

# Glyoxalase 1 and protein kinase C $\lambda$ as potential therapeutic targets for late-stage breast cancer

HITOMI MOTOMURA<sup>1</sup>, AYAKA OZAKI<sup>1</sup>, SHOMA TAMORI<sup>1</sup>, CHOTARO ONAGA<sup>1</sup>, YUKA NOZAKI<sup>1</sup>, YUKO WAKI<sup>1</sup>, RYOKO TAKASAWA<sup>2</sup>, KAZUMI YOSHIZAWA<sup>2</sup>, YASUNARI MANO<sup>2</sup>, TSUGUMICHI SATO<sup>2</sup>, KAZUNORI SASAKI<sup>3</sup>, HITOSHI ISHIGURO<sup>4,5</sup>, YOHEI MIYAGI<sup>6</sup>, YOJI NAGASHIMA<sup>7</sup>, KOUJI YAMAMOTO<sup>8</sup>, KEIKO SATO<sup>9</sup>, TAKEHISA HANAWA<sup>2</sup>, SEI-ICHI TANUMA<sup>1,10</sup>, SHIGEO OHNO<sup>3,11</sup> and KAZUNORI AKIMOTO<sup>1</sup>

Departments of <sup>1</sup>Medicinal and Life Sciences, and <sup>2</sup>Pharmacy, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba 278-8510; <sup>3</sup>Department of Molecular Biology, Yokohama City University, School of Medicine; <sup>4</sup>Department of Urology, Yokohama City University Graduate School of Medicine, Kanagawa 236-0004; <sup>5</sup>Photocatalyst Group, Research and Development Department, Kanagawa Institute of Industrial Science and Technology, Kanagawa 210-0821; <sup>6</sup>Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, Kanagawa 241-8515; <sup>7</sup>Department of Surgical Pathology, Tokyo Women's Medical University Hospital, Tokyo 162-8666; <sup>8</sup>Department of Biostatistics, Yokohama City University, School of Medicine, Kanagawa 236-0004; <sup>9</sup>Department of Information Sciences, Faculty of Science and Technology; <sup>10</sup>Department of Genomic Medicinal Science, Research Institute for Science and Technology, Organization for Research Advancement, Tokyo University of Science, Chiba 278-8510; <sup>11</sup>Department of Cancer Biology, Institute for Diseases of Old Age, Juntendo University School of Medicine, Tokyo 113-8421, Japan

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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 C $\lambda$  (PKC $\lambda$ ) is also upregulated in various types of cancer and is involved in cancer progression. In the present study, the association between enhanced glycolysis and PKC $\lambda$  in breast cancer was investigated. In human breast cancer, high GLO 1 expression was associated with high PKC $\lambda$  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 PKC $\lambda$  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 PKC $\lambda$  inhibitor) reduced both cell viability and tumor-sphere formation. These results suggested that GLO 1 and PKC $\lambda$  were cooperatively involved in cancer progression and contributed to a poor prognosis in breast cancer. In conclusion, GLO 1 and PKC $\lambda$  serve as potentially effective therapeutic targets for treatment of late-stage human breast cancer.

## 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

*Correspondence to:* Professor Kazunori Akimoto, Department of Medicinal and Life Sciences, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan  
E-mail: akimoto@rs.tus.ac.jp

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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) isoforms, PKC $\lambda$ , 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). PKC $\lambda$  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, TGF $\beta$  and IL1 $\beta$  induce PKC $\lambda$  phosphorylation and promote PKC $\lambda$ -dependent proliferation, invasiveness and metastasis by inducing NF- $\kappa$ B p65 nuclear translocation (30). c-Met and PKC $\lambda$  are cooperatively involved in cellular viability and tumor formation in basal-like breast cancer cells (42). PKC $\lambda$  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 PKC $\lambda$  via PI3-kinase (43-45), and PKC $\lambda$  subsequently binds to and regulates p70 S6 kinase (46). PKC $\lambda$  also activates the Rac1-Pak-Mek1/2-Erk1/2 signaling pathway, which is associated with lung cancer cell proliferation and tumorigenicity (47). PKC $\lambda$  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). PKC $\lambda$  regulates the translocation of GLUT1 from intracellular vesicles to the plasma membrane in 3T3-L1 adipocytes (51). However, the role of PKC $\lambda$  in the enhanced glycolysis seen in cancer cells remains unclear.

In the present study, the association between the levels of GLO 1 and PKC $\lambda$  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- $\mu$ m 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-PKC $\lambda$  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  $\kappa$  Isotype Control (eBMG2b; 1:500; cat. no. 14-4732-82; eBioscience; Thermo Fisher Scientific, Inc.) and mouse IgG1  $\kappa$  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-PKC $\lambda$  mAb (1:50), rabbit anti-GLO 1 pAb (1:200; cat. no. A1932; ABClonal, Inc.), mouse IgG2b  $\kappa$  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 PKC $\lambda$  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 PKC $\lambda$  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 clinico-pathological 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 PKC $\lambda$  (reporter, A\_23\_P18392) mRNA expression are presented using log<sub>2</sub> 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-PKC $\iota$  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- $\beta$ -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<sup>3</sup>/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<sup>3</sup>/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  $\geq 50 \mu\text{m}$  in diameter were counted. Numerical values of the test groups are shown relative to the untreated cells. Cell Titer-Glo<sup>®</sup> 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 PKC $\lambda$  expression, statistical significance was calculated using the  $\chi^2$ -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  $\pm$  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  $\pm$  standard error of the mean of three independent experiments. Data for the Cell Titer-Glo<sup>®</sup> luminescence assay is presented as the mean  $\pm$  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,  $\alpha$ -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 PKC $\lambda$  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). PKC $\lambda$  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 PKC $\lambda$  were colocalized in breast cancer cells (Fig. 1C). To evaluate the relationship between GLO 1 and PKC $\lambda$ , their signal intensities were quantified (Fig. 1A and B). High expression of GLO 1 was significantly correlated with high expression of PKC $\lambda$  in breast cancer tissue (Fig. 1D, P<0.01,  $\chi^2$ -test; Fig. 1E, r=0.71, P<0.01).



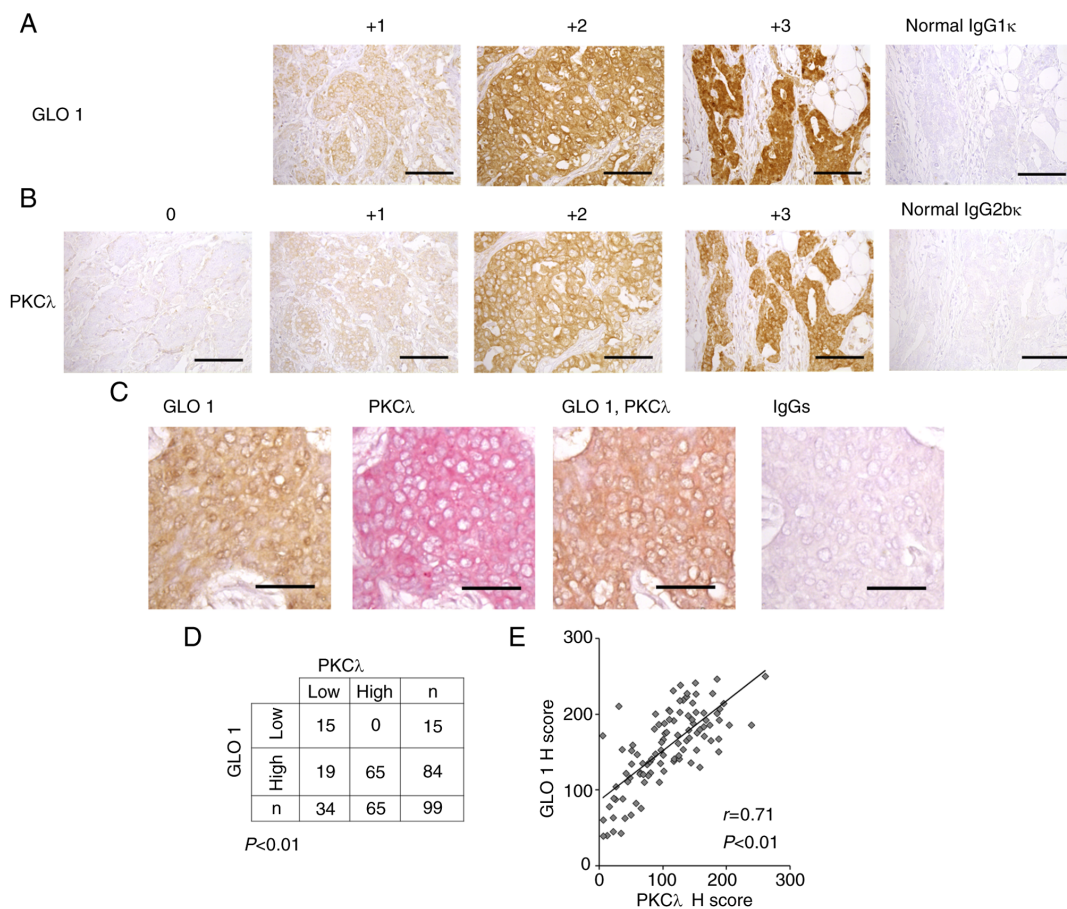


Figure 1. Overexpression of GLO 1 is associated with PKC $\lambda$  expression in breast cancer. Representative IHC images of (A) GLO 1 and (B) PKC $\lambda$  staining in breast cancer tissues. Scale bars, 100  $\mu$ m. (C) Double staining using IHC. Scale bar, 50  $\mu$ m. (D)  $\chi^2$ -test based on protein GLO 1 and PKC $\lambda$  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 PKC $\lambda$  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  $\alpha$ -level was fixed at 0.05, and  $P < 0.05$  was considered to indicate a statistically significant difference. GLO 1, glyoxalase 1; PKC $\lambda$ , protein kinase C $\lambda$ ; IHC, immunohistochemistry;  $r$ , Spearman's rank correlation coefficient.

*GLO 1 expression is correlated with PKC $\lambda$  expression at the mRNA level in human breast cancer.* To further examine the relationship between *GLO 1* and *PKC $\lambda$*  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, *PKC $\lambda$*  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 *PKC $\lambda$*  expression in all patients and in patients with early-stage (stage 0-II) tumors (Fig. 2A), but was not correlated with *PKC $\lambda$*  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 *PKC $\lambda$*  gene expression. Nonetheless, these results, together with the IHC findings, suggest that *GLO 1* and *PKC $\lambda$*  may be cooperatively involved in certain cases of breast cancer.

*High GLO 1 and PKC $\lambda$  expression is correlated with a poorer prognosis in patients with stage III-IV tumors.* Next, we examined whether high *GLO 1* and *PKC $\lambda$*  expression is correlated with

prognosis (Figs. 2B-D and S2; Tables I and SIV). Examination of the prognosis of patients with *GLO 1*<sup>high</sup> and *PKC $\lambda$* <sup>high</sup> breast cancer revealed that *GLO 1*<sup>high</sup> was not associated with a poorer OS amongst all patients (Fig. 2B) (21). It was previously shown that overexpression of *PKC $\lambda$*  and its signaling promotes TNBC growth and metastasis (30). Consistent with this finding, patients classed as *PKC $\lambda$* <sup>high</sup> 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 *PKC $\lambda$*  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*<sup>high</sup>, *PKC $\lambda$* <sup>high</sup> or *GLO 1*<sup>high</sup> *PKC $\lambda$* <sup>high</sup>) exhibited similar clinical outcomes to those expressing lower levels of these genes (Fig. 2C), as did those with late-stage tumors (*GLO 1*<sup>high</sup>; Fig. 2D). However, patients with late-stage tumors (*PKC $\lambda$* <sup>high</sup>) had a worse OS (Fig. 2D) (42). Furthermore, patients with *GLO 1*<sup>high</sup> *PKC $\lambda$* <sup>high</sup> tumors had poorer prognoses than patients with *GLO 1*<sup>high</sup> *PKC $\lambda$* <sup>low</sup> (adjusted by Holm's method;  $P = 0.015$ ) and *GLO 1*<sup>low</sup> *PKC $\lambda$* <sup>low</sup> ( $P = 0.040$ ), but not *GLO 1*<sup>low</sup> *PKC $\lambda$* <sup>high</sup> ( $P = 0.83$ ) tumors. Multivariable Cox regression analysis revealed that *GLO 1*<sup>high</sup> *PKC $\lambda$* <sup>high</sup> was associated with poorer clinical outcomes than *GLO 1*<sup>low</sup> *PKC $\lambda$* <sup>low</sup> (Table I; HR=2.36;



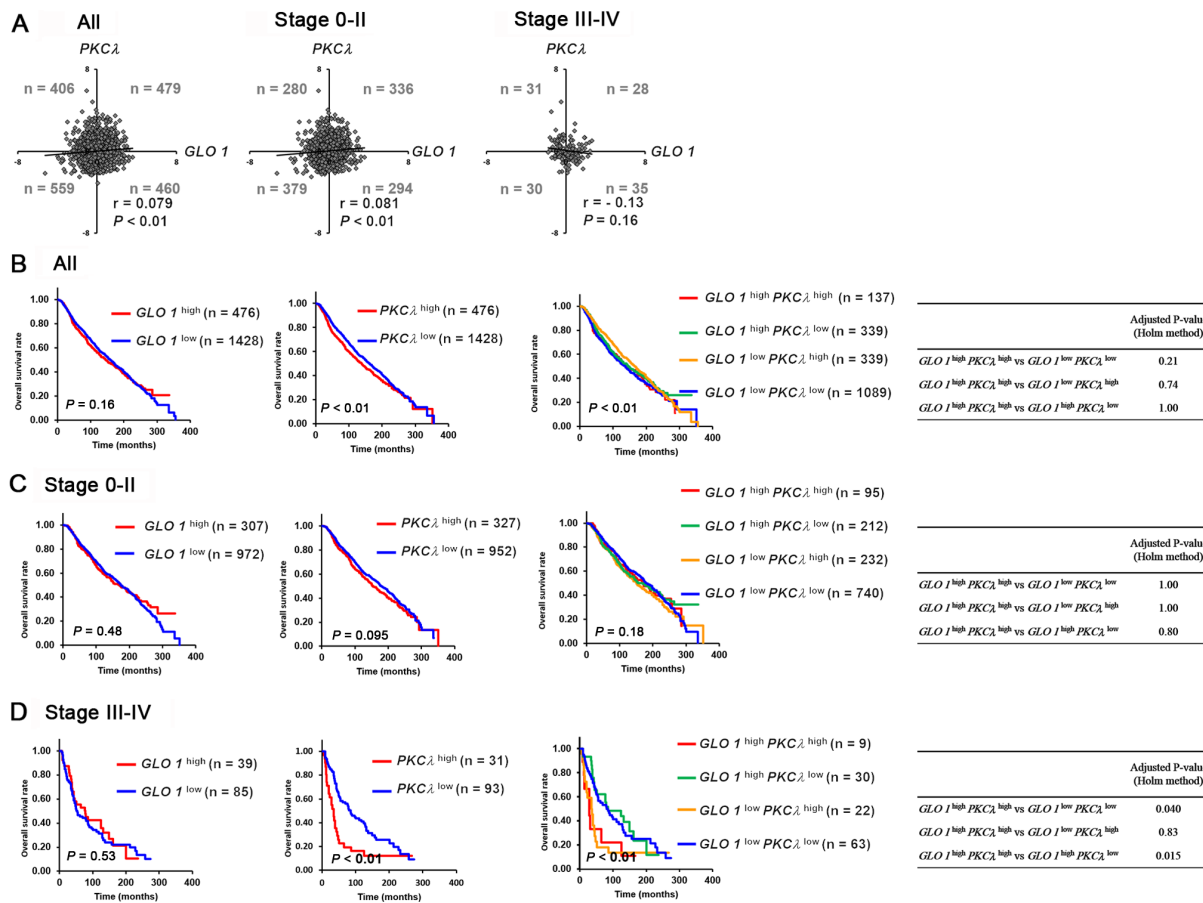


Figure 2. Co-expression of *GLO 1* and *PKCλ* is associated with a poor prognosis in late-stage tumors. (A) Scatter plots showing the correlation between *GLO 1* and *PKCλ* expression in all patients with breast cancer, stage 0-II patients and stage III-IV patients. The *r* and *P*-values are indicated. *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  $\alpha$ -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*<sup>high</sup> *PKCλ*<sup>low</sup> (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*<sup>high</sup> *PKCλ*<sup>high</sup> 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*<sup>high</sup> *PKCλ*<sup>high</sup> was larger than the proportion classed as *GLO 1*<sup>low</sup> *PKCλ*<sup>low</sup>, irrespective of whether they had early- or late-stage tumors (Fig. S3). All patients with breast cancer classed as *GLO 1*<sup>high</sup> *PKCλ*<sup>high</sup> primarily had luminal B type breast cancer (43.1%, 59/137), whereas those with *GLO 1*<sup>low</sup> *PKCλ*<sup>low</sup> 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*<sup>high</sup> *PKCλ*<sup>high</sup> primarily had luminal B breast cancer (48.4%, 46/95), and those with *GLO 1*<sup>low</sup> *PKCλ*<sup>low</sup> primarily had luminal A breast cancer (45.2%, 333/737). However, amongst patients with late-stage lesions, those classed as *GLO 1*<sup>high</sup> *PKCλ*<sup>high</sup> 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*<sup>low</sup> *PKCλ*<sup>low</sup> (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*<sup>high</sup> *PKCλ*<sup>high</sup>. In our previous study, it was shown that *GLO 1* expression is upregulated in basal-like breast cancer cells, 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 *PKC $\lambda$*  expression and breast cancer in all patients, and in patients stratified by stage (0-II and III-IV).

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

<sup>a</sup>Hazard ratio adjusted by age estimated using Cox proportional hazard model. GLO 1, glyoxalase 1; PKC $\lambda$ , protein kinase C $\lambda$ .

50, 75 and 100  $\mu$ M TLSC702 were used in the present study (21). To inhibit PKC $\lambda$ , ATM was used (5 and 10  $\mu$ M), which interferes with the PB1-PB1 domain interactions between PKC $\lambda$  and Par6 and induces apoptosis (62,63). As a result of the inhibitory effect of 0.5, 1, 5 and 10  $\mu$ M ATM on the colony formation ability of MDA-MB-157 cells, it was found that 5 and 10  $\mu$ M 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  $\mu$ M) (21). By contrast, inhibition of PKC $\lambda$  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  $\mu$ M; 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 PKC $\lambda$  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  $\geq 50 \mu$ m in diameter. Furthermore, a combination of TLSC702 and ATM inhibited the formation of tumor-spheres  $\geq 50 \mu$ m in diameter (Fig. 4A and B;  $P < 0.05$ ). Cell Titer-Glo<sup>®</sup> 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 PKC $\lambda$  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 PKC $\lambda$  expression at the protein and mRNA levels in breast cancer and that patients with breast cancer classed as *GLO 1*<sup>high</sup> *PKC $\lambda$* <sup>high</sup> had poorer clinical outcomes for late-stage tumors. GLO 1 and PKC $\lambda$  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 PKC $\lambda$  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 PKC $\lambda$  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 PKC $\lambda$  expression exhibited poorer overall survival compared with patients expressing lower levels of these genes. Therefore, GLO 1 and PKC $\lambda$  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*.

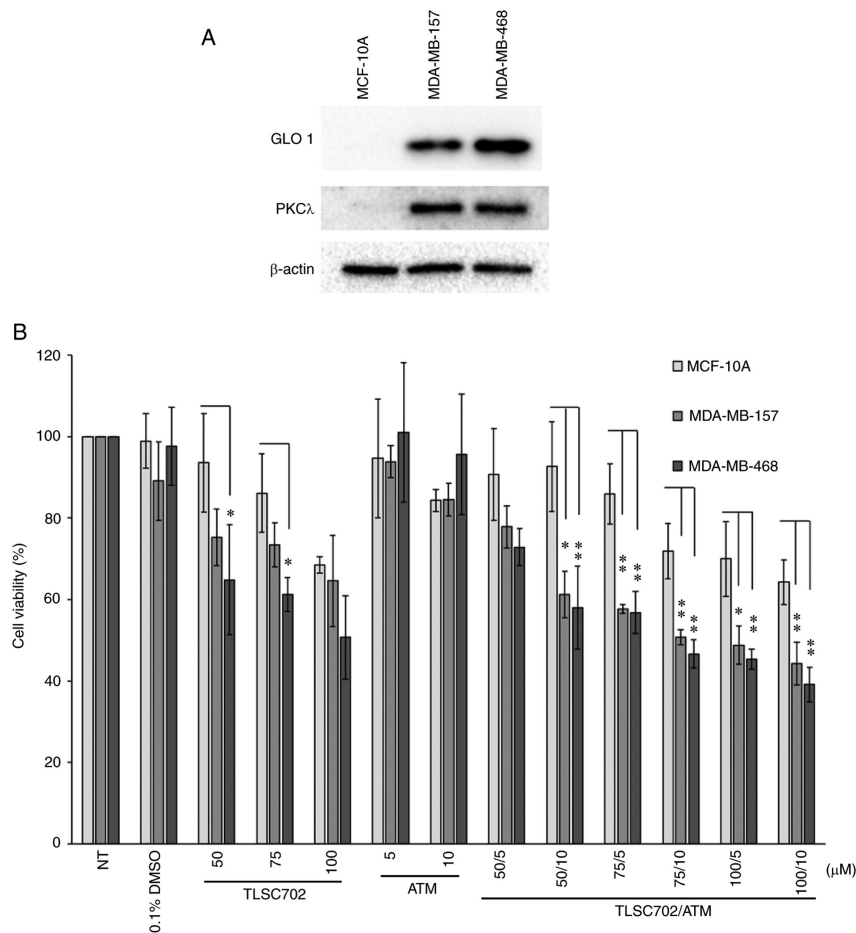


Figure 3. TLSC702 and ATM suppress the viability of breast cancer cells. (A) GLO 1 and PKC $\lambda$  protein expression in MCF-10A human normal-like mammary epithelial cells and in MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cells were analyzed by immunoblotting.  $\beta$ -actin was used 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  $\mu$ M) and/or ATM (5 or 10  $\mu$ M) was assessed using WST-8 assays. Values of the experimental groups are expressed relative to the untreated cells. Data are presented as the mean  $\pm$  standard deviation of three independent experiments and were compared using a Tukey's test. \* $P$ <0.05, \*\* $P$ <0.01. The  $\alpha$ -level was fixed at 0.05, and  $P$ <0.05 was considered to indicate a statistically significant difference. ATM, aurothiomalate; GLO 1, glyoxalase 1; PKC $\lambda$ , protein kinase  $\lambda$ .

The prognosis of patients with stage 0-II breast cancer with *GLO*  $I^{\text{high}}$  *PKC* $\lambda^{\text{high}}$  did not differ significantly from those with *GLO*  $I^{\text{low}}$  *PKC* $\lambda^{\text{low}}$ . Notably, amongst patients with stage III-IV tumors, those with *GLO*  $I^{\text{high}}$  *PKC* $\lambda^{\text{high}}$  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*  $I^{\text{high}}$  and *GLO*  $I^{\text{low}}$  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*  $I^{\text{high}}$  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 PKC $\lambda$

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, PKC $\lambda$  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*  $I^{\text{high}}$  *PKC* $\lambda^{\text{high}}$  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*  $I^{\text{high}}$  *PKC* $\lambda^{\text{high}}$ . This result suggests that GLO 1 and PKC $\lambda$  are cooperatively involved in the progression of basal-like breast cancer. Moreover, the GLO 1 inhibitor TLSC702 and the PKC $\lambda$  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-PKC $\lambda$  complex interacts with epithelial cell transforming sequence 2 to activate Rac1 during cancer cell proliferation (65), and PKC $\lambda$  activates the Rac1-Pak-Mek1/2-Erk1/2 signaling pathway in lung cancer cell growth and tumorigenicity (47). PKC $\lambda$  modulates c-Myc



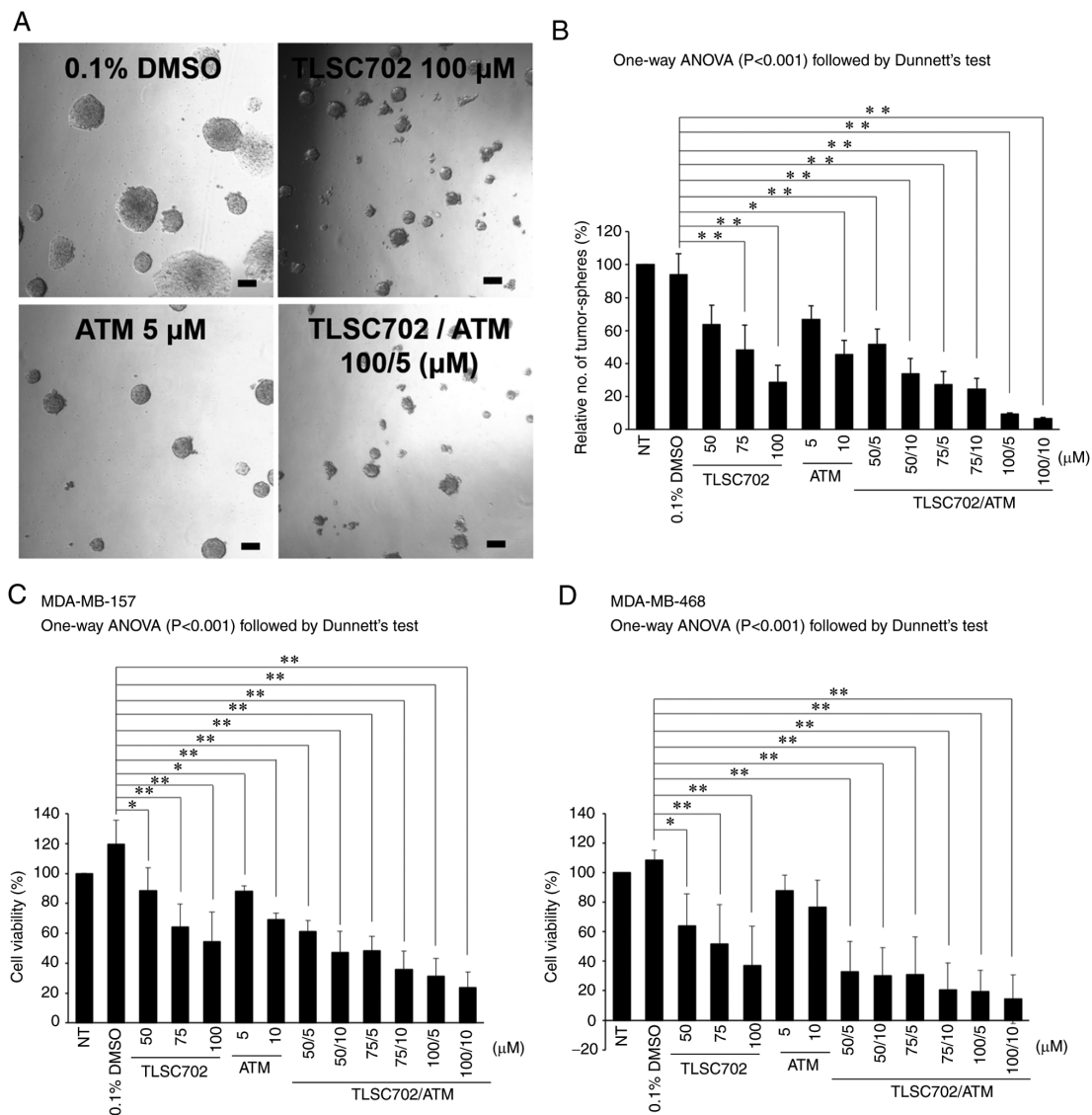


Figure 4. TLSC702 and ATM suppress tumor-sphere formation and breast cancer cell viability. (A) Representative images of tumor-spheres composed of MDA-MB-157 cells. Scale bar, 50  $\mu$ m. (B) Treatment for 6 days with TLSC702 (50, 75 or 100  $\mu$ M) and/or ATM (5 or 10  $\mu$ M) suppressed tumor-sphere 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  $\mu$ M) and/or ATM (5 or 10  $\mu$ M), as indicated. Data for the tumor-sphere assay are presented as the mean  $\pm$  standard error of the mean of three independent experiments. Data for the Cell Titer-Glo<sup>®</sup> luminescence assay are presented as the mean  $\pm$  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  $\alpha$ -level was fixed at 0.05, and  $P < 0.05$  was considered to indicate a statistically significant difference. ATM, aurothiomalate; GLO 1, glyoxalase 1; PKC $\lambda$ , protein kinase C $\lambda$ .

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, PKC $\lambda$  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 PKC $\lambda$  in the progression of ALDH1-positive breast CSCs.

PKC $\lambda$  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 PKC $\lambda$  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 PKC $\lambda$  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). PKC $\lambda$  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, PKC $\lambda$

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 PKC $\lambda$  and then interacts directly with the PKC $\lambda$  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 PKC $\lambda$  expression in several basal-like breast cancer types, it may be that PKC $\lambda$  regulates glucose uptake by GLUT1, leading to increased glycolysis catalyzed in part by GLO 1.

Expression of PKC $\zeta$ , 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 PKC $\zeta$  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 PKC $\zeta$  may contribute to the regulation of glycolysis in breast cancer. In addition, PKC $\zeta$  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, PKC $\zeta$  directly phosphorylates and inhibits the enzymatic activity of PHGDH, which suppresses metabolic reprogramming of glycolytic intermediates (75). This suggests that both PKC $\lambda$  and PKC $\zeta$  are associated with glycolysis, directly and indirectly, at different stages in breast cancer development/progression.

Expression levels of GLO 1 and PKC $\lambda$  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 PKC $\lambda$  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 PKC $\lambda$  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*<sup>high</sup> PKC $\lambda$ <sup>high</sup> 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 PKC $\lambda$  remain to be determined.

In conclusion, the levels of *GLO 1* and PKC $\lambda$  expression were shown to be correlated with breast cancer. Patients with late-stage tumors who were classed as *GLO 1*<sup>high</sup> PKC $\lambda$ <sup>high</sup> 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 PKC $\lambda$  inhibitor, reduced both cell viability and tumor-sphere formation in basal-like breast cancer cells. It thus appears that GLO 1 and PKC $\lambda$  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 PKC $\lambda$  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.

## References

- World Cancer Research Fund (WCRF): Breast cancer statistics. Breast cancer is the most common cancer in women worldwide. WCRF, London, 2018. <https://www.wcrf.org/dietandcancer/cancer-trends/breast-cancer-statistics>.
- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68: 394-424, 2018.
- Maughan KL, Lutterbie MA and Ham PS: Treatment of breast cancer. *Am Fam Physician* 81: 1339-1346, 2010.
- van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, *et al*: A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347: 1999-2009, 2002.
- Perou CM, Sørli T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, *et al*: Molecular portraits of human breast tumours. *Nature* 406: 747-752, 2000.
- Sørli T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, *et al*: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 98: 10869-10874, 2001.
- Herschkovitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, *et al*: Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* 8: R76, 2007.
- Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, *et al*: Basal-like and triple-negative breast cancers: A critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol* 24: 157-167, 2011.
- Warburg O, Wind F and Negelein E: The metabolism of tumors in the body. *J Gen Physiol* 8: 519-530, 1927.
- Gatenby RA and Gillies RJ: Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4: 891-899, 2004.
- Sakamoto H, Mashima T, Sato S, Hashimoto Y, Yamori T and Tsuruo T: Selective activation of apoptosis program by S-p-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells. *Clin Cancer Res* 7: 2513-2518, 2001.
- Cheng WL, Tsai MM, Tsai CY, Huang YH, Chen CY, Chi HC, Tseng YH, Chao IW, Lin WC, Wu SM, *et al*: Glyoxalase-I is a novel prognosis factor associated with gastric cancer progression. *PLoS One* 7: e34352, 2012.
- Ranganathan S and Tew KD: Analysis of glyoxalase-I from normal and tumor tissue from human colon. *Biochim Biophys Acta* 1182: 311-316, 1993.
- Zhang S, Liang X, Zheng X, Huang H, Chen X, Wu K, Wang B and Ma S: Glol genetic amplification as a potential therapeutic target in hepatocellular carcinoma. *Int J Clin Exp Pathol* 7: 2079-2090, 2014.
- Baunacke M, Horn LC, Trettner S, Engel KM, Hemdan NY, Wiechmann V, Stolzenburg JU, Bigl M and Birkenmeier G: Exploring glyoxalase 1 expression in prostate cancer tissues: Targeting the enzyme by ethyl pyruvate defangs some malignancy-associated properties. *Prostate* 74: 48-60, 2014.
- Kreycy N, Gotzian C, Fleming T, Flechtenmacher C, Grabe N, Plinkert P, Hess J and Zaoui K: Glyoxalase 1 expression is associated with an unfavorable prognosis of oropharyngeal squamous cell carcinoma. *BMC Cancer* 17: 382, 2017.
- Zou XY, Ding D, Zhan N, Liu XM, Pan C and Xia YM: Glyoxalase I is differentially expressed in cutaneous neoplasms and contributes to the progression of squamous cell carcinoma. *J Invest Dermatol* 135: 589-598, 2015.
- Bair WB III, Cabello CM, Uchida K, Bause AS and Wondrak GT: GLO1 overexpression in human malignant melanoma. *Melanoma Res* 20: 85-96, 2010.
- Rulli A, Carli L, Romani R, Baroni T, Giovannini E, Rosi G and Talesa V: Expression of glyoxalase I and II in normal and breast cancer tissues. *Breast Cancer Res Treat* 66: 67-72, 2001.
- Peng HT, Chen J, Liu TY, Wu YQ, Lin XH, Lai YH and Huang YF: Up-regulation of the tumor promoter Glyoxalase-I indicates poor prognosis in breast cancer. *Int J Clin Exp Pathol* 10: 10852-10862, 2017.
- Tamori S, Nozaki Y, Motomura H, Nakane H, Katayama R, Onaga C, Kikuchi E, Shimada N, Suzuki Y, Noike M, *et al*: Glyoxalase 1 gene is highly expressed in basal-like human breast cancers and contributes to survival of ALDH1-positive breast cancer stem cells. *Oncotarget* 9: 36515-36529, 2018.
- Zhang D, Tai LK, Wong LL, Chiu LL, Sethi SK and Koay ES: Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. *Mol Cell Proteomics* 4: 1686-1696, 2005.
- Gandalovičová A, Vomastek T, Rosel D and Brábek J: Cell polarity signaling in the plasticity of cancer cell invasiveness. *Oncotarget* 7: 25022-25049, 2016.
- Akimoto K, Mizuno K, Osada S, Hirai S, Tanuma S, Suzuki K and Ohno S: A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. *J Biol Chem* 269: 12677-12683, 1994.
- Suzuki A, Akimoto K and Ohno S: Protein kinase C lambda/iota (PKClambda/iota): A PKC isotype essential for the development of multicellular organisms. *J Biochem* 133: 9-16, 2003.
- Ohno S: Intercellular junctions and cellular polarity: The PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* 13: 641-648, 2001.
- Parker PJ, Justilien V, Riou P, Linch M and Fields AP: Atypical protein kinase C $\iota$  as a human oncogene and therapeutic target. *Biochem Pharmacol* 88: 1-11, 2014.
- Murray NR, Jamieson L, Yu W, Zhang J, Gökmen-Polar Y, Sier D, Anastasiadis P, Gatalica Z, Thompson EA and Fields AP: Protein kinase C $\iota$  is required for Ras transformation and colon carcinogenesis in vivo. *J Cell Biol* 164: 797-802, 2004.
- Kojima Y, Akimoto K, Nagashima Y, Ishiguro H, Shirai S, Chishima T, Ichikawa Y, Ishikawa T, Sasaki T, Kubota Y, *et al*: The overexpression and altered localization of the atypical protein kinase C lambda/iota in breast cancer correlates with the pathologic type of these tumors. *Hum Pathol* 39: 824-831, 2008.
- Paul A, Gunewardena S, Stecklein SR, Saha B, Parelkar N, Danley M, Rajendran G, Home P, Ray S, Jokar I, *et al*: PKC $\lambda/\iota$  signaling promotes triple-negative breast cancer growth and metastasis. *Cell Death Differ* 21: 1469-1481, 2014.
- Mizushima T, Asai-Sato M, Akimoto K, Nagashima Y, Taguri M, Sasaki K, Nakaya MA, Asano R, Tokinaga A, Kiyono T, *et al*: Aberrant expression of the cell polarity regulator aPKC $\lambda/\iota$  is associated with disease progression in cervical intraepithelial Neoplasia (CIN): A possible marker for predicting CIN prognosis. *Int J Gynecol Pathol* 35: 106-117, 2016.
- Tokinaga-Uchiyama A, Mizushima T, Akimoto K, Nagashima Y, Sasaki K, Nakaya MA, Ohashi K, Kubota K, Maruyama Y, Kato H, *et al*: Aberrant nuclear localization of aPKC $\lambda/\iota$  is associated with poorer prognosis in uterine cervical cancer. *Gynecol Pathol* 38: 301-309, 2019.
- Baba J, Kioi M, Akimoto K, Nagashima Y, Taguri M, Inayama Y, Aoki I, Ohno S, Mitsudo K and Tohrai I: Atypical protein kinase C $\lambda/\iota$  expression is associated with malignancy of oral squamous cell carcinoma. *Anticancer Res* 38: 6291-6297, 2018.
- Ishiguro H, Akimoto K, Nagashima Y, Kagawa E, Sasaki T, Sano JY, Takagawa R, Fujinami K, Sasaki K, Aoki I, *et al*: Coexpression of aPKC $\lambda/\iota$  and IL-6 in prostate cancer tissue correlates with biochemical recurrence. *Cancer Sci* 102: 1576-1581, 2011.
- Ishiguro H, Akimoto K, Nagashima Y, Kojima Y, Sasaki T, Ishiguro-Imagawa Y, Nakaigawa N, Ohno S, Kubota Y and Uemura H: aPKClambda/iota promotes growth of prostate cancer cells in an autocrine manner through transcriptional activation of interleukin-6. *Proc Natl Acad Sci USA* 106: 16369-16374, 2009.
- Regala RP, Weems C, Jamieson L, Khoo A, Edell ES, Lohse CM and Fields AP: Atypical protein kinase C  $\iota$  is an oncogene in human non-small cell lung cancer. *Cancer Res* 65: 8905-8911, 2005.
- Eder AM, Sui X, Rosen DG, Nolden LK, Cheng KW, Lahad JP, Kango-Singh M, Lu KH, Warneke CL, Atkinson EN, *et al*: Atypical PKC $\iota$  contributes to poor prognosis through loss of apical-basal polarity and cyclin E overexpression in ovarian cancer. *Proc Natl Acad Sci USA* 102: 12519-12524, 2005.



38. Scotti ML, Bamlet WR, Smyrk TC, Fields AP and Murray NR: Protein kinase Ciota is required for pancreatic cancer cell transformed growth and tumorigenesis. *Cancer Res* 70: 2064-2074, 2010.
39. Kato S, Akimoto K, Nagashima Y, Ishiguro H, Kubota K, Kobayashi N, Hosono K, Watanabe S, Sekino Y, Sato T, *et al*: aPKC $\lambda$ /i is a beneficial prognostic marker for pancreatic neoplasms. *Pancreatol* 13: 360-368, 2013.
40. Takagawa R, Akimoto K, Ichikawa Y, Akiyama H, Kojima Y, Ishiguro H, Inayama Y, Aoki I, Kunisaki C, Endo I, *et al*: High expression of atypical protein kinase C lambda/iota in gastric cancer as a prognostic factor for recurrence. *Ann Surg Oncol* 17: 81-88, 2010.
41. Nozaki Y, Motomura H, Tamori S, Kimura Y, Onaga C, Kanai S, Ishihara Y, Ozaki A, Hara Y, Harada Y, *et al*: High PKC $\lambda$  expression is required for ALDH1-positive cancer stem cell function and indicates a poor clinical outcome in late-stage breast cancer patients. *PLoS One* 15: e0235747, 2020.
42. Motomura H, Nozaki Y, Onaga C, Ozaki A, Tamori S, Shiina TA, Kanai S, Ohira C, Hara Y, Harada Y, *et al*: High expression of c-Met, PKC $\lambda$  and ALDH1A3 predicts a poor prognosis in late-stage breast cancer. *Anticancer Res* 40: 35-52, 2020.
43. Akimoto K, Takahashi R, Moriya S, Nishioka N, Takayanagi J, Kimura K, Fukui Y, Osada SI, Mizuno K, Hirai SI, *et al*: EGF or PDGF receptors activate atypical PKClambda through phosphatidylinositol 3-kinase. *EMBO J* 15: 788-798, 1996.
44. Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, Miyake K, Sano W, Akimoto K, Ohno S and Kasuga M: Requirement of atypical protein kinase lambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol* 18: 6971-6982, 1998.
45. Farese RV: Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states. *Am J Physiol Endocrinol Metab* 283: E1-E11, 2002.
46. Akimoto K, Nakaya M, Yamanaka T, Tanaka J, Matsuda S, Weng QP, Avruch J and Ohno S: Atypical protein kinase C lambda binds and regulates p70 S6 kinase. *Biochem J* 335: 417-424, 1998.
47. Regala RP, Weems C, Jamieson L, Copland JA, Thompson EA and Fields AP: Atypical protein kinase Ciota plays a critical role in human lung cancer cell growth and tumorigenicity. *J Biol Chem* 280: 31109-31115, 2005.
48. Riddell M, Nakayama A, Hikita T, Mirzapoourshafiyi F, Kawamura T and Pasha A: aPKC controls endothelial growth by modulating c-Myc via FoxO1 DNA-binding ability. *Nat Commun* 9: 5357, 2018.
49. Szablewski L: Expression of glucose transporters in cancers. *Biochim Biophys Acta* 1835: 164-169, 2013.
50. Hussein YR, Bandyopadhyay S, Semaan A, Ahmed Q, Albashiti B, Jazaerly T, Nahleh Z and Ali-Fehmi R: Glut-1 expression correlates with basal-like breast cancer. *Transl Oncol* 4: 321-327, 2011.
51. Bosch RR, Bazuine M, Span PN, Willems PH, Olthoff AJ, van Rennes H, Maassen JA, Tack CJ, Hermus AR and Sweep CG: Regulation of GLUT1-mediated glucose uptake by PKClambda-PKCbeta(II) interactions in 3T3-L1 adipocytes. *Biochem J* 384: 349-355, 2004.
52. Varghese F, Bukhari AB, Malhotra R and De A: IHC Profiler: An open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One* 9: e96801, 2014.
53. Nozaki Y, Tamori S, Inada M, Katayama R, Nakane H, Minamishima O, Onodera Y, Abe M, Shiina S, Tamura K, *et al*: Correlation between c-Met and ALDH1 contributes to the survival and tumor-sphere formation of ALDH1 positive breast cancer stem cells and predicts poor clinical outcome in breast cancer. *Genes Cancer* 8: 628-639, 2017.
54. Sato K and Akimoto K: Expression levels of KMT2C and SLC20A1 identified by information-theoretical analysis are powerful prognostic biomarkers in estrogen receptor-positive breast cancer. *Clin Breast Cancer* 17: e135-e142, 2017.
55. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, *et al*: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486: 346-352, 2012.
56. Pereira B, Chin SF, Rueda OM, Vollen HK, Provenzano E, Bardwell HA, Pugh M, Jones L, Russell R, Sammut SJ, *et al*: The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes. *Nat Commun* 7: 11479, 2016.
57. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al*: The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2: 401-404, 2012.
58. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, *et al*: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6: pii, 2013.
59. Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. *Nature* 490: 61-70, 2012.
60. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A and Chinnaiyan AM: ONCOMINE: A cancer microarray database and integrated data-mining platform. *Neoplasia* 6: 1-6, 2004.
61. Takasawa R, Tao A, Saeki K, Shionozaki N, Tanaka R, Uchiro H, Takahashi S, Yoshimori A and Tanuma S: Discovery of a new type inhibitor of human glyoxalase I by myricetin-based 4-point pharmacophore. *Bioorg. Med Chem Lett* 21: 4337-4342, 2011.
62. Stallings-Mann M, Jamieson L, Regala RP, Weems C, Murray NR and Fields AP: A novel small-molecule inhibitor of protein kinase Ciota blocks transformed growth of non-small-cell lung cancer cells. *Cancer Res* 66: 1767-1774, 2006.
63. Trani M, Sorrentino A, Busch C and Landström M: Pro-apoptotic effect of aurothiomalate in prostate cancer cells. *Cell Cycle* 8: 306-313, 2009.
64. Chiavarina B, Nokin MJ, Durieux F, Bianchi E, Turtoi A, Peulen O, Peixoto P, Irigaray P, Uchida K, Belpomme D, *et al*: Triple negative tumors accumulate significantly less methylglyoxal specific adducts than other human breast cancer subtypes. *Oncotarget* 5: 5472-5482, 2014.
65. Justilien V and Fields AP: Ect2 links the PKCiota-Par6alpha complex to Rac1 activation and cellular transformation. *Oncogene* 28: 3597-3607, 2009.
66. Guo Y, Zhang Y, Yang X, Lu P, Yan X, Xiao F, Zhou H, Wen C, Shi M, Lu J and Meng QH: Effects of methylglyoxal and glyoxalase I inhibition on breast cancer cells proliferation, invasion, and apoptosis through modulation of MAPKs, MMP9, and Bcl-2. *Cancer Biol Ther* 17: 169-180, 2016.
67. Singh S, Brocker C, Koppaka V, Chen Y, Jackson BC and Matsumoto A: Aldehyde dehydrogenases in cellular responses to oxidative/electrophilic stress. *Free Radic Biol Med* 56: 89-101, 2013.
68. Kang Y, Edwards LG and Thornalley PJ: Effect of methylglyoxal on human leukaemia 60 cell growth: Modification of DNA G1 growth arrest and induction of apoptosis. *Leuk Res* 20: 97-405, 1996.
69. Liu L, Lei B, Wang L, Chang C, Yang H, Liu J, Huang G and Xie W: Protein kinase C-iota-mediated glycolysis promotes non-small-cell lung cancer progression. *Oncotargets Ther* 12: 5835-5848, 2019.
70. Tisdale EJ: Glyceraldehyde-3-phosphate dehydrogenase is phosphorylated by protein kinase Ciota/lambda and plays a role in microtubule dynamics in the early secretory pathway. *J Biol Chem* 277: 3334-3341, 2002.
71. Tisdale EJ: Rab2 interacts directly with atypical protein kinase C (aPKC) iota/lambda and inhibits aPKCiota/lambda-dependent glyceraldehyde-3-phosphate dehydrogenase phosphorylation. *J Biol Chem* 278: 52524-52530, 2003.
72. Kim JY, Valencia T, Abu-Baker S, Linares J, Lee SJ, Yajima T, Chen J, Eroshkin A, Castilla EA, Brill LM, *et al*: c-Myc phosphorylation by PKC $\zeta$  represses prostate tumorigenesis. *Proc Natl Acad Sci USA* 110: 6418-6423, 2013.
73. Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA and Dang CV: Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* 275: 21797-21800, 2000.
74. Gjyshi O, Bottero V, Veettil MV, Dutta S, Singh VV and Chikoti L: Kaposi's sarcoma-associated herpesvirus induces Nrf2 during de novo infection of endothelial cells to create a microenvironment conducive to infection. *PLoS Pathog* 10: e1004460, 2014.
75. Ma L, Tao Y, Duran A, Llado V, Galvez A, Barger JF, Castilla EA, Chen J, Yajima T, Porollo A, *et al*: Control of nutrient stress-induced metabolic reprogramming by PKC $\zeta$  in tumorigenesis. *Cell* 152: 599-611, 2013.
76. Franchet C, Duprez-Paumier R and Lacroix-Triki M: Molecular taxonomy of luminal breast cancer in 2015. *Bull Cancer* 102 (6 Suppl 1): S34-S46, 2015 (In French).
77. Liu Z, Zhang XS and Zhang S: Breast tumor subgroups reveal diverse clinical prognostic power. *Sci Rep* 4: 4002, 2014.

