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Inhibition of Glutaminolysis Inhibits Cell Growth *via* Down-regulating Mtorc1 Signaling in Lung Squamous Cell Carcinoma

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Abstract. *Background/Aim: Inhibition of glutaminolysis has been reported as a promising therapeutic strategy to target several solid carcinomas. We aimed to investigate the effects of glutaminolysis on cell proliferation in lung squamous cell carcinoma cell lines and to explore the potential of targeting glutaminolysis as an anticancer strategy. Materials and Methods: Glutamine (Gln) dependence was assessed in six lung squamous cell carcinoma cell lines. Cell proliferation, mammalian target of rapamycin complex 1 (mTORC1) activity and the induction of autophagy were assessed after inhibition of glutaminolysis via Gln depletion or glutaminase (GLS) inhibition. Results: Five of six lung squamous cell carcinoma cell lines exhibited glutamine- dependence. The extent of dependence was correlated with the mRNA levels of GLS1/GLS2. Inhibition of glutaminolysis inhibited cell proliferation by down-regulating of mTORC1 signaling and inducing autophagy in Gln-dependent lung squamous cell carcinoma cell lines. Conclusion: Targeting glutaminolysis may represent a potential therapeutic strategy for the treatment of Gln-dependent lung squamous cell carcinomas.*

Lung cancer is the leading cause of cancer-related death worldwide in both men and women (1). Some molecular targeted drugs have improved survival in non-small cell lung carcinoma (NSCLC) but, mostly, in the context of adenocarcinoma (2). Such studies for lung squamous cell carcinoma are quite limited; therefore, there is an urgent need to identify novel molecular targets for the treatment of this disease (3).

It has recently been shown that targeting cancer cell metabolism can be an effective form of cancer treatment (4). The most famous theory of cancer cell metabolism is the "Warburg effect" in which cancer cells consume glucose at an extremely high rate and secrete large amounts of lactate, even in the presence of oxygen (5). Glutaminolysis has been shown to be an essential source of nitrogen for nucleotide and protein synthesis, as well as a modulator of protein translation *via* regulation of the mammalian target of rapamycin complex 1 (mTORC1) (6). During glutaminolysis, glutamine (Gln) enters the cells and is converted into glutamate by glutaminase (GLS). Glutamate is then converted into α−ketoglutarate, which enters the tricarboxylic acid (TCA) cycle and may later exit the cycle as malate to be processed into lactate (7). Recent studies have shown that inhibition of glutaminolysis is a promising new therapeutic strategy for treating acute myeloid leukemia (AML) (8, 9). Two isoforms of GLS have been identified in human cells: GLS1 and GLS2. It has been shown that regulation of GLS1 and glutaminolysis is under oncogenic control in cancer. The oncogenic transcription factor c-Myc enhances mitochondrial GLS1 expression and glutamine metabolism by suppressing miR-23a/b (10). Silencing *GLS1* significantly decreases the proliferation of prostate cancer cells *in vitro* (11), Ehrlich ascites tumor cells both *in vitro* and *in vivo* (12) and T98G glioblastoma cells (13). Expression of GLS2 is regulated by the *p53* gene (14).

Target of rapamycin (TOR) is a serine/threonine kinase that is highly conserved between yeasts and humans (15). Activation of the mammalian TOR (mTOR) signaling pathway in mammalian cells promotes cell growth and metabolism and inhibits apoptosis. It is reported that glutaminolysis can stimulate lysosomal translocation and activation of mTORC1, which controls cell growth and autophagy (16).

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Key Words: Lung squamous cell carcinoma, molecular targeted therapy, glutaminolysis, glutaminase, BPTES, mTORC1.

In the present study, we investigated the effects and underlying molecular mechanisms of glutaminolysis in six human lung squamous cell carcinoma cell lines by measuring cell proliferation under conditions of Gln depletion and by application of the GLS1-selective inhibitors [bis-2-(5 phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulfide] (BPTES) and 5-[3-Bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2, 2-dimethylbenzo[a]phenanthridin-4(1H)-one (compound 968) (17-19).

Materials and Methods

Cell lines and cell culture. Five lung squamous cell carcinoma cell lines (Sq-1, LK-2, LC-1/sq, EBC-1 and RERF-LC-AI) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The QG56 line was generously provided by Professor Gemma from the Nippon Medical School (Tokyo, Japan). All cell lines were grown in RPMI-1640 media (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and cultured at 37˚C in a humidified incubator in the presence of 5% CO_2.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA) and reverse transcriptase reactions were performed using 2 μg aliquots of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using TaqMan Universal PCR Master Mix and the TaqMan Gene Expression Assay (Applied Biosystems) in an ABI 7900HT sequence detection system (Applied Biosystems). The TaqMan Gene Expression Assay identifications (IDs) of detected genes were Hs00248163_m1 (*GLS1*), Hs00998733_m1 (*GLS2*) and Hs02758991_g1 (*GAPDH*). *GAPDH* was used as an internal control.

Western blot analysis. Whole-cell extracts prepared using a protein extract kit (Active Motif, Tokyo, Japan) were loaded onto a NuPAGE 4%–12% Bis–Tris gel (Invitrogen, Carlsbad, CA, USA), subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) iBlot Gel Transfer Stacks (Invitrogen). After blotting, membranes were blocked in 1% non-fat milk for 1 h and then probed with the following antibodies overnight at 4˚C: A rabbit monoclonal antibody against p-S6 (Cell Signaling Technologies, Danvers, MA, USA); a mouse monoclonal antibody against S6 (Cell Signaling); a rabbit polyclonal antibody against GLS2 (both from Abcam, Cambridge, MA, USA); and a rabbit polyclonal antibody against LC3-II (Cell Signaling). Membranes were washed four times for 10 min each with TBS-T and incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. A rabbit monoclonal antibody against β-actin (Cell Signaling) was used as an internal control. Antibody detection was performed using a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA).

AlamarBlue cell viability assay. Cells were seeded into a 96-well plate in RPMI-1640 containing 10% FBS (Gibco). After 24 h, the medium was carefully removed, replaced with 200 μl RPMI containing Gln or without Gln (Gibco) and cultured for 72 or 120 h. Next, 20 μl of AlamarBlue Cell Viability Assay Reagent (Life Technologies, Zug, Switzerland) was added and the cells were incubated for another 4 h. The samples were assayed in a spectrophotometer with a plate reader set at 570 nm excitation and 595 nm emission wavelengths.

GLS1 inhibitors' study. Cells were seeded into a 96-well plate in RPMI-1640 medium containing 10% FBS at a growth rate-dependent density. After 24 h, the medium was replaced with 200 μL RPMI-1640 supplemented with 10% (v/v) FBS and different concentrations of BPTES (0, 10, 20 and 40 μM) or compound 968 (0, 1, 5, 10, 20 and 40 μM). The cells were cultured for another 5 days before a cell viability assay was performed. Each experiment was performed in six replicate samples and repeated three times.

Knockdown assay. *GLS2* knockdown was achieved using *GLS2* siRNA (Life Technologies; #4392420); the negative control siRNA was also from Life Technologies. siRNA (10 or 50 nM) was transiently transfected into QG56 cells using a reverse transfection protocol with lipofectamine RNAiMAX, according to the manufacturer's protocol (Invitrogen). After 24 h, the medium was replaced with RPMI with or without Gln and culture was continued for 72 or 120 h, followed by a cell viability assay using AlamarBlue. Alternatively, after 24 h of knockdown, cells were subjected to Gln-depleted conditions for 24 h or were incubated with 10 μM BPTES or 10 μM compound 968 for 48 h and the cell proteins were subjected to Western blotting.

Autophagy measurements. RERF-LC-AI and QG56 cells were seeded into a 6-well plate in complete RPMI medium. After 24 h, the medium was replaced with Gln-depleted RPMI medium for 2 h or 10 μM BPTES-containing medium for 22 h. Then, the cells were cultured for a further 2-h period in the same medium with or without lysosomal protease inhibitors (10 μg/mL E64d and pepstatin). Cell lysates were subjected to NuPAGE followed by immunoblot analysis with the anti-LC3 antibody.

Statistical analysis. Data are presented as the mean±standard deviation. One-way analysis of variance (ANOVA) and Tukey-Kramer tests were used to assess the significance of differences. A *p*-value less than 0.05 was deemed to indicate statistical significance.

Results

Glutamine depletion decreases cell proliferation in five of six lung squamous cell carcinoma cell lines. Six lung squamous cell carcinoma cell lines were cultured in complete or Gln-depleted media for up to 120 h. As shown in Figure 1A, the proliferation of Sq-1, LK-2, LC-1/sq, EBC-1 and RERF-LC-AI cells was significantly decreased in Gln-depleted medium (*$p<0.05$). In the EBC-1 and RERF-LC-AI cell lines, cell proliferation was inhibited by over 50% after 72 h and by 70% after 120 h. The Sq-1, LK-2 and LC-1/sq cell lines exhibited reduced proliferation (30-47% inhibition) after 72 h and a 47-62% reduction in proliferation after 120 h. It was considered that inhibition >70% indicated high-level Gln-dependence and inhibition of 30-70% intermediate-level

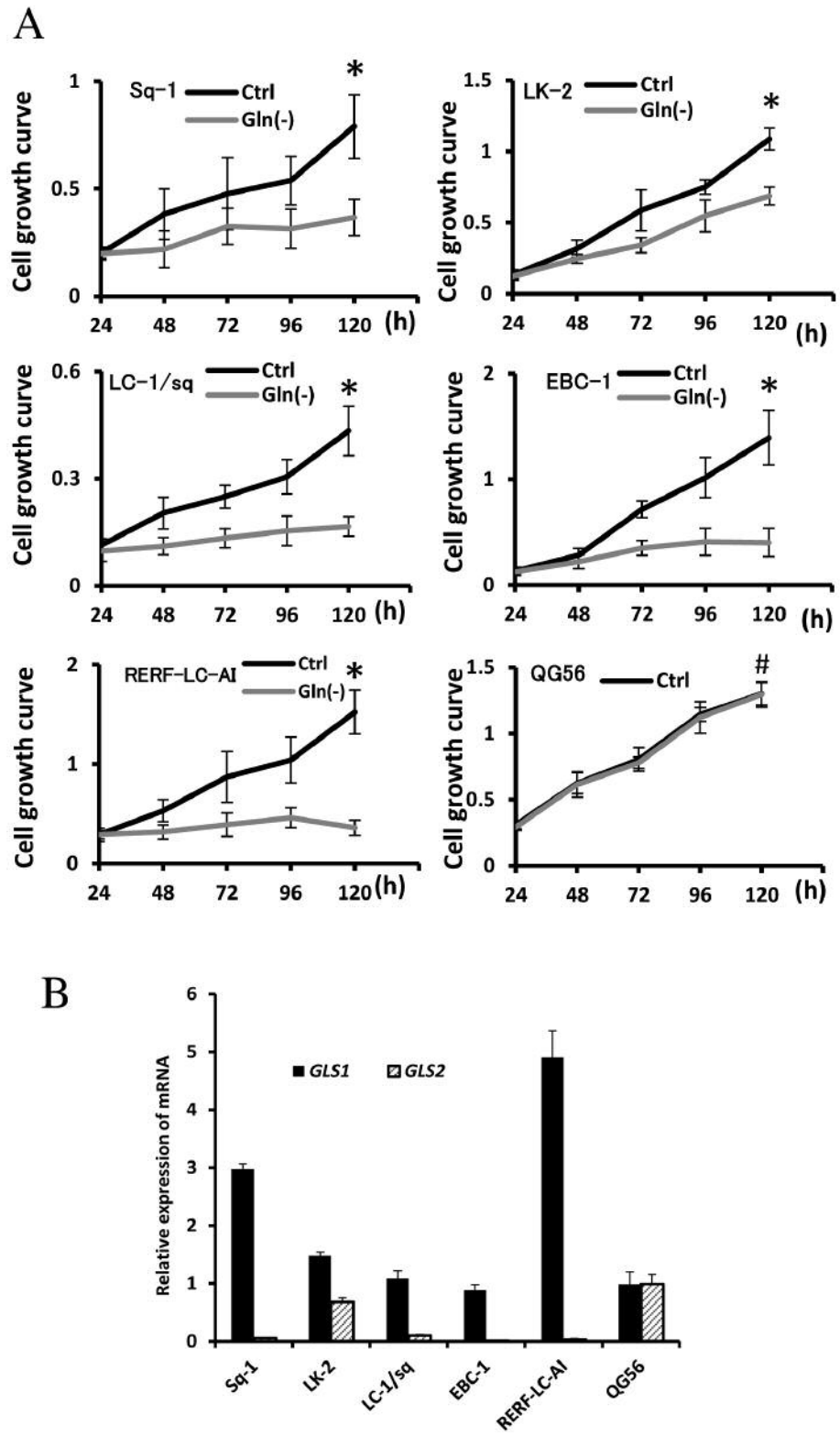

Figure 1. *Glutaminolysis profiles of six human lung squamous cell carcinoma cell lines. (A) Effect of glutamine depletion on cell proliferation. Cells were grown for the indicated times in complete or Gln-depleted medium. Cell growth was assessed by the AlamarBlue assay. The data points shown represent the mean±SD of three independent experiments (n=6, *p<0.01, #p>0.05 compared to control). (B) GLS1 and GLS2 mRNA expression was measured by real-time PCR. Expression ratios were normalized to the GAPDH expression level. Data represent the mean±SD of three independent experiments.*

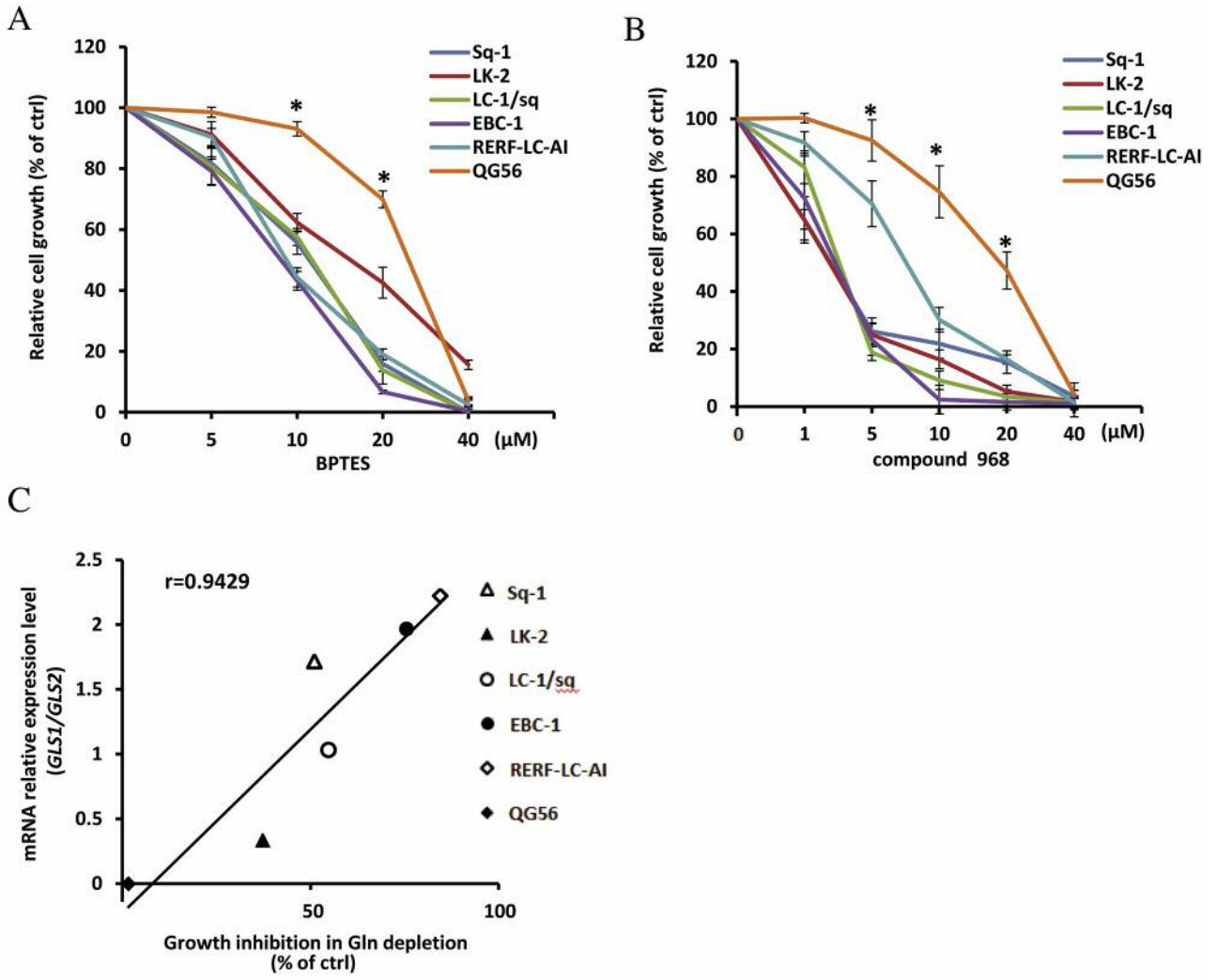

Figure 2. *The effect of glutaminase inhibition on cell growth in six human lung squamous cell carcinoma cell lines. (A, B) The GLS1-selective inhibitors BPTES and compound 968 inhibited cell growth. Cells were grown for 5 days in control medium or medium containing different concentrations of BPTES (5, 10, 20 or 40 μM) or compound 968 (1, 5, 10, 20 or 40 μM). Cell growth was assessed by the AlamarBlue assay. The data points shown represent the mean±SD of three independent experiments (n=6, *p<0.01, QG56 compared to all other conditions). (C) Correlation between mRNA expression (GLS1/GLS2) and growth inhibition under glutamine-depleted conditions for 5 days (Spearman's rank correlation coefficient: r=0.9429, p<0.05). The y-axis has a logarithmic scale.*

dependence (7). In this study, EBC-1 and RERF-LC-AI were considered to be highly Gln-dependent, whereas Sq-1, LK-2 and LC-1/sq were considered to have Gln dependence at intermediate levels. In contrast, no significant inhibition of cell proliferation was observed in the QG56 cell line at either 72 or 120 h (Figure 1A, $^{\#}p>0.05$). Therefore, this cell line was considered Gln-independent.

mRNA expression ratio of GLS1/GLS2 is essential for Gln dependence in lung squamous cell carcinoma cell lines. As shown in Figure 1B, the relative mRNA level of *GLS1* was markedly higher than that of *GLS2* in all cell lines, except for QG56. It is reported that GLS1 is the key enzyme associated with Gln dependence in NSCLC (7). Therefore, we hypothesized that GLS1 may be also essential to glutaminolysis in lung squamous cell carcinoma cell lines. The effect of GLS1 on growth of lung squamous cell carcinoma cell lines was then studied using the GLS1-specific inhibitor BPTES. Cells were incubated with different concentrations of BPTES (0, 5, 10, 20 and 40 μM) for 5 days. The proliferation of the Gln-dependent cell lines RERF-LC-AI and EBC-1 was strongly inhibited (RERF-LC-

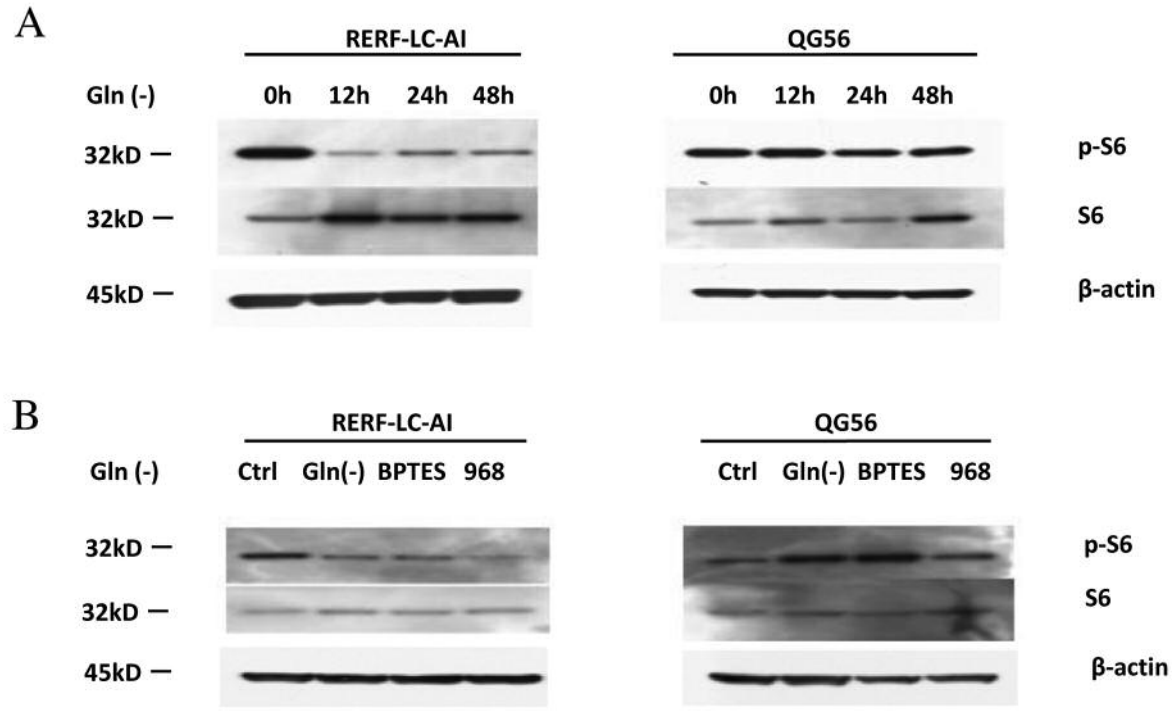

Figure 3. *Inhibition of glutaminolysis alters mTORC1 activity in Gln-dependent and -independent cell lines. (A) Phosphorylation status of S6 in the Gln-dependent cell line RERF-LC-AI and the Gln-independent cell line QG56 upon glutamine withdrawal for 0, 12, 24 and 48 h. (B) Phosphorylation status of S6 in the Gln-dependent cell line RERF-LC-AI and the Gln-independent cell line QG56 cultured under glutamine-depleted conditions for 24 h or treated with 10 μM BPTES or 10 μM compound 968 for 48 h.*

AI: 55.76±3.18% for 10 μM BPTES and 81.00±1.76% for 20 μM BPTES; EBC-1: 56.83±3.07% for 10 μM BPTES and 93.36±0.57% for 20 μM BPTES). In the Gln-independent cell line, QG56, inhibition of cell proliferation was only 7.93±2.34% in 10 μM BPTES and 30.11±2.77% in 20 μM BPTES (*$p<0.05$, compared with RERF-LC-AI and EBC-1). We also confirmed the effect of GLS1 inhibition on cell growth using another GLS1-selective inhibitor, compound 968. When Gln-dependent cell lines were cultured with 5, 10 or 20 μM of compound 968, proliferation was greatly inhibited compared to that of QG56 cells (Figure 2B, *$p<0.01$). These results indicate that inhibition of GLS1 inhibits cell proliferation in Gln-dependent lung squamous cell carcinoma cell lines.

In contrast to these findings, no significant correlation was found between *GLS1* mRNA expression levels and cell growth inhibition by Gln depletion (data not shown). It is interesting that the relative expression of *GLS1* mRNA is nearly equal in the EBC-1 and QG56 cell lines (Figure 1B), yet the level of Gln dependence is extremely different, as shown in Figure 1A. To investigate this phenomenon, the mRNA expression ratio of *GLS1*/*GLS2* in the lung squamous cell carcinoma cell lines was evaluated, revealing that the *GLS1*/*GLS2* ratio strongly correlates with Gln dependence (Figure 2C). The Spearman's rank correlation coefficient indicates that the *GLS1*/*GLS2* ratio perfectly and positively correlates with cell growth inhibition under Gln-depleted conditions (Rs=0.942, $p<0.05$). RERF-LC-AI served as a representative Gln-dependent cell line and QG56 as a Gln-independent cell line in subsequent experiments. To confirm the importance of the *GLS1*/*GLS2* ratio, we used RNA silencing to determine whether GLS2 inhibition in QG56 cells conferred Gln-dependence and suppressed cell proliferation. As shown in Figure 4B, after knockdown of GLS2 protein expression using *GLS2* siRNA, QG56 cell proliferation decreased to 83.8 and 69% of the initial level 72 and 120 h later, respectively (*$p<0.01$ *versus* the untransfected or control siRNA groups). Cell proliferation was further inhibited when the cells were subjected to Gln depletion after *GLS2* knockdown, especially when the culture time was prolonged to 120 h (▲$p>0.05$ *versus* the *GLS2* siRNA group, **$p<0.01$ *versus* the *GLS2* siRNA group).

Inhibition of glutaminolysis suppresses mTORC1 activity in Gln-dependent cell line but not in Gln-independent cell line. mTORC1 activity was analyzed by assessing the extent of

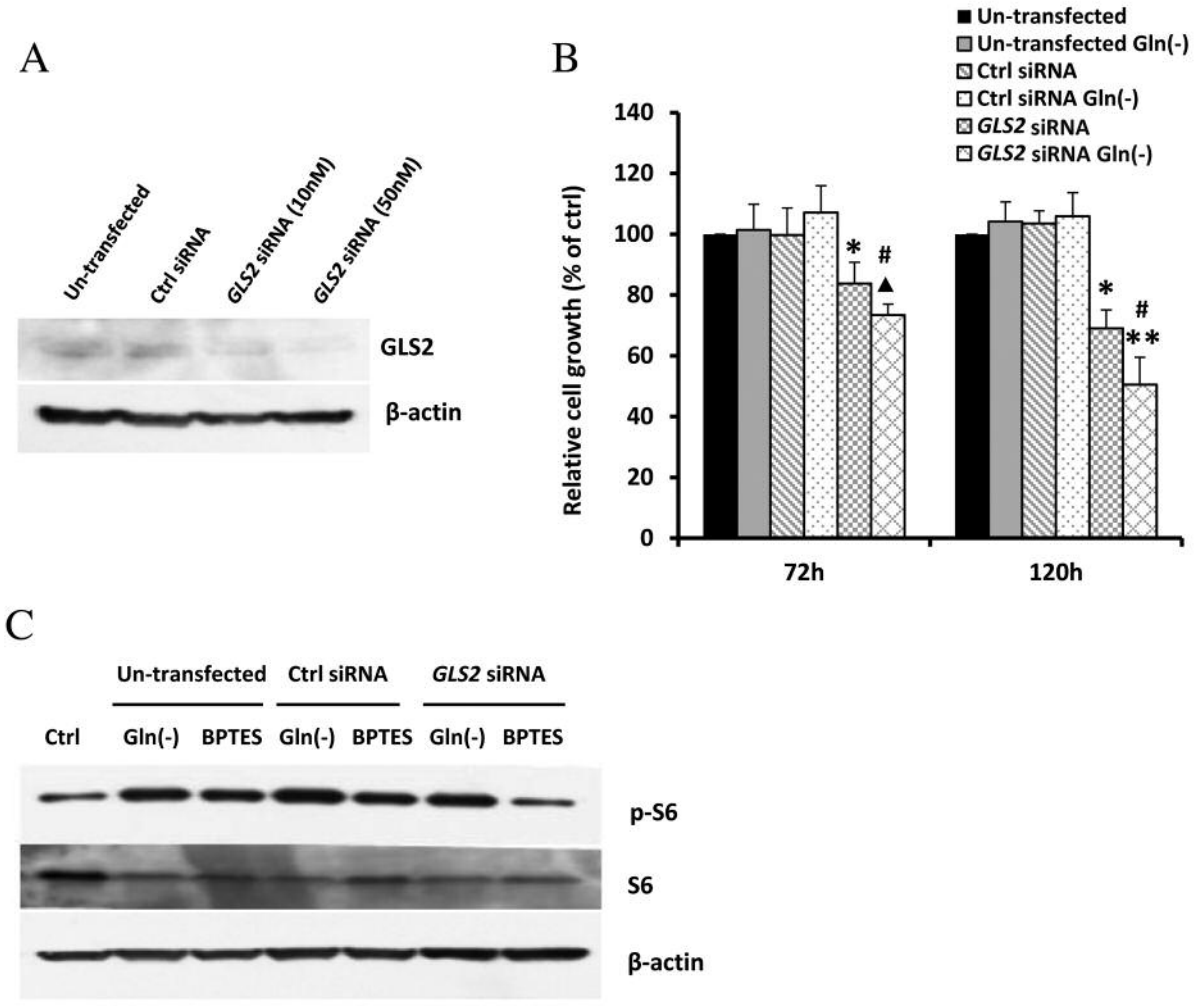

Figure 4. *GLS2 knockdown confers an element of Gln dependence in the Gln-independent cell line QG56. (A) Western blots measuring GLS2 protein levels after knockdown using control siRNA or GLS2 siRNA (10 or 50 nM) in QG56 cells. (B) After knockdown with control siRNA or 50 nM GLS2 siRNA, QG56 cells were grown for 3 or 5 days in complete or Gln-depleted medium. Cell growth was assessed using the AlamarBlue assay. The data represent the means±SDs of those of three independent experiments (n=6, *p<0.01 compared with untransfected or control siRNA-treated cells; #p<0.01 compared with untransfected or control siRNA-treated cells; ▲p>0.05 compared with the GLS2 siRNA-treated group; **p<0.01 compared with the GLS2-treated siRNA group). (C) After knockdown using control siRNA or 50 nM GLS2 siRNA, QG56 cells were cultured under glutamine-depleted conditions for 24 h or in the presence of 10 μM BPTES or 10 μM compound 968 for 48 h. The level of phosphorylated S6 was analyzed by western blotting.*

phosphorylation of S6 ribosomal protein (p-S6), one of the major downstream substrates of the mTOR pathway. The Gln-dependent cell line RERF-LC-AI exhibited decreased levels of S6 phosphorylation when cultured in Gln-depleted medium for 12, 24 and 48 h, whereas the levels of phosphorylated S6 in the Gln-independent QG56 cell line exhibited negligible changes (or even increased in extent) at all tested time points (Figure 3A). After treatment with 10 μM BPTES or compound 968 for 48 h, the levels of phosphorylated S6 were inhibited in RERF-LC-AI cells, but not in QG56 cells (Figure 3B). These data indicate that suppression of glutaminolysis inhibited mTORC1 activation in Gln-dependent cell lines but had no significant inhibitory effect in the QG56 cell line. However, after knockdown of GLS2 (using *GLS2* siRNA) in QG56 cells, the levels of phosphorylated S6 were inhibited, especially when the cells were further treated with BPTES (Figure 4C).

Inhibition of glutaminolysis induces autophagy in Gln-dependent cell line. After confirming the inhibitory effect of the mTORC1 pathway in Gln-dependent lung squamous cell carcinoma cell lines by inhibiting glutaminolysis, we

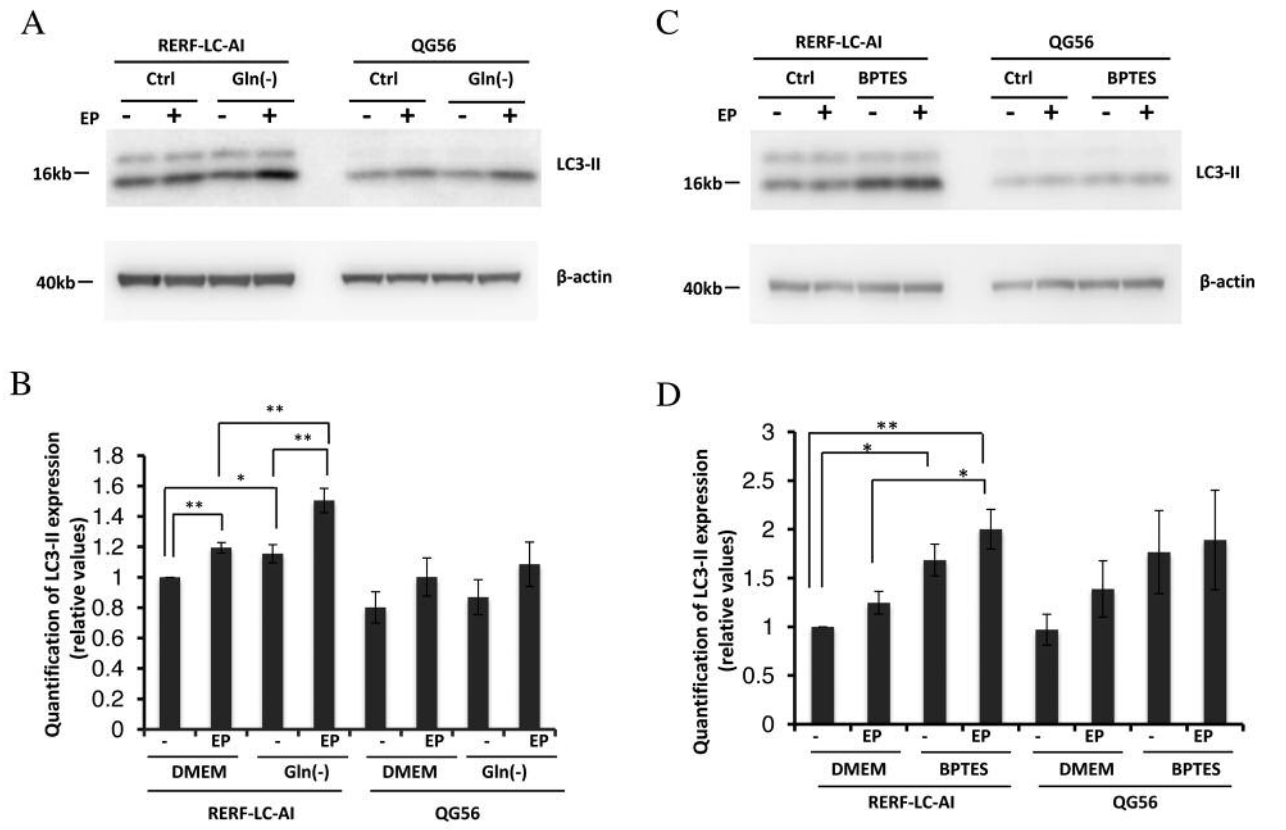

Figure 5. *Inhibition of glutaminolysis induces autophagy in a Gln-dependent cell line. (A) RERF-LC-AI and QG56 cell lines treated with glutamine depletion for 24 h. Endogenous LC3-II levels were analyzed by immunoblotting. (B) Quantification of five independent experiments (n=5, *p<0.05, **p<0.01). (C) RERF-LC-AI and QG56 cell lines treated with 10 μM BPTES for 4 h. Endogenous LC3-II levels were analyzed by immunoblotting. (D) Quantification of five independent experiments (n=5, *p<0.05, **p<0.01).*

investigated whether autophagy was activated in this cell line under these conditions. To date, LC3 is a reliable marker of autophagosomes (20, 21). The level of LC3-II correlates significantly with autophagosome formation due to its association with the autophagosome membrane and is, thus, commonly used as an indicator of autophagic activity. E64d and pepstatin A were used to inhibit lysosomal enzymes and impede autophagosome clearance. As shown in Figure 5A and 5B, the Gln-dependent cell line RERF-LC-AI showed increased LC3-II accumulation when cultured in Gln-depleted medium. The accumulation of LC3-II was further enhanced when E64d and pepstatin A were applied (*$p<0.05$, **$p<0.01$). Conversely, there was no statistically significant difference in the accumulation of LC3-II in the Gln-independent cell line QG56 (Figure 5A and B). Treatment with BPTES also increased LC3-II accumulation in RERF-LC-AI (Figure 5C and 5D, $p<0.05$, **$p<0.01$) but not in QG56 ($p>0.05$) cells. These data indicate that inhibition of glutaminolysis increases autophagic activity in Gln-dependent lung squamous cell carcinoma cell line.

Discussion

In the present study, we investigated the role played by glutaminolysis in human lung squamous cell carcinoma cell lines. The results indicate that disruption of glutamine metabolism could be an effective therapeutic approach for the treatment of Gln-dependent lung squamous cell carcinoma.

GLS is a key enzyme for glutamine metabolism playing a key role in oncogenic transformation (12, 22) and in cancer cell growth and proliferation (13, 23, 24). Inhibition of GLS is currently being explored as a promising cancer therapeutic strategy; however, a major challenge is how to efficiently predict which tumors will respond to GLS inhibition (25). Increased GLS1 expression has been shown to be an adequate indicator in different tumors (26). GLS1 inhibition showed antileukemic activity in AML by activating mitochondrial apoptosis and synergizing with BCL-2 inhibition (9). In NSCLC, it has been demonstrated

that GLS1 is the key enzyme associated with Gln dependence and that the GLS1 inhibitor BPTES has an inhibitory effect on cell growth and proliferation in Gln-dependent NSCLC cell lines *in vitro* (7). In a similar manner, we have shown that the majority of lung squamous cell carcinoma cell lines tested (five of six) are Gln-dependent and BPTES strikingly inhibits the growth and proliferation of these lines. However, the Gln-dependent cell line EBC-1 displayed the same level of GLS1 expression as that of the Gln-independent cell line, QG56 (Figure 1B). Therefore, in these cell lines, increased GLS1 expression alone is not an indicator of Gln dependence. We observed that the relative mRNA levels of *GLS1* were much higher than those of *GLS2*, except in the QG56 cell line. It is reported that GLS1 expression is primarily up-regulated in rapidly proliferating cells, whereas increased GLS2 expression is associated with slowly proliferating cells, seemingly characteristic of resting or quiescent cells (26-28). In this context, the co-expression pattern of *GLS1* and *GLS2* may be useful for evaluating the effect of glutaminolysis on cell growth and proliferation. We next analyzed the mRNA expression ratio of *GLS1*/*GLS2* in lung squamous cell carcinoma cell lines and found that it was strongly correlated with Gln dependence. Higher *GLS1*/*GLS2* mRNA expression ratios were strongly associated with Gln dependence and a compromise in cell growth upon GLS1 inhibition. Interestingly, *GLS2* knockdown conferred an element of Gln dependence; the Gln-independent cell line QG56exhibited reduced proliferation and decreased mTORC1 activity (Figure 4B and 4C). These data are in line with those of a recent report that GLS2 inhibition reduced carcinoma cell proliferation and colony formation and inhibited mTORC1 activity (29). Thus, our data show that the degree of Gln dependence can be predicted based on the mRNA expression ratio of *GLS1*/*GLS2*. Further studies will be needed to determine the percentage threshold of *GLS1* among total *GLS* mRNA expression to predict the Gln dependence of cancer cells.

mTORC1 promotes cell growth (30, 31) and inhibits autophagy (32). These processes are regulated by glutaminolysis, which is upstream of the Rag GTPase and mTORC1 pathways (16). Therefore, it is plausible that inhibition of glutaminolysis might have an anticancer effect. The GLS1-selective inhibitors BPTES and compound 968 are effective tools when exploring GLS activities. Similarly, our data demonstrate that inhibition of glutaminolysis by Gln depletion or GLS1 inhibitors inhibit cell growth and proliferation *via* down-regulation of mTORC1 signaling and induction of autophagy in Gln-dependent lung squamous cell carcinoma cell lines.

To our knowledge, this study is the first to describe the effect of glutaminolysis on cell growth and proliferation in lung squamous cell carcinoma cell lines. Our study indicates that the mRNA expression ratio of *GLS1*/*GLS2* may be a useful indictor of Gln dependence. Furthermore, the data described herein support the use of GLS1 inhibitors as a potential targeted therapeutic strategy in Gln-dependent lung squamous cell carcinoma.

Conflicts of Interest

No Author has any conflicts of interest to report.

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