Protein kinase C α is involved in the regulation of AXL receptor tyrosine kinase expression in triple-negative breast cancer cells

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Abstract. AXL receptor tyrosine kinase is overexpressed in triple-negative breast cancer (TNBC), and has a function in cancer progression and metastases. However, the mechanism underlying AXL gene regulation in TNBC remains unknown. In this study, the involvement of protein kinase C α (PKC α) in the expression of AXL was investigated in human TNBC cells. The microarray data from other studies showed that PKCa is significantly correlated with AXL expression in TNBC cell lines. Tissue array analysis also confirmed their correlation in TNBC. The PKCa inhibitor Go6976 was used to treat MDA-MB-231 and Hs578T TNBC cells, which resulted in decreased expression of AXL and epithelia-mesenchymal transition-related gene vimentin, and decreased cell proliferation. An MZF-1 acidic domain fragment (MZF-1 peptide), which was designed to downregulate PKCa expression, was transfected into the cells and resulted in inhibition of AXL expression. This effect was reversed by co-treatment with the constitutive form of PKCa. Moreover, the downregulation of PKCa was also confirmed by treatment with TAT-fused

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MZF-1 peptide. Thus, the current study proposes that AXL may be correlated with PKC α -dependent TNBC cells, and could be modulated by MZF-1 peptides.

Introduction

AXL, a member of the Tyro-AXL-Mer receptor tyrosine kinases family, is not only involved in mesenchymal and neural development, but is also associated with various high-grade cancers and can independently predict poor overall survival of patients with breast cancer (1,2). AXL is present predominately in triple-negative breast cancer (TNBC) (3). It diversifies epidermal growth factor receptor (EGFR) signaling and limits the response to EGFR-targeted inhibitors (4). However, there is limited research regarding AXL gene regulation in TNBC.

Protein kinase C α (PKC α), which is a member of the PKC family, is correlated with carcinogenesis and breast cancer development. A previous study indicated that PKC α expression is higher in TNBC than that in non-TNBC (5). Its overexpression in breast cancer cells has been shown to be associated with invasive growth in two genetic models of epithelia-mesenchymal transition (EMT) (6), increased molecular potential for anti-estrogen resistance and cell growth (7), and endocrine resistance in the clinic (8-10). Furthermore, decreased breast cancer cell malignancy and increased anticancer drug sensitivity can be achieved through PKC α inhibition (10-12).

Other studies have indicated that AXL gene expression induced by the PKC activator phorbol 12-myristate 13-acetate in leukemia cells is mediated by AP-1 motifs (13), and its downregulation in chronic myeloid leukemia cells can be achieved by transfection with PKC α/β small interfering RNA (14). Thus, as PKC α and AXL have similar functions in breast cancer, there is a possible correlation between PKC α and the AXL signaling pathway in the induction of cell malignancy in certain types of cancer. In this study, the

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involvement of PKC α was determined in AXL expression in human TNBC cells. Results from the microarray and tissue array analysis, and comparative analysis with PKC α inhibitor Go6976 demonstrated that PKC α was significantly correlated with AXL expression in human TNBC cells. Furthermore, an MZF-1 acidic domain fragment (MZF-1 peptide) that was designed to downregulate PKC α expression also inhibited AXL expression. This study proposes a novel therapeutic strategy for TNBC involving the use of PKC α -AXL signaling.

Materials and methods

Materials. The following antibodies were used in the present study: Polyclonal mouse anti-PKC α (cat. no. 610108; BD Biosciences, San Jose, CA, USA), polyclonal rabbit anti-AXL (cat. no. GTX108560; GeneTex, Inc., Irvine, CA, USA), anti-vimentin (cat. no. 5741; Cell Signaling Technologies Inc., Beverly, MA, USA) and monoclonal mouse anti- β -actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-labeled anti-mouse (cat. no. W4021) and anti-rabbit (cat. no. W4011) secondary antibodies were obtained from Promega Corporation (Madison, WI, USA). Go6976 was obtained from Calbiochem (La Jolla, CA, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

Microarray data searching. The microarray raw data were searched in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-TABM-157 for 26 TNBC cell lines.

Tissue array. The array slides (BR1503b) were purchased from US Biomax Inc. (Rockville, MD, USA). Detailed information for this array can be viewed at http://www. biomax.us/tissue-arrays/. There were seven breast intraductal carcinoma and 60 breast invasive ductal carcinoma slides, which contained 30 TNBC duplicate cores per case. Each specimen was represented by two cores, and prepared from paraffin-embedded specimens, deparaffinized in xylene, and rehydrated through an alcohol series. The sections were then incubated with 3% H_2O_2 for 5 min. After washing with phosphate-buffered saline (PBS), the sections were heated to boiling in EDTA solution (1 mM EDTA, 0.1% NP-40; pH 8.0) for 5 min in a microwave oven. This non-competitive inhibition procedure was repeated once after an interruption of 10 min.

After cooling for 20 min, the sections were washed three times in PBS for 5 min and then incubated in PBS with 3% Invitrogen fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 25 min. The sections were washed with PBS and incubated with purified polyclonal antibodies against PKC α or AXL [10 ng/ml PBS plus 0.2% bovine serum albumin; BSA (Sigma-Aldrich)] at room temperature for 1 h. After washing three times in PBS for 5 min, the sections were incubated with biotinylated-labeled goat anti-rabbit IgG (dilution, 1:2,000; Sigma-Aldrich) at room temperature for 30 min. The sections were then washed with PBS and incubated with ABC reagent (Avidin/Biotin kit; Vector Laboratories, Inc., Burlingame, CA, USA) conjugated with peroxidase at room temperature for 30 min. PKC α or AXL antigen staining was visualized by adding 3,3'-diaminobenzidine substrate (Sigma-Aldrich). The reaction was terminated by rinsing the sections in distilled water.

The sections were counterstained with Gill's hematoxylin V (Mute Pure Chemicals Ltd., Tokyo, Japan), dehydrated in graded alcohol, and cleared with xylene prior to mounting with Malinol (Mute Pure Chemicals Ltd.). Immunoreactivity was examined with a BX40 system microscope (Olympus, Tokyo, Japan) with a CCD DPII Camera (Olympus, Tokyo, Japan). Images were analyzed by Image-Pro Plus software version 4.5 (Media Cybernetics, Silver Spring, MD, USA). The intensity was scored as '1+,' '2+,' '3+,' and '4+' for none, weak, moderate, and strong staining, respectively.

Cell culture. Hs578T and MDA-MB-231 breast cancer cell lines were included in the present study. The cells were purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan), cultured with Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12, respectively Gibco/Invitrogen; Thermo Fisher Scientific, Inc.), and supplemented with 10% FBS, 100 U/ml penicillin G, and 100 μ g/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere containing 5% CO₂ at 37°C.

Transfection. Lipofectin (Sigma-Aldrich) was used to perform transfection. Cells were cultured in a 60-mm dish containing 10% FBS-DMEM at 37°C, incubated for 24 h before rinsing with serum-free DMEM, and transferred into 1 ml serum-free DMEM containing 15 μ g/ml Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.) and 5 or 10 μ g of the indicated plasmid. After a 6-h incubation, 1 ml DMEM supplemented with 10% FBS was added to the medium. After incubation for another 18 h, the medium was replaced with fresh 10% FBS-DMEM before 48 h of incubation. The cells were then lysed for western blot analysis.

Western blot analysis. The cultured cells were washed twice with PBS and lysed with a lysing buffer containing 50 mM Tris/HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40 and 0.3% sodium deoxycholate. The cell lysates were centrifuged at 12,000 x g and 4°C for 15 min. The supernatant was collected and the protein concentration was determined by the Bradford method. Equal amounts of protein extracts (50 μ g) were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma-Aldrich) and blotted onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with BSA, the membrane was incubated with specific anti-PKC α (1:5,000), anti-AXL (1:1,000) and anti-\beta-actin (1:10,000) antibodies. The blots were then washed three times in 50 ml buffer for 10 min and incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:3,000) at room temperature for 2 h. Proteins were detected using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech).

Cell proliferation assay. Cell proliferation was determined by an MTT assay (Sigma-Aldrich). The cells were seeded in 24-well plates at $1x10^4$ cells/well, and cultured in Dulbecco's

Table I. Expression of AXL in breast cancer tissue with high and low expression of PKC α (n=30).

Level of PKCa ^a	No. of patients	AXL expression ^b	
Low	8	1.50±0.19°	
High	22	2.68±0.12	

^aExpression of PKC α . A score of 1 or 2 was designed as 'low' expression; and a score of 3 or 4 was designed as 'high'. ^bMean \pm standard error of the mean. ^cP<0.01. Statistical analyses were performed by the Mann-Whitney U test. PKC α , protein kinase C α .



Figure 1. Correlation between the expression of PKC α and AXL in TNBC cell lines. The microarray raw data were obtained from ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-TABM-157 for 26 TNBC cell lines. P<0.0001 using linear regression test. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.

modified Eagle's medium (DMEM) containing 10% FBS at 37°C overnight. These cells were treated with various doses of Go6976 and incubated for 24 or 48 h. After incubation, the medium was replaced with fresh medium, and the cells were incubated with 5 mg/ml MTT (Sigma-Aldrich) for 4 h prior to dissolving in 1 ml isopropanol for 10 min. The optical density at 570 nm was then measured using a spectrophotometer (Synergy 2 multi-mode reader; BioTek Instruments, Inc., Winooski, VT, USA).

Plasmid construction. Vectors containing myc-MZF-1 (amino acids 60-72) were constructed by expressing MZF-1 (amino acids 60-72; SDLRSEQDPTDED encoded from 1,268 to 1,306 bp) in a pcDNA3.1/myc-His vector. DNA fragments of MZF-1 (forward: 5'-GGAATTCCAAGCGATCTGAGG AGTGAACAGGACCCCACGGACGAGGATCCCAAGCTT GGG-3' and reverse: 5'-CCCAAGCTTGGGATCCTCGTC CGTGGGGTCCTGTTCACTCCTCAGATCGCTTGGAAT TCCA-3') were synthesized by MDBio, Inc. (Rockville, MD, USA) and cloned through ligation into the *Eco*RI-*Hin*dIII sites of the pcDNA3.1/myc-His vector.

Vector containing full length PKC α -c-myc was constructed by expressing PKC α (28-2,046 bp) in a pcDNA3.1/myc-His vector. The open reading frame of the human PKC α (GenBank accession no. X52479.1) gene was obtained from MDA-MB-231 cells by reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR. An aliquot of total RNA (0.5 μ g) was reverse transcribed using 0.5 μ M oligo d(T) primers in a reaction solution (50 μ l) containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 10 mM DTT, 10 units RNase inhibitor (Promega Corporation), 0.8 mM total dNTPs, and 200 units of Maloney murine leukemia virus reverse transcriptase (Promega Corporation). The sample was incubated at 42°C for 1 h and at 99°C for 5 min before cooling on ice for 10 min.

The RT product (2 μ l) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris-HCl and 2 mM MgCl₂) to a final volume of 50 μ l, containing 0.5 μ M dNTPs (final concentration, 0.8 mM) and 0.5 units of Super-Therm Taq DNA polymerase [Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa]. PCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems; Thermo Fisher Scientific, Inc.). For each experiment, up to 40 cycles were performed to avoid reaching the PCR plateau values. The PCR products were analyzed by 1.2% agarose gel electrophoresis and direct visualization using SYBR Green I (Cambrex Bio Science Rockland Ltd., Rockland, ME, USA) staining. The agarose gels were scanned and analyzed using the Kodak Scientific 1D Imaging system (Kodak, Rochester, NY, USA). The specificity of the cDNA was also evaluated via DNA sequence analysis (data not shown).

The primers were as follows: 5'-GGAATTCCAATG GCTGACGTTTTCCCGGGC-3' (containing an EcoRI site) and 5'-CCCAAGCTTTACTGCACTCTGTAAGATGGG-3' (containing a HindIII site). The PKC α fragment was constructed with *Eco*RI and *Hind*III, and the fragment was isolated and cloned through ligation into the *Eco*RI-*Hind*III sites of the mammalian expression system vector pcDNA3.1/myc-His(-)B vector (Invitrogen; Thermo Fisher Scientific, Inc.).

TAT-fused peptide design. The TAT-fused peptides were designed such that the TAT moiety corresponded to amino acid residues 48-57 of the HIV TAT protein (15), The TAT-fused peptide MZF-1 fragment (60-72; YGRK-KRRQRRGGGDEDTPDQESRLDS) was purchased from MDBio, Inc. (Rockville, MD, USA).

Statistical analysis. Microarray data analyses were performed using a linear regression test. Tissue array data analyses were performed by the Mann-Whitney U tests and Fisher's exact test, and data are expressed as the mean \pm standard error of the mean. Cell proliferation and western blotting results were analyzed by analysis of variance and Student's t-test was used for the two-group comparisons. Analyse-it software (http://analyse-it.com/) was used for performing statistical analysis and P<0.05 was considered to indicate a statistically significant difference.

Results

 $PKC\alpha$ is correlated with AXL expression in TNBC. Previous microarray data was analyzed and a significant correlation between PKC α and AXL expression in human TNBC cell lines was demonstrated (Fig. 1). Tissue array results confirmed

Level of AXL ^b		
Low (n)	High (n)	P-value ^c
13	13	NS
3	1	
14	11	NS
2	3	
3	4	NS
13	10	
10	9	NS
6	5	
	Level o Low (n) 13 3 14 2 3 13 10 6	

Table II. Association between the expression levels of AXL and the clinical characteristics of patients with breast cancer (n=30).

^aClinicopathological data were download from US Biomax Inc. (http://www.biomax.us/tissue-arrays/Breast/BR1503b). ^bExpression of AXL. A score of 1 or 2 score was designed as 'low'; and 3 or 4 score was designed as 'high'. ^cStatistical analyses were performed by the Fisher exact test. P<0.05 was considered significant. NS, not significant.



Figure 2. Expression of PKC α and AXL in TNBC (magnification, x40). The array slides (BR1503b) were purchased from US Biomax Inc. (Rockville, MD, USA). The slides were prepared and stained with antibodies against PKC α and AXL. The intensity was scored as '1+,' '2+,' '3+,' and '4+' for no, weak, moderate and strong staining, respectively. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.

the same phenomenon in TNBC (Fig. 2 and Tables I and II). Data showed that the higher expression of AXL was correlated with elevated PKC α expression in TNBC. No significant association was identified between AXL expression and all clinicopathological features and biomarkers.

AXL expression is inhibited by Go6976 in TNBC cells. To further determine the correlation between PKC α and AXL expression, a selective PKC α/β inhibitor, Go6976, was used to treat the breast cancer cells. The results show that Go6976, at a gradient concentration of 10-1,000 nM, significantly inhibited TNBC MDA-MB-231 cell proliferation from 92 to 41% of the control group (Fig. 3), and TNBC Hs578T cell proliferation from 78 to 29%. AXL and vimentin expression also decreased in a dose-dependent manner (Fig. 4), but there were no changes in PKC α expression.

PKCa and AXL are downregulated by an MZF-1 peptide in TNBC cells. In a previous study, it was demonstrated that PKCa is regulated by MZF-1 and Elk-1 (16). Recently, data has shown that MZF-1 and Elk-1 form a heterodimer and a peptide (MZF-1 peptide) matching one of their binding domains (Lee *et al*, unpublished data), which is the binding site for Elk-1. When saturation of the Elk-1 binding site was induced by transfection with this peptide into the MDA-MB-231 and Hs578T cells, PKCa and AXL expression was observed to be decreased (Fig. 5). This was reversed by co-treatment with full length of PKCa (Fig. 6). When the same cells were treated



Figure 3. Changes in cell proliferation of TNBC cells following treatment with Go6976. After Go6976 (0-1,000 nM) was added to the subculture medium of breast cancer Hs578T and MBA-MD-231 cells for 72 h, the cell proliferation was detected by an MTT assay. Dimethyl sulfoxide served as the control solution. The data are presented as the mean \pm standard error of the mean (n=3 in each group). *P<0.05, **P<0.01 compared with the 0 nM Go6976-treated group.



Figure 4. Changes in PKC α , AXL and vimentin expression following treatment with Go6976 in TNBC cells. After Go6976 (0 nM to 1,000 nM) was added to the subculture medium of breast cancer Hs578T and MBA-MD-231 cells for 3 days, the expression of PKC α , AXL and vimentin were detected by western blot analysis. Vehicle (dimethyl sulfoxide) served as a control. β -actin was used as a housekeeping gene. Data are a representative of three independent experiments. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.

with a TAT-fused peptide, which is a TAT peptide fused to the fragment of the MZF-1 acidic domain, inhibition was also observed (Fig. 7), confirming the correlation between PKC α and AXL.

Discussion

The present microarray data show that PKC α was significantly correlated with AXL expression in the TNBC cell lines. Tissue array data showed that AXL was correlated with elevated PKC α expression in TNBC, suggesting that AXL and PKC α synergize to promote breast cancer malignant progression. These results were consistent with the findings that AXL downregulation in



Figure 5. AXL and PKC α expression decreased in MZF-1 peptide-transfected TNBC cells. The Hs578Tv and MDA-MB-231v stable cloned cells are the Hs578TT and MDA-MB-231 cells transfected with the empty vector, respectively. The Hs578Tm1 and Hs578Tm2 stably cloned cells are the Hs578T cells transfected with the MZF-1 acidic domain-derived peptide (aa 60-72)-vector. The MDA-MB-231m1 and MDA-MB-231m2 stable cloned cells are the MDA-MB-231 cells transfected with the MZF-1 acidic domain-derived peptide (aa 60-72)-vector. The MDA-MB-231m1 and MDA-MB-231m2 stable cloned cells are the MDA-MB-231 cells transfected with the MZF-1 acidic domain-derived peptide (aa 60-72)-vector. The expression levels of PKC α and AXL were detected by western blot analysis. β -actin was designed as a housekeeping gene. Data are representative of three independent experiments. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.



Figure 6. AXL expression was reversed by constitutively active PKC α in MZF-1 peptide-transfected TNBC cells. The MZF-1 acidic domain-derived vector-transfected TNBC stable clone Hs578Tm2 and MDA-MB-231m2 cells were transfected with 10 μ g of the empty vector or constitutively active PKC α for 3 days. The cell lysates were used to detect the expressions of PKC α and AXL by western blot analysis. β -actin was designed as a housekeeping gene. Data are representative of three independent experiments. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.



Figure 7. AXL expression is decreased in TAT-fused peptide-treated TNBC cells. After the Tat-fused peptide (50 and 100 μ M) was added to the subculture medium of Hs578T and MBA-MD-231 cells for 3 days, the cell lysates were used to detect the expression of PKC α and AXL by western blot analysis. β -actin was designed as a housekeeping gene. Data are representative of three independent experiments. PKC α , protein kinase C α ; TNBC, triple-negative breast cancer.

leukemia is achieved by transfection with PKC α/β small interfering RNA (14). In addition, inactivation of AXL caused by monoclonal antibodies and RNA interference attenuates tumor growth and reduces cell survival (15,17). Furthermore, treatment with the PKC α inhibitor Go6976, demonstrated that AXL and the EMT-related gene, vimentin, could be regulated by PKC α in TNBC cells. These findings suggest the involvement of AXL signaling in PKC α -dependent TNBC.

Furthermore, the decrease of AXL expression was correlated with PKC α downregulation in MZF-1 peptide-treated TNBC cells. Moreover, the EMT-related gene vimentin and slug expression, cell migration, tumorigenicity, and drug-resistance of the same cells were also previously shown to be reduced (Lee *et al*, unpublished data). These findings suggest that PKC α may promote AXL expression and EMT in TNBC cells. By contrast, Tam *et al* (18) found that encoding EMT-related genes in non-cancer stem cells can induce PKC α expression causing their transformation into cancer stem cells. However the data from the present study demonstrates an opposite effect, where the PKC α -induced EMT is mediated through the AXL signaling pathway, can also occur in TNBC cells.

AXL has been reported to be induced by PKC through the PKC/mitogen-activated protein kinase (MAPK)/AP-1 signaling axis in leukemia (13). Moreover, AXL gene expression can be regulated by MZF-1 in chronic myeloid leukemia, as well as microRNA-34a and EMT-related genes in breast cancer (19-21). This suggests that some of the above-mentioned MAPK and microRNA genes, including AXL, may be downstream of PKC α , as it also regulates claudin-1 via the Snail- and MAPK-dependent pathways during EMT (22), and are associated with invasive growth in two genetic models of EMT (6). Thus, although the molecular mechanism of AXL expression regulated by PKC α in TNBC cells remains unclear, it was suggested that PKC α -induced AXL expression may be mediated through multiple signaling pathways.

Gjerdrum *et al* (2) did not identify any significant association between AXL expression and important clinicopathological features, such as tumor diameter, histological grade, expression of estrogen and progesterone receptors, and auxiliary lymph node status. Moreover, AXL expression was not significantly associated with Her2 status, E-cadherin expression, markers of basal differentiation (cytokeratin 5/6 or P-cadherin), or tumor cell proliferation by Ki-67 staining (2). The present data confirmed the absence of significant associations between AXL expression and all clinicopathological features and biomarkers.

In lung and bladder cancer, like breast cancer, AXL has been correlated with poor diagnosis and its inhibition has been demonstrated to be an important mechanism in inhibiting cancer progression (23). Moreover, AXL and vimentin expression levels and cell proliferation in A549 and HT5637 cell lines can also be inhibited by Go6976 (data not shown). In conclusion, these results confirm the correlation between AXL and PKC α , and suggest PKC α -AXL signaling may be a treatment target, particularly in malignant cancer cells. Furthermore, the present findings provide the basis for additional research into the treatment of TNBC.

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