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ORIGINAL ARTICLE

Glucose transporter 1 regulates the proliferation and cisplatin sensitivity of esophageal cancer

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Abstract

Glucose transporter 1 (GLUT1) expression is a prognostic marker for esophageal squamous cell carcinoma (ESCC). Recent work on GLUT1 and development of specific inhibitors supports the feasibility of GLUT1 inhibition as a treatment for various cancers. The anti–proliferative effects of GLUT1-specific small interfering RNA (siRNA) and a GLUT1 inhibitor were evaluated in ESCC cell lines. Expression of pro–proliferative and anti–proliferative signaling and effector molecules was examined by western blotting and quantitative RT-PCR. GLUT1 expression in pretreatment clinical biopsy samples was measured by immunohistochemistry and correlated with various clinicopathological parameters and response to chemotherapy. The reduction in standardized uptake value (SUV) of ^{18}F-fluoro-deoxyglucose was calculated using the formula: ([pretreatment SUV_{max} − posttreatment SUV_{max}]/pretreatment SUV_{max}) × 100. GLUT1-specific siRNA expression in ESCC cells inhibited their proliferation, increased expression of p27kip, and decreased expression of cyclin-dependent kinase 6, pyruvate kinase muscle isozyme M2, lactate dehydrogenase A and phospho-ERK1/2. Suppression of GLUT1 by siRNA increased low-dose cisplatin-induced inhibition of proliferation of TE-11 ESCC cells, which express high GLUT1 levels. Similarly, BAY-876, a GLUT1 inhibitor, enhanced cisplatin-mediated inhibition of ESCC cell proliferation. GLUT1 expression in pretreatment biopsy samples was associated with the response to chemotherapy as well as the pathological tumor stage and histological response grade after esophagectomy. Finally, GLUT1-negative tumors showed a significantly larger reduction in SUV_{max} (61.2% ± 4.5%) compared with GLUT1-positive tumors (46.2% ± 4.4%). GLUT1 expression may be a surrogate marker of response to chemotherapy, and inhibition of GLUT1 may be a potential novel therapy for ESCC patients.

KEYWORDS
BAY-876, chemotherapy, GLUT1, GLUT1 inhibitor, PET

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1 | INTRODUCTION

Esophageal carcinoma is an aggressive disease with a tendency to spread both locoregionally and distally, and it is generally treated with a multidisciplinary approach.[1] The Japan Clinical Oncology Group Study JCOG-9204 showed that postoperative chemotherapy with 2 courses of 5-fluorouracil (5-FU) and cisplatin significantly prolonged the survival of patients with node-positive stage II/III esophageal squamous cell carcinoma (ESCC) compared with surgery alone.[2] Moreover, ESCC patients treated with preoperative chemotherapy survived longer than those receiving postoperative chemotherapy in the JCOG-9907 study.[3] Based on these findings, the current standard treatment for resectable stage II/III ESCC in Japan is chemotherapy followed by surgery. Despite treatment with multiple anticancer drugs, however, the disease still progresses in some patients. The underlying mechanisms that determine the sensitivity/resistance of ESCC to anticancer agents remains unclear.

Cisplatin cytotoxicity is mediated via formation of cisplatin–DNA adducts,[4] which leads to irreparable DNA damage. Many chemotherapeutic drugs and radiation therapy induce oxidative stress in targeted cells; indeed, reactive oxygen species are required for radiation-induced DNA damage. Therefore, accumulation of antioxidants (eg, lactate) may induce or enhance resistance to radiation and some chemotherapies.[5] Cancer cells exhibit altered glucose metabolism, termed the Warburg effect, which is defined as increased uptake of glucose and conversion to lactate under conditions of adequate oxygen tension.

Expression of glucose transporter type 1 (GLUT1) is affected by environmental and cancer-specific metabolic events. GLUT1 expression is normally restricted to erythrocytes, endothelial cells at the blood–brain barrier and the placenta, and it is generally absent from normal epithelial cells. However, elevated GLUT1 expression has been observed in many epithelial malignancies, and high GLUT1 levels have been reported to be a prognostic marker for esophageal cancer.[6] Analysis of datasets from The Cancer Genome Atlas confirmed that patients with head and neck squamous cell carcinoma with a favorable immune and metabolic gene signature (high CD8A, high COX5B and low GLUT1) had better short-term and long-term survival compared with patients with an unfavorable signature.[7] Positive GLUT1 expression has also been associated with tumor regression grade and may be a useful predictive marker for response to chemoradiotherapy in rectal cancer.[8] However, the relationship between GLUT1 expression and chemotherapy resistance in ESCC remains unclear. The crystal structure of human GLUT1 has been reported,[9] and a potent GLUT1 inhibitor, BAY-876, was identified in a screen of approximately 3 million compounds.[10] These and other recent studies have suggested that GLUT1 inhibition may be a feasible cancer treatment.

One important goal for esophageal cancer therapy is the discovery of novel agents that can overcome the resistance and/or improve the sensitivity of tumor cells to anticancer agents. Here, we investigated the anti–proliferative effects of inhibiting GLUT1 function via genetic (small interfering RNA [siRNA]) and pharmacological (BAY-876) approaches in human ESCC cell lines. We also demonstrate that GLUT1 inhibition increases ESCC cellular sensitivity to cisplatin and that GLUT1 expression in clinical biopsy samples correlates with patient response to chemotherapy. The results of this study may enable the development of GLUT1 inhibitors as a novel therapeutic strategy for ESCC.

2 | MATERIALS AND METHODS

2.1 | Patients

We consecutively enrolled 105 node-positive ESCC patients who were treated with modified DCF (mDCF; 60 mg/m^2 docetaxel on day 1; 350 mg/m^2 5-FU and 6 mg/m^2 cisplatin on days 1-5) at Kumamoto University Hospital from January 2008 to December 2012. All patients underwent upper gastroenterological fiberscope, enhanced computed tomography imaging from neck to abdomen, and ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG-PET) for tumor staging according to the TNM classification. Endoscopically biopsied tumor specimens (n = 105) were collected before initiation of mDCF, and the samples were paraffin embedded for immunohistochemical analysis. Of the initial 105 patients, 8 did not complete chemotherapy, and 97 patients underwent 2 courses of mDCF followed by post–chemotherapy FDG-PET. Sixty-one of these 97 patients then underwent radical esophagectomy (see scheme in Figure S1). Clinical data were collected retrospectively for all 105 patients and included age, gender and clinical TNM classification (criteria of the International Union Against Cancer, 7th edition). In addition, pathological lymph node metastasis, depth of invasion and histological response grade were obtained for the 61 patients who underwent surgery. Tumor regression was designated Grade 0, 1a, 1b, 2 or 3 based on the percentage necrotic area in the residual tumor and the histological response criteria for drug and radiotherapy: Grade 0, no regression; Grade 1a, extremely mild effect (tumor degeneration or necrosis by <1/3); Grade 1b, mild effect (tumor degeneration, necrosis, or fusion between 1/3 and 2/3); Grade 2, significant effect (remarkable tumor degeneration, necrosis, fusion, or tumor reduction by >2/3); and Grade 3, complete response (tumor disappearance, or tumor necrosis with rearranged granulation tissue or a fibrotic lesion). Informed consent was obtained from all patients, and the study was approved by the Institute Review Board of the Graduate School of Medical Science, Kumamoto University (approval number: 236; 2 August 2008).

2.2 | Analysis of response to therapy by ^{18}F-fluorodeoxyglucose positron emission tomography

Several studies have reported that a decrease in FDG uptake (maximal standardized uptake value, SUV_{max}) after chemotherapy is associated with an anti–proliferative effect.[11] The percentage reduction in SUV_{max} of the primary tumors was calculated for patients who underwent FDG-PET before and after chemotherapy using the

formula: ([pretreatment SUV_{max} – posttreatment SUV_{max}]/pretreatment SUV_{max}) × 100.

2.3 | Cell lines and treatments

Five ESCC cell lines (TE-1, TE-4, TE-8, TE-10 and TE-11) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, and the Riken BioResource Center Cell Bank. The cell lines were tested and authenticated using the Cell ID System (Promega, Madison, WI, USA). BAY-876 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was prepared in DMSO.

2.4 | Cell proliferation analysis

Cell proliferation was measured using a kit based on the water-soluble tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium (WST-8) according to the manufacturer's instructions (Dojin Chemicals, Tokyo, Japan). ESCC cells were cultured overnight in 96-well plates at a density of 1-5 × 10^3 cells per well. The number of surviving cells was assessed by determining the absorbance at 450 nm.

2.5 | Transfection of small interfering RNA

Small interfering RNA (siRNA) for GLUT1 and control nontargeting siRNA was obtained from ambion (Carlsbad, CA, USA). Stealth RNA interference sequences (GLUT1 siRNA#1 and siRNA#2) are shown in Figure S2. Non-silencing control siRNA, with no sequence homology to any known human gene sequence, was used as a control for non-specific effects in all experiments. Human esophageal cancer cells were transfected with siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen, CA, USA) following the manufacturer's instructions. Two days after transfection, the efficacy of siRNA knockdown was assessed using quantitative RT-PCR (qRT-PCR) and immunoblotting.

2.6 | Quantitative RT-PCR

RNA was isolated from the cell lines using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.[12] The expression levels of GLUT1, cyclin-dependent kinase 4 (CDK4), CDK6, p21cip1, p27kip, p53, GLUT3, hexokinase 2 (HK2), pyruvate kinase muscle isozyme M2 (PKM2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase isozyme-1 (PDK1) were determined by qRT-PCR using TaqMan probes and the reactions were run on a LightCycler 480 System II (Roche Diagnostic, Basel, Schweiz). All qRT-PCR were performed in triplicate. mRNA levels were normalized to β-actin mRNA.

2.7 | Western blot analysis

Cells and tumors were lysed in RIPA buffer (25 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl) supplemented with 2 mmol/L EDTA and Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo). The membranes were probed with primary antibodies overnight at 4°C, followed by incubation with a 1:1500 dilution of peroxidase-conjugated anti–rabbit IgG antibody. Detection was accomplished with an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

2.8 | Immunohistochemical staining of glucose transporter 1

Paraffin blocks of biopsy specimens were cut into 4-μm-thick sections and mounted on slides. Antigen was retrieved by microwaving the sections in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 5 minutes at 25°C. Sections were then incubated overnight at 4°C with polyclonal mouse anti–GLUT1 antibody (ab40084; Abcam, Cambridge, UK) diluted 1:200 with 0.1 mol/L PBS (pH 7.4). After washing, the sections were incubated with HRP-labeled polymer (EnVision+ kit; Dako, Carpinteria, CA, USA) for 60 minutes at 25°C and then incubated with 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 0.05 mol/L Tris-HCl (pH 7.6) for 15 minutes at 25°C.

2.9 | Evaluation of glucose transporter type 1 expression

Immunohistochemical staining of GLUT1 expression was evaluated by an experienced pathologist blinded to the clinical data. GLUT1 immunoreactivity was considered positive when a homogeneous intense staining was observed in the cell membranes of cancer cells, as previously reported.[13] The percentage of total cancer cells positive for GLUT1 in 3 randomly chosen high-power fields was calculated, and the average value was scored on a 3-point scale: weak, 0%-10%; moderate, 11%-50%; and strong, 51%-100%.

2.10 | Statistical analysis

Data are presented as the mean ± standard deviation (SD). Group means were compared using 2-tailed paired or unpaired Student's *t*-tests, as appropriate, based on the results of *F*-tests. The Mann–Whitney *U*-test was used for non-normally distributed data. Categorical variables were compared using the χ^2-test or Fisher's exact test as indicated. Statistical significance was defined as a *P-value* < 0.05. All data were processed and analyzed using JMP11 software (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | Glucose transporter 1 expression and the anti–proliferative effects of siRNA-mediated glucose transporter 1 silencing in esophageal squamous cell carcinoma cell lines

To initiate our investigation of the effects of GLUT1 inhibition in ESCC, we analyzed GLUT1 protein and mRNA levels in

FIGURE 1 Glucose transporter 1 (GLUT1) expression and the effects of siRNA-mediated GLUT1 inhibition. A, Quantitative RT-PCR (qRT-PCR) analysis of GLUT1 mRNA levels in esophageal squamous cell carcinoma (ESCC) cell lines, normalized to β-Actin levels. B, Western blot analysis of GLUT1 protein levels. β-Actin was probed as a loading control. C-E, qRT-PCR analysis of GLUT1 mRNA levels (C), western blot analysis of GLUT1 protein in TE-11 cells (D), and cell proliferation assay (E) of ESCC cell lines after transfection with control or GLUT1-targeting siRNA. F, Western blot analysis of PARP in TE-11 cells after transfection with control or GLUT1-targeting siRNA. G, qRT-PCR analysis of cell cycle-related genes in TE-8 and TE-11 cells after transfection with control or GLUT1-targeting siRNA. Graphs show the mean ± SD of n = 3 replicates. N.S., not significant, *$P < 0.05$ by Student's *t*-test

5 human ESCC cell lines. qRT-PCR and western blot analysis showed that the GLUT1 mRNA levels (Figure 1A) and protein levels (Figure 1B) were consistent between cell lines, with TE-11 cells showing the highest expression level. We next transfected TE-11, TE-10 and TE-8 cells (high, medium and low GLUT1 levels, respectively) with 2 GLUT1-specific siRNA or a scrambled control, and then determined the efficiency of silencing by RT-PCR. We found that GLUT1 mRNA (Figure 1C) and protein (Figure 1D) were strongly suppressed by both of the GLUT1-specific siRNA. We then examined the effects of GLUT1 silencing using a WST-9 cell proliferation assay. Indeed, TE-8, TE-10 and TE-11 cell proliferation was significantly reduced by transfection of GLUT1-targeting siRNA compared with control siRNA (Figure 1E), indicating that GLUT1 is required for ESCC cell proliferation. Based on these analyses, we selected TE-8 and TE-11 cells, which express low and high GLUT1 levels, respectively, to investigate the mechanism underlying the anti–proliferative effects of GLUT1 inhibition.

3.2 | Involvement of cell cycle-dependent kinases in the anti–proliferative effects of glucose transporter 1 inhibition

To clarify the mechanism by which GLUT1 silencing inhibits the proliferation of ESCC cells, we examined cell apoptosis by western blot analysis of cleaved poly (ADP-ribose) polymerase (cleaved PARP) levels and flow cytometric analysis of caspase-3 activity. These assays showed that neither PARP expression (Figure 1F) nor caspase-3 activity were influenced by GLUT1 siRNA expression, indicating that the anti–proliferative effect did not result from increased apoptosis. We next assessed whether expression of cell cycle-related genes was perturbed by GLUT1 inhibition. RT-PCR analysis of CDK4, CDK6, p21cip, p27kip and p53 showed that siRNA mediated GLUT1 silencing. CDK4 and p21cip levels were not significantly changed after using siRNA2 for GLUT1, suggesting the anti–proliferating effect of GLUT1 siRNA may result from decreased CDK6 and increase p27kip expression in TE-11 cells. A significant reduction in

CDK6 mRNA and a significant increase in p27kip mRNA levels were detected in both TE-11 and TE-8 cells. The inhibition of TE-11 proliferation was stronger than that of TE-8 proliferation after using GLUT1 siRNA, as shown by changes to CDK6 and p27kip expression (Figure 1G). These data indicate that GLUT1 inhibition affects cell cycle regulation in ESCC cells.

3.3 | Enhanced effects of cisplatin after inhibition of glucose transporter 1 expression in esophageal squamous cell carcinoma cells

We analyzed the association between GLUT1 expression and inhibitory concentration (IC) 50 values of cisplatin in 17 esophageal cancer cell lines using an online database (GSE36133 and the Genomics of Drug Sensitivity in Cancer). We divided the 17 cell lines into 2 groups according to high and low GLUT1 expression, based on average expression levels. The average ± SD of cisplatin IC50 values in cell lines with high and low expression of GLUT1 was 28.3 ± 8.2 and 18.3 ± 11.6 μmol/L, respectively, representing a tendency for the IC50 value to be lower in cell lines with low GLUT1 expression ($P = 0.0697$; Figure 2A). TE-8 cells showed low GLUT1 expression and a cisplatin IC50 value of 10.1 μmol/L, while TE-11 cells showed high GLUT1 expression and a cisplatin IC50 value of 20.4 μmol/L.

We next investigated whether the anti–tumor activity of cisplatin was affected by GLUT1 expression levels using GLUT1 siRNA. For this, TE-8 and TE-11 cells were transfected with GLUT1 siRNA for

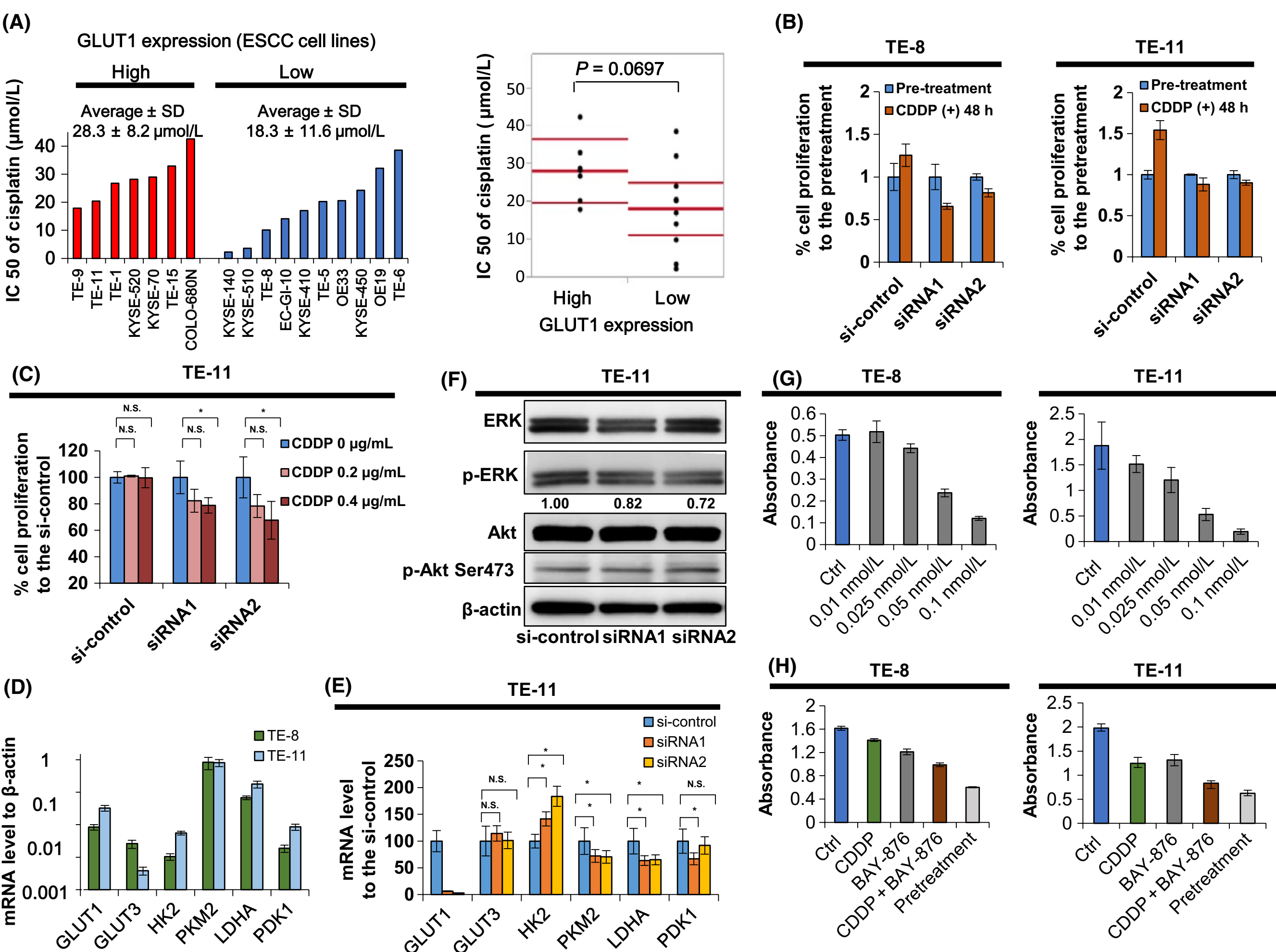

FIGURE 2 Anti–proliferative effects of cisplatin in combination with genetic or pharmacological inhibition of glucose transporter 1 (GLUT1). A, The association between GLUT1 expression and the inhibitory concentration (IC) 50 value of cisplatin in 17 esophageal cancer cell line using online data base. The 17 cell lines were divided into 2 groups according to high and low GLUT1 expression, based on average expression levels. B, Cell proliferation assay of TE-8 and TE-11 cell lines transfected with control or GLUT1-targeting siRNA and then incubated with cisplatin for 48 h. C, Cell proliferation assay of TE-11 cells transfected with control or GLUT1-targeting siRNA and then incubated with 0, 0.2 and 0.4 μg/mL cisplatin for 48 h. D, qRT-PCR analysis of glycolysis-related gene expression in TE-8 and TE-11 cells. E, qRT-PCR analysis of glycolysis-related gene expression in TE-11 cells transfected with control or GLUT1-targeting siRNA. F, Western blot analysis of total and phosphorylated (p-) ERK and AKT in TE-11 cells after transfection with control or GLUT1-targeting siRNA. G, Cell proliferation assay of TE-8 and TE-11 cell lines after treatment with DMSO (Ctrl) or the indicated concentrations of BAY-876 for 48 h. H, Cell proliferation assay of TE-8 and TE-11 cell lines after treatment with DMSO, 0.5 μg/mL cisplatin, 0.025 nmol/L BAY-876 or 0.5 μg/mL cisplatin plus 0.025 nmol/L BAY-876 for 48 h. Graphs show the mean ± SD of n = 3 replicates. N.S., not significant, $*P < 0.05$ by Student's *t*-test

48 hours and then incubated with 8 μg/mL cisplatin for an additional 48 hours. Indeed, the number of cancer cells gradually increased despite the presence of cisplatin after treatment with control siRNA, but the number of cancer cells was markedly reduced by transfection with GLUT1-targeting siRNA (Figure 2B). Moreover, very low doses of cisplatin (0.2 and 0.4 μg/mL) had a strong inhibitory effect on the proliferation of TE11 cells transfected with GLUT1-specific siRNA, but not with control siRNA (Figure 2C), indicating that GLUT1 silencing enhances the potency of cisplatin.

3.4 | Expression of glycolysis-related proteins in esophageal squamous cell carcinoma cells after glucose transporter 1 silencing

Tumor expression of a number of glycolysis-related proteins, including GLUT1, GLUT3, HK2, PKM2, LDHA and PDK1, is reportedly associated with the prognosis of patients with various gastrointestinal cancers.[14] Therefore, we next asked whether GLUT1 silencing influenced the expression of these proteins in ESCC cells. Of note, PKM2 and LDHA mRNA levels were highly expressed in both TE-8 and TE-11 cells (Figure 2D). We detected no effects of GLUT1 silencing on the expression of these genes in TE-8 cells, which express low GLUT1 levels (data not shown). In TE-11 cells, however, HK2 mRNA levels were significantly increased, whereas PKM2 and LDHA mRNA levels were significantly decreased and GLUT3 expression was unaffected in GLUT1-silenced cells compared with control cells (Figure 2E). We also examined activation of the mitogen-activated protein kinase (MAPK) pathway, which has been reported to be associated with PKM2 and GLUT1 expression.[15,16] We found that phosphorylation of extracellular signal-regulated kinase (ERK)1/2 on Thr202/Tyr204 was reduced in TE-11 cells transfected with GLUT1 siRNA compared with control siRNA, whereas phosphorylation of AKT on Ser473 was unaffected (Figure 2F). Taken together, these data indicate that GLUT1 knockdown affects signaling through the MAPK pathway and expression of glycolytic enzymes, suggesting that suppression of glycolysis is a potential mechanism for the anti–tumor activity of cisplatin.

3.5 | Additive anti–proliferative effects of the glucose transporter 1 inhibitor BAY-876 in combination with cisplatin

As an alternative method of suppressing GLUT1 activity, we tested the effects of the small molecule inhibitor BAY-876. This compound inhibited the proliferation of TE-8 and TE-11 cells in a dose-dependent manner, with almost complete inhibition in the presence 0.1 nmol/L BAY-876 (Figure 2G). Similar to the effects of GLUT1-specific siRNA, we also found that BAY-876 treatment increased the sensitivity of TE-8 and TE-11 cells to cisplatin. Thus, addition of 0.5 μmol/L cisplatin, 0.025 nmol/L BAY-876 or 0.025 nmol/L BAY-876 plus 0.5 μmol/L cisplatin reduced the proliferation of TE-8 cells by 79.9% ± 2.3%, 59.9% ± 4.9% and 38.1% ± 3.1% (average ± SD), respectively, and of TE-11 cells by 46.0% ± 6.5%, 51.0% ± 8.2% and 15.4% ± 3.7%, respectively, compared with the vehicle-treated control cells (Figure 2H). These data suggest that the small molecule inhibitor BAY-876 exerts anti–proliferative effects on ESCC cells at low concentrations and acts in an additive manner when combined with cisplatin.

3.6 | Association between pretreatment glucose transporter 1 expression levels and the reduction in FDG SUV_{max} in esophageal squamous cell carcinoma patients after chemotherapy

Having established that GLUT1 inhibition can enhance the sensitivity of ESCC cells to cisplatin in vitro, we next asked whether tumor levels of GLUT1 could predict the response of patients to platinum-based therapy. GLUT1 expression was analyzed by immunohistochemistry in primary ESCC tumor biopsies from 97 patients prior to mDCF chemotherapy, and positive staining was detected in 49 (50.5%) of samples (Figure 3A). GLUT1 expression was not significantly associated with any pretreatment clinicopathological factors (Table 1). Interestingly, patients with GLUT1-negative tumors showed a significantly larger reduction in SUV_{max} in the post–chemotherapy FDG-PET compared with patients with GLUT1-positive tumors ($P = 0.0202$, 61.2% ± 4.5% and 46.2% ± 4.4%, respectively; Figure 3B). Of the 97 patients who were evaluated by FDG-PET, 61 underwent esophagectomy. This patient subset showed a similar reduction in FDG SUV_{max} ($P = 0.0099$, 62.6% ± 5.7% and 43.5% ± 5.4% for GLUT1-negative and GLUT1-positive patients, respectively; Figure 3C). Finally, we found that the pathological tumor stage ($P = 0.0069$) and the histological response grade ($P = 0.0292$) were significantly associated with GLUT1 expression in the pretreatment biopsy samples (Table 2 and Figure 3D).

4 | DISCUSSION

In this study, we demonstrated the anti–proliferative effect of inhibition of GLUT1 expression and activity and the association between sensitivity to chemotherapy and GLUT1 expression in ESCC cell lines. Silencing of GLUT1 resulted in a significant reduction in CDK6, PKM2, LDHA and phospho-ERK1/2 expression and a significant upregulation of p27kip. Furthermore, inhibition of GLUT1 by siRNA or BAY-876 increased the sensitivity of ESCC cells to low-dose cisplatin. Finally, we showed that GLUT1 expression in pretreatment biopsy samples was significantly associated with the patient response to chemotherapy.

We previously demonstrated that positive GLUT1 expression was associated with depth of invasion and vascular invasion in ESCC and was also a biomarker of hematogenous recurrence.[13] Therefore, in the present study, we investigated the anti–proliferative effect of inhibiting GLUT1 expression. Previous work had shown that mutations in cell cycle regulatory genes are common in ESCC. For example, in 1 study, 2 key regulatory proteins, *CDKN2A/2B* and *CCDN1*,

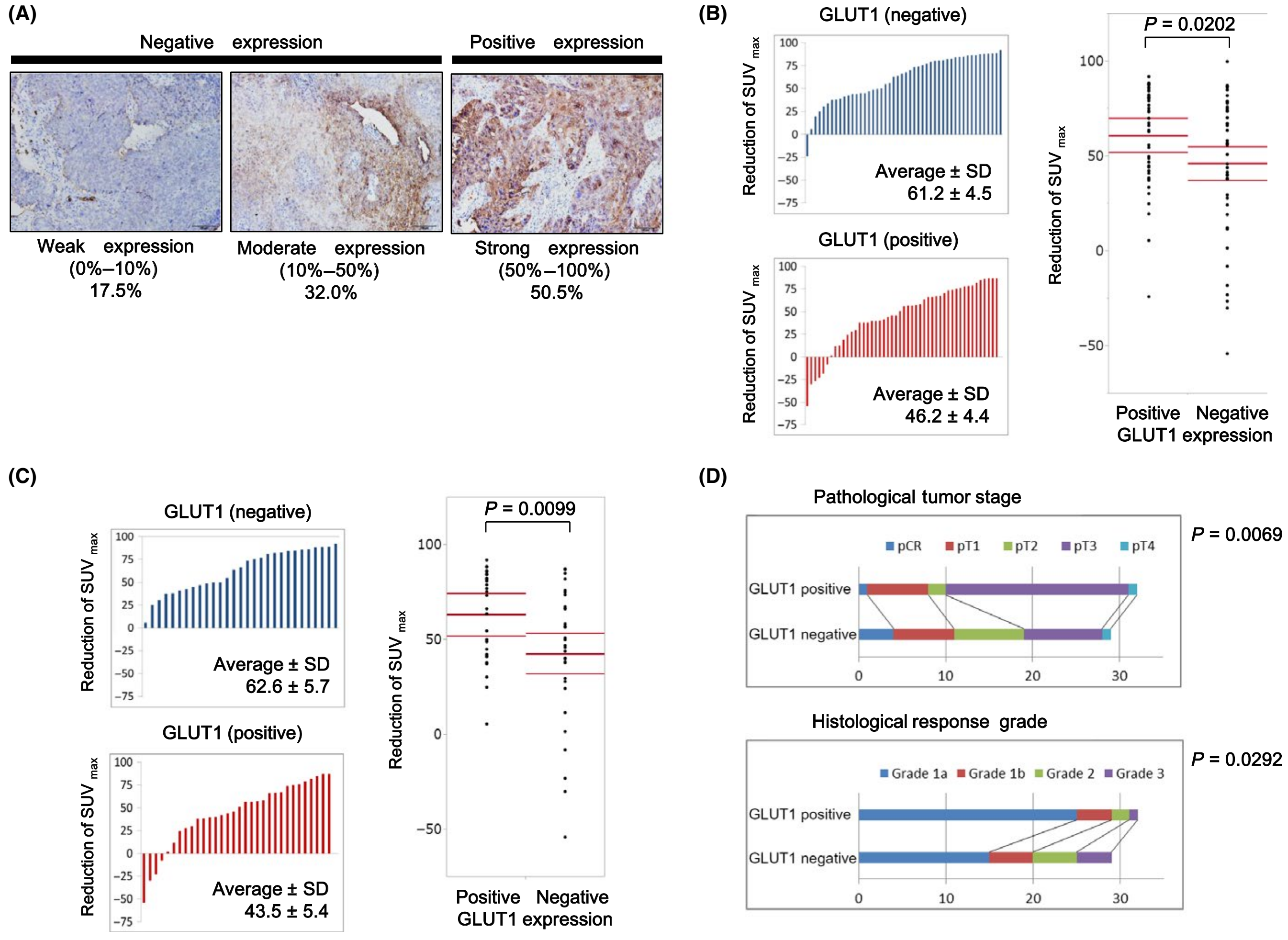

FIGURE 3 Expression of glucose transporter 1 (GLUT1) in esophageal squamous cell carcinoma (ESCC) biopsy samples before chemotherapy and relationship to clinicopathological factors. A, Immunohistochemical staining of GLUT1 in biopsy samples from ESCC patients showing weak, moderate and strong GLUT1 expression. B, Reduction in FDG SUV_{max} in the 97 patients (GLUT1-negative, n = 48; GLUT1-positive, n = 49) who completed 2 courses of chemotherapy. C, Reduction in FDG SUV_{max} in the 61 patients (GLUT1-negative, n = 29; GLUT1-positive, n = 32) who underwent 2 courses of chemotherapy followed by radical surgery. D, Pathological tumor stage and histological response grade of the GLUT1-positive (n = 32) and GLUT1-negative (n = 29) tumors

were deleted in 47.9% and amplified in 46.5%, respectively, of ESCC tumors.[17] *CDKN2A/2B* is also deleted in the TE-11 ESCC cell line employed here. CDK6 is a serine/threonine kinase that forms active complexes with cyclin D1 and promotes cellular proliferation by phosphorylating and inactivating key substrates. CDK6 overexpression is associated with poor survival and may be a marker of aggressive behavior in esophageal cancer.[18] Inhibitors of cyclin D-associated kinases have been proposed as potential cancer therapeutics.[19] p27kip also plays a key role in coordinating CDK activity during the cell cycle. In this study, we found that CDK6 and p27kip expression was decreased and increased, respectively, in TE-8 and TE-11 cells after inhibition of GLUT1 expression, suggesting that these proteins may be involved in the anti–proliferative effect of inhibiting GLUT1 expression.

Glucose transporter 1 (GLUT1) expression is regulated by the activity of many genes, including hypoxia-inducible factor-1, MYC and PKM2.[14] In human cancer cells, signaling via the epidermal growth factor receptor induces ERK-dependent phosphorylation of PKM2, leading to PKM2 nuclear translocation and upregulation of GLUT1 and LDHA expression.[9] High expression of GLUT1 is associated with resistance to chemoradiotherapy in ESCC,[20] rectal cancer[8] and oral squamous cell carcinoma.[21] However, the mechanism by which low GLUT1 expression is linked to chemosensitivity is unclear. We demonstrated that suppression of GLUT1 in TE-11 cells caused downregulation of phospho-ERK1/2, PKM2 and LDHA, which could reflect that a reduction in glycolytic activity LDHA expression is associated with chemosensitivity in breast cancer.[22] Esophageal cell lines with low expression of GLUT1 tended to have higher sensitivity to cisplatin than those with high expression of GLUT1 based on online database findings. In addition, we found that the anti–proliferative effect of cisplatin was increased after inhibition of GLUT1 expression via genetic or pharmacological approaches.

The GLUT family of proteins comprises 14 members in 3 classes: 1 (GLUT1–4 and 14), 2 (GLUT5, 7, 9 and 11) and 3 (GLUT6, 8, 10 and

TABLE 1 Association between GLUT1 expression and clinical factors for patients who underwent 2 courses of chemotherapy (n = 97)

Factors	Total N = 97	GLUT1 expression Positive N = 49	Negative N = 48	*P*-value
Age (years old)				
<65	39	19 (39%)	20 (42%)	0.7715
≥65	58	30 (61%)	28 (58%)	
Gender				
Male	81	39 (80%)	42 (88%)	0.2941
Female	16	10 (20%)	6 (13%)	
Tumor location				
Ce + Ut + Mt	69	34 (33%)	35 (27%)	0.7013
Lt + Ae	28	15 (67%)	13 (73%)	
Depth of invasion				
cT1-2	24	10 (20%)	14 (29%)	0.3168
cT3-4	73	39 (80%)	34 (71%)	
cM				
Absent	86	42 (86%)	44 (92%)	0.3524[a]
Present	11	7 (14%)	4 (8%)	
Operation				
Not done	36	17 (35%)	19 (40%)	0.7732
Done	61	32 (65%)	29 (60%)	

Ae, abdominal esophagus; Ce, cervical esophagus; cM, clinical distant metastasis; Lt, lower thoracic esophagus; Mt, middle thoracic esophagus; Ut, upper thoracic esophagus.
[a]Fisher's exact test.

TABLE 2 Association between GLUT1 expression and clinicopathological factors for patients who underwent esophagectomy after 2 courses of chemotherapy (n = 61)

Factors	Total N = 61	GLUT1 expression Positive N = 32	Negative N = 29	*P*-value
Age (years old)				
<65	30	15 (47%)	15 (52%)	0.7051
≥65	31	17 (53%)	14 (48%)	
Gender				
Male	55	29 (91%)	26 (90%)	1.0000[a]
Female	6	3 (9%)	3 (10%)	
Tumor location				
Ce + Ut + Mt	44	22 (69%)	22 (76%)	0.5352
Lt + Ae	17	10 (31%)	7 (24%)	
cT (pre-CT)				
cT1-2	18	7 (22%)	11 (38%)	0.1688
cT3-4	43	25 (78%)	18 (62%)	
Depth of invasion				
pT1-2	29	10 (31%)	19 (66%)	0.0069[b]
pT3-4	32	22 (69%)	10 (34%)	
Lymph node metastasis				
Absent	13	8 (25%)	5 (17%)	0.5606[a]
Present	42	22 (69%)	20 (69%)	
Histological response grade				
Grade Ia	40	25 (78%)	15 (52%)	0.0292[b]
Grade Ib + II + III	21	14 (44%)	7 (24%)	

Ae, abdominal esophagus; Ce, cervical esophagus; CT, chemotherapy; cT, clinical tumor stage; Lt, lower thoracic esophagus; Mt, middle thoracic esophagus; Ut, upper thoracic esophagus.
[a]Fisher's exact test.
[b]Statistically significant.

12, and H+/myo-inositol transporter). Previous work evaluating datasets from the Genome Expression Omnibus Found that GLUT1 and GLUT3 is expressed at higher levels in esophageal cancer tissue than in normal tissue (tumor to normal tissue ratios: 2.44, 95% confidence intervals [CI] 1.78-3.34 for GLUT1; 1.96, 1.23-3.13 for GLUT3; and <1.5 for all other GLUT proteins).[14] GLUT1 and glycolytic enzymes associated with catabolizing glucose are transcriptionally regulated by HIF1A and MYC oncogenes such as HIF1A and MYC, which are activated in cancers with increased GLUT1 expression. GLUT3 expression has also been associated with poor prognosis in various cancers.[23] However, we did not detect a compensatory upregulation of GLUT3 expression in ESCC cells after inhibiting GLUT1. In contrast, HK2, which catalyzes the conversion of glucose to glucose-6-phosphate, was upregulated in GLUT1-silenced cells. High expression of HK2 confers a poor prognosis in hepatocellular cancer[24] and gastric cancer.[25] We have also found that the HK2 inhibitor 3-bromopyruvate (50 μmol/L) inhibits the proliferation of TE-8 and TE-11 cells (data not shown), suggesting that the observed increase in HK2 might represent a compensatory mechanism to regulate glycolysis after inhibition of GLUT1 expression.

The histological response grade and pathological tumor stage are reliable parameters to estimate the chemotherapy response in patients who undergo surgical resection. A decrease in FDG uptake during neoadjuvant therapy is predictive of response and survival in esophageal cancer.[11] GLUT1-positive tumors may have lower sensitivity for cisplatin than GLUT1-negative tumors, as indicated by the fact that GLUT1-positive tumors increased despite being treated with anticancer drugs. In accordance with tumor progression, GLUT1-positive tumors increased the uptake of glucose. However, the proliferation of GLUT1-negative tumors was inhibited by cisplatin, so the uptake of glucose may be decreased. We speculate that the difference in the reduction of SUVmax between GLUT1-positive and GLUT1-negative tumors is associated with the sensitivity to chemotherapy. We found that the GLUT1 expression level in pretreatment biopsy specimens was associated with the response to chemotherapy according to the historical response grade,

pathological tumor stage and reduction in FDG SUV_{max}. Therefore, pretreatment tumor expression of GLUT1 may be a useful biomarker for estimating the response to chemotherapy.

Several small molecule GLUT1 inhibitors have been investigated for ESCC therapy. In a nude mouse study, daily intraperitoneal injection of 10 mg/kg WZB117 for 10 weeks resulted in >70% reduction in the volume of human lung cancer xenografts.[26] However, this beneficial effect was accompanied by a body weight loss of approximately 1-2 g, aberrant lymphocyte and platelet counts, and hyperglycemia.[26] Of the several additional small molecule GLUT1 inhibitors described in the literature, 50 μmol/L resveratrol,[27] 30 μmol/L WZB117[26] and 30 μmol/L salicylketoxime[28] have all shown efficacy in vitro. However, BAY-876 shows a high selectivity and affinity for GLUT1,[10] and we found that 0.1 nmol/L BAY-876 exerted a strong anti–proliferative effect on the ESCC cell lines examined here.

In conclusion, this study demonstrated that downregulation of GLUT1 expression had a strong anti–proliferative effect in ESCC cells and also improved their sensitivity to cisplatin. These results suggest that GLUT1 inhibitor, alone and in combination with cisplatin, could have potential utility as a therapy for ESCC. Moreover, our observations indicate that the pretreatment level of GLUT1 in ESCC tumors could be a predictive biomarker of the therapy response of patients with high GLUT1-expressing tumors.

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DISCLOSURE

The authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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