results obtained from flow cytometry analysis, MTT assays showed that the anti-proliferation effects of glucosamine were reduced by hypoxic stress and this hypoxia-induced resistance was markedly ablated by HIF-1α downregulation in both A549 and HepG2 cells (Fig. 7D). Lastly, it was found that 3-BrPA, HKII inhibitor, also effectively reversed hypoxia-induced glucosamine resistance in both A549 and HepG2 cells (Fig. 7E). Collectively, these results suggested that glucosamine exerts its antiproliferative activity by inhibiting HKII within the cell and that the induction of HIF-1α and transactivation of its target genes, particularly HKII, are responsible for hypoxia-induced glucosamine resistance (Fig. 8).

Discussion

A number of cancer cells exhibit elevated glycolysis even in the presence of oxygen, relying more heavily on this process to produce ATP compared with normal cells. This phenomenon, known as the Warburg effect, has become a key focus in cancer treatment strategies, particularly through the development of glycolytic inhibitors (15,23). Meanwhile, glycolysis is more than just an energy-generating pathway; it plays a crucial role in regulating various cellular processes necessary for cancer cell growth and physiology (49,50). Hence, glycolysis inhibition has been studied as an antiproliferative strategy. Although the inhibitory effects of glucosamine on glycolysis and tumor growth were reported in the late 1900s (27,32), the precise mechanisms of action of glucosamine driving antiproliferative effects have not been fully understood and thus, the present study investigated the antiproliferative mechanism of glucosamine.

At first, to gain a broader understanding of the mechanism behind glucosamine's antiproliferative effects, microarray technology, a powerful tool for high-throughput screening

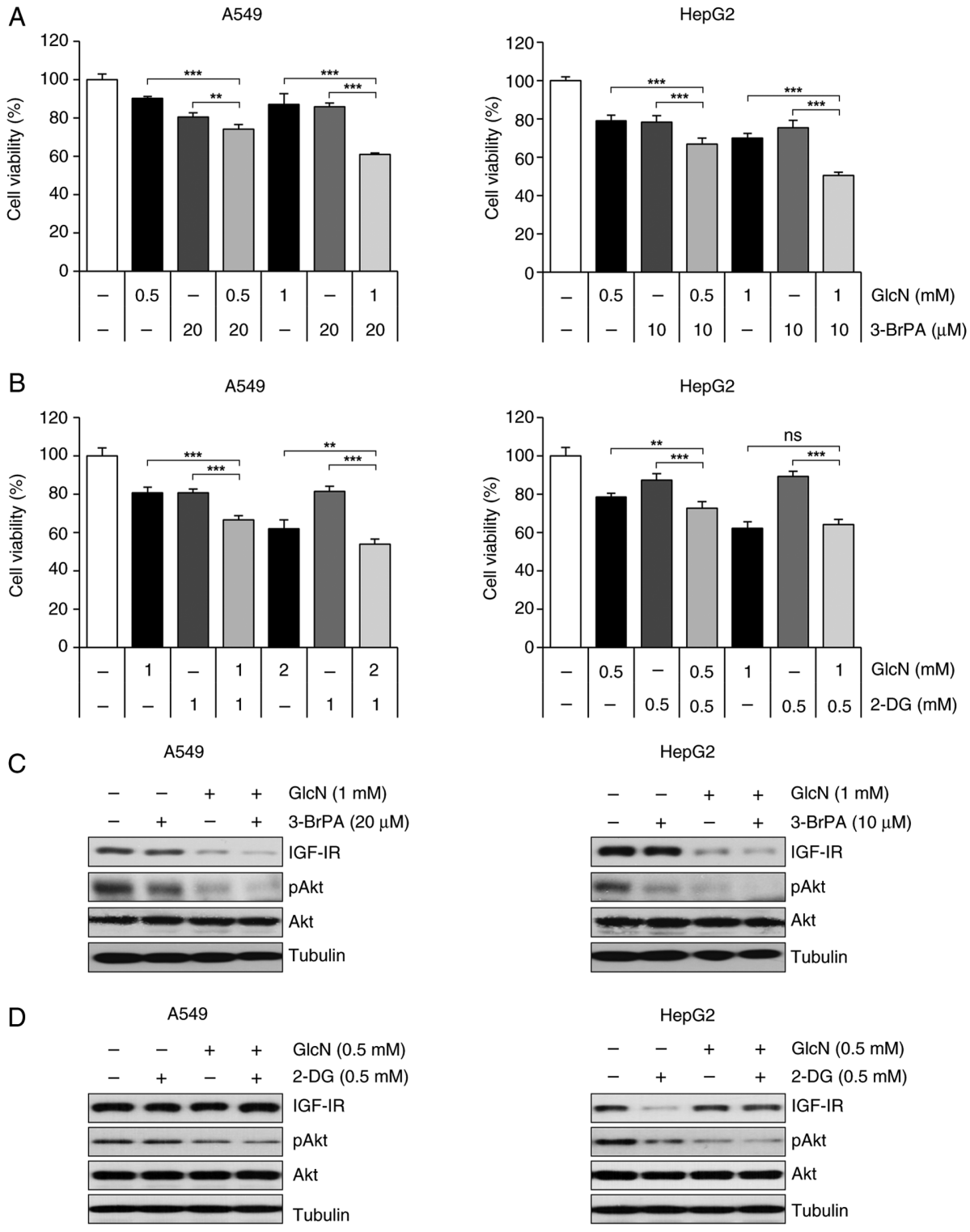

Figure 6. Combined effects of glycolysis inhibitors and GlcN. Cell viability of A549 and HepG2 cells after treated with GlcN combined with (A) 3-BrPA or (B) 2-DG for 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (C, D) Western blot analysis of IGF-1R, p-Akt and Akt expression levels in HepG2, Hep3B and A549 cells after treatment with GlcN combined with 3-BrPA (C) or 2-DG (D) for 24 h. **P<0.01, ***P<0.001, ns, no significance; GlcN, glucosamine; IGF-1R, insulin-like growth factor 1 receptor; p-, phosphorylated.

was employed. By using a whole-genome chip, the present study aimed to identify changes in the gene expression profile of A549 cells following the introduction of glucosamine. Genes upregulated by glucosamine by more than two-fold were primarily associated with pro-apoptosis and cell death, while those downregulated by more than two-fold were mostly linked to cell cycle regulation and DNA damage response. Additionally, glucosamine was found to influence genes involved in carbohydrate metabolism, biomolecule biosynthesis and glycosylation; processes related to glycolysis. In particular, the result showing that genes related to glycosylation were regulated by glucosamine is supported by previous

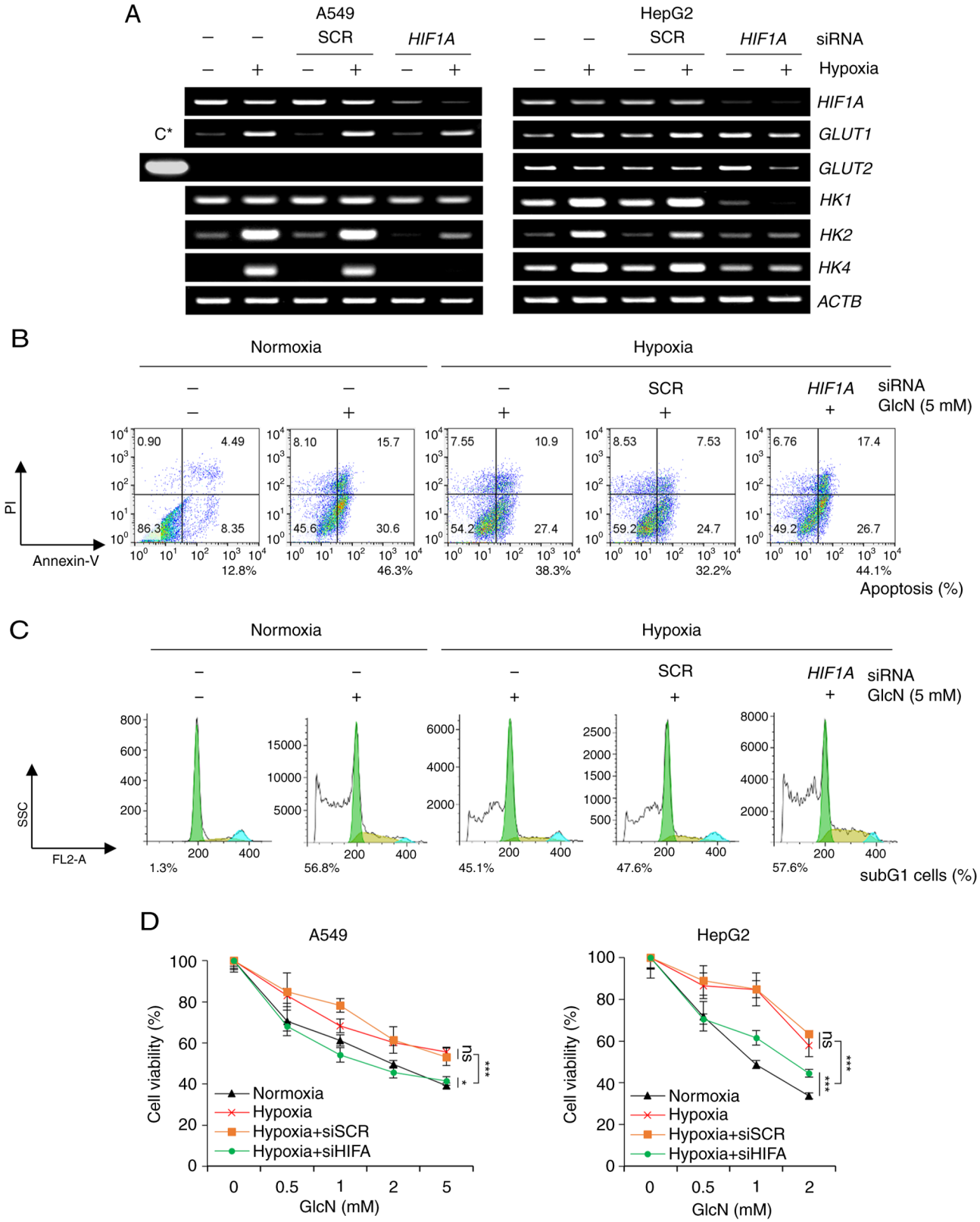

Figure 7. Continued.

reports demonstrating that glucosamine induces abnormal glycosylation of the IGF-1R prototype and COX-2 and eventually decreases their molecular mass (9,35). KEGG pathway analysis revealed that glucosamine downregulated the overall glycolysis pathway, while some enzymes regulating the entry into glycolysis were upregulated, possibly as a compensatory response to glycolysis inhibition. This compensatory mechanism is consistent with findings from previous studies using other glycolysis inhibitors (51,52). Given these findings, along with glucosamine's structural similarity to glucose and its

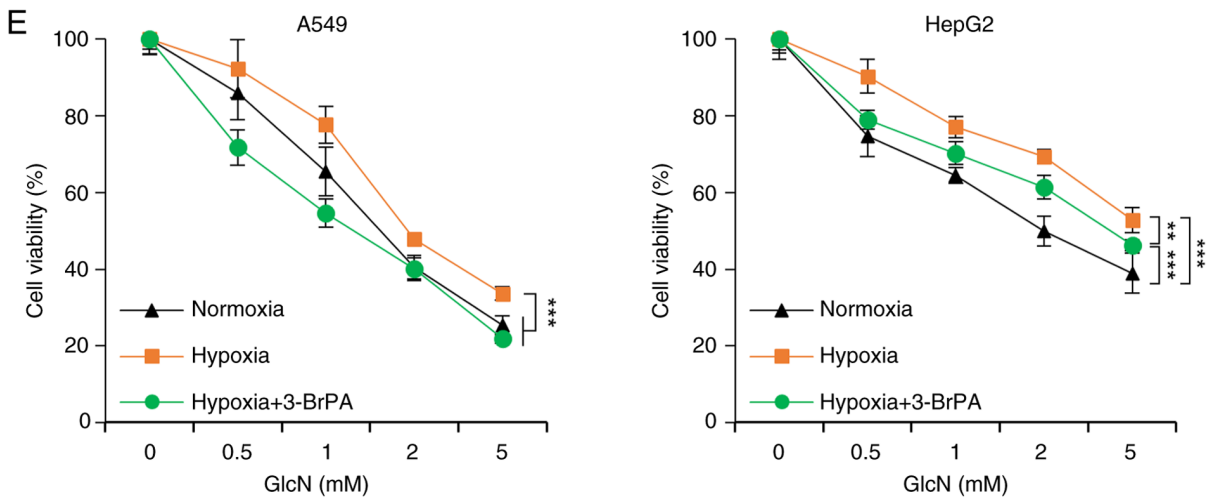

Figure 7. Anticancer effects of GlcN are regulated through changing of HK levels during hypoxia. (A) Semiquantitative PCR of *HIF1A*, *GLUT1*, *GLUT2* and HK isoforms of A549 and HepG2 cells. Cells were transfected with siSCR or si*HIF1A* and then cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 h. HepG2 sample (C*) in Fig. 3A was used as a positive control for *GLUT2* expression in the left panel. *ACTB* served as a loading control. Flow cytometric analysis of (B) apoptosis and (C) cell cycle in A549 cells. Cells were transfected with siSCR or si*HIF1A* for 24 h and further incubated under normoxic or hypoxic conditions for 24h followed by glucosamine treatment for additional 48 h. Subsequently, cells were stained with Annexin V-FITC and PI. Apoptosis (%) indicates sum of Annexin V single positive proportion and Annexin V-PI double positive proportion. Cell cycle analysis was performed with linearized value of PI intensity and population of subG1 cells is presented. (D) Cell viability of A549 and HepG2 cells. Cells were transfected with siSCR or si*HIF1A* for 24 h and further incubated under normoxic or hypoxic conditions for 24 h followed by GlcN treatment for additional 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. (E) Cell viability of A549 and HepG2 cells. Cells were incubated under normoxic or hypoxic conditions for 24 h followed by GlcN and 3-BrPA (10 or 20 μM for A549 or HepG2 cells, respectively) treatment for additional 48 h. Cell viability was measured by MTT assay and results are presented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ns, no significance; GlcN, glucosamine; HK, hexokinase; si, small interfering.

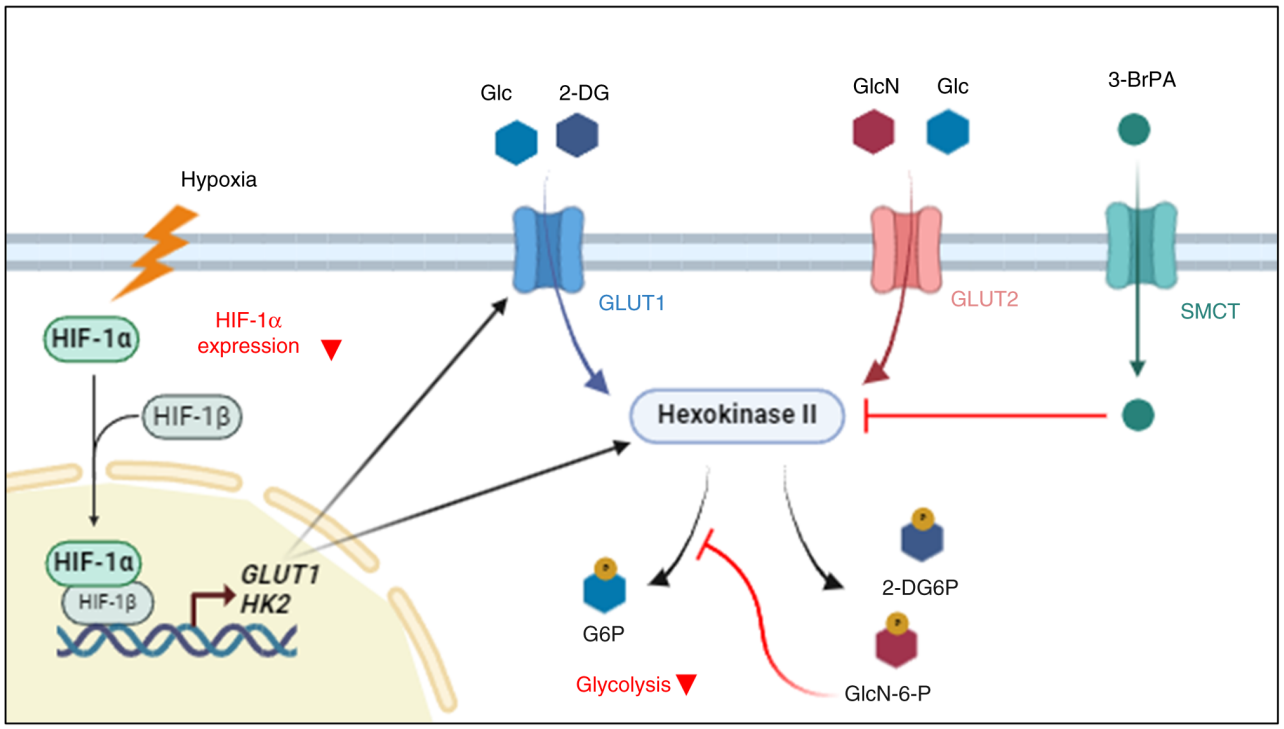

Figure 8. Schematic representation of the glycolysis inhibition effect by GlcN in cancer cells. GlcN is mainly transported into cancer cells through GLUT2. Within cytoplasm, GlcN can inhibit the glycolysis of cancer cells by acting as competitor to glucose. As HIF-1α enhances glycolysis through increasing GLUT1 and HK expression, inhibition of HIF-1α by glucosamine can also contribute to the reduction in glycolysis. Eventually, ablated glycolysis by glucosamine leads cancer cell death. GlcN, glucosamine; GLUT, glucose transporter; hypoxia-inducible factor 1α; HK, hexokinase; Glc, glucose.

previously reported effect of reducing ATP levels in cancer cells, it is suggested that glycolysis is the main target pathway of glucosamine.

Glucosamine, structurally similar to glucose except for its NH_2 group, has been shown to compete with glucose in carbohydrate metabolism (23). According to this, glucose

competitively inhibited glucosamine-induced antiproliferative effects and restored glycolysis as indicated by the recovery of lactate production that had been suppressed by glucosamine. Since glucosamine is known to inhibit the IGF-1R/Akt pathway (9), it was found that increasing glucose restored IGF-1R and p-Akt expression levels reduced by glucosamine. The results supported that glucosamine inhibited the rate of proliferation of cancer cell lines mainly by competing with glucose during glycolysis.

Based on the structural similarity of glucosamine and glucose, it was predicted that plasma membranes, especially where glucose transports exist, would be the battlefield of glucosamine and glucose. Among GLUT isoforms, expression levels in liver cancer cell lines of *GLUT2*, which is responsible for transportation of glucosamine as well (11), were much higher compared with other cell lines, while GLUT1 expression levels were similar between types of cancer. These GLUT2-rich liver cancer cell lines (HepG2 and Hep3B) consumed more glucosamine and were more sensitive to glucosamine than other GLUT2-poor cell lines. In addition, glucosamine consumption and the antiproliferative effects of glucosamine were abrogated by inhibiting GLUT2 with its specific inhibitor (Phloretin) or si*GLUT2* transfection. Thus, so far, it can be said that GLUT2 is the main gate for glucosamine to get into cells and high dose of glucose can keep glucosamine from entering into cells at GLUT2. Previous studies report that cancer cells have higher GLUT2 expression levels compared with normal cells (37,53). These facts suggested that the amount of GLUT2 expression in each cancer cell can be a crucial biomarker in determining the sensitivity against glucosamine and also proves that glucosamine is more effective against cancer cells than normal cells.

Glucosamine and glucose not only have a similar structure but are also catalyzed by the same enzyme, HKs and are converted to GlcN-6-phosphate and Glc-6-phosphate, respectively, after transport into the cell (33). The lower the expression of HKII, the greater the inhibitory effect of glucosamine on glycolysis. Compared with the known glycolysis inhibitors, 3-BrPA and 2-DG, the effective dose range of glucosamine was similar to 2-DG, not to 3-BrPA which is entered into cells by SMCT rather than GLUTs, demonstrating that glucosamine inhibits glycolysis by competing with glucose as does 2-DG, rather than by directly inhibiting HKs. The synergistic effect observed when two drugs with different mechanisms of action are combined suggests distinct pathways (54). While the combination of glucosamine and 3-BrPA showed enhanced antiproliferative effects, the same was not observed with 2-DG. Considering these results and the structure of glucosamine, it can be inferred that the mechanism of action of glucosamine is probably different from that of 3-BrPA and similar to that of 2-DG.

Various former studies reveal that HIF-1α regulates the expression level of HKs and GLUT1 efficiently by working as a transcription factor (17,19,55). Furthermore, the increased stability of HIF-1α under hypoxic conditions has been reported to influence sensitivity of types of cancer to a number of anticancer agents (56,57). In particular, studies have demonstrated that the antiproliferative effect of 2-DG is decreased when there are high levels of HIF-1α and that this phenomenon is related to increased levels of HKs, which are induced by HIF-1α (21). The present study showed that A549 cells, which have low basal levels of HKII and lactate, inhibited glycolysis and decreased cell proliferation when treated with glucosamine. Under hypoxic conditions, *GLUT2* expression levels were relatively constant in HepG2 cells, suggesting that the uptake amount of glucosamine would not be changed by HIF-1α activation. However, activated HIF-1α induced HKII expressions in lung and liver cancer cell lines and this activated HIF-1α/HKII pathway made cancer cell lines more resistant to glucosamine. In addition, genetically depleting *HIF1A* and pharmacologically inhibiting HKII markedly restored glucosamine-induced antiproliferative effects.

In humans, cohort studies found that regularly taking glucosamine as a supplement reduced the incidence and mortality of colorectal cancer, lung cancer and kidney cancer and the overall cancer mortality could be 6% lower compared with non-users (58,59). We previously reported *in vivo* anti-tumor effects of glucosamine using a xenograft mouse model (9). Furthermore, the present results supported the relationship between glucosamine intake and lower cancer mortality with the molecular mechanism of glucosamine-induced antiproliferative effects. Collectively, glucosamine can act like a roadblock on the path of glycolysis, competing with glucose for HK, slowing down the process like a rival runner in a race for the same baton. Nevertheless, the present study was limited by its exclusive use of cell line models, which may not fully reflect *in vivo* conditions. Further studies should focus on validating these findings in more physiologically relevant models and exploring the broader therapeutic potential of glucosamine under diverse biological conditions. Such efforts could clarify its efficacy across different tumor types and microenvironments, ultimately strengthening its clinical applicability.

In conclusion, the present study indicated that glucosamine reduced growth, decreased cell viability and impaired glucose metabolism in the studied cancer cell lines. Moreover, GLUT2 and HKII were identified as biomarkers determining sensitivity to glucosamine. While the precise molecular mechanisms underlying the antiproliferative effects of glucosamine remain to be fully elucidated, the present study provided significant insights by demonstrating its mode of action as a glucose metabolism inhibitor in cancer cells. Furthermore, the findings suggested potential therapeutic markers for identifying types of cancer or characteristics that are most responsive to glucosamine, thereby offering a rationale for its use in combination therapy. These findings contributed to expanding the potential of glucosamine as an effective anticancer agent.

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Availability of data and materials

Microarray datasets generated in the present study has been submitted to ArrayExpress (accession number: E-MTAB-14871), and publicly accessible under this

URL: https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-14871. Other data generated in the present study may be requested from the corresponding author.

Authors' contributions

SP, KS and JK participated in the study design, conducted the experiments, collected and analyzed the data, and drafted the initial manuscript. JK and SO supervised the study, contributed to the study design and data interpretation, and critically revised the manuscript for important intellectual content. All authors confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. ViroCure Inc. had no commercial interest in this study.

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