Glyoxalase 1 and protein kinase Cλ as potential therapeutic targets for late-stage breast cancer

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Abstract. Cancer cells upregulate the expression levels of glycolytic enzymes in order to reach the increased glycolysis required. One such upregulated glycolytic enzyme is glyoxalase 1 (GLO 1), which catalyzes the conversion of toxic methylglyoxal to nontoxic S-D-lactoylglutathione. Protein kinase Cλ (PKCλ) is also upregulated in various types of cancer and is involved in cancer progression. In the present study, the association between enhanced glycolysis and PKCλ in breast cancer was investigated. In human breast cancer, high GLO 1 expression was associated with high PKCλ expression at the protein ($P<0.01$) and mRNA levels ($P<0.01$). Furthermore, Wilcoxon and Cox regression model analysis revealed that patients with stage III-IV tumors with high GLO 1 and PKCλ expression had poor overall survival compared with patients expressing lower levels of these genes [$P=0.040$ (Gehan-Breslow generalized Wilcoxon test) and $P=0.031$ (hazard ratio, 2.36; 95% confidence interval, 1.08-5.16), respectively]. Treatment of MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cells with TLSC702 (a GLO 1 inhibitor) and/or aurothiomalate (a PKCλ inhibitor) reduced both cell viability and tumor-sphere formation. These results suggested that GLO 1 and PKCλ were cooperatively involved in cancer progression and contributed to a poor prognosis in breast cancer. In conclusion, GLO 1 and PKCλ serve as potentially effective therapeutic targets for treatment of late-stage human breast cancer.

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Introduction

Breast cancer is the second most common type of cancer worldwide (1), and the most common type of cancer in women, with an estimated 2.09 million new cases (24.2% of all cases of cancer in women), and 0.6 million cancer-related deaths annually (2). The prognosis for breast cancer patients is generally good; however, patients with late-stage tumors (stages III and IV) have significantly shorter overall survival (OS) (3). This is due to the fact that late-stage breast cancers are often resistant or less responsive to conventional medical approaches, such as conventional surgery, chemotherapy and radiotherapy, and exhibit a high rate of both recurrence and metastasis (3). Thus, novel pharmacological approaches are required to manage late-stage cancer.

Breast cancer can be classified, based on gene expression patterns (PAM 50), into at least six subtypes: Normal-like, luminal A, luminal B, HER2-enriched, claudin-low and basal-like (4-7). Based on receptor expression status, breast cancer can also be classified immunohistochemically as estrogen receptor (ER)-positive and/or progesterone receptor (PgR)-positive type, HER2-positive type, and triple-negative

type (ER-negative, PgR-negative, HER2-negative; TNBC). TNBC has the poorest prognosis amongst the different breast cancer subtypes, and 70-80% of TNBCs are basal-like breast cancer (8).

Metabolic reprogramming leading to increased glycolysis, termed the Warburg effect, is a characteristic feature of cancer cells (9). This enhancement of glycolysis in cancer cells contributes to their proliferation, migration, survival and drug resistance (10). In addition, glyoxalase 1 (GLO 1), which catalyzes the conversion of methylglyoxal (MG), a toxic byproduct of glycolysis, to non-toxic S-D-lactoylglutathione, is upregulated in several types of malignancy, including lung, stomach, colon, liver, prostate, oropharyngeal, skin and breast cancer (11-21). GLO 1 is essential for the survival of aldehyde dehydrogenase 1 (ALDH1)-positive breast cancer stem cells, and operates in a caspase-3-dependent manner (21). In addition, HER2/neu signaling regulates GLO 1 expression in HER2-positive tissues and cell lines (22). However, the signal transduction mechanisms of GLO 1 in breast cancer remain unclear.

It is well established that the majority of cancer cells are derived from epithelial cells, and defects in cell polarity are a characteristic feature of cancer cells (23). One of the atypical protein kinase C (aPKC) isotypes, PKCλ/ι, is known to be involved in cellular responses that include determination of cell polarity, as well as cell proliferation, survival, chemotaxis and migration (24-26). PKCλ is overexpressed in several types of cancer, including breast cancer (27-42), and is known to be involved in cancer progression, contributing to poor clinical outcomes (32-42). In TNBC cells, TGFβ and IL1β induce PKCλ phosphorylation and promote PKCλ-dependent proliferation, invasiveness and metastasis by inducing NF-κB p65 nuclear translocation (30). c-Met and PKCλ are cooperatively involved in cellular viability and tumor formation in basal-like breast cancer cells (42). PKCλ is also essential for the survival of ALDH1-positive breast cancer stem cells in a caspase-3-dependent manner (41,42). EGF, platelet-derived growth factor and insulin collectively activate PKCλ via PI3-kinase (43-45), and PKCλ subsequently binds to and regulates p70 S6 kinase (46). PKCλ also activates the Rac1-Pak-Mek1/2-Erk1/2 signaling pathway, which is associated with lung cancer cell proliferation and tumorigenicity (47). PKCλ phosphorylates FoxO1 and modulates the DNA-binding ability of c-Myc, promoting cellular proliferation in angiosarcoma (48). Glucose transporter 1 (GLUT1) facilitates glucose transport and its expression is increased in several types of cancer (including breast cancer), where it is involved in enhanced glycolysis and cancer progression (49,50). PKCλ regulates the translocation of GLUT1 from intracellular vesicles to the plasma membrane in 3T3-L1 adipocytes (51). However, the role of PKCλ in the enhanced glycolysis seen in cancer cells remains unclear.

In the present study, the association between the levels of GLO 1 and PKCλ expression in human breast cancer was investigated, and their impact on the prognoses of patients with late-stage breast cancer was assessed.

Materials and methods

Immunohistochemistry (IHC). Specimens used for IHC were prepared at the Kanagawa Cancer Center Research Institute from archives of surgically removed and formalin-fixed, paraffin-embedded breast cancer tissues in the Pathology Department. With the approval of the Research Ethics Committee, these prepared specimens were used in the present study through the Kanagawa Cancer Research and Information Association, which has since been dissolved and its duties transferred to the Kanagawa Cancer Center Research Institute Biospecimen Center (approval no. 3-2009). The clinicopathological data of the patients from whom the samples were obtained are summarized in Table SI. TNM stage data is lacking 27% because the data is already anonymized. The research protocol used was also approved by the Institutional Ethics Committees of Tokyo University of Science (approval nos. 13003, 15006 and 16038), and all patients provided consent for the use of their tissue samples for research purposes.

IHC was performed as previously described (29,31-35,39,40). Briefly, 4-μm thick paraffin embedded sections were deparaffinized, rehydrated in a descending series of ethanol solutions and autoclaved (120˚C for 20 min) in 10 mmol/l citrate buffer (pH 6.0) for antigen retrieval. The semi-serially prepared sections (adjacent sections) were then immersed in 0.3% hydrogen peroxide at room temperature for 30 min to quench the intrinsic peroxidase activity before incubation with a primary antibody at 4˚C overnight. The antibodies used in the present study were: Mouse anti-PKCι mAb (1:250; cat. no. 610176; BD Biosciences), mouse anti-GLO 1 mAb (1:2,000; cat. no. NBP1-19015; Novus Biologicals, Inc.), mouse IgG2b κ Isotype Control (eBMG2b; 1:500; cat. no. 14-4732-82; eBioscience; Thermo Fisher Scientific, Inc.) and mouse IgG1 κ Isotype Control (P3.6.2.8.1) (1:1,000; cat. no. 14-4714-82; eBioscience; Thermo Fisher Scientific, Inc.). The labeled antigens were visualized using a Histo Fine kit (Nichirei) and DAB plus (Dako; Agilent Technologies, Inc.). The sections were counterstained with hematoxylin. The antibodies used for double staining were: Mouse anti-PKCι mAb (1:50), rabbit anti-GLO 1 pAb (1:200; cat. no. A1932; ABclonal, Inc.), mouse IgG2b κ Isotype Control and normal rabbit IgG (1:952; cat. no. PM035; MBL). The labeled antigens were visualized using a Histo Fine alkaline phosphatase kit and DAB plus. The sections were counterstained with hematoxylin.

IHC scoring. To evaluate the expression of GLO 1 and PKCλ proteins using IHC, ImageJ version 1.51u was used (National Institutes of Health) with the IHC Profiler plugin (52). The scoring system was based on the classification calculated from the IHC Profiler (+3, high-positive; +2, positive; +1, low-positive; and 0, negative). Signal intensity of GLO 1 was classified into color density as follows; +3, High positive; +2, Positive; and +1, Low positive. Signal intensity of PKCλ was classified into color density as follows: +3, High positive; +2, Positive; +1, Low positive; and 0, Negative. Signal intensities were categorized as high (+3 or +2) or low (+1 or 0). H-scores of the scatter plot data were based on calculated values from the IHC Profiler.

Analysis of gene expression in the breast cancer dataset from the molecular taxonomy of breast cancer international consortium (METABRIC). Gene expression data was downloaded from cBioportal and analyzed as previously described (21,41,42,53,54). Briefly, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset (55,56) was downloaded from cBioPortal (cbioportal.

org/; last entry, 25th November 2019) (57,58). The clinicopathological data of the patients are summarized in Table SII. The median age at the time of diagnosis was 61.8 years (age range, 21.9-96.3 years). Gene expression levels were classified as high if they were in the top 25% of Z-scores; or otherwise, they were classed as low.

Analysis of gene expression in the breast cancer dataset from The Cancer Genome Atlas (TCGA). Briefly, gene expression microarray datasets from TCGA were downloaded from Oncomine (oncomine.org; Compendia Bioscience, 28th January 2021) (59,60), and the breast cancer dataset (n=459) was obtained. Levels of GLO 1 (reporter, A_32_P53822) and PKCλ (reporter, A_23_P18392) mRNA expression are presented using $\log_2$ median-centered ratio boxplots for normal vs. cancerous tissues.

Cell culture. The MCF-10A human normal-like (non-transformed) mammary epithelial cell line and the MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cell lines were obtained from the American Type Culture Collection. MCF-10A cells were grown in mammary epithelial cell growth medium (MEGM; Lonza Group, Ltd.) according to instructions from ATCC. The cancer cell lines were cultured as previously described (21,41,42,53). Mycoplasma testing was performed on all the cell lines used.

Inhibitory compounds. 3-(1,3-Benzothiazol-2-yl)-4-(4-methoxyphenyl) but-3-enoic acid (TLSC702) was purchased from Namiki Shoji Co., Ltd. and dissolved in DMSO. Aurothiomalate (ATM) was purchased from Calbiochem (Merck KGaA) and dissolved in water.

Immunoblot analysis. Immunoblotting was performed as previously described (21,41,42,53). The primary antibodies used were: Mouse anti-PKCι mAb (1:5,000; cat. no. 610176; BD Biosciences), mouse anti-GLO 1 mAb (1:2,000; cat. no. sc-133144, Santa Cruz Biotechnology, Inc.) and mouse anti-β-actin mAb (1:20,000; cat. no. 60008-1-Ig, ProteinTech Group Inc.). The secondary antibody used was a goat anti-mouse IgG horseradish peroxidase-conjugate (1:5,000; cat. no. 7076S; Cell Signaling Technology, Inc.).

WST-8 assay. WST-8 assays were performed according to the manufacturer's protocol, and as previously described (21,42,53). Briefly, cells ($5x10^3$/well) were seeded into 96-well plates (Sigma-Aldrich; Merck KGaA) and incubated for 24 h. Inhibitors were then added to the culture medium, and the cells were incubated for an additional 3 days, after which cell viability was assessed using a Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Inc.). The formazan dye formed was measured using Sunrise Remote (Tecan Group, Ltd.) at 450 nm. Assays with MCF-10A cells were performed in MEGM supplemented with 10% FBS. Assays using the cancer cell lines were performed in DMEM supplemented with 10% FBS. Numerical values of the test groups were expressed relative to the control cell (no drug).

Tumor-sphere culture. Tumor-spheres were grown as previously described (21,41,42,53). Briefly, cells ($1x10^3$/well) were cultured in 96-well ultralow attachment plates (Greiner Bio-One) and treated with inhibitors for 6 days. Images were taken through an inverted microscope (Leica Microsystems, Inc.), and the numbers of tumor-spheres ≥50 μm in diameter were counted. Numerical values of the test groups are shown relative to the untreated cells. Cell Titer-Glo® luminescence assays (Promega Corporation) were performed according to the manufacturer's protocol, and as previously described (21,53). Values for test groups are shown relative to cells in the absence of the drug.

Statistical analysis. For correlation between protein GLO 1 and PKCλ expression, statistical significance was calculated using the χ^2-test with Yates' correction. H-scores of the scatter plot data were based on calculated values from the IHC Profiler. Spearman's rank correlation coefficients (r) and P-value are indicated. In the analysis of gene expression, Pearson's correlation coefficients (r) and P-value are indicated. P-values were calculated using a test for non-correlation. In the analysis of gene expression, survival curves were plotted using the Kaplan-Meier method, and P-values were calculated using the Gehan-Breslow generalized Wilcoxon test to weight early death points. Multiplicity was adjusted using the Holm's method for post-hoc analysis. A multivariable Cox regression model was used to evaluate the effect of gene expression and to estimate the adjusted hazard ratios (HRs) with age as a confounding factor. P-values for comparison of gene expression are presented using the Kruskal-Wallis test with the Steel-Dwass test. Statistical analysis was performed using BellCurve for Excel version 2.11 (SSRI). Data for the WST-8 assay is presented as the mean ± standard deviation of three independent experiments. Differences between groups were compared using Tukey's test. Data for the tumor-sphere assay is presented as the mean ± standard error of the mean of three independent experiments. Data for the Cell Titer-Glo® luminescence assay is presented as the mean ± standard deviation of three independent experiments. Statistical significance was calculated using one-way ANOVA followed by Dunnett's test. For any of the analyses above, α-level was fixed at 0.05, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

GLO 1 expression is positively correlated with PKCλ expression in breast cancer. GLO 1 protein expression in both the cytosol and nucleus of breast cancer cells was detected, and the results were consistent with earlier observations (Fig. 1A) (20). There were no 0 image samples for GLO 1 protein. GLO 1 protein expression was detected in all breast cancer samples, and the results were consistent with a previous report (20). PKCλ was also localized in the cytosol and nucleus of breast cancer cells (Fig. 1B) as reported in our previous study (29). Double IHC staining showed that GLO 1 and PKCλ were colocalized in breast cancer cells (Fig. 1C). To evaluate the relationship between GLO 1 and PKCλ, their signal intensities were quantified (Fig. 1A and B). High expression of GLO 1 was significantly correlated with high expression of PKCλ in breast cancer tissue (Fig. 1D, $P<0.01$, χ^2-test; Fig. 1E, $r=0.71$, $P<0.01$).

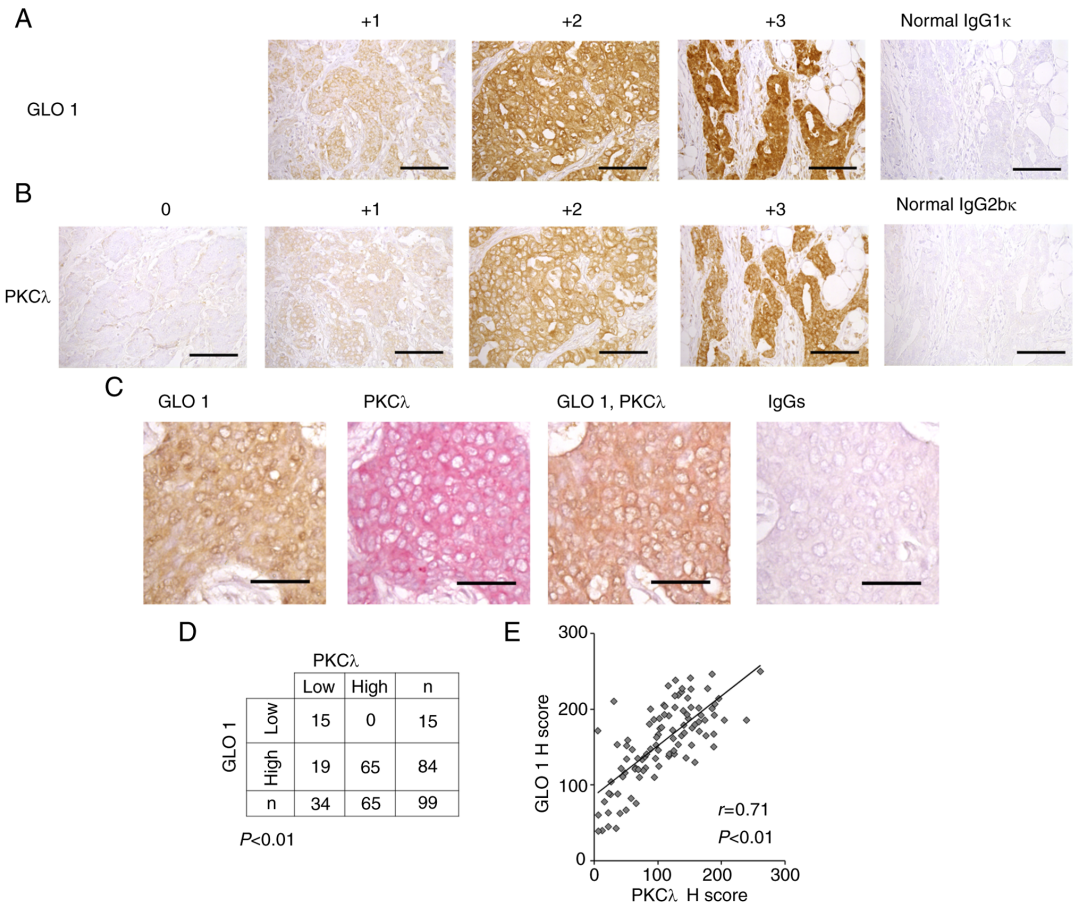

Figure 1. Overexpression of GLO 1 is associated with PKCλ expression in breast cancer. Representative IHC images of (A) GLO 1 and (B) PKCλ staining in breast cancer tissues. Scale bars, 100 μm. (C) Double staining using IHC. Scale bar, 50 μm. (D) χ^2-test based on protein GLO 1 and PKCλ expression. Color densities were categorized as high (+3 or +2) or low (+1 or 0). (E) Scatter plot showing the correlation between GLO 1 and PKCλ protein expression in patients with breast cancer. H-scores of the scatter plot data were based on calculated values from the IHC Profiler. r and the P-value are indicated. The α-level was fixed at 0.05, and P<0.05 was considered to indicate a statistically significant difference. GLO 1, glyoxalase 1; PKCλ, protein kinase Cλ; IHC, immunohistochemistry; r, Spearman's rank correlation coefficient.

GLO 1 expression is correlated with PKCλ expression at the mRNA level in human breast cancer. To further examine the relationship between *GLO 1* and *PKCλ* at the mRNA level, mRNA expression data from the METABRIC breast cancer dataset (n=1,904) was downloaded from cBioPortal (Fig. 2A). In our previous study, it was shown that *GLO 1* expression is higher in breast cancer compared with normal tissue samples (21). In addition, GLO 1 gene expression was enhanced at all breast cancer tumor stages compared with normal tissue samples. Similarly, *PKCλ* expression was significantly higher in breast cancer tissues and at all tumor stages compared with the normal tissues (41) (Fig. S1). Scatter plot analysis indicated that *GLO 1* expression was weakly correlated with *PKCλ* expression in all patients and in patients with early-stage (stage 0-II) tumors (Fig. 2A), but was not correlated with *PKCλ* expression in patients with late-stage (stage III-IV) tumors (Fig. 2A). As shown in Table SIII, none of the clinicopathological parameters were correlated with high *GLO 1* and *PKCλ* gene expression. Nonetheless, these results, together with the IHC findings, suggest that GLO 1 and PKCλ may be cooperatively involved in certain cases of breast cancer.

High GLO 1 and PKCλ expression is correlated with a poorer prognosis in patients with stage III-IV tumors. Next, we examined weather high GLO 1 and PKCλ expression is correlated with prognosis (Figs. 2B-D and S2; Tables I and SIV). Examination of the prognosis of patients with *GLO 1*^{high} and *PKCλ*^{high} breast cancer revealed that *GLO 1*^{high} was not associated with a poorer OS amongst all patients (Fig. 2B) (21). It was previously shown that overexpression of PKCλ and its signaling promotes TNBC growth and metastasis (30). Consistent with this finding, patients classed as *PKCλ*^{high} had worse OS (Fig. 2B; P<0.01) (42). In our previous study, it was shown that patients with stage III or IV cervical cancer with high PKCλ expression had a worse clinical outcome (32). Thus, tumors were classified into early-stage (stage 0-II) and late-stage (stage III-IV) tumors, and the difference in survival was assessed using Kaplan-Meier analysis. Patients with early-stage tumors (*GLO 1*^{high}, *PKCλ*^{high} or *GLO 1*^{high} *PKCλ*^{high}) exhibited similar clinical outcomes to those expressing lower levels of these genes (Fig. 2C), as did those with late-stage tumors (*GLO 1*^{high}; Fig. 2D). However, patients with late-stage tumors (*PKCλ*^{high}) had a worse OS (Fig. 2D) (42). Furthermore, patients with *GLO 1*^{high} *PKCλ*^{high} tumors had poorer prognoses than patients with *GLO 1*^{high} *PKCλ*^{low} (adjusted by Holm's method; P=0.015) and *GLO 1*^{low} *PKCλ*^{low} (P=0.040), but not *GLO 1*^{low} *PKCλ*^{high} (P=0.83) tumors. Multivariable Cox regression analysis revealed that *GLO 1*^{high} *PKCλ*^{high} was associated with poorer clinical outcomes than *GLO 1*^{low} *PKCλ*^{low} (Table I; HR=2.36;

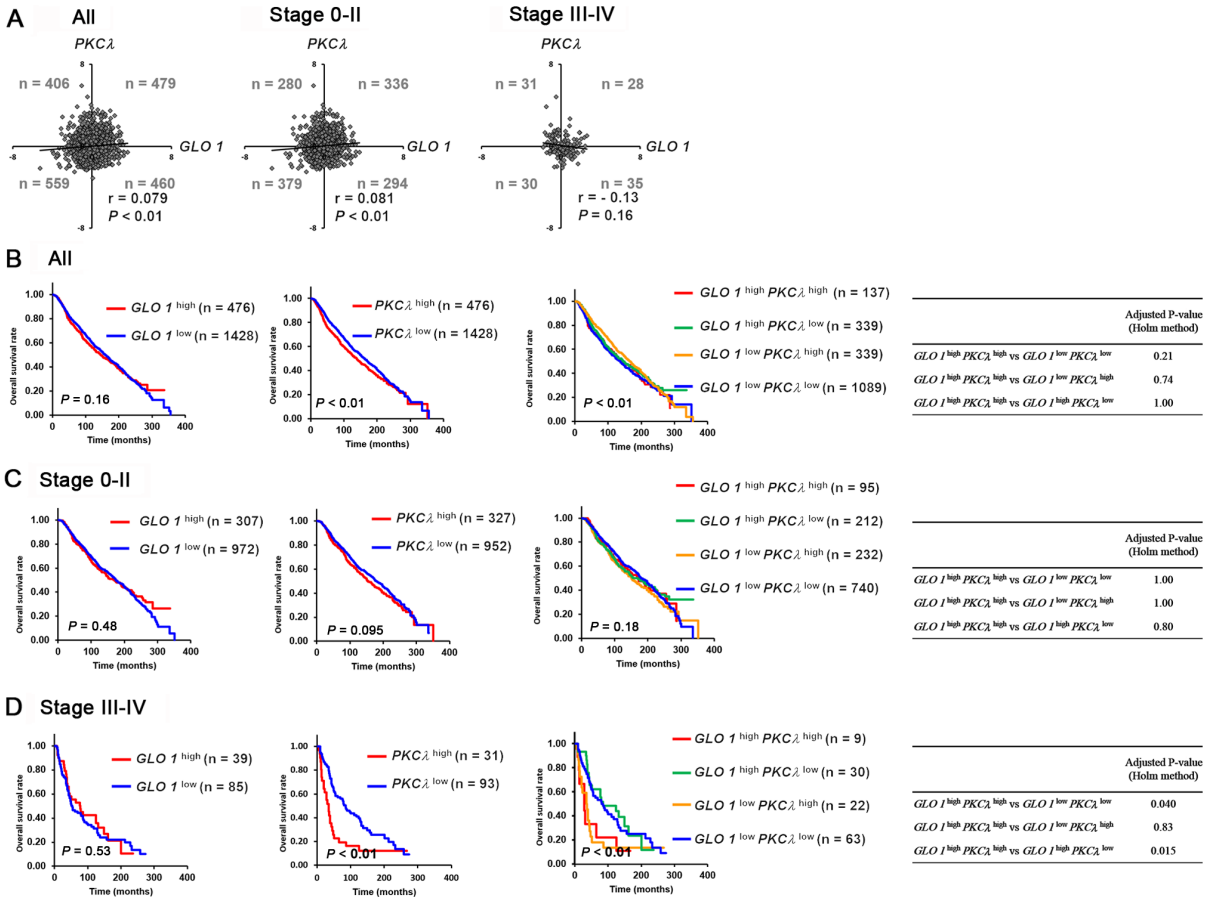

Figure 2. Co-expression of *GLO 1* and *PKCλ* is associated with a poor prognosis in late-stage tumors. (A) Scatter plots showing the correlation between *GLO 1* and *PKCλ* expression in all patients with breast cancer, stage 0-II patients and stage III-IV patients. The r and P-values are indicated. P-values were calculated using a test for non-correlation. (B-D) Kaplan-Meier analyses of the effect of high *GLO 1* and *PKCλ* expression on overall survival amongst (B) all patients, (C) stage 0-II patients and (D) stage III-IV patients. P-values were calculated using a Gehan-Breslow generalized Wilcoxon test. Adjusted P-values were calculated using Holm's method for post hoc analysis. The α-level was fixed at 0.05, and P<0.05 was considered to indicate a statistically significant difference. GLO 1, glyoxalase 1; PKCλ, protein kinase Cλ; r, Pearson's correlation coefficient.

95% CI, 1.08-5.16; P=0.03) and *GLO 1*^{high} *PKCλ*^{low} (Table I; HR=3.25, 95% CI, 1.26-8.35; P=0.01) in those with late-stage tumors. Furthermore, normal-like breast cancer patients classified as *GLO 1*^{high} *PKCλ*^{high} also exhibited a worse prognosis (Fig. S2; adjusted by Holm's method; P=0.016 and Table SIV; HR=9.11, 95% CI, 2.07-40.15; P<0.01). Thus, high PKCλ expression, regardless of GLO 1 expression level, contributes to poor clinical outcome. Of note, GLO 1 protein (Fig. 1) and mRNA (Fig. S1) expression was considerably higher in breast cancer, reflecting enhanced glycolysis. Therefore, GLO 1 and PKCλ may function cooperatively to promote cancer progression, and contribute to worse clinical outcomes in patients with late-stage breast cancer.

TLSC702 and ATM suppresses breast cancer cell viability. Amongst patients with basal-like breast tumors, the proportion of patients classed as *GLO 1*^{high} *PKCλ*^{high} was larger than the proportion classed as *GLO 1*^{low} *PKCλ*^{low}, irrespective of whether they had early- or late-stage tumors (Fig. S3). All patients with breast cancer classed as *GLO 1*^{high} *PKCλ*^{high} primarily had luminal B type breast cancer (43.1%, 59/137), whereas those with *GLO 1*^{low} *PKCλ*^{low} primarily had luminal A type breast cancer (42.3%, 459/1,084) (Fig. S3). Similarly, amongst patients with early-stage lesions, those classed as *GLO 1*^{high} *PKCλ*^{high} primarily had luminal B breast cancer (48.4%, 46/95), and those with *GLO 1*^{low} *PKCλ*^{low} primarily had luminal A breast cancer (45.2%, 333/737). However, amongst patients with late-stage lesions, those classed as *GLO 1*^{high} *PKCλ*^{high} exhibited higher incidences of luminal A or basal-like type breast cancer (luminal A, 33.3%, 3/9; basal-like, 33.3%, 3/9) compared with patients classed as *GLO 1*^{low} *PKCλ*^{low} (luminal A, 28.6%, 18/63; basal-like, 6.3%, 4/63). As shown in Fig. S4, 38% (76/199) of patients with basal-like type cancer were classed as *GLO 1*^{high} *PKCλ*^{high}. In our previous study, it was shown that *GLO 1* expression is upregulated in basal-like breast cancer, and that inhibition of GLO 1 suppresses basal-like breast cancer cell viability (21). To further clarify the effects of inhibiting GLO 1 and PKCλ in basal-like breast cancer cells, MCF-10A human normal-like (non-transformed) mammary epithelial cells were compared with MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cells (Fig. 3) (21). Expression levels of both GLO 1 and PKCλ were higher in MDA-MB-157 and MDA-MB-468 cells than in MCF-10A cells (Fig. 3A). TLSC702 has previously been shown to inhibit GLO 1 activity and induce MG accumulation and apoptosis in cancer cells (21,61). Based on the inhibitory effects of TLSC702 on the viability of MCF-10A, MDA-MB-157 and MDA-MB-468 cells, concentrations of

Table I. Multivariable Cox regression analysis of the association between *GLO 1* and *PKCλ* expression and breast cancer in all patients, and in patients stratified by stage (0-II and III-IV).

Comparison	Hazard ratio[a] (95% confidence interval)	P-value
All		
GLO 1^{high} vs. *GLO 1*^{low}	1.06 (0.93-1.22)	0.39
PKCλ^{high} vs. *PKCλ*^{low}	1.20 (1.05-1.38)	<0.01
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{low}	1.18 (0.94-1.49)	0.15
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{high}	0.95 (0.74-1.22)	0.69
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{high} *PKCλ*^{low}	1.08 (0.84-1.40)	0.54
Stage 0-II		
GLO 1^{high} vs. *GLO 1*^{low}	0.99 (0.83-1.18)	0.92
PKCλ^{high} vs. *PKCλ*^{low}	1.14 (0.97-1.35)	0.12
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{low}	1.03 (0.77-1.37)	0.86
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{high}	0.85 (0.62-1.17)	0.32
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{high} *PKCλ*^{low}	0.98 (0.71-1.36)	0.91
Stage III-IV		
GLO 1^{high} vs. *GLO 1*^{low}	0.89 (0.57-1.39)	0.62
PKCλ^{high} vs. *PKCλ*^{low}	2.23 (1.41-3.54)	<0.01
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{low}	2.36 (1.08-5.16)	0.03
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{low} *PKCλ*^{high}	1.04 (0.44-2.43)	0.93
GLO 1^{high} *PKCλ*^{high} vs. *GLO 1*^{high} *PKCλ*^{low}	3.25 (1.26-8.35)	0.01

[a]Hazard ratio adjusted by age estimated using Cox proportional hazard model. GLO 1, glyoxalase 1; PKCλ, protein kinase Cλ.

50, 75 and 100 μM TLSC702 were used in the present study (21). To inhibit PKCλ, ATM was used (5 and 10 μM), which interferes with the PB1-PB1 domain interactions between PKCλ and Par6 and induces apoptosis (62,63). As a result of the inhibitory effect of 0.5, 1, 5 and 10 μM ATM on the colony formation ability of MDA-MB-157 cells, it was found that 5 and 10 μM ATM markedly suppressed colony formation compared with the untreated control group (unpublished data). The inhibitor concentrations used in the present study were based on these findings. WST-8 assays showed that MCF-10A cells were less sensitive to GLO 1 inhibition than the two cancer cell lines, consistent with our previous study (Fig. 3B; TLSC702 50 and 75 μM) (21). By contrast, inhibition of PKCλ using ATM did not significantly affect the cell viability of any of the three cell types assessed (Fig. 3B). In addition, the combination of TLSC702 and ATM decreased the viability of the two cancer cell lines, which was reduced to a greater degree than that of the control cells (TLSC702/ATM, 50/10, 75/5, 75/10, 100/5 and 100/10 μM; Fig. 3B).

TLSC702 and ATM suppresses tumor-sphere formation in basal-like breast cancer cells. In vitro tumor-sphere formation was assessed using MDA-MB-157 and MDA-MB-468 cells to determine the roles of GLO 1 and PKCλ in the tumorigenicity of basal-like breast cancer cells. As shown in Fig. 4A and B, treatment with TLSC702 or ATM reduced the relative numbers of tumor-spheres ≥50 μm in diameter. Furthermore, a combination of TLSC702 and ATM inhibited the formation of tumor-spheres ≥50 μm in diameter (Fig. 4A and B; P<0.05). Cell Titer-Glo® assays also showed that the combination of TLSC702 and ATM suppressed the viability of MDA-MB-157 and MDA-MB-468 cells (Fig. 4C and D; P<0.05). Taken together, these results along with the results of the viability assays suggest that GLO 1 and PKCλ are cooperatively involved in cancer progression and survival of basal-like breast cancer cells.

Discussion

In the present study, it was shown that high GLO 1 expression was correlated with high PKCλ expression at the protein and mRNA levels in breast cancer and that patients with breast cancer classed as *GLO 1*^{high} *PKCλ*^{high} had poorer clinical outcomes for late-stage tumors. GLO 1 and PKCλ exhibit low frequencies of gene amplification and mutations in with breast cancer (21,41). It thus appears that in breast cancer, higher GLO 1 and PKCλ mRNA expression and activation reflect higher transcriptional activity rather than gene amplification or mutations. In stage III-IV patients, the IHC value (based on tissue acquisition) is relatively low, and disease burden is approximated using imaging methods, such as CT or PET. However, the present cohort data lacks CT/PET confirmation. Therefore, GLO 1 and PKCλ may not be suitable prognostic factors in stage III-IV patients. However, late-stage breast cancer cases are often resistant or less responsive to conventional medical approaches (3). In the present study, patients with stage III-IV tumors with high GLO 1 and PKCλ expression exhibited poorer overall survival compared with patients expressing lower levels of these genes. Therefore, GLO 1 and PKCλ may potentially serve as effective therapeutic targets for late-stage human breast cancer. However, there is a lack of *in vivo* studies of breast cancer using TLSC702 and ATM, thus the use of both inhibitors requires further investigation *in vivo*.

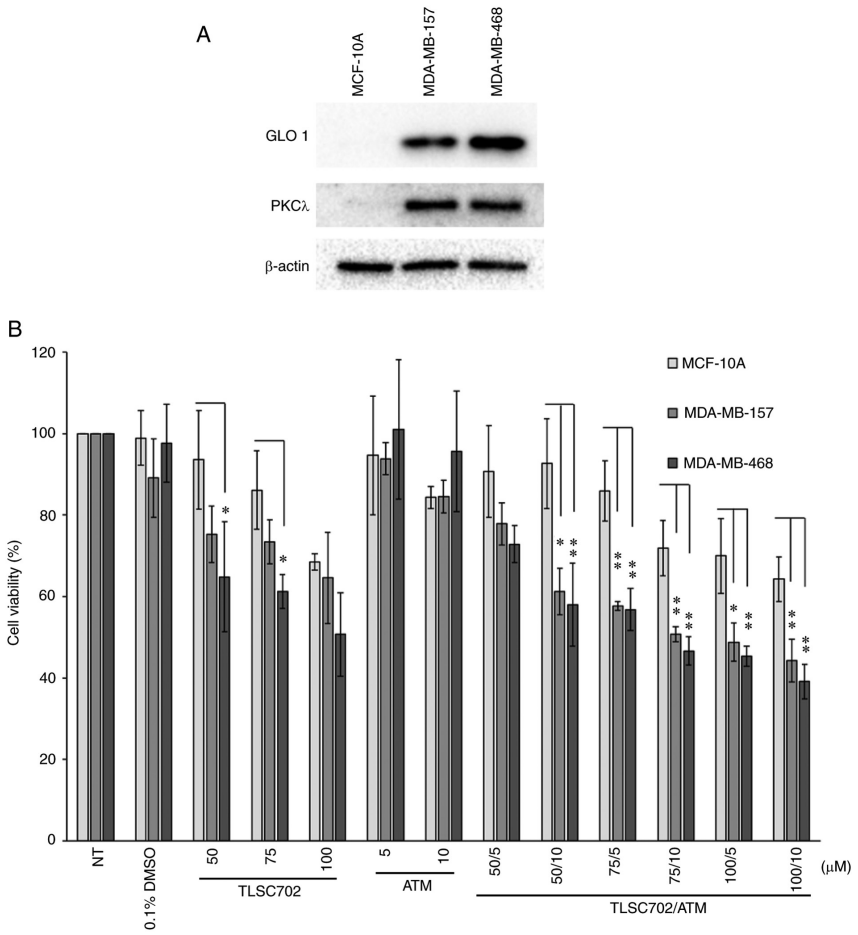

Figure 3. TLSC702 and ATM suppress the viability of breast cancer cells. (A) GLO 1 and PKCλ protein expression in MCF-10A human normal-like mammary epithelial cells and in MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cells were analyzed by immunoblotting. β-actin was used as the internal control. (B) Viability of MCF-10A, MDA-MB-157 and MDA-MB-468 cells treated for 3 days with or without TLSC702 (50, 75 or 100 μM) and/or ATM (5 or 10 μM) was assessed using WST-8 assays. Values of the experimental groups are expressed relative to the untreated cells. Data are presented as the mean ± standard deviation of three independent experiments and were compared using a Tukey's test. *P<0.05, **P<0.01. The α-level was fixed at 0.05, and P<0.05 was considered to indicate a statistically significant difference. ATM, aurothiomalate; GLO 1, glyoxalase 1; PKCλ, protein kinase Cλ.

The prognosis of patients with stage 0-II breast cancer with *GLO 1*^{high} *PKCλ*^{high} did not differ significantly from those with *GLO 1*^{low} *PKCλ*^{low}. Notably, amongst patients with stage III-IV tumors, those with *GLO 1*^{high} *PKCλ*^{high} status exhibited poorer prognoses. To support increased glycolysis, glycolytic enzymes are upregulated in cancer cells. In breast cancer, one such enzyme is GLO 1 (19-21), and MG, an intermediate metabolite of glycolysis, induces GLO 1 expression (64). In the present study, GLO 1 protein expression was detected in all breast cancer samples. These results are in line with a previous report (20). Fig. S1 also showed that GLO 1 gene expression is enhanced at all tumor stages of breast cancer in comparison with normal tissues. Thus, GLO 1 mRNA and protein expression is considerably high in breast cancer, reflecting an increased level of glycolysis. Therefore, it is considered that there is no difference in prognosis between *GLO 1*^{high} and *GLO 1*^{low} even when classified by high and low expression in breast cancers in which GLO 1 expression was essentially high. This is in line with the results that we previously analyzed using same gene expression data set, which the prognosis of patients with *GLO 1*^{high} was not associated with a poorer OS amongst breast cancer patients (21). Thus, the results of the current study suggest that both GLO 1 and PKCλ may be cooperatively involved in breast cancer progression, and contribute to poor prognosis.

Basal-like breast cancer, the majority of cases of which are TNBC, has the poorest clinical outcomes amongst all breast cancer subtypes (6). Notably, TNBC cells show higher GLO 1 expression levels, higher GLO 1 activity and lower accumulation of a MG-arginine adduct, Arg-pyrimidine (64). Notably, PKCλ is upregulated in patients with TNBC (30), and it was confirmed that the fraction of stage III-IV basal-like breast cancer cases with *GLO 1*^{high} *PKCλ*^{high} were enriched compared with all other breast cancer subtypes. Thirty eight percent of patients (76/199) with basal-like type cancer were classed as *GLO 1*^{high} *PKCλ*^{high}. This result suggests that GLO 1 and PKCλ are cooperatively involved in the progression of basal-like breast cancer. Moreover, the GLO 1 inhibitor TLSC702 and the PKCλ inhibitor ATM suppressed the viability of MDA-MB-157 and MDA-MB-468 basal-like breast cancer cells and tumor-sphere formation using these cells. The Par6-PKCλ complex interacts with epithelial cell transforming sequence 2 to activate Rac1 during cancer cell proliferation (65), and PKCλ activates the Rac1-Pak-Mek1/2-Erk1/2 signaling pathway in lung cancer cell growth and tumorigenicity (47). PKCλ modulates c-Myc

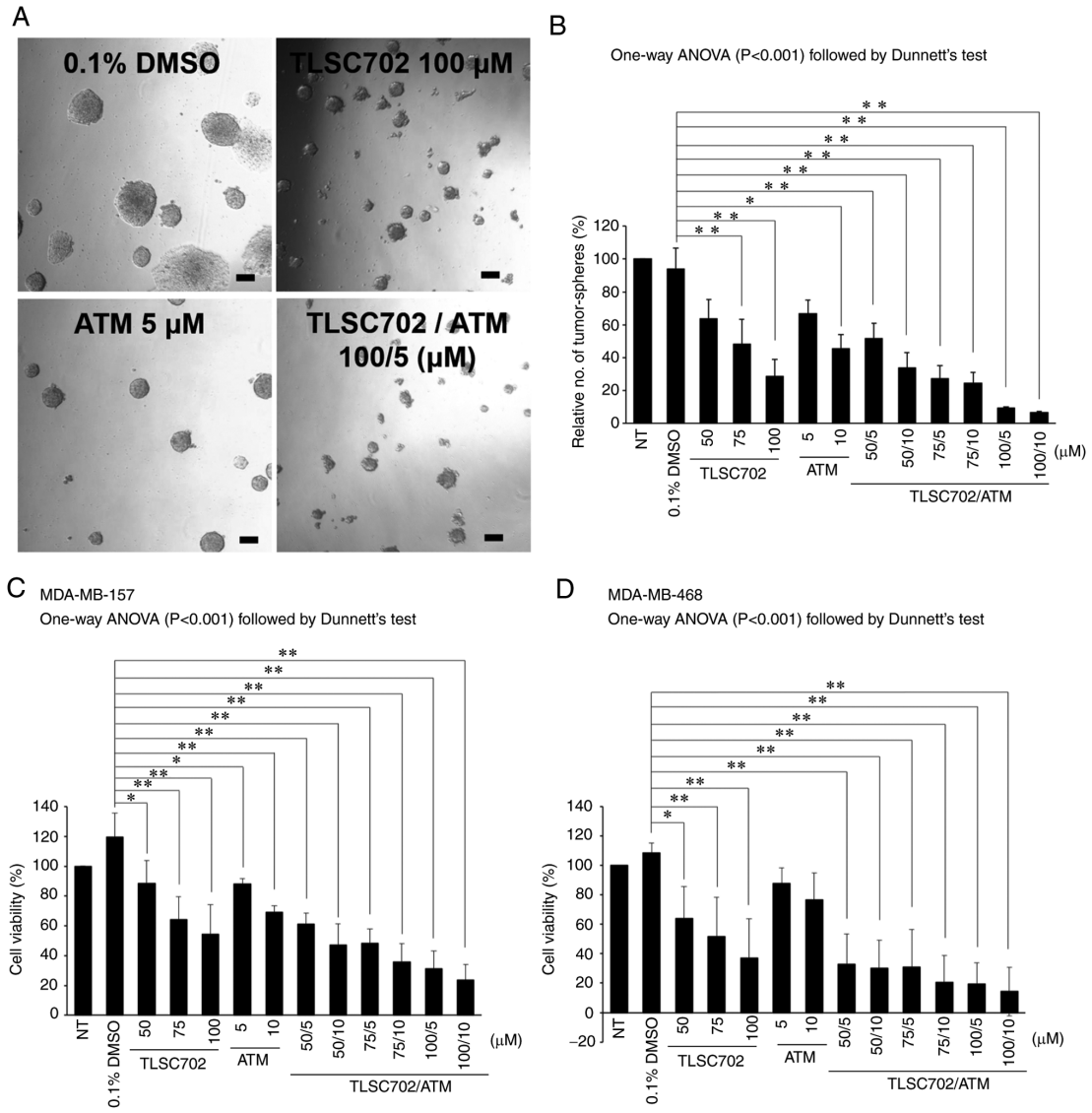

Figure 4. TLSC702 and ATM suppress tumor-sphere formation and breast cancer cell viability. (A) Representative images of tumor-spheres composed of MDA-MB-157 cells. Scale bar, 50 μm. (B) Treatment for 6 days with TLSC702 (50, 75 or 100 μM) and/or ATM (5 or 10 μM) suppressed tumor-sphere formation. (C and D) Cell titer GLO assays of ATP levels measured as an index of cell viability of (C) MDA-MB-157 or (D) MDA-MB-468 cells. Cells were treated for 6 days with or without TLSC702 (50, 75 or 100 μM) and/or ATM (5 or 10 μM), as indicated. Data for the tumor-sphere assay are presented as the mean ± standard error of the mean of three independent experiments. Data for the Cell Titer-Glo® luminescence assay are presented as the mean ± standard deviation of three independent experiments and were compared using one-way ANOVA (P<0.001) followed by Dunnett's test. *P<0.05, **P<0.01. The α-level was fixed at 0.05, and P<0.05 was considered to indicate a statistically significant difference. ATM, aurothiomalate; GLO 1, glyoxalase 1; PKCλ, protein kinase Cλ.

via FoxO1 DNA-binding ability and contributes to cell growth of angiosarcoma (48). MG, which induces expression and activity of GLO 1, also induces phosphorylation of Erk1/2 (66). GLO 1 modulates cell viability and tumor formation in ALDH1-positive breast cancer stem cells (CSCs) (21). Moreover, PKCλ also modulates cell viability, Caspase 3-dependent apoptosis and tumor formation in an Akt independent manner in ALDH1-positive breast CSCs (41,42). The results of the present study suggested that GLO 1 is also functionally associated with PKCλ in the progression of ALDH1-positive breast CSCs.

PKCλ is essential for cancer cell survival of ALDH1-positive breast CSCs by maintaining low levels of ROS (41). ALDH1 serves a role in the detoxification of toxic aldehyde intermediaries generated by ROS-induced peroxidation of intracellular lipids (67). Conversely, GLO 1 detoxifies MG, a cytotoxic byproduct of glycolysis that induces apoptosis (68). Inhibition of GLO 1 reduces cell viability and induces apoptosis in ALDH1-positive breast CSCs (21). In the present study, the combination of TLSC702 and ATM suppressed viability of basal-like breast cancer cells. Therefore, GLO 1 and PKCλ may be involved in cell viability by maintaining lower intracellular ROS levels and/or detoxification of MG. However, the detailed relationship between GLO 1 and PKCλ in ALDH1-positive breast CSCs remains to be determined.

GLUT1 facilitates glucose transport, and its expression is increased in several types of cancers, including basal-like breast cancer, where it is involved in cancer progression (49,50). PKCλ is involved in GLUT1 translocation from intracellular vesicles to the plasma membrane in 3T3-L1 adipocytes (51), where it increases glucose accumulation and promotes cell growth via upregulation of GLUT1 (69). Furthermore, PKCλ

is involved in insulin-dependent glucose-uptake by GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes (44,45). In addition, GAPDH is phosphorylated by PKCλ and then interacts directly with the PKCλ regulatory domain to promote microtubule nucleation. (70,71). *GLO 1* is also overexpressed in basal-like breast cancer (21). Given the high levels of *GLO 1* and *PKCλ* expression in several basal-like breast cancer types, it may be that PKCλ regulates glucose uptake by GLUT1, leading to increased glycolysis catalyzed in part by GLO 1.

Expression of PKCζ, another aPKC isotype, is also upregulated in breast cancer tissues compared with normal tissues derived from the same patient (41). c-Myc is reportedly phosphorylated by PKCζ in prostate cancer (72), and c-Myc is known to directly transactivate glucose metabolic genes, including GLUT1, PFK-1, GAPDH and enolase, and to increase glucose uptake (73). Thus, c-Myc phosphorylation by PKCζ may contribute to the regulation of glycolysis in breast cancer. In addition, PKCζ phosphorylates Nrf2, which regulates glucose-6-phosphate dehydrogenase (G6PD) gene expression (74). G6PD dehydrogenizes glucose-6-phosphate, an intermediate of glycolysis, and is the rate limiting enzyme in the pentose phosphate pathway (PPP). PPP generates glyceraldehyde 3 phosphate (GAP), an intermediate metabolite of glycolysis, and GAP is converted into MG. Furthermore, despite nutrient stress, PKCζ directly phosphorylates and inhibits the enzymatic activity of PHGDH, which suppresses metabolic reprogramming of glycolytic intermediates (75). This suggests that both PKCλ and PKCζ are associated with glycolysis, directly and indirectly, at different stages in breast cancer development/progression.

Expression levels of GLO 1 and PKCλ mRNA are correlated in ER- and/or PgR-positive and luminal B type breast cancer. Given that luminal B type breast cancer exhibits expression of ER and/or PgR, the correlation between *GLO 1* and *PKCλ* in luminal B tumors may be related to the ER and/or PgR positivity of those tumors. The luminal B subtype is associated with poorer clinical outcomes compared with the luminal A subtype (76). These results therefore suggest that high expression of *GLO 1* and *PKCλ* may be contributed to cancer progression in luminal B.

Kaplan-Meier and multivariable Cox regression analyses showed that normal-like breast cancer patients classified as *GLO 1*^{high} *PKCλ*^{high} also exhibited a worse prognosis. Earlier studies reported that patients with normal-like tumors had a better prognosis compared with patients with other breast cancer subtypes (6). However, unlike other breast cancer subtypes, which have well-described molecular characteristics, the significant features of the normal-like subtype are largely unknown (77), and the roles of GLO 1 and PKCλ remain to be determined.

In conclusion, the levels of *GLO 1* and *PKCλ* expression were shown to be correlated with breast cancer. Patients with late-stage tumors who were classed as *GLO 1*^{high} *PKCλ*^{high} had a poorer prognosis and accounted for a large percentage of cases of basal-like breast cancer. In addition, TLSC702, a GLO 1 inhibitor, and ATM, a PKCλ inhibitor, reduced both cell viability and tumor-sphere formation in basal-like breast cancer cells. It thus appears that GLO 1 and PKCλ are cooperatively involved in cancer progression and contribute to poorer clinical outcomes in late-stage breast cancer patients. It is therefore suggested that GLO 1 and PKCλ are potentially effective therapeutic targets for treatment of late-stage breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HM, AO, ST and YW performed the experiments. HM, AO and KA confirmed the authenticity of all the raw data. HM and AO analyzed the data using IHC Profiler. HM, AO, ST, CO, YNo, YW, YMa, TS, KeS and KYa performed the bioinformatics analysis. RT, KYo, TH, KaS, HI, YMi, YNa, SIT and SO contributed to acquisition of data, and analysis and interpretation of data. HM and KA conceived the study. HM drafted the manuscript. HM, AO, KaS, HI, SO and KA contributed to discussion and review of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol of the present study was reviewed and approved by the Institutional Review Board of Kanagawa Cancer Center Hospital (approval no. 3-2009; Kanagawa, Japan). Written informed consent for participation was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all patients for publication.

Competing interests

The authors declare that they have no competing interests.

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