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Running title: Glycolysis promotes gastric cancer progression via PI3K/AKT

High glycolysis phenotype influences malignant progression and poor prognosis of gastric cancer through the PI3K/AKT pathway

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Gastric cancer (GC) is a prevalent gastrointestinal malignancy, with metabolic reprogramming, particularly glycolysis, playing a critical role in cancer cell stemness. However, the interaction between glycolysis and GC prognosis, along with its underlying mechanisms, remains poorly understood. This study aimed to systematically analyze the prognostic significance of glycolysis in GC and explore its functional impact. A glycolysis-related gene score was constructed using bioinformatics to assess glycolysis levels based on differentially expressed genes between GC and normal tissues. A nomogram model was developed to predict clinical prognosis, and the functional phenotypes of GC cell lines cultured under high and low glucose conditions were evaluated using metabolite detection and extracellular acidification rate (ECAR) measurements. Enrichment analyses identified key signaling pathways, which were further validated by western blot. Results showed that elevated glycolysis was associated with larger tumor size and poorer prognosis in GC patients. The nomogram demonstrated strong predictive accuracy. High glucose culture promoted glucose consumption, lactate production, ATP generation, and ECAR, enhancing epithelial-mesenchymal transition and malignant progression via the PI3K/AKT pathway. In conclusion, high glycolysis is linked to poor prognosis in GC and drives metastasis and stemness through the PI3K/AKT signaling pathway, highlighting its potential as a prognostic marker and therapeutic target.

Key words: gastric cancer; glycolysis; prognostic; hyperglycemia; PI3K/AKT

Gastric cancer (GC) is the fifth most common malignancy and the fourth leading cause of cancer-related mortality [1, 2]. Despite the decreased global incidence of GC [3], it remains highest in East Asian countries, including China [4]. The efficacy of traditional clinical treatments for GC, including surgery and chemoradiotherapy, is limited, resulting in poor patient prognoses and stagnant five-year survival rates. The primary reasons for the limited efficacy include the complex GC driver genes, high intra-tumor and inter-tumor heterogeneity, and the presence of cancer stem cells (CSCs). CSCs, in particular, contribute to metastasis, recurrence, and drug resistance [5]. Metabolism plays a crucial role in GC and impacts patient prognosis [6]. Epithelial-mesenchymal transition (EMT) represents a reversible cellular program that may serve as a pivotal early stage of tumor metastasis [7]. Furthermore, hyperglycemia promotes cell invasion and metastasis in various cancers [8]. However, the mechanisms underlying this phenomenon remain unclear.

Metabolic reprogramming, an emerging hallmark of cancer [9], has gained substantial attention in the last few years. The relationship between metabolic reprogramming and cancer has been extensively validated, and glycolysis is a crucial pathway for energy metabolism reprogramming in tumor cells. Tumor cells exhibit significantly elevated glycolysis levels, preferentially utilizing this pathway for energy production under both hypoxic and aerobic environments [10]. Metabolic reprogramming has been observed in vivo in various tumor types and is closely associated with the maintenance of CSCs, cancer progression, metastasis, and drug resistance [11]. Metabolism, particularly glycolysis, is intricately linked with EMT and stemness in tumor cells [12, 13]. Alterations in glycolysis levels in tumor cells are associated with stemness-associated characteristics exhibited by the entire tumor cell population [14]. Under normal circumstances, cells are not affected by any changes in intracellular glucose metabolism due to changes in blood sugar in the body. However, tumor cells are highly sensitive to fluctuations in glucose concentration in the external environment, thereby regulating intracellular glucose metabolism and changing their metabolic mode to ensure survival and further metastasis and proliferation. However, the regulation of glycolysis in tumor cells and its role in controlling EMT and metastasis remain unclear.

Consequently, we investigated the effects of elevated glucose levels on EMT and stemness in tumors. In this study, the effects of high glucose levels on GC cell metastasis and stemness were evaluated using both clinical data and biological experiments. Furthermore, the objective of this

study was to gain preliminary insight into the molecular mechanisms through which high glucose exerts these effects. Our results have significant clinical implications for identifying specific targets and inhibitors related to tumor metabolism and stemness [11]. Thus, our findings provide valuable insights into GC that may contribute to developing new therapeutic strategies.

Materials and methods

Cell lines and culture. The human GC cell lines MGC803 and HGC27 were obtained from the Chinese Academy of Sciences (China). The cells were cultured in DMEM complete media containing 10% fetal bovine serum, 1% L-glutamine, penicillin, and streptomycin. All cells were cultured in a 37 °C incubator with 5% CO_2.

Data processing and establishment of the GRG score model. Gene expression data were extracted from The Cancer Genome Atlas (TCGA) database. The "limma" package was used to select differentially expressed genes (DEGs) between the tumor and normal tissues with the following criteria: FDR-adjusted p-values < 0.05 and |fold change| > 1.5. The Molecular Signatures Database (MSigDB) v4.0 was searched for glycolysis-related gene sets (HALLMARK_GLYCOLYSIS). Gene set enrichment analysis (GSEA) was performed on DEGs to identify pathways and networks potentially involved in GC progression. From the 4266 DEGs, 54 GRGs were identified using a Venn diagram. Next, Cox regression analysis and Least Absolute Shrinkage and Selection Operator (LASSO) regression were performed to identify glycolytic target genes associated with overall survival (OS) to establish GRG score model. The GRG scores for patients with stomach adenocarcinoma (STAD) were calculated based on the coefficients (Coefi) and expression levels (Expri) of the prospective prognostic GRGs using the following formula: $\text{GRG score} = \sum_{i=1}^{n} \text{Coefi} \times \text{Expri}$.

Construction and evaluation of the GRG prognostic model. Data from the TCGA database were used as the training set. The median GRG score (median=0.033) was used as the cutoff to divide 355 TCGA-STAD patients into high and low glycolysis groups. Kaplan-Meier curves were plotted to compare the OS between the high and low GRG score groups. Time-dependent receiver operating characteristic (ROC) curves were used to evaluate the predictive ability of the GRG score model. To validate the prognostic model, the same process was carried out on a testing dataset from the Gene Expression Omnibus (GEO) (GSE62254, N=300).

Development of a nomogram based on GRG scores and clinical factors in TCGA-STAD. The "compare Groups" R package was used to compare the predictive efficacy of the GRG score with clinical characteristics. Cox regression analysis was applied to evaluate the independent prognostic value of the GRG scores and other clinical features. A nomogram was constructed using the R package "rms" and "regplot" to predict prognosis, based on GRG scores and clinical features. Calibration plots of the nomograms were created to assess the predictive accuracy of the nomogram using the "caret" package. ROC curves were used to validate the predictive ability of the nomogram.

GRG score model differential analysis and enrichment analysis. The TCGA-STAD dataset was divided into 2 groups based on the median glycolysis score. DEGs were selected using the following criteria: FDR-adjusted p-values < 0.05 and |fold change| > 1.5 between the high and low GRG score group. The differential biological effects and signaling pathways between the high and low glycolysis groups in the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were evaluated using the "cluster Profiler" package in R. GSEA was employed to assess whether expression of specific gene sets from the MSigDB collection (h.all.v2023.1.Hs.symbols.gmt) were significantly different between the high and low glycolysis groups.

Calculation of EMT and stemness scores. The pan-cancer 78-gene EMT signature was downloaded from the EMTome online database (https://www.emtome.org/) [15]. The expression levels of the 78 genes were calculated as the sum across all samples. The top 10 mesenchymal genes (MGene_top10) and the top 10 epithelial genes (EGene_top10) were defined. The EMT score for each sample (panCancer_EMTscore) was calculated as the difference in expression values between the MGene_top10 and EGene_top10. A higher EMT score indicates a more mesenchymal phenotype and a less epithelial phenotype. The stemness score (ssGSVA score) was constructed with the "GSVA" R package using 109 cancer stem cell-related genes compiled from previous research [16].

Glucose consumption and lactate production measurement. Cells were seeded into 6-well plates. After 24 h, 2 ml of fresh medium was used instead. Following a fixed incubation period, the cell culture supernatant was collected. Glucose consumption was assessed using a colorimetric method according to the instructions of the Glucose and Sucrose Assay Kit (Sigma-Aldrich, #MAK013,

USA). Lactate production was measured using the Lactate Assay Kit II (Abcam, #ab65331, UK). Glucose consumption and lactate production were normalized to the number of cells (μmol/10^6 cells).

Adenosine 5'-triphosphate (ATP) production measurement. Cells were seeded into 6-well plates. After 24 h, 2 ml of fresh medium was used instead. Following a fixed incubation period, the cells were collected, and ATP production was measured using the ATP Assay Kit (Beyotime, #S0027, China). ATP production was normalized to the control group (nmol/mg protein).

Extracellular acidification rate (ECAR) measurement. Cells were seeded in XF96 plates (15,000 cells/well) and incubated overnight. After washing, the cells were incubated in a 37 °C CO_2-free incubator for 60 min. Glucose (10 mM), oligomycin (1 μM), and 2-deoxyglucose (2-DG, 50 mM) were added, and ECAR was measured at the specified time points. The ECAR was assessed using the Seahorse XFe96 analyzer (Agilent Technologies Inc., USA) to evaluate glycolytic flux.

Oxygen consumption rate (OCR) measurement. Cells were seeded in XF96 plates (15,000 cells/well) and incubated overnight. After washing, the cells were incubated in a 37 °C CO_2-free incubator for 60 min. Oligomycin (1 μM), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 μM), and rotenone/antimycin A (0.5 μM) were sequentially added, and OCR was measured at the specified time points. OCR was determined using the Seahorse XFe96 analyzer (Agilent Technologies Inc., USA) to assess mitochondrial respiration.

Cell invasion and migration assay. To assess cell migration and invasion, Transwell™ chambers (24-well inserts; pore size, 8 μm; Corning, USA) were either left uncoated or coated with diluted Matrigel (BD Biosciences, USA). Serum-starved cells (2×10^4) were seeded in the upper chamber with serum-free medium, while the lower chamber contained complete medium with varying glucose concentrations. After 24 h of incubation at 37 °C, the cells that had migrated through the membrane were fixed, stained, photographed, and counted. Quantitative analysis was then performed to assess cell migration and invasion.

Cell proliferation analysis. Cells cultured in high- and low-glucose environments were seeded in 96-well plates (approximately 5,000 cells/well) and cultured in media with different glucose concentrations. Using the IncuCyte S3 platform (Sartorius, Göttingen, Germany), phase contrast images were collected from two regions within each well at 3 h intervals using a 10× objective. The IncuCyte S3 image analysis software was set to detect cell edges and determine their confluence

166 percentage. Proliferation curves were plotted based on the confluence percentage over time to
167 evaluate cell proliferation capacity.
168 **Sphere formation assay.** A solution of 0.8% methylcellulose was prepared [14]. and 2 ml of the
169 solution was added to each well of a low-adhesion 24-well plate. Cells were cultured in high- and
170 low-glucose environments during their logarithmic growth phase and harvested. The cell density
171 was adjusted to 5×10^4 cells/ml and 10 μl of the cell suspension was added to the methylcellulose
172 in each well. Cells were incubated at 37 °C with 5% CO_2 for 7-14 days. Photograph and count the
173 number and size of spheres under an inverted fluorescence microscope.
174 **Colony formation assay.** Cells are cultured in high- and low-glucose environments during their
175 logarithmic growth phase. The cell density was adjusted to 5 × 103 cells/ml and 20 μl of the cell
176 suspension was added to each well of a 6-well plate and gently shaken to disperse the cells. Every 3
177 days, 1 ml of the corresponding fresh medium was added. Cells were incubated at 37 °C until
178 visible colonies formed. The colonies were fixed with 2 ml of 4% formaldehyde (Solarbio, China)
179 for 30 min, stained with 2 ml of 0.1% crystal violet (Solarbio, China) for 30 min, rinsed with PBS,
180 air dried, and photographed. Count the number of colonies formed in each group.
181 **Protein extraction and western blot.** Cells cultured in high- and low-glucose environments were
182 lysed to extract total protein. The proteins were separated using SDS-PAGE and transferred to
183 membranes, followed by the addition of ECL detection reagent for visualization. Western blot band
184 grayscale values were quantified using ImageJ software. Target protein band densities were
185 normalized to the corresponding β-actin internal control.
186 The following primary antibodies were used: CD44 (#ab157107), SOX2 (#ab97959), and Oct4
187 (#ab18976), were from Abcam (USA), and vimentin (#5741S), β-actin (#4970S), E-cadherin
188 (#3195S), N-cadherin (#13116S), Snail (#3879S), Nanog (#3580S), p-PI3K (#4228S), PI3K
189 (#4249S), p-AKT (#4060S) and AKT (#2920S) were from Cell Signaling Technology (USA).
190 Secondary antibodies were HRP-labeled goat anti-rabbit and anti-mouse IgG (Jackson, USA).
191 **Statistical analysis.** Statistical analysis and data visualization were performed using R version
192 4.2.0, SPSS 25.0, and GraphPad Prism 8.0. All experiments were repeated three times, and the data
193 are presented as means±standard deviation ($\bar{x} \pm s$). Normality and homogeneity of variance
194 analyses were conducted on the data. $P < 0.05$ was considered statistically significant.
195

Results

Impact of the high glycolysis model on prognosis in patients with GC. Using TCGA data, we identified DEGs between GC and normal tissues (Supplementary Figure S1A). Based on the glycolysis gene set from the hallmark database, glycolysis genes were significantly enriched in GC patients (Supplementary Figure S1B). After integrating DEGs from TCGA, we selected 54 glycolysis-related DEGs in tumor tissues versus normal tissues for further study (Supplementary Figure S1C). After filtering through LASSO and multivariable Cox regression analysis (Supplementary Figures S1D, S1E), 4 genes (STC1, VCAN, SOX9, and AK4) significantly associated with OS were identified ($p < 0.05$). GRG scores were calculated using the following formula: GRG score=(0.1908 × STC1)+(0.1034 × VCAN)+(−0.1660 × SOX9)+(0.1216 × AK4). STAD patients were divided into high and low glycolysis groups based on the GRG scores. The heatmap displays the expression profiles of the four genes. Compared to the low glycolysis group, the expression levels of AK4, STC1, and VCAN were higher in the high glycolysis group, whereas SOX9 expression was lower in the high glycolysis group (Figure 1A).

The relationship between the survival status and time of GC patients was ranked by GRG scores (Figure 1B). Based on the Kaplan-Meier survival analysis, the predictive model exhibited strong prognostic capability, with the low glycolysis group showing a higher survival rate and patients in the high glycolysis group showing shorter survival times ($p < 0.005$; Figure 1C). The predictive ability of the prognostic model was validated using the 1-year, 3-year, and 5-year ROC curves; the AUC values of the ROC curves were all above 0.6, indicating that the GRG score model accurately predicted prognosis in patients with GC (Figure 1D).

The ACGR cohort (GSE62254, N=300) from the GEO database was selected as the testing gene set to verify the GRG scoring model using the same methodology. The distribution of the four GRG-related genes in the heatmap plot (Figure 1E) and the survival status stratified by median GRG scores (median=0.411; Figure 1F) were similar to the previous findings. The Kaplan-Meier survival curves demonstrated that the survival rate of the low glycolysis group was higher than the survival rate of the high glycolysis group (Figure 1G), consistent with the results from the TCGA-STAD. Additionally, the GRG score-based model exhibited good sensitivity and specificity, with AUC values above 0.6 in ROC curves (Figure 1H). Further analysis of the relationship between the GRG score and clinical pathological characteristics revealed that high glycolysis levels

were associated with larger tumor size in patients with GC (Supplementary Table S1, Supplementary Figure S2A). Meanwhile, the TCGA project has classified gastric cancer into four molecular subtypes including genome stable (GS), MSI, EBV, and chromosomal instability (CIN). We found that the distribution of GRGscore varies significantly among different molecular subtypes and higher GRG score cases were concentrated on the subtypes of GS (Supplementary Figures S2B, S2C).

Nomogram for predicting prognosis in GC patients combining glycolysis and clinical characteristics. A glycolysis-clinical nomogram was developed to predict individual survival rates based on glycolysis and clinical factors. First, Cox regression analyses were conducted to evaluate the GRG score and other clinical features. The univariate Cox results indicated that age, stage, Tumor node metastasis (TNM) classification, and the GRG score, were associated with OS (Figure 2A). More total points were associated with shorter 1-year, 3-year, and 5-year survival times (Figure 2B). Multivariate regression revealed only 3 independent prognostic factors (Supplementary Table S1). Calibration curves comparing the predicted survival times with observed 1-year, 3-year, and 5-year survival times (Figure 2C) indicated that the nomogram prediction model accurately predicted the survival time of patients with GC within a 5-year period.

The GRG score model exhibited better predictive accuracy compared to clinical features such as tumor grade and TNM classification. The nomogram model combining the GRG score with significant clinical features from the multivariate Cox analysis exhibited the highest AUC (> 0.7) (Figure 2D). Thus, combining glycolysis and clinical factors reliably predicted patient prognosis, highlighting the clinical significance of glycolysis.

High and low glucose culture environments induce different glycolytic phenotypes in GC cells. To verify the impact of glycolysis on the malignant GC phenotype, MGC803 and HGC27 GC cell lines were cultured in media with 25 mM, 15 mM, and 5.5 mM glucose to establish cell lines with varying glycolytic phenotypes. Treatment with different glucose concentrations significantly altered the glycolytic levels in GC cells. A high-glucose environment significantly increased glucose consumption and the production of major glycolytic products, including lactate and ATP (Figures 3A-3C). High-glucose culture also significantly elevated the ECAR levels in GC cells (Figure 3D). Conversely, a low-glucose environment significantly reduced glucose consumption, lactate production, ATP generation, and ECAR in GC cells. We further performed mitochondrial oxygen

consumption rate (OCR) assays in MGC803 and HGC27 cells. The results demonstrated that neither basal respiration, maximal respiratory capacity, nor ATP-linked respiration showed significant alterations under different glucose concentrations (5.5 mM, 15 mM, and 25 mM) (Supplementary Figure S3). These results indicate that high glucose environment induces a high-glycolytic phenotype and low glucose environment induces a low-glycolytic phenotype in GC cells. Thus, these culture conditions were used for subsequent cell models.

High-glucose environment promotes malignant phenotypes in GC cells. Changes in the functional phenotypes of GC cells in high- and low-glucose culture environments were determined. The proliferation rates of GC cells cultured in a high-glucose environment were significantly higher than the proliferation rates of cells cultured in a low-glucose environment (Figure 4A). Colony formation assays demonstrated that the number of colonies formed increased with higher glucose concentrations (Supplementary Figure S4A). Thus, increased glycolysis significantly enhances the proliferative capacity of GC cells. The numbers of migrating and invading cells were significantly higher in the high-glucose culture group compared with the numbers in the low-glucose culture group, indicating that the migration and invasion abilities of GC cells increased under high-glucose conditions (Figure 4B, Supplementary Figure S4B). Sphere formation assays indicated that the number of spheres formed by GC cells significantly increased in cells cultured in high-glucose concentrations compared with the number of spheres in cells cultured in low-glucose conditions, indicating that the stem cell-like properties of GC cells were enhanced in a high-glucose environment (Figure 4C). Overall, these results demonstrate that increased glycolysis levels are associated with the malignant phenotype of GC cells, including metastasis and stemness, and a high-glucose environment promotes these malignant traits.

High-glucose environment promotes GC progression via the PI3K/AKT pathway. To further investigate the mechanisms by which high glucose regulates GC progression, DEGs in GC patients with high and low glycolysis in the TCGA-STAD dataset were analyzed. 2248 genes were upregulated and 165 genes were downregulated in the high glycolysis group compared to the low glycolysis group (Figure 5A). DEGs were mainly enriched in biological functions such as extracellular matrix, cell adhesion, and migration, according to GO analysis (Supplementary Figure S5A). And KEGG analysis demonstrated that DEGs were mainly enriched in the PI3K/AKT pathway (Figure 5B). GSEA analysis further confirmed that the PI3K/AKT (Figure 5C) was

significantly enriched in GC with high glycolysis. This was further validated by western blot analysis (Figure 5D, Supplementary Figure S6A), which demonstrated increased activation of the PI3K/AKT in GC cells with increasing glucose concentrations in the culture environment. Collectively, these results suggest that high glycolysis in GC enhances malignant phenotypes such as metastasis and stemness by activating the PI3K/AKT signal pathway.

High-glucose environment promotes EMT and stemness in GC. The GSEA analysis indicated that the EMT and P53 pathways were significantly enriched in the high glycolysis group within the hallmark gene sets (Supplementary Figure S5B). Based on previous findings on the functional phenotypes of GC cells, it is tenable to hypothesize that a high-glucose environment influences EMT and stemness in GC. The EMTome database [15] was utilized to download a pan-cancer 78-gene EMT signature, which was employed to construct an EMT score. EMT scores were significantly elevated in the high glycolysis group compared with the EMT scores in the low glycolysis group (Figure 6A), and EMT scores significantly correlated with GRG scores (Figure 6B). Expression levels of EMT-related markers (N-cadherin, vimentin, and snail) were significantly higher in GC cells cultured in a high-glucose environment compared with cells cultured in a low-glucose environment (Figure 6C, Supplementary Figure S6B), confirming that increased glycolysis promotes EMT in GC cells.

A stemness score was constructed using 109 tumor stem cell genes, as previously described [16]. Stemness scores were significantly elevated in the high glycolysis group compared to the scores in the low glycolysis group (Figure 6D); GRG scores significantly correlated with stemness (Figure 6E). WB analysis confirmed the stemness-related molecular phenotype in GC cells. The expression levels of stemness-related markers (CD44, OCT4, and SOX2) were significantly higher in GC cells cultured in a high-glucose environment compared with the expression levels in GC cells cultured in a low-glucose environment (Figure 6F, Supplementary Figure S6C). Thus, elevated glycolysis influences stemness in GC cells.

Discussion

Despite recent advances in the diagnosis and treatment of GC, drug resistance is associated with low survival rates. Thus, identifying novel therapeutic targets and strategies for patients with GC is urgently needed [17]. High blood glucose levels are associated with poorer prognoses in cancer

patients, including those with GC [18, 19]. Diabetes is known to increase the risk of various cancers, such as liver, pancreatic, colorectal, and GCs. Most cancer patients with uncontrolled plasma glucose levels have poorer prognoses and shorter survival times [20, 21]. This highlights the impact of glucose metabolism on tumor stemness and metastasis [22, 23].

Increasing evidence suggests that relying on single clinical factors or individual gene characteristics often results in poor predictive performance. Now, we can focus on identifying a series of the most critical genes related to patient survival predictions, rather than conducting broad explorations. We constructed a high glycolysis score model (GRG score) based on clinical data and demonstrated that high glucose levels affect the prognosis of patients with GC, consistent with previous research findings [22, 23]. Subsequent survival analysis revealed that patients in the high GRG score group had worse prognoses. This result was validated with an independent ACGA cohort. The distribution of GRG scores differed significantly across molecular subtypes, with enrichment of high glycolysis in the GS subtype. These findings suggest that metabolic phenotypes may complement genomic classifications and offer an additional layer of tumor stratification, potentially paving the way for refined prognostic and therapeutic frameworks. GRG scores and clinical characteristics were combined into a nomogram. The nomogram is an effective tool for the clinical diagnosis and treatment of GC patients. These results indicate that GRG scores and the nomogram strongly predict the prognosis of patients with GC and can guide clinical treatment decisions [24].

Subsequently, a high-glucose state was simulated in vitro by culturing cells in a high-glucose medium, thereby mimicking hyperglycemia. Prolonged exposure to elevated glucose levels increased glycolysis and enhanced malignancy-related phenotypes associated with cancer stemness [18]. The proliferation, migration, invasion, and self-renewal abilities of GC cells were significantly enhanced when exposed to high glucose. Active glycolysis is a hallmark of malignancy, typically accompanied by elevated levels of glycolytic enzymes and corresponding metabolites. Nevertheless, the precise mechanisms by which glycolysis contributes to tumorigenesis are unclear [25]. Increasing evidence indicates that metabolism, particularly glycolysis, and cancer stemness are intricately intertwined processes within tumor tissues [26]. Tumor cells exhibit active glycolytic activity and a strong dependence on glycolysis [27]. Abnormal increases in glycolytic intermediates or products are markers of enhanced cancer stemness and chemoresistance [13]. Additionally, CSCs exhibit significantly increased glucose uptake and lactate production and reduced mitochondrial

respiration [28]. Leveraging these metabolic changes may provide effective targeted therapeutic strategies, reducing the risk of recurrence and metastasis [29].

Regarding the molecular mechanisms of glucose-driven oncogenesis, glucose is widely recognized to promote cancer progression through various metabolic pathways. Tumor cells are capable of sensing extracellular glucose fluctuations and adaptively modulating intracellular metabolic flux, thereby regulating glycolytic activity and influencing stemness-related properties that contribute to tumor initiation and progression. In this study, we evaluated the effects of different glucose concentrations on glycolysis and oxidative phosphorylation (OXPHOS) by measuring ECAR and OCR, respectively. The results demonstrated that OXPHOS activity remained relatively stable across the tested glucose concentrations, whereas glycolytic activity, as indicated by ECAR, exhibited more pronounced dynamic changes in response to extracellular glucose levels.

Previous studies have shown that tumor cells display highly dynamic metabolic adaptations to glucose availability, with the magnitude and direction of these shifts largely dependent on tumor type and metabolic plasticity. For instance, in glioblastoma, lower glucose concentrations (100 mg/L) are associated with increased OCR, suggesting compensatory reliance on OXPHOS when glycolytic flux is restricted. In contrast, at higher glucose levels (1,000-4,500 mg/l), glycolytic reserve increases while OCR decreases, indicating a metabolic shift toward glycolysis [30]. Conversely, in breast cancer cells, glucose deprivation suppresses both ECAR and OCR, accompanied by ATP depletion, lactate reduction, mitochondrial depolarization, and activation of pyroptotic pathways [31]. These findings underscore the significant intertumoral variability in metabolic regulation and OXPHOS dependency.

Our findings further support the notion that gastric cancer cells exhibit a distinct form of metabolic flexibility, maintaining relatively stable mitochondrial respiration while dynamically adjusting glycolytic activity in response to extracellular glucose availability. These observations complement the broader understanding that glucose promotes cancer progression through multiple metabolic pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, glycosylation, and lipid synthesis. Such metabolic reprogramming contributes to the activation of oncogenic signaling pathways, enhancing tumor cell proliferation, metastasis, drug resistance, and angiogenesis. Furthermore, high glucose levels can upregulate key enzymes in glucose metabolism, leading to increased glycolysis, glycosylation modifications, lactate production, and lipid synthesis. These

alterations in energy metabolism activate signaling pathways associated with invasive tumor phenotypes [18, 32], promoting tumor cell proliferation, metastasis, drug resistance, and angiogenesis. Hyperglycemia, both directly and indirectly, is associated with an increased risk for carcinogenesis. The mechanisms through which hyperglycemia contributes to carcinogenic pathways are numerous and complex. These include direct or indirect DNA damage, reactive oxygen species (ROS) formation, mutation accumulation, impaired DNA repair, and aberrant regulation of oncogenes and tumor suppressor genes [33]. These metabolic reprogramming and tumor microenvironment modifications ultimately promote cancer development and progression. The p53 checkpoint bypasses independent of mutations may represent the carcinogenic origin and targetable susceptibility of glucose-driven cancers [34]. Additionally, glucose can promote cancer without being metabolized. Glucose functions as an oncogenic signaling molecule that directly binds to NSUN2, inhibiting the activation of the STING pathway. Suppression of the STING pathway impedes the activation and infiltration of antitumor T cells, ultimately promoting cancer initiation and progression [35].

Energy-responsive growth signaling pathways are implicated in metabolic reprogramming to support abnormal proliferation and metastasis [36, 37]. As a key oncogenic signaling pathway, PI3K/AKT has emerged as an important therapeutic target in cancer treatment due to its central role in regulating tumor cell proliferation, survival, metabolism, and resistance. Our results demonstrate the effects of high glucose-mediated glycolysis on PI3K/AKT signaling in GC. This finding validates the impact of glycolysis on EMT and stemness-related biological and molecular phenotypes in tumor cells, consistent with previous studies [38]. Notably, several PI3K inhibitors have already been approved for clinical use in certain cancer types, and multiple PI3K/AKT-targeted agents are currently under active clinical investigation [39, 40]. Meanwhile, it is essential to acknowledge the constraints of our study when interpreting the results. The culturing of GC cells in varying glucose concentrations can only simulate a high-glucose environment and does not accurately represent the in vivo hyperglycemic environment. In addition, the current study is limited by the availability of cell models, and future investigations incorporating low-passage primary gastric cancer cells or patient-derived models would provide more clinically relevant insights. Furthermore, given that our analysis relies solely on retrospective TCGA data, future prospective clinical studies are needed to validate the relationship between blood glucose levels,

therapeutic response, and tumor progression.

In conclusion, we demonstrate that elevated glycolysis is associated with worse clinical prognoses in patients with GC. A predictive model combining glycolysis and clinical features are developed and validated to assess patient prognosis. Additionally, we simulate a high-glucose environment by culturing GC cells in various glucose concentrations. This approach demonstrates that the high-glycolysis phenotype induced by a high-glucose environment significantly impacts malignant characteristics such as metastasis and stemness in GC cells. This mechanism may involve the PI3K/AKT pathway, which promotes EMT and regulates GC stemness by influencing glycolysis levels. Moving forward, we plan to investigate the mechanistic role of PI3K/AKT signaling through pharmacological inhibition and assess the therapeutic potential of combined targeting of glycolysis and the PI3K/AKT pathway. Our findings highlight the necessity for clinical attention to GC patients with hyperglycemia and present novel strategies and therapeutic targets for GC diagnosis and treatment by elucidating the underlying mechanisms.

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Figure Legends

Figure 1. Glycolysis-related gene (GRG) score analyses in patients with gastric cancer (GC) in the training and testing sets based on the four-GRGs. A) Heatmap of the four-GRG expression profile. B) Distribution of GRG scores per patient. C) Survival status and survival times of GC patients according to GRG scores. D) Kaplan-Meier plots showing overall survival (OS) in high- and low-GRG score groups. E, F) External validation of the GRG score model using expression data from the ACGA database.

Figure 2. Combining glycolysis and clinical features to construct a nomogram for predicting the prognosis of GC patients. A) Forest plot illustrating the univariate Cox regression of the GRG

scores and corresponding clinical features. B) Age, TNM, stage, and GRG score were employed in the Nomogram, and the total score was used to predict the 1-, 3-, and 5-year prognosis of patients with GC. C) Calibration curve of the Nomogram to predict survival within 5 years. D) Receiver operating characteristic curves (ROC) curves for predicting prognosis using different predictive models at 1 year, 3 years, and 5 years.

Figure 3. High and low glucose culture conditions induce high and low glycolytic phenotypes in GC cells. A) Glucose consumption, B) lactate production, C) intracellular adenosine 5'-triphosphate (ATP) production, and D) extracellular acidification rate (ECAR) in MGC803 and HGC27 cells cultured in different glucose concentrations. Data are shown as means±SD. $^{**}p < 0.01$, $^{***}p < 0.001$

Figure 4. Culturing in different glucose concentrations affects the stem-related features of GC cells. A) Cell proliferation capacity, B) self-renewal ability (scale bar, 1000 μm), and (C) invasion ability (scale bar, 100 μm) of MGC803 and HGC27 cells cultured in different glucose concentrations. Data are presented as means±SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 5. High-glucose environment culture contributes to the progression of GC via the PI3K/AKT pathway. A) Volcano plot showing the distribution of differentially expressed genes (DEGs) in high and low glycolysis-related gene (GRG) score groups. B) KEGG enrichment analysis of DEGs between the high and low GRG score groups. C) GSEA analysis of PI3K/AKT genes between the high and low GRG score groups. D) Western blots showing phosphorylation of proteins in the PI3K/AKT pathway in GC cells cultured in different glucose concentrations.

Figure 6. High-glucose environment promotes epithelial-mesenchymal transition (EMT) and stemness in GC. A) Violin plot comparing the distribution of the EMT scores in the high- and low-GRG score groups. B) Correlation between the EMT and GRG scores in the TCGA database. C) Western blots showing expression of EMT-related markers in GC cells cultured in different glucose concentrations. D) Violin plot analysis comparing stemness scores in the high- and low-GRG score groups. E) Correlation between stemness and GRG scores in the TCGA database. F) Western blots

596 showing expression of stemness markers in GC cells cultured in different glucose concentrations.

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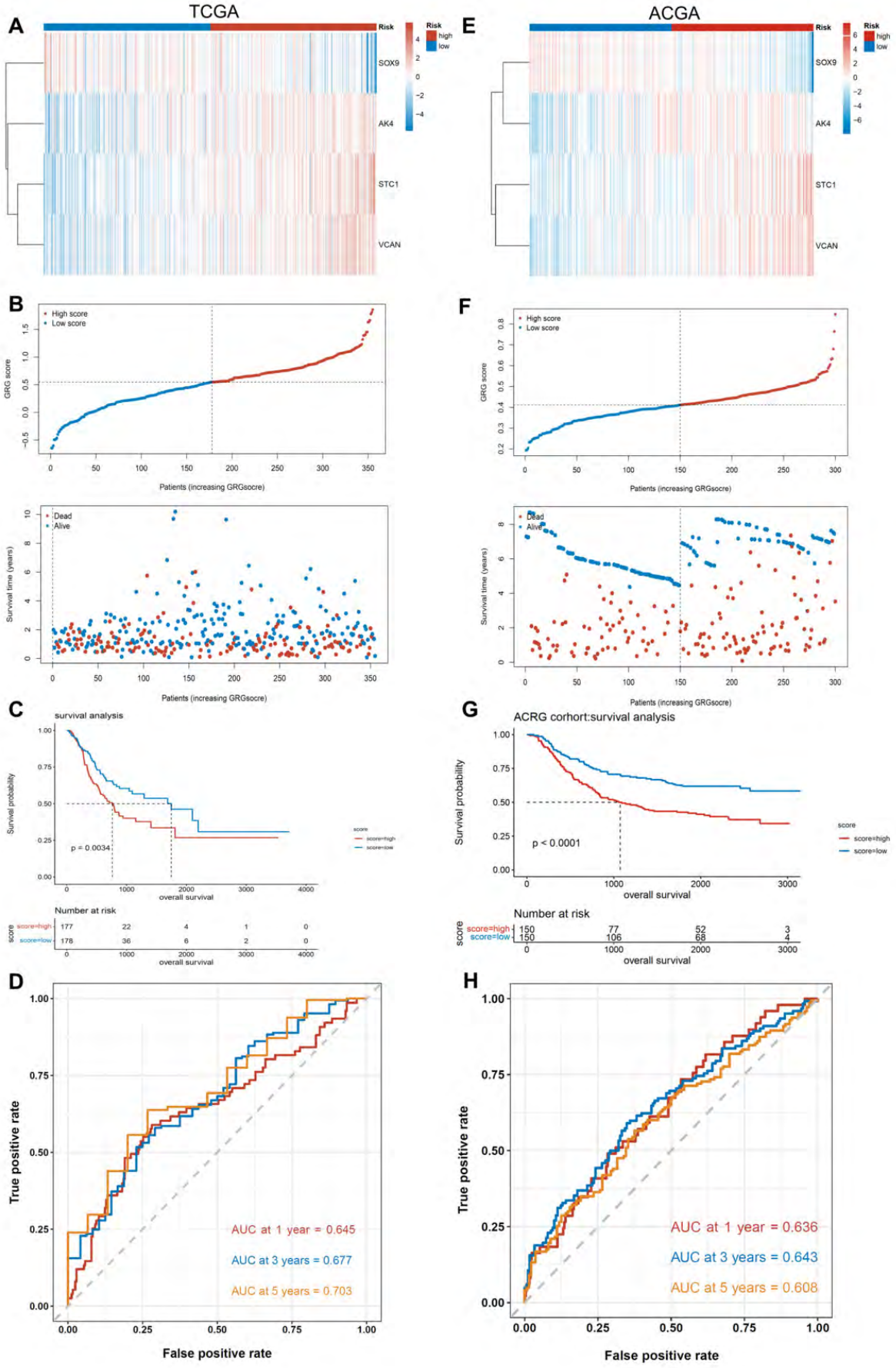

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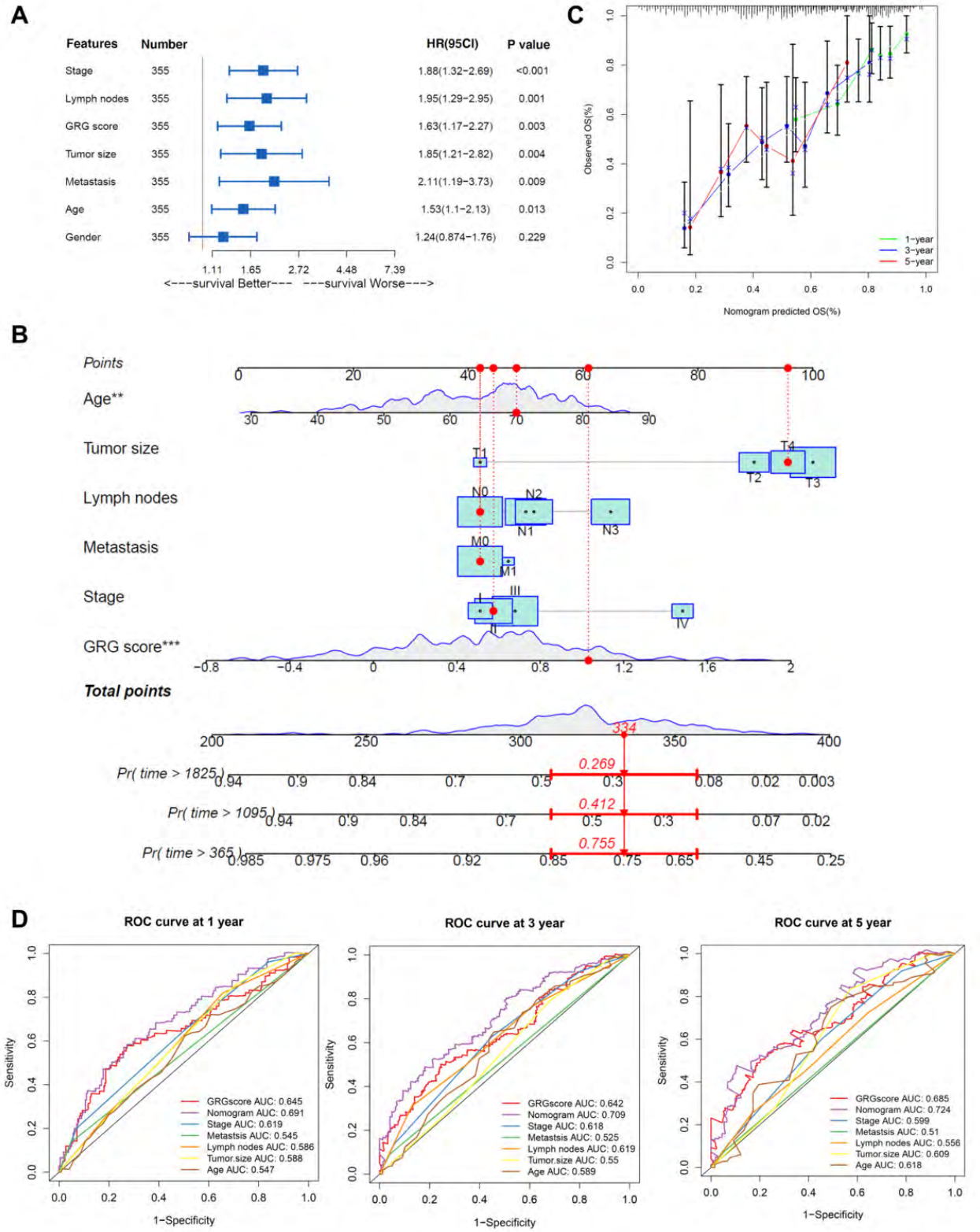

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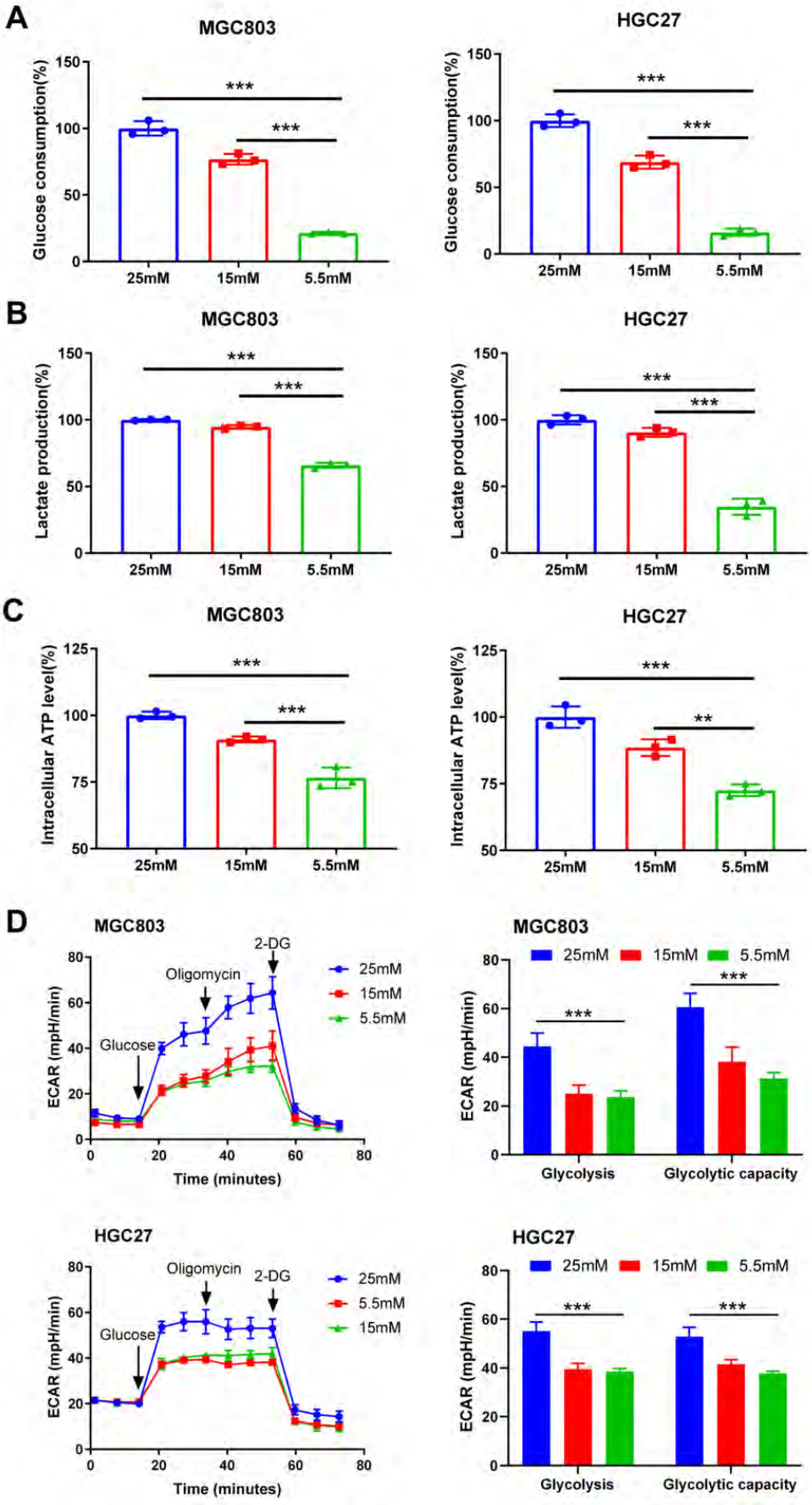

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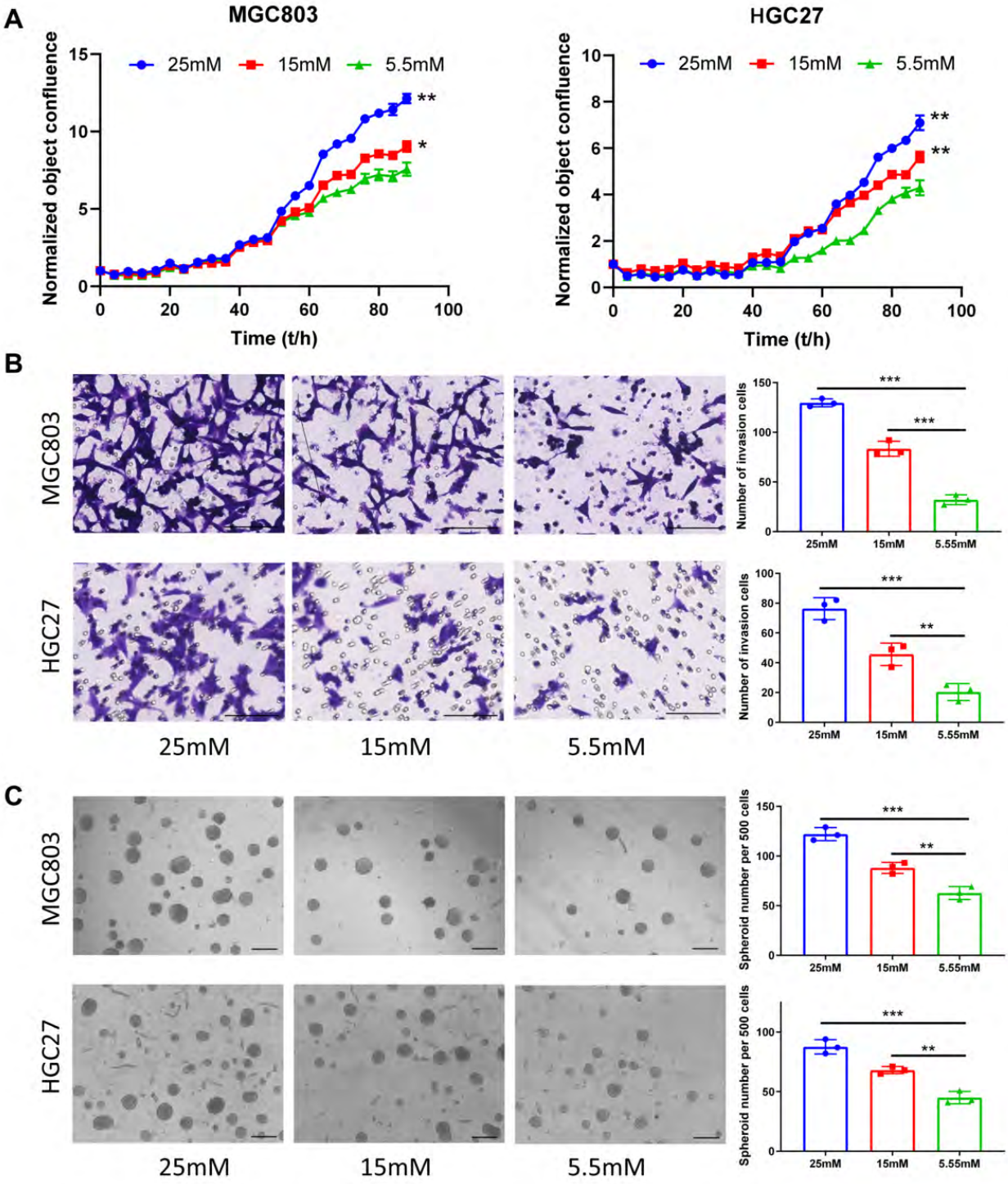

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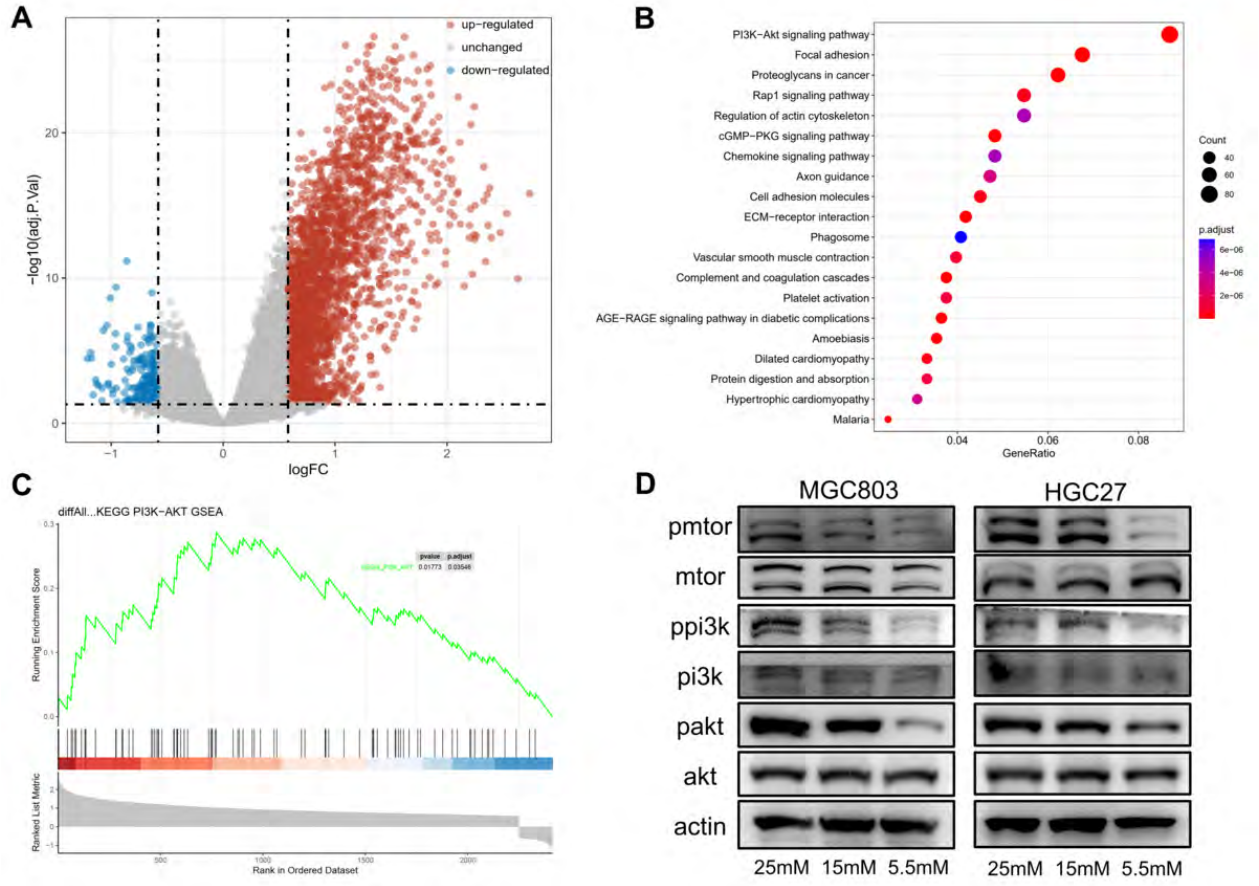

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