High Expression of *c-Met*, *PKCλ* and *ALDH1A3* Predicts a Poor Prognosis in Late-stage Breast Cancer

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Abstract. *Background/Aim:* Co-expression of *c-Met* and *ALDH1A3* indicates a poor prognosis in stage III-IV breast cancers and contributes to cell proliferation and tumor formation by *ALDH1*-positive breast CSCs. *PKCλ* is overexpressed and contributes to a poor prognosis in several cancers. *Materials and Methods:* A breast cancer genomics data set (METABRIC, n=2509) was downloaded and analyzed, as was the effect *c-Met* and *PKCλ* inhibitors on *ALDH1^{high}* cell viability and tumor-sphere formation. *Results:* *c-Met* expression correlates with expression of *PKCλ* in breast cancer. Stage III-IV breast cancer patients with *c-Met^{high} PKCλ^{high} ALDH1A3^{high}* have a poorer prognosis than patients with *c-Met^{low} PKCλ^{low} ALDH1A3^{low}*. Foretinib and auranofin suppressed cell viability and tumor-sphere formation by *ALDH1^{high}* cells. These results suggest that *c-Met* and *PKCλ* are cooperatively involved in cancer progression and contribute to poor prognoses in breast cancer. *Conclusion:* *c-Met* and *PKCλ* are potentially useful prognostic markers and therapeutic targets in late-stage breast cancer.

Breast cancer is the second most frequently diagnosed cancer worldwide, and the most commonly occurring cancer among women, with 2.09 million new cases (24.2% of all cancers in

women) and 0.6 million cancer-related deaths annually (1). Breast cancers are classified based on their gene expression pattern (PAM 50) into at least six subtypes, including normal-like, luminal A, luminal B, HER2-enriched, claudin-low and basal-like (2-5). Among these, basal-like breast cancers have stem-like properties and a poor prognosis (4). Nonetheless, the prognosis for breast cancer patients is good overall, though it is significantly poorer for patients with late-stage tumors (stage III or IV) (6). This is in large part because late-stage breast cancers are often resistant to standard medical treatments, such as conventional surgery, chemotherapy, and radiotherapy, which is reflected in their recurrence and metastasis (6). Consequently, the new pharmacological approach to managing late-stage breast cancers is greatly needed.

Tumors are composed of populations of cancer cells and distinct cancer stem cells (CSCs), which are largely undifferentiated tumorigenic cells that exhibit such stem-like properties as self-renewal and multipotency (7, 8). Most CSCs are resistant to conventional chemo- and radiotherapies, and the development of targeted therapies against CSCs is very much needed to improve clinical outcomes. CSCs within breast tumors can be identified based on their expression of CD44, CD24 and aldehyde dehydrogenase 1 (ALDH1) (9, 10). ALDH1 is an enzyme that converts aldehydes to carboxylic acids and is abundant in normal stem/progenitor cells, and various CSCs, including those in breast cancers (9, 11). Among the *ALDH1* gene family, *ALDH1A1* and *ALDH1A3* are known to be CSC markers in several cancers (12-17). In particular, *ALDH1A3* reportedly contributes significantly to the ALDH1 activity detected in breast cancer cells, and its expression correlates significantly with cancer type, tumor grade and metastasis in breast cancer patients (16-19).

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c-Met is a receptor-type tyrosine kinase that is involved in a wide range of cellular functions, including proliferation, motility, migration, invasion and tumor angiogenesis (20, 21). c-Met is reported to be highly expressed and aberrantly activated in a variety of cancers, including breast cancer (20-22). Moreover, high *c-Met* expression reportedly correlates with expression of the CSC markers *CD133*, *CD44*, and *ALDH1* (18, 23-25), and appears to be involved in various processes in breast and head neck CSCs (18, 25). *c-Met* is enriched in late-stage basal-like breast cancers, which exhibit stem cell properties and strongly express *ALDH1A3* (18). High *c-Met* and *ALDH1A3* expression in late-stage breast tumors is predictive of a poor clinical outcome (18), but the relationship between c-Met and other signaling molecules in ALDH1-positive breast CSCs remains unclear.

PKC λ is one of the atypical protein kinase C (aPKC) subfamily proteins and is known to be involved in cellular processes contributing to cell polarity, proliferation, survival, chemotaxis, and migration (26-28). PKC λ is overexpressed in many human cancers, including breast cancer (29-40), where it is known to be involved in cancerous progression and to contribute to a poor clinical outcome (33-40). In ovarian cancer, lung cancer and glioblastoma, PKC λ is reportedly involved in cell proliferation and tumor formation by stem-like cells (41-44). However, the role of PKC λ in ALDH1-positive breast CSCs remains to be elucidated. In the present study, therefore, we investigated the relations among *c-Met*, PKC λ and *ALDH1A3* in breast cancer. Our findings suggest that c-Met and PKC λ are potentially useful prognostic markers and therapeutic targets in late-stage breast cancers.

Materials and Methods

Analysis of cancer genomics data. Gene expression data were downloaded from the cBioportal and analyzed as previously described (18, 19, 45). Briefly, the Molecular Taxonomy of Breast Cancer International Consortium dataset (METABRIC, n=2509) and The Cancer Genome Atlas (TCGA) provisional datasets for several cancers were downloaded from cBioPortal (46, 47). For breast cancer, we classified tumor stage as early stage (Tumor stage 0-II) and late stage (Tumor stage III-IV). We defined the optimal cutoffs for the high and low expression groups using receiver operator characteristic (ROC) curves based on the expression of genes *versus* patient overall survival status. Values of *p* less than 0.05 were considered significant. Survival curves were plotted using the Kaplan-Meier method. For breast cancer, *p*-values were calculated using the Gehan-Breslow generalized Wilcoxon test; for other cancers, *p*-values were calculated using the log-rank test. A multivariate Cox regression model was used to evaluate the influence of gene expression and to estimate adjusted hazard ratios by age as a confounding factor. Values of copy-number alterations are shown as 2=high level amplification; 1=gain; 0=neutral/no change; -1=hemizygous deletion; -2=homozygous deletion. Statistical analysis was performed using BellCurve for Excel ver 2.11. (SSRI, Tokyo, Japan).

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 (18, 19).

Inhibitory compounds. Foretinib (c-Met inhibitor) was purchased from Selleck (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO). Auranofin (PKC λ inhibitor) was purchased from Santa Cruz Inc. (Dallas, TX, USA) and dissolved in DMSO.

ALDEFLUOR assay. ALDH1^{high} cells were isolated from MDA-MB 157 cells using an ALDEFLUOR assay kit (Stem Cell Technology, Vancouver, BC, Canada) according to the manufacturer's instructions. 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.

WST-8 assay. WST-8 assays were performed as previously described (18, 19). Briefly, cells (1×10³/well) were seeded into 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 24 h. Inhibitors were then added to the culture medium, and the cells were incubated for an additional 7 days, after which cell viability was assessed using Cell Count Reagent SF (Nacalai tesque, Kyoto, Japan). The formazan dye formed was measured using a Sunrise Remote (TECAN, Männedorf, Switzerland) at 450 nm. Numerical values of the test groups are expressed as relative to cells in the absence of drug. Statistical significance was determined using Tukey's test. Data are presented as mean±SE of three independent experiments.

Tumor-sphere culture. Tumor-spheres were grown as previously described (18, 19). Briefly, cells (1×10³/well) were seeded into 96-well ultralow attachment plates (Greiner Bio-One, Kremsmünster, Austria) and incubated for 24 h. Cells were cultured for 6 days while being treated with inhibitors. Images were taken through an inverted microscope (DMIL LED, Leica, Wetzlar, Germany), and the areas of tumor-spheres were determined using ImageJ software. Statistical significance was determined using Tukey's test. The areas of tumor-spheres are presented as mean±SE of three independent experiments. The number of tumor-spheres are presented as mean±SD of three independent experiments.

Immunoblot. Immunoblotting was performed as previously described (18, 19). Mouse anti-PKC λ (λ) mAb was purchased from BD Biosciences (San Jose, CA, USA). Rabbit anti-c-Met pAb was from Santa Cruz Inc. (Dallas, TX, USA). Rabbit anti-phospho-Met (Tyr1234/1235) mAb was from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-ALDH1A3 pAb was from Invitrogen (Waltham, MA, USA). Mouse anti-β-actin mAb was from Proteintech Group, Inc. (Rosemont, IL, USA). Goat anti-mouse IgG and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugate were from Cell Signaling Technology (Danvers, MA, USA).

Results

***c-Met* correlates with PKC λ in breast cancer.** We previously reported that *c-Met* is elevated in basal-like breast cancers (18), while PKC λ was shown to be overexpressed in triple-negative (ER-negative, PgR-negative, HER2-negative) breast

Figure 1. Correlation between c-Met and PKC λ expression in breast cancer. (A) Correlation between c-Met and PKC λ in all breast cancer patients at early tumor stages (0-II) and late tumor stages (III-IV). (B) Correlation between c-Met and PKC λ expression in the indicated breast cancer subtypes. Values are shown as scattered plots. Pearson's correlation coefficient (r) and p -value are indicated.

cancer (TNBC) (31). Approximately, 70-80% of basal-like breast cancers fall into the TNBC category (48). We therefore examined the correlation between c-Met and PKC λ expression in breast cancer. We found that c-Met expression correlated with expression of PKC λ in all patients (Figure 1A) and in several breast cancer subtypes, including normal-like, luminal B, claudin-low, and basal-like (Figure 1B). Our earlier report showed that in basal-like breast cancers, c-Met expression is higher at later tumor stages (stage III-IV) (18). In the present study, we found that the correlation between c-Met and PKC λ expression was stronger at late tumor stages than at early tumor stages (Figure 1A). On the other hand, PKC ζ , another aPKC subfamily member, did not correlate with c-Met in all patients or for any breast cancer subtype (Figure 2A and B).

Late-stage breast cancer patients with c-Met^{high} PKC λ ^{high} have a poorer prognosis. We next assessed the prognosis of patients with c-Met^{high} PKC λ ^{high} (Figures 3 and 4). Kaplan-Meier analysis showed that patients with c-Met^{high} PKC λ ^{high} had poorer clinical outcomes than those with c-Met^{low} PKC λ ^{low} for all tumors (Figure 3A, $p=0.017$), late-stage tumors (Figure 3C, $p<0.01$), and normal-like tumors (Figure 4, $p=0.042$). Multivariable Cox regression analysis also showed that c-Met^{high} PKC λ ^{high} was predictive of a poorer prognosis than c-Met^{low} PKC λ ^{low} in all tumors (Table I, HR=1.25, 95%CI=1.04-1.51, $p=0.017$), late-stage tumors (Table I, HR=2.28, 95%CI=1.15-4.54, $p=0.019$), normal-like tumors (Table II, HR=2.45, 95%CI=1.10-5.48, $p=0.029$), and luminal A tumors (Table II, HR=1.72, 95%CI=1.14-2.60,

Figure 2. $PKC\zeta$ expression does not correlate with $c\text{-Met}$ expression in breast cancer. (A) Correlation between $c\text{-Met}$ and $PKC\zeta$ in all breast cancer patients at early tumor stages (0-II) and late tumor stages (III-IV). (B) Correlation between $c\text{-Met}$ and $PKC\zeta$ expression in the indicated breast cancer subtypes. Values are shown as scattered plots. Pearson's correlation coefficient (r) and p -value are indicated.

$p < 0.01$). These results suggest that $c\text{-Met}$ and $PKC\lambda$ act cooperatively towards cancer progression.

Late-stage breast cancer patients with $c\text{-Met}^{\text{high}}$ $PKC\lambda^{\text{high}}$ $ALDH1A3^{\text{high}}$ have a poorer prognosis. We have previously shown that late-stage breast cancer patients with $c\text{-Met}^{\text{high}}$ $ALDH1A3^{\text{high}}$ have a poorer prognosis than those with $c\text{-Met}^{\text{low}}$ $ALDH1A3^{\text{low}}$ (18). Here, we assessed the prognosis of patients with $c\text{-Met}^{\text{high}}$ $PKC\lambda^{\text{high}}$ $ALDH1A3^{\text{high}}$ (Figures 5 and 6). We found that late-stage patients with $c\text{-Met}^{\text{high}}$ $PKC\lambda^{\text{high}}$ $ALDH1A3^{\text{high}}$ had a poorer prognosis than those

with $c\text{-Met}^{\text{low}}$ $PKC\lambda^{\text{low}}$ $ALDH1A3^{\text{low}}$ (Figure 5C, $p < 0.01$). Moreover, multivariate Cox regression analysis showed that $c\text{-Met}^{\text{high}}$ $PKC\lambda^{\text{high}}$ $ALDH1A3^{\text{high}}$ was predictive of a poorer prognosis than $c\text{-Met}^{\text{low}}$ $PKC\lambda^{\text{low}}$ $ALDH1A3^{\text{low}}$ in all tumors (Table I, HR=1.55, 95%CI=1.21-1.99, $p < 0.01$), early-stage tumors (Table I, HR=1.44, 95%CI=1.07-1.94, $p = 0.018$), late-stage tumors (Table I, HR=2.47, 95%CI=1.08-5.65, $p = 0.031$), and luminal A tumors (Table II, HR=1.79, 95%CI=1.07-2.99, $p = 0.026$). These results suggest that $c\text{-Met}$ and $PKC\lambda$ act cooperatively towards the progression of ALDH1-positive breast CSCs.

A

ALL

B

Stage 0 - II

C

Stage III - IV

Figure 3. High c-Met and PKC λ expression leads to poorer prognoses in late-stage cancers. Kaplan-Meier analysis of the effect of high c-Met and PKC λ expression on overall survival among all patients (A), Stage 0-II patients (B), and Stage III-IV patients (C). p -Value was calculated by the Gehan-Breslow generalized Wilcoxon test.

Auranofin suppresses ALDH1^{high} cell viability and tumor-sphere growth. To examine the effects of c-Met and PKC λ co-expression in ALDH1-positive breast CSCs, we used the MDA-MB 157 human basal-like breast cancer cell line as a model. These cells express c-Met (18), phospho-Met (18),

PKC λ and ALDH1A3 (19) (Figure 7A). ALDH1^{high} cells derived from MDA-MB 157 exhibit such CSC-like properties as self-renewal, differentiation, and tumorigenesis (18). We have previously shown that the c-Met inhibitor foretinib suppresses cell viability and tumor-sphere

Normal-like

Luminal A

Luminal B

HER2-enriched

Figure 4. Continued

Claudin-low

Basal-like

Figure 4. Kaplan-Meier analysis of the effect on overall survival of c-Met and PKC λ gene expression in the indicated breast cancer subtypes. p-Value was calculated by the Gehan-Breslow generalized Wilcoxon test.

formation by ALDH1^{high} cells (18). In addition, the PKC λ inhibitor auranofin suppressed oncosphere formation and tumor growth by ovarian tumor-initiating cells (41). Consistent with these earlier observations, WST-8 assays showed that foretinib suppresses the cell viability of ALDH1^{high} MDA-MB 157 cells (IC₅₀=0.79 μ M, Figure 7B), as did auranofin (IC₅₀=0.24 μ M, Figure 7C). However, the inhibitory combined effects of foretinib and auranofin on ALDH1^{high} cell viability were neither additive nor synergistic as compared to the effect of each drug separately (Figure 7D).

To further assess the roles of c-Met and PKC λ in the tumorigenesis mediated by ALDH1-positive breast CSCs, we examined the effects of foretinib and auranofin on *in vitro* tumor-sphere formation. We found that foretinib and auranofin each suppressed tumor-sphere formation by ALDH1^{high} cells (Figure 7E and F). As in the WST-8 assays, the combined inhibitory effects of foretinib and auranofin on tumor-sphere formation by ALDH1^{high} cells were neither additive nor synergistic (Figure 7E and F). Moreover, the two drugs had no effect on tumor-sphere numbers when administered separately or together (Figure 7G). These

results suggest that c-Met and PKC λ are cooperatively involved in ALDH1-positive breast CSC proliferation and tumor growth, acting *via* the same signaling pathway.

Discussion

Basal-like breast cancers are associated with poorer clinical outcomes than other breast cancer subtypes (4). c-Met mRNA is enriched in basal-like breast cancers as compared to other subtypes (18), while PKC λ is overexpressed in TNBC, which overlaps with basal-like breast cancers (31). We confirmed that in stage III-IV cancers, the fraction of basal-like breast cancer was significantly higher in c-Met^{high} PKC λ ^{high} patients than in c-Met^{low} PKC λ ^{low} patients (Figure 8A). As shown in Figure 1B, the majority of basal-like breast cancer patients highly expressed c-Met and PKC λ (51.8%, n=103 of 199). Furthermore, the fraction of stage III-IV basal-like breast cancer patients with c-Met^{high} PKC λ ^{high} ALDH1A3^{high} was much larger than the fraction with c-Met^{low} PKC λ ^{low} ALDH1A3^{low} patients (Figure 8B). In addition, late-stage patients with c-Met^{high} PKC λ ^{high} ALDH1A3^{high} had poorer prognoses than those with c-Met^{low}

Table I. Multivariable Cox regression analysis of the association between *c-Met*, *PKCλ* and *ALDH1A3* expression and breast cancer in all patients and patients with stage 0-II or III-IV cancers.

		Hazard ratio*	95% Confidence interval	p-Value
ALL	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.02	(0.90-1.15)	0.79
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.22	(1.06-1.39)	<0.01
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.94	(0.83-1.06)	0.32
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	1.25	(1.04-1.51)	0.017
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	1.55	(1.21-1.99)	<0.01
Stage 0-II	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.03	(0.89-1.20)	0.67
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.15	(0.98-1.36)	0.094
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.92	(0.79-1.07)	0.30
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	1.24	(0.99-1.55)	0.064
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	1.44	(1.07-1.94)	0.018
Stage III-IV	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.10	(0.71-1.70)	0.68
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	2.23	(1.41-3.54)	<0.01
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	1.07	(0.70-1.64)	0.75
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	2.28	(1.15-4.54)	0.019
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	2.47	(1.08-5.65)	0.031

*Hazard ratio adjusted by age estimated using Cox proportional hazard model.

Table II. Multivariable Cox regression analysis of the association between *c-Met*, *PKCλ* and *ALDH1A3* and the indicated breast cancer subtypes.

		Hazard ratio*	95% Confidence interval	p-Value
Normal-like	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.10	(0.69-1.75)	0.70
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.89	(0.94-3.80)	0.073
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.85	(0.46-1.55)	0.59
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	2.45	(1.10-5.48)	0.029
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	2.75	(0.63-11.96)	0.18
Luminal A	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.12	(0.90-1.40)	0.31
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.24	(0.95-1.62)	0.12
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.91	(0.74-1.12)	0.37
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	1.72	(1.14-2.60)	<0.01
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	1.79	(1.07-2.99)	0.026
Luminal B	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	0.94	(0.73-1.20)	0.61
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.06	(0.83-1.36)	0.65
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.93	(0.72-1.20)	0.57
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	0.93	(0.64-1.35)	0.70
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	1.21	(0.67-2.18)	0.53
HER2-enriched	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	1.20	(0.87-1.67)	0.27
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	0.94	(0.67-1.33)	0.73
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.95	(0.66-1.36)	0.77
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	1.14	(0.70-1.84)	0.60
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	1.36	(0.67-2.75)	0.40
Claudin-low	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	0.77	(0.50-1.19)	0.24
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.10	(0.68-1.78)	0.70
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	0.70	(0.43-1.13)	0.14
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	0.83	(0.45-1.53)	0.55
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	0.65	(0.24-1.75)	0.40
Basal-like	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	0.76	(0.51-1.12)	0.17
	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	1.08	(0.74-1.58)	0.68
	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	1.28	(0.79-2.06)	0.31
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	0.87	(0.53-1.43)	0.59
	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}	0.83	(0.33-2.09)	0.70

*Hazard ratio adjusted by age estimated using Cox proportional hazard model.

A

ALL

B

Stage 0 - II

C

Stage III - IV

Figure 5. High c-Met, PKC λ and ALDH1A3 expression leads to poor prognoses in late-stage cancers. Kaplan-Meier analysis of the effect of high c-Met, PKC λ and ALDH1A3 expression on overall survival among all patients (A), Stage 0-II patients (B), and Stage III-IV patients (C). p-Value was calculated by the Gehan-Breslow generalized Wilcoxon test.

Normal-like

Luminal A

Luminal B

HER2-enriched

Figure 6. Continued

$PKC\lambda^{\text{low}} ALDH1A3^{\text{low}}$ (Figure 5C). Taken together, these results strongly suggest that c-Met and PKC λ are cooperatively involved in cancer progression mediated by ALDH1-positive CSCs in late-stage basal-like breast cancer. The Kaplan-Meier analysis summarized in Table III indicates

that cancer patients with $c\text{-Met}^{\text{high}} PKC\lambda^{\text{high}}$ have poorer clinical outcomes. The patients' cancers included head and neck squamous cell carcinoma ($p < 0.01$), pancreatic adenocarcinoma ($p < 0.01$) and lung adenocarcinoma ($p < 0.01$). Similarly, patients with $c\text{-Met}^{\text{high}} PKC\lambda^{\text{high}}$

Figure 6. Kaplan-Meier analysis of the effect on overall survival of c-Met, PKC λ and ALDH1A3 gene expression in the indicated breast cancer subtypes. p-Value was calculated by the Gehan-Breslow generalized Wilcoxon test.

ALDH1A3^{high} also had poorer clinical outcomes. In these cases, the patients' cancers included head and neck squamous cell carcinoma ($p=0.026$), cervical squamous cell carcinoma and endocervical adenocarcinoma ($p=0.032$), pancreatic adenocarcinoma ($p<0.01$), bladder urothelial carcinoma ($p<0.01$), lung adenocarcinoma ($p=0.033$) and ovarian serous cystadenocarcinoma ($p<0.01$) (Table III). These results suggest that the contributions of c-Met and PKC λ to the progression of ALDH1-positive CSCs is not limited to breast cancer, and that c-Met and PKC λ levels may be useful prognostic markers and therapeutic targets in a variety of cancers.

In the present study, we showed that c-Met^{high} correlates with PKC λ ^{high} in breast cancer (Figure 1) and that breast cancer patients with c-Met^{high} PKC λ ^{high} have poorer clinical outcomes for all tumors and for late-stage tumors (Figure 3). c-Met gene amplification and mutations occur in cancers (49, 50). In breast cancers, however, amplification of c-Met copy number was low (Figure 9A and B). Likewise, we found that no c-Met missense, in-frame or truncation mutations occurred in breast cancers (Table IV). It thus appears that in breast cancer, higher c-Met mRNA expression and activation reflect higher transcription rather than gene amplification or mutation. By contrast, amplification and gain of c-Met copy number frequently occurs in glioblastoma multiforme (high

level amplification; 2.8%, gain; 75.2%) and testicular germ cell cancer (gain; 64.7%) (Figure 9C). In addition, c-Met mutation and overexpression is seen in stage III-IV lung carcinoma (49). The c-Met mutation rate is less than 5% in other cancers (Table IV). It appears, therefore, that higher c-Met expression reflects increased transcription rather than gene amplification or mutation in various cancers and that it contributes to the progression of these cancers.

Hepatocyte growth factor (HGF), a c-Met ligand, enhances CXCR4 expression *via* PKC ζ and promotes breast cancer invasion and metastasis (51). We found that expression of PKC ζ does not correlate with c-Met expression in all patients and breast cancer subtypes (Figure 2). By contrast, c-Met correlated with PKC λ in all patients and breast cancer subtypes (Figure 1). These results suggest that PKC λ , but not PKC ζ , is involved in c-Met-dependent cell proliferation and tumor formation, as well as other actions of ALDH1-positive breast CSCs. Remarkably, PKC λ controls the Notch signal pathway, a key driver of stemness in KRAS-mediated lung adenocarcinoma (LADC) (43) and in glioblastoma (44). In addition, PKC λ controls signaling by SOX2-hedgehog acyl transferase (HHAT), a master transcriptional regulator of stemness, in lung squamous cell carcinoma (42). Importantly, the stemness markers *Notch1*, *Notch3*, *Sox2* and *ALDH1A3* are all enriched in grade 3

Figure 7. Foretinib and auranofin suppress cell viability and tumor-sphere formation by ALDH1^{high} cells. (A) c-Met, phospho-Met, PKC λ and ALDH1A3 protein expression in MDA-MB 157 and MDA-MB 468 human basal-like breast cancer cell lines. β -actin was used as an internal control. (B) Cell viability of ALDH1^{high} cells isolated from MDA-MB 157 cells treated for 7 days with foretinib (0.03, 0.1, 0.3, 1, 3 and 10 μ M) was assessed in WST-8 assays. Values for the test groups are expressed relative to cells treated with 0.1% DMSO. (C) Cell viability of ALDH1^{high} cells isolated from MDA-MB 157 cells treated for 7 days with auranofin (0.03, 0.1, 0.3, 1, 3 and 10 μ M) was assessed using WST-8 assays. Values for the test groups are expressed relative to cells treated with 0.06% DMSO. (D) Cell viability of ALDH1^{high} cells isolated from MDA-MB 157 cells treated for 7 days with or without foretinib (0.1, 0.3, 1 μ M) and/or auranofin (0.1, 0.3, 1 μ M) was assessed using WST-8 assays. Values for the test groups are expressed relative to cells treated with 0.01% DMSO. (E) Representative images of tumor-spheres formed by ALDH1^{high} cells isolated from MDA-MB 157 cells treated for 6 days with foretinib and/or auranofin. Bars: 100 μ m. (F), (G) Foretinib and/or auranofin suppressed tumor-sphere formation measured as tumor-sphere area (F) or number (G). Values for the test groups are expressed relative to cells treated with 0.01% DMSO. (B), (C), (D), (F): Data represent the mean $\pm$ SE (three independent experiments). (G): Data represent the mean $\pm$ SD (three independent experiments). (D), (F), (G): Statistical significance was determined by Tukey's test. p-Value (**p<0.01, *p<0.05); vs. 0.01% DMSO. N.S.: Not significant.

Figure 8. High fraction of basal-like breast cancers among breast cancer patients with *c-Met*^{high} *PKCλ*^{high} or *c-Met*^{high} *PKCλ*^{high} *ALDH1A3*^{high}. (A) Fractions (%) of patients with the indicated breast cancer subtypes expressing *c-Met*^{high} *PKCλ*^{high} or *c-Met*^{low} *PKCλ*^{low} in all patients and each tumor stage breast cancer. (B) Fractions (%) of patients with the indicated breast cancer subtypes expressing *c-Met*^{high} *PKCλ*^{high} *ALDH1A3*^{high} or *c-Met*^{low} *PKCλ*^{low} *ALDH1A3*^{low} in all patients and each tumor stage breast cancer.

Table III. Patients with *c-Met*^{high} *PKCλ*^{high} or *c-Met*^{high} *PKCλ*^{high} *ALDH1A3*^{high} have poorer prognoses in several cancers.

	p-Value				
	<i>c-Met</i> ^{high} vs. <i>c-Met</i> ^{low}	<i>PKCλ</i> ^{high} vs. <i>PKCλ</i> ^{low}	<i>ALDH1A3</i> ^{high} vs. <i>ALDH1A3</i> ^{low}	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low}	<i>c-Met</i> ^{high} <i>PKCλ</i> ^{high} <i>ALDH1A3</i> ^{high} vs. <i>c-Met</i> ^{low} <i>PKCλ</i> ^{low} <i>ALDH1A3</i> ^{low}
Head and neck squamous cell carcinoma n=530	<0.01	0.017	0.28	<0.01	0.026
Uterine corpus endometrial carcinoma n=548	0.96	0.90	0.10	0.88	0.13
Cervical squamous cell carcinoma and endocervical adenocarcinoma n=310	0.10	0.92	0.17	0.64	0.032
Pancreatic adenocarcinoma n=186	<0.01	<0.01	<0.01	<0.01	<0.01
Prostate adenocarcinoma n=499	0.37	0.018	0.93	0.38	0.81
Liver hepatocellular carcinoma n=442	0.16	<0.01	0.063	0.35	0.73
Uveal melanoma n=80	0.10	0.090	0.15	0.81	0.29
Stomach adenocarcinoma n=478	0.27	0.56	0.027	0.85	0.25
Glioblastoma multiforme n=604	0.59	0.58	0.47	0.70	0.19
Colorectal adenocarcinoma n=640	0.42	0.11	0.26	0.48	0.083
Adrenocortical carcinoma n=92	0.016*	0.024	0.90	0.96	0.97
Cholangiocarcinoma n=51	0.81	0.58	0.57	0.63	0.97
Bladder urothelial carcinoma n=413	0.15	0.032	0.012	0.052	<0.01
Pheochromocytoma and paraganglioma n=184			No data		
Lung squamous cell carcinoma n=511	0.60	0.29	<0.01	0.38	0.094
Lung adenocarcinoma n=586	0.17	<0.01	0.59	<0.01	0.033
Ovarian serous cystadenocarcinoma n=606	0.25	0.18	<0.01	0.073	<0.01
Lymphoid neoplasm diffuse large B-cell lymphoma n=48	0.26	0.20	0.58	0.90	0.28
Acute myeloid leukemia n=200	0.51	<0.01*	0.63	0.22	0.21
Thyroid carcinoma n=516	0.072	0.27	0.37	0.75	0.13
Testicular germ cell cancer n=156	0.45	0.88	0.98	0.32	1.00

*Good prognosis.

tumors, which are a major component of basal-like breast cancers (19).

The inhibitory effects of foretinib and auranofin on *ALDH1*^{high} cell viability and tumor-sphere formation were neither additive nor synergistic (Figure 7). This suggests that *PKCλ* is a major downstream mediator of *c-Met* signaling in *ALDH1*-positive breast CSCs. *c-Met* activation also leads to PI3-kinase activation, and the stimulatory effect of *PKCλ* on cell proliferation is PI3-kinase-dependent (52-54). In addition, PI3-kinase activates the small GTP protein *rac* (55). HGF/*c-Met* also mediated *rac* activation and breast cancer migration, while epithelial cell transforming sequence 2 (*Ect2*) interacts with the *Par6-PKCλ* complex to activate *rac* during cancer cell proliferation (56). This suggests the presence of a *c-Met*-PI3-kinase-*Par6/PKCλ/Ect2-rac* signaling axis in *ALDH1*-positive breast CSCs.

Conclusion

In summary, we showed that high *c-Met* expression correlates with high *PKCλ* expression in breast cancer. In addition, patients with late-stage tumors showing high expression of *c-*

Table IV. Fraction (%) of the indicated cancers exhibiting *c-Met* mutation.

	Mutation %
Breast cancer n=2509	0.00
Head and neck squamous cell carcinoma n=530	0.75
Uterine corpus endometrial carcinoma n=548	2.37
Cervical squamous cell carcinoma and endocervical adenocarcinoma n=310	0.65
Pancreatic adenocarcinoma n=186	0.54
Prostate adenocarcinoma n=499	0.80
Liver hepatocellular carcinoma n=442	0.45
Uveal melanoma n=80	0.00
Stomach adenocarcinoma n=478	1.46
Glioblastoma multiforme n=604	0.33
Colorectal adenocarcinoma n=640	0.63
Adrenocortical carcinoma n=92	0.00
Cholangiocarcinoma n=51	0.00
Bladder urothelial carcinoma n=413	0.97
Pheochromocytoma and paraganglioma n=184	0.00
Lung squamous cell carcinoma n=511	0.78
Lung adenocarcinoma n=586	2.73
Ovarian serous cystadenocarcinoma n=606	0.66
Lymphoid neoplasm diffuse large B-cell lymphoma n=48	4.17
Acute myeloid leukemia n=200	0.00
Thyroid carcinoma n=516	0.19
Testicular germ cell cancer n=156	0.00

Figure 9. Copy number status of c-Met expression in breast cancer and other cancers. (A) Copy number status of c-Met expression in all breast cancer patients and in patients with stage 0-II or stage III-IV tumors. (B) Copy number status of c-Met expression in patients with the indicated breast cancer subtypes. (C) Copy number status of c-Met expression in the indicated cancers. Values: 2=high level amplification; 1=gain, 0=neutral/no change, -1=hemizygous deletion, -2=homozygous deletion.

Met, *PKC λ* and *ALDH1A3* had poorer prognoses. Treating *ALDH1^{high}* cells with a c-Met or *PKC λ* inhibitor suppressed cell viability and tumor-sphere formation. These results suggest that c-Met and *PKC λ* in *ALDH1*-positive breast

CSCs are involved in tumor progression and contribute to a poor prognosis in breast cancer. We therefore suggest that c-Met and *PKC λ* are useful prognostic markers and therapeutic targets in late-stage breast cancers.

Conflicts of Interest

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

Authors' Contributions

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

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