GLO 1 and PKC λ Regulate ALDH1-positive Breast Cancer Stem Cell Survival

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Abstract. *Background/Aim:* We examined the inhibitory effects of both glyoxalase 1 (GLO 1) and protein kinase C (PKC) λ in aldehyde dehydrogenase 1 (ALDH1)-positive breast cancer stem cells (CSCs). *Materials and Methods:* Breast cancer genomics datasets (TCGA, n=593; METABRIC, n=1904) were downloaded and statistically analyzed. The effects of GLO 1 and PKC λ on trypan blue staining and tumor-sphere formation by ALDH1^{high} cells derived from triple negative breast cancer (TNBC) and basal-like breast cancer were examined. *Results:* GLO 1^{high}, PKC λ ^{high}, and ALDH1A3^{high} tumors were enriched in stage I/II/III/IV samples, associated with the HER2 and TNBC subtypes according to receptor status, and associated with the HER2-enriched and basal-like subtypes according to PAM50. Inhibition of either GLO 1 (TLSC702) or PKC λ (ANF) suppressed tumor-sphere formation and enhanced death in ALDH1^{high} cells. TLSC702 also effectively inhibited tumor-sphere formation and induced death in PKC λ knockout ALDH1^{high} cells. *Conclusion:* GLO 1 and PKC λ are important for the survival of ALDH1-positive breast CSCs, and may represent potential therapeutic targets for the treatment of ALDH1-positive breast CSCs.

Breast cancer is the second most frequently diagnosed cancer worldwide (1). There are approximately 2.26 million new cases of breast cancer, which account for 24.5% of all cases of cancer in women (Global Cancer Statistics 2020) (2). In addition, breast cancer is responsible for approximately 680,000 cancer-associated deaths annually (2). Breast cancer is typically classified based on its receptor status and specific gene expression signature (PAM50) (3, 4). In terms of receptor status, breast cancer is categorized into the estrogen receptor (ER)-positive, progesterone receptor (PgR)-positive, human epidermal growth factor receptor 2 (HER2)-positive or triple-negative breast cancer (TNBC; which is negative for ER, PgR, and HER2) subtypes (5, 6). TNBC has the poorest prognosis among the four types of breast cancers, which is most likely due to its stem-like properties (7). Using PAM50 gene expression analysis, breast cancers can also be classified into ≥ 6 subtypes, namely normal-like, luminal A, luminal B, HER2-enriched, claudin-low, and basal-like (3, 4). Among these subtypes, basal-like breast cancer is associated with poorer clinical outcomes, which is also at least partially due to its stem-like properties (3, 8, 9). Therefore, basal-like breast cancer has been frequently found to be either resistant or less responsive to conventional therapeutic approaches, including conventional surgery, chemotherapy and radiotherapy, resulting in high rates of recurrence and metastasis (10). In particular, 70-80% of basal-like breast cancers have also been reported to be of the TNBC subtype (11). Therefore, novel therapeutic targets for the effective treatment of TNBC and basal-like breast cancers are in demand.

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Cancer stem cells (CSCs) are undifferentiated cells with stem-like properties, including self-renewal, multipotency and the ability to promote tumorigenesis (12, 13). Since the majority of CSCs are resistant to conventional chemotherapy and radiotherapy, a deeper understanding of the mechanisms that underlie these CSC properties will assist in the identification of novel therapeutic targets against CSCs (14). Aldehyde dehydrogenase 1 (ALDH1) is an enzyme that converts aldehydes to carboxylic acids and is particularly abundant in normal stem/progenitor cells and various CSCs (15, 16). Within the ALDH1 family, ALDH1A1 and ALDH1A3 have been previously found to be CSC markers in several types of cancer (17-22). In breast cancer, ALDH1A3 contributes significantly to ALDH1 activity (22). In patients with breast cancer, the expression of ALDH1A3 is observed to be significantly associated with cancer type, tumor grade, metastasis, and prognosis (9, 21-24).

A characteristic feature of cancer cells is to undergo metabolic reprogramming to increase and favor glycolysis, in a phenomenon known as the Warburg effect (25). Of note, CSCs exhibit higher rates of glycolysis compared to non-CSCs (24, 26, 27). Glyoxalase 1 (GLO 1) is an enzyme that breaks down the cytotoxic compound methylglyoxal (MG), which is a byproduct of glycolysis that can induce apoptosis (28, 29). GLO 1 is found to be up-regulated in a number of different cancer types, including breast cancer (24, 30-37). In addition, GLO 1 activity is essential for the survival of ALDH1-positive breast CSCs (24). However, the relationship between GLO 1 and other signaling molecules in ALDH1-positive breast CSCs remains unclear.

The majority of cancer cells are derived from epithelial cells, and defects in cell polarity are a characteristic feature of cancer cells (38). PKC λ /I belongs to the atypical protein kinase C (aPKC) subfamily, which is a Ser/Thr kinase that regulates cell polarity (39, 40). PKC λ is up-regulated in several types of cancer, including breast cancer, and is involved in cancer progression, contributing to poor clinical outcomes (9, 37, 41-53). PKC λ is involved in the regulation of cell polarity, proliferation, chemotaxis, and migration (9, 37, 48, 49, 53-55). PKC λ is also essential for the survival of ALDH1-positive breast CSCs (9, 53). However, the relationship between GLO 1 and PKC λ in ALDH1-positive breast CSCs remains unclear. Therefore, in the present study, we investigated the relationship among GLO 1, PKC λ , and ALDH1A3 in breast cancer. Our findings suggest that GLO 1 and PKC λ are potentially useful therapeutic targets in ALDH1-positive breast CSCs.

Materials and Methods

Analysis of cancer genomics data. Gene expression microarray datasets of breast cancer samples (n=593) from The Cancer Genome Atlas were downloaded from Oncomine in January 2021 (56, 57). Expression levels of GLO 1 (reporter, A_32_P53822), PKC λ

(reporter, A_23_P18392), and ALDH1A3 (reporter, A_23_P205950) mRNA are presented using the log2 median-centered ratio for both normal and cancerous tissues. Another set of gene expression data was downloaded from the cBioportal in June 2021 and analyzed as previously described (9, 23, 24, 37, 53, 58, 59). Briefly, the Molecular Taxonomy of Breast Cancer International Consortium dataset (n=1,904) was downloaded from cBioPortal (60, 61). Tumor-stage information is composed of 1,893 patients. The number of patients in each stage patients are the following: stage 0: 4, stage I: 475, stage II: 800, stage III: 115, stage IV: 9. Gene expression levels were classified as high if Z-score>0. Three-dimensional scatterplots were produced using the JMP 14 statistical software (SAS Institute Inc., NC, USA).

Cell culture. The MDA-MB-157 and MDA-MB-468 human basal-like breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were then cultured as previously described (9, 23, 24, 37, 53, 59). Control knockout (KO) and PKC λ KO clones established using the CRISPR-Cas9 system were cultured and maintained as previously described (9).

Inhibitory compounds. 3-(1,3-Benzothiazol-2-yl)-4-(4-methoxyphenyl) but-3-enoic acid (TLSC702) was purchased from Namiki Shoji Co., Ltd. (Tokyo, Japan) and dissolved in DMSO. Auranofin (ANF) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and dissolved in DMSO.

ALDEFLUOR assay. ALDH1^{high} cells were isolated from MDA-MB-157 and MDA-MB-468 cells using an ALDEFLUORTM assay kit (Stem Cell Technology, Vancouver, BC, Canada) according to the manufacturer's protocol as previously described (9, 23, 24, 53, 59). As a negative control for the ALDEFLUOR assay, cells were incubated with the ALDH1 inhibitor diethylaminobenzaldehyde (DEAB). Approximately 5-10% of the total were sorted as ALDH1^{high} cells by the cell sorter (FACS Aria III, BD Biosciences, San Jose, CA, USA), taking the negative control into consideration.

Tumor-sphere culture. Tumor-spheres were grown as previously described (9, 23, 24, 37, 53, 59). Briefly, cells (1x10³/well) were seeded into 96-well ultralow attachment plates (Greiner Bio-One, Kremsmünster, Austria) and incubated for 24 h. The cells were cultured for 6 days in the presence of TLSC702 and ANF. Images were taken through an inverted microscope (DMIL LED, Leica, Wetzlar, Germany) and the area of tumor-spheres was determined using ImageJ software version 1.53e (National Institutes of Health, MD, USA). Cell clusters that comprised more than 4 cells were defined as a sphere. The area of the MDA-MB-157 sphere was more than 4 cells (1256 μ m²), whereas that of the MDA-MB-468 sphere was more than 4 cells (314 μ m²). IC₅₀ values were calculated based on the group without drug. The area and the number of tumor-spheres are presented as the mean $\pm$ SD from three independent experiments.

Western blotting. Western blotting was performed as previously described (9, 23, 24, 37, 53). Cells were dissolved in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.5 w/v% sodium deoxycholate, 0.1 w/v% SDS, 1.0 w/v% Nonidet P-40]. Cell lysate proteins were electrophoresed by SDS-PAGE (8% or 12.5% gel) and transferred to Immobilon-P Transfer Membranes (Millipore, Burlington, MA, USA). The mouse anti-GLO 1 monoclonal antibody

(mAb) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); the mouse anti-PKC ϵ mAb was purchased from BD Biosciences (San Jose, CA, USA). Rabbit anti-ALDH1A3 polyclonal antibody (pAb) was obtained from Invitrogen, Thermo Fisher Scientific, Inc. (Waltham, MA, USA); the mouse anti- β -actin mAb was purchased from ProteinTech Group, Inc. (Rosemont, IL, USA); and the goat anti-mouse IgG and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Trypan blue assay. Trypan blue assay was performed as previously described (9,24). Briefly, ALDH1^{high} cells were seeded into 12-well plates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a density of 1×10^4 cells/well and incubated for 24 h. ALDH1^{high} cells were treated with 75 μ M TLSC702 or 0.075% DMSO as a control and incubated for a further 3 days.

Caspase-3/7 fluorometric assay. Apo-ONE[®] Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI, USA) was performed as previously described (24). Briefly, ALDH1^{high} cells were seeded into 96-well black plates (Corning, NY, USA) at a density of 1×10^3 cells/well and incubated for 20 h. ALDH1^{high} cells were treated with 75 μ M TLSC702 or 0.075% DMSO as a control and incubated for 2 days. Fluorescence (excitation, 485 nm; emission, 535 nm) was measured using a fluorescence plate reader (ARVO; PerkinElmer, Inc., Waltham, MA, USA).

Statistical analysis. Statistical analysis was performed using BellCurve for Excel version 2.14 (SSRI, Tokyo, Japan). α -level was fixed at 0.05 and $p < 0.05$ was considered to indicate a statistically significant difference. Tumor sphere assays of statistical significance were determined by Dunnett's test. Trypan blue assay and Caspase-3/7 fluorometric assay results were analysed by Tukey's test.

Results

Tumors with high expression levels of GLO 1, PKC λ , and ALDH1A3 are highly represented in tumor stages I-IV. GLO 1 is frequently found to be up-regulated in breast cancer and is essential for the survival of ALDH1-positive breast CSCs (24). PKC λ has also been revealed to be up-regulated in breast cancer and can regulate the survival of ALDH1-positive breast CSCs (9, 53). In addition, GLO 1 and PKC λ are involved in the regulation of cell viability and tumor-sphere formation in breast cancer (37). However, the relationship between GLO 1 and PKC λ in ALDH1-positive breast CSCs remains unclear. Therefore, we first analyzed the data of a population of patients with GLO 1^{high} PKC λ ^{high} ALDH1A3^{high} breast cancer within the two datasets downloaded. As shown in Figure 1, the GLO 1^{high} PKC λ ^{high} ALDH1A3^{high} expression profile is frequently observed in breast cancer [TCGA; normal tissues; 0% (0/61), breast cancers; 25.8% (137/532), METABRIC; breast cancers 11.0% (209/1904)], which can be detected from the early stages of development onwards [stage 0; 0% (0/4); stage I, 10.7% (51/475); stage II, 11.6% (93/800); stage III, 14.8% (17/115); stage IV; 11.1% (1/9)]. These results suggest that

GLO 1 and PKC λ are cooperatively involved in the progression of ALDH1-positive breast CSCs from the early tumorigenic stages onwards.

Tumors with a GLO 1^{high}, PKC λ ^{high} and ALDH1A3^{high} profile are enriched among the HER2 and TNBC subtypes. Next, we examined the population of patients with the GLO 1^{high} PKC λ ^{high} ALDH1A3^{high} expression profile among the various breast cancer subtypes in terms of receptor status. As shown in Figure 2A, patients with GLO 1^{high} PKC λ ^{high} ALDH1A3^{high} breast cancer were associated with the HER2 and TNBC subtypes [ER and/or PgR, 8.0% (118/1,478); HER2, 23.7% (56/236); TNBC, 17.4% (52/299)]. These results suggest that GLO 1 and PKC λ are cooperatively involved in the progression of HER2-positive or triple-negative ALDH1-positive breast CSCs.

Tumors with the GLO 1^{high}, PKC λ ^{high}, and ALDH1A3^{high} profile are enriched among the HER2-enriched and basal-like subtypes according to the PAM50 classification. As shown in Figure 2B, patients with GLO 1^{high} PKC λ ^{high} ALDH1A3^{high} breast cancer also appeared to mostly exhibit the basal-like subtype [normal-like, 5.7% (8/140); luminal A, 9.7% (66/679); luminal B, 6.7% (31/461); HER2-enriched, 17.7% (39/220); basal-like 22.6% (45/199), claudin-low, 10.1% (20/199)]. These results suggest that GLO 1 and PKC λ are cooperatively involved in the progression of ALDH1-positive breast CSCs of the HER2-enriched and basal-like subtypes. These results in terms of the two aforementioned classification strategies suggest that high expression levels of GLO 1, PKC λ and ALDH1A3 serve important roles in the progression of HER2-positive breast cancer and TNBC in addition to breast cancer of the HER2-enriched and basal-like subtypes. The majority of breast cancers in the HER2-positive category can be divided into the luminal B and HER2-enriched subtypes, whilst most TNBCs overlap with the basal-like subtype (11, 62). Agents targeting the HER2 molecule have been developed and applied clinically against HER2-positive and HER2-enriched breast cancer (63). However, specific therapeutic targets for TNBC and basal-like breast cancer remain elusive. Therefore, we next focused on investigating the roles of GLO 1 and PKC λ expression in ALDH1-positive breast CSCs in TNBC/basal-like breast cancer cell lines.

TLSC702 and ANF suppress viability and tumor-sphere formation by ALDH1^{high} breast cancer cells. We next examined the roles of GLO 1 and PKC λ in ALDH1-positive breast CSCs using the MDA-MB-157 and MDA-MB-468 human TNBC/basal-like breast cancer cell lines. These cells lines express GLO 1, PKC λ , and ALDH1A3 (9, 24, 37, 53). Combined treatment with the GLO 1 inhibitor TLSC702 and the PKC λ inhibitor aurothiomalate (ATM) was previously

Figure 1. Three dimensional scatterplots of GLO 1, PKC λ , and ALDH1A3 expression in normal and breast cancer tissues. Each graph is shown as a three-dimensional scatterplot representing the levels of GLO 1, PKC λ , and ALDH1A3 gene expression. ● represents patients with GLO 1^{high}, PKC λ ^{high} and ALDH1A3^{high} profiles, whilst ○ represents patients with other GLO 1, PKC λ and ALDH1A3 profiles. The number indicates the ratio of patients with GLO 1^{high}, PKC λ ^{high} and ALDH1A3^{high} profiles. (A) Comparison of gene expression between normal and tumor tissues using The Cancer Genome Atlas dataset downloaded from Oncomine. (B) Comparison of gene expression between all patients and patients with different stages of breast cancer using the Molecular Taxonomy of Breast Cancer International Consortium dataset. GLO 1: Glyoxalase 1; PKC λ : protein kinase C λ ; ALDH1: aldehyde dehydrogenase 1.

Figure 2. Three dimensional scatterplots of GLO 1, PKC λ , and ALDH1A3 in the various breast cancer subtypes. Each graph is shown as a three-dimensional scatterplot representing GLO 1, PKC λ , and ALDH1A3 gene expression. ● represents patients with GLO 1^{high}, PKC λ ^{high} and ALDH1A3^{high} profiles, whilst ○ represents patients with other GLO 1, PKC λ and ALDH1A3 profiles. The number indicates the ratio of patients with GLO 1^{high}, PKC λ ^{high} and ALDH1A3^{high}. Comparison of GLO 1, PKC λ and ALDH1A3 among the breast cancer subtypes according to the (A) receptor status or (B) PAM50 results using the Molecular Taxonomy of Breast Cancer International Consortium dataset. GLO 1: Glyoxalase 1; PKC λ : protein kinase C λ ; ALDH1: aldehyde dehydrogenase 1.

found to suppress the viability and tumor-sphere formation of MDA-MB-157 cells (37). Furthermore, TLSC702 has been demonstrated to induce MG accumulation and apoptosis in non-small lung cancer cells (29). ATM interferes with the Phox and Bem1 (PB1)-PB1 domain interactions between with PAR6 and PKC λ , which results in apoptosis (64, 65). ALDH1^{high} cells derived from MDA-MB-157 and MDA-MB-468 cells exhibit CSC-like properties, including self-renewal, differentiation and tumorigenesis (23). In our previous study, TLSC702 was found to reduce cell viability, whilst suppressing tumor-sphere formation by ALDH1^{high} breast cancer cells (IC₅₀ of ALDH1^{high} MDA-MB-157, 145.4 μ M; IC₅₀ of ALDH1^{high} MDA-MB-468, 67.5 μ M) (24). In addition, the PKC λ inhibitor ANF, which is a small molecule gold compound in the same chemical class as ATM, can also suppress oncosphere formation and tumor growth by ovarian tumor-initiating cells (66). Our previous study has shown that ANF can suppress tumor-sphere formation by ALDH1^{high} breast cancer cells (IC₅₀ of ALDH1^{high} MDA-MB-157, 0.24 μ M) (53). Consistent with these previous observations, according to Figure 3, we revealed that the combination of TLSC702 and ANF significantly decreased both the number and size of tumor-spheres formed by ALDH1^{high} MDA-MB-157 and MDA-MB-468 cells. These results suggest that GLO 1 and PKC λ are both involved in the regulation of cell viability and tumor-sphere formation of ALDH1-positive breast CSCs.

TLSC702 suppresses cell viability and tumor-sphere formation of PKC λ KO ALDH1^{high} cells. Small molecule gold compounds such as ATM and ANF inhibit the interaction of PB1-PB1 domain between PAR6 and PKC λ , but also inhibit the interaction of different targets with the PB1 domain to affect various cellular responses (41, 64-68). In addition, ANF also induces anti-cancer and anti-inflammatory effects *via* mediating various cellular signals (69, 70). Therefore, to exclude the possibility of off-target effects mediated by ANF, we next performed experiments using PKC λ KO MDA-MB-157 cells (Figure 4). Knocking out PKC λ in ALDH1^{high} MDA-MB-157 cells led to a decrease in both the number and size of the tumor-spheres (9). Treating PKC λ KO ALDH1^{high} MDA-MB-157 cells with TLSC702 led to a significant reduction in both the size and number of the tumor-spheres (Figure 4B and C). As shown in Figure 4B, PKC λ KO ALDH1^{high} cells were more sensitive to TLSC702 than control KO ALDH1^{high} cells at low concentrations (IC₅₀ of ALDH1^{high} control KO 108.4 μ M, IC₅₀ of ALDH1^{high} PKC λ KO; 67.0 μ M). These results suggest that GLO 1 and PKC λ are both involved in the regulation of cell viability and tumor-sphere formation of ALDH1-positive breast CSCs.

GLO 1 and PKC λ cooperatively regulate ALDH1^{high} cell survival. GLO 1 inhibition by TLSC702 can induce apoptosis

in ALDH1^{high} cells (24). PKC λ deficient ALDH1^{high} cells also exhibit reduced cell viability and increased levels of cell death (9). Therefore, to examine the reason underlying the TLSC702-mediated inhibition of PKC λ KO ALDH1^{high} MDA-MB-157 tumor-sphere formation, we performed trypan blue and caspase-3/7 fluorometric assays. Treating PKC λ KO ALDH1^{high} MDA-MB-157 cells with TLSC702 led to an increase in the number of trypan blue-positive cells and caspase-3 activity (Figure 4D and E). These results suggest that GLO 1 and PKC λ cooperate in promoting cell survival in ALDH1-positive breast CSCs by suppressing apoptosis.

Discussion

TNBC and basal-like breast cancers exhibit stem-like properties and are typically associated with poorer prognoses (3, 7-9). High expression levels of both GLO 1 and PKC λ indicate poorer clinical outcomes in stage III/IV breast tumors, which include high population of basal-like tumors (37). The incidence of patients with stage III breast cancer with high levels of *GLO 1*, *PKC λ* , and *ALDH1A3* expression were found to be slightly higher compared with that in other stages (Figure 1B). This may be due to late-stage breast cancer frequently showing stem-like properties, a characteristic that is also shared by basal-like breast cancer (9). Our recent study revealed that tumors of the *GLO 1*^{high} *PKC λ* ^{high} profile are associated with poorer prognoses in late-stage breast cancer (37). Kaplan-Meier analysis showed that tumors with a *GLO 1*^{high} *PKC λ* ^{high} *ALDH1A3*^{high} profile indicated poorer clinical outcomes compared with those with a *GLO 1*^{low} *PKC λ* ^{low} *ALDH1A3*^{low} profile ($p < 0.01$, Gehan-Breslow generalized Wilcoxon test, unpublished data). However, Cox regression analysis showed that tumors with a *GLO 1*^{high} *PKC λ* ^{high} *ALDH1A3*^{high} profile indicated only the tendency of poor prognosis without significance (hazard ratio=2.07; 95% confidence interval=0.63-6.78, unpublished data). Development of pharmacological treatment methods for late-stage breast cancer is important for the supplementation of conventional surgery and radiotherapy, as distant and widespread metastasis occurs following treatment (71). Therefore, *GLO 1* and *PKC λ* may be potential therapeutic targets for ALDH1A3-positive CSCs during late-stage breast cancer. *GLO 1*^{high} *PKC λ* ^{high} *ALDH1A3*^{high} tumors constituted more than 10% of the population from the early stages of I and II through to the later stages of III/IV disease (Figure 1B). Therefore, GLO 1 and PKC λ may also serve as potential therapeutic targets for ALDH1A3-positive CSCs at all stages of breast cancer, except for stage 0. In this study, we subsequently examined the effects of GLO 1 and PKC λ on ALDH1-positive breast CSCs derived from TNBC and basal-like breast cancer cell lines. Tumors of the HER2-positive and HER2-enriched subtypes also have high ratios of the *GLO 1*^{high} *PKC λ* ^{high}

Figure 3. TLSC702 and ANF suppress tumor-sphere formation by ALDH1^{high} cells. (A) Representative images of tumor-spheres composed of ALDH1^{high} MDA-MB-157 cells. Scale bar, 100 μm. TLSC702 and/or ANF suppressed tumor-sphere formation by ALDH1^{high} MDA-MB-157 cells both in terms of (B) tumor-sphere area and (C) the number of tumor-spheres formed. TLSC702 and/or ANF suppressed tumor-sphere formation by ALDH1^{high} MDA-MB-468 cells both in terms of the (D) tumor-sphere area and (E) the number of tumor-spheres formed. Values for the test groups are expressed relative to cells treated with 0.15% DMSO. Data represent the mean±SD from three independent experiments. Statistical significance was determined using a Dunnett's test. ** $p<0.01$ and * $p<0.05$ vs. 0.15% DMSO. ANF: Auranofin; ALDH1: aldehyde dehydrogenase 1.

Figure 4. TLSC702 treatment reduces viability and tumor-sphere formation by PKCλ KO ALDH1^{high} cells. (A) Measurement of GLO 1, PKCλ and ALDH1A3 protein expression in control KO and PKCλ KO MDA-MB-157 cells. TLSC702 suppressed tumor-sphere formation by ALDH1^{high} MDA-MB-157 control KO and PKCλ KO cell both in terms of (B) tumor-sphere area and (C) the number of tumor-spheres formed. Data represent the mean±SD from three independent experiments. Statistical significance was determined using a Dunnett's test. **p<0.01 and *p<0.05 vs. control KO cells. (D) Trypan blue assays of ALDH1^{high} MDA-MB-157 control KO and PKCλ KO cells, after incubation with 0.075% DMSO (control) or 75 μM TLSC702 for 3 days. (E) Caspase-3/7 activity in ALDH1^{high} cells derived from ALDH1^{high} MDA-MB-157 control KO and PKCλ KO cells, after incubation with 0.075% DMSO (control) or 75 μM TLSC702 for 2 days. GLO 1: Glyoxalase 1; PKCλ: protein kinase Cλ; ALDH1: aldehyde dehydrogenase 1; KO: knockout.

ALDH1A3^{high} profile (Figure 2). In HER2-positive tissues and cell lines, HER2/neu signaling regulates GLO 1 expression (72). In addition, *PKCλ* and stemness markers *ALDH1A3*, *CD133*, and *OCT4* were previously found to be highly expressed in HER2-enriched breast cancer (9). HER2 regulates mammary stem/progenitor cell proliferation in breast CSCs (73, 74). Therefore, the role of GLO 1 and *PKCλ* in ALDH1-positive CSCs from HER2-enriched breast cancer warrants further study.

Combined inhibition of both GLO 1 and *PKCλ* decreased the number and size of the tumor-spheres formed by ALDH1^{high} cells (Figure 3). TLSC702 can induce MG accumulation and apoptosis in cancer cells (29). MG contains both ketone and aldehyde structures and is normally broken down by ALDH1 under physiological conditions (75). In addition, ALDH1 serves a role in the detoxification of toxic aldehyde intermediaries generated by the reactive oxygen species (ROS)-induced peroxidation of intracellular lipids (76). By contrast, *PKCλ* regulates cell survival in ALDH1-positive breast CSCs by maintaining low ROS levels (9). Therefore, GLO 1 and *PKCλ* may be cooperatively involved in the regulation of cell viability and tumor formation by reducing intracellular ROS levels, whilst also removing toxic aldehyde compounds, such as MG, from ALDH1-positive breast CSCs.

GLO 1 maintains cell viability and promotes tumor formation by ALDH1-positive breast CSCs by suppressing caspase 3-dependent cell death (24). Using a similar mechanism, *PKCλ* can also regulate cell survival and tumor formation in ALDH1-positive breast CSCs (9). In addition, results from the present study showed that *PKCλ* KO ALDH1^{high} MDA-MB-157 cells treated with TLSC702 increased the number of trypan blue-positive cells and cleaved caspase-3 activity. Therefore, this suggests that GLO 1 and *PKCλ* regulate cell survival and tumor formation in ALDH1-positive breast CSCs through a caspase-3 dependent mechanism.

GLO 1 activity was found to be enhanced in ALDH1^{high} CSCs compared with that in ALDH1^{low} cells (24). Inhibition of both GLO 1 and *PKCλ* suppressed tumor-sphere formation by ALDH1^{high} cells in this study. *PKCλ* can up-regulate the expression of GLUT1, which can translocate from intracellular vesicles to the plasma membrane (77), where it promotes glucose uptake and promotes cell proliferation (78). Therefore, *PKCλ* may promote glucose uptake and induce GLO 1 activation in ALDH1-positive breast CSCs.

In Bcr-Abl⁺ leukemia cells, hypoxia treatment resulted in increased GLO 1 expression and enzymatic activity, which in turn promoted cell viability and tumor formation (79). *PKCλ* has also been documented to regulate cell proliferation and tumor formation in stem-like cells derived from ovarian cancer, lung cancer, and glioblastoma (66, 80-82). The role of GLO 1 and *PKCλ* in the CSCs of these cancers remains elusive. In addition, our recent study showed that *PKCλ* and

c-Met cooperatively regulate cell viability and tumor formation by ALDH1-positive breast CSCs (53). Therefore, it can be hypothesized that other signaling pathways that can crosstalk with *PKCλ* are potentially involved in modulating the physiology of ALDH1-positive breast CSCs, which requires further study.

Conclusion

We showed that patients with high expression levels of *GLO 1*, *PKCλ*, and *ALDH1A3* were associated with the TNBC and basal-like subtypes. Inhibition of both GLO 1 and *PKCλ* in ALDH1^{high} cells leads to the suppression of tumor-sphere formation and increased cell death. These results suggest that GLO 1 and *PKCλ* play an important role in mediating cell survival in ALDH1-positive breast CSCs. We therefore conclude that GLO 1 and *PKCλ* can potentially be effective therapeutic targets for the treatment of ALDH1-positive breast CSCs.

Conflicts of Interest

The Authors state that they have no conflicts of interest to declare in regard to this study.

Authors' Contributions

H.M., S.T., M.Y., A.N., C.O., A.O. and Y.H. performed the experiments; H.M., S.T., M.Y., A.N. and Y.H. analyzed the data; H.M., S.T. and Ke.S. performed bioinformatics; R.T., Y.H., T.H., Y.H., S.-I.T., Ka.S. and S.O. supplied experimental materials and resources; H.M., M.Y., A.N., Y.X. and K.A. conceived the study; H.M. drafted the manuscript; H.M., S.T., M.Y., A.N., Y.X., Y.M., T.S., Ke.S., Ka.S., S.O. and K.A. contributed to discuss and review the final manuscript; all the Authors approved the final manuscript.

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