Protein kinase C isoform expression as a predictor of disease outcome on endocrine therapy in breast cancer

J W Assender, J M W Gee, I Lewis, I O Ellis, J F R Robertson, R I Nicholson

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See end of article for authors' affiliations

Correspondence to:
Dr J Assender, CRUK
Institute of Cancer Studies,
University of Birmingham,
Edgbaston, Birmingham B15
2TT, UK; J.W.Assender@
bham.ac.uk

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Background: Although *in vitro* breast cancer models have demonstrated a role for protein kinase C (PKC) α and δ isoforms in endocrine insensitivity and resistance respectively, there is currently little clinical evidence to support these observations.

Aims: To define the pattern of PKC α and δ expression using breast cancer cell lines, with and without endocrine resistance, and also breast cancer samples, where expression can be correlated with clinicopathological and endocrine therapy outcome data.

Methods: PKC isoform expression was examined in tamoxifen responsive, oestrogen receptor positive (ER⁺), ER⁺ acquired tamoxifen resistant (TAM-R) and oestrogen receptor negative (ER⁻) cell lines by western blotting and immunocytochemical analysis. PKC isoform expression was then examined by immunohistochemistry in archival breast cancer specimens from primary breast cancer patients with known clinical outcome in relation to endocrine response and survival on therapy.

Results: ER⁺ breast cancer cell lines expressed considerable PKC- δ but barely detectable levels of PKC- α , whereas ER⁻ cell lines expressed PKC- α but little PKC- δ . ER⁺ acquired TAM-R cell lines expressed substantial levels of both PKC- α and δ . In clinical samples, high PKC- δ expression correlated to endocrine responsiveness whereas PKC- α expression correlated to ER negativity. PKC- δ was an independent predictor of duration of response to therapy. Patients showing a PKC- δ ⁺/PKC- α ⁻ phenotype had a six times longer endocrine response than patients with the PKC- δ ⁺/PKC- α ⁺ phenotype (equating to tamoxifen resistance *in vitro*).

Conclusions: Levels of PKC- α and δ expression appear to be indicative of response to anti-oestrogen therapy and could be useful in predicting a patient's suitability for endocrine therapy.

Anti-hormone therapies such as tamoxifen are widely used to treat breast cancer patients.¹ A small but significant number of patients receiving tamoxifen however will not respond or will develop resistance.^{2–3} Many mechanisms have been suggested which may play a role in tamoxifen resistance but the mechanisms have not yet been fully elucidated.^{4–7} Although rapid progress is being made in understanding the biology of oestrogen receptor (ER) function, the only predictive markers for endocrine therapy that currently yield sufficient levels of evidence to be recommended for routine practice, are ER and progesterone receptors, and to a lesser extent HER-2 status.¹ Better ways of predicting which patients are suitable for endocrine therapy would prove useful in the fight against breast cancer. Expression of the signal transduction molecule, protein kinase C (PKC) is increased in breast cancer models of poor prognosis; for example, ER⁻ cell lines express significantly more PKC than ER⁺ cell lines.^{8–9} However, multiple isoforms of PKC exist, with variation in their expression profile and mechanism of activation.^{10–12} We have previously shown that ER⁺ MCF-7 cell lines have high PKC- δ and low PKC- α expression, whereas ER⁻ MDA-MB-231 cells have high PKC- α and low PKC- δ expression. A wealth of literature now support these observations^{13–16} linking PKC- α expression to loss of ER expression and adverse cellular features,^{13–15} and there is also emerging data that PKC- δ expression can relate to loss of endocrine sensitivity *in vitro*.¹⁷ These studies did not however investigate the effect of PKC- δ expression on clinical outcome. Moreover, a recent clinical study showed PKC- α to be decreased in advanced breast cancer samples,¹⁸ suggesting that laboratory observations may not translate to the clinic.

We have therefore established cell line models of tamoxifen resistance,^{19–20} and used these models and well-characterised clinical specimens^{21–22} with known response to endocrine

therapy, to study PKC expression. We have shown that PKC- α and δ may prove useful in predicting whether patients will respond or not to endocrine therapy.

MATERIALS AND METHODS

Cell culture

For experimental purposes ER⁺ and ER⁻ breast cancer cell lines were grown in phenol red-free RPMI medium supplemented with 5% activated charcoal stripped, steroid depleted fetal calf serum (ssFCS), 200 mM glutamine and antibiotics (10 IU/ml penicillin, 10 μ g/ml streptomycin). Acquired TAM-R cell lines were established by culturing MCF-7¹⁹ or T47D²⁰ cells in 10⁻⁷ M 4-hydroxy-tamoxifen for 6 months. These resistant cells over-express EGF receptor,¹⁹ and have enhanced MAPK signalling,^{20–23–24} enhanced growth characteristics,^{20–25} and increased Src activity versus their parental lines.²⁶

Western analysis

Cells were lysed in buffer (4°C) and acetone precipitated as previously described.²⁷ Protein (40 μ g) was run on an 8% SDS-PAGE gel and blotted onto membranes. Non-specific binding was blocked with 5% non-fat milk in Tris-buffered saline (TBS; 10 mM Tris pH 7.5, 100 mM NaCl) containing 0.1% Tween-20. Primary monoclonal antibodies (Transduction Laboratories; α = IgG_{2b} clone 3, δ = IgG_{2b} clone 14) were diluted according to manufacturer's instructions in TBS containing 1% bovine serum albumin (BSA), and incubated with the blot for 3 hours.

Abbreviations: AP-1, activator protein-1 complex; BSA, bovine serum albumin; DAB, diaminobenzidine-tetrahydrochloride; EGF, epidermal growth factor; ER, oestrogen receptor; ERK2, extracellular signal-regulated kinase 2; HR, hazard ratio; MAPK, mitogen activated protein kinase; NHS, normal human serum; PBS, phosphate buffered saline; PKC, protein kinase C; TAM-R, tamoxifen resistant; TBS, Tris buffered saline

Figure 1 Western blot comparison of protein kinase C (PKC) α and δ expression in oestrogen receptor positive (ER⁺) cell lines (MCF-7, T47D), their tamoxifen resistant derivative cell lines (MTR and TTR) and the ER⁻ MDA-MB-231 cell line (231). The resistant cell lines have increased expression of both PKC isoforms compared to the parental cell lines. Equality of protein loading was checked by confirming equality of β -actin expression between samples. Figure representative of at least three independent experiments.

at 22°C, then overnight at 4°C. The membrane was washed 6×5 min in TBS-Tween before incubating in secondary antibody (mouse horseradish peroxidase conjugated, Amersham International, 1 hour). The blot was washed 6×5 min in TBS-Tween; antibody binding was detected using the SuperSignal WEST DURA chemiluminescence system (Pierce).

Immunocytochemical examination of paraffin embedded cells

Cells were concentrated by centrifugation (1000 g for 5 min), then fixed in 4% formal-saline for 4 h. Fixed cells were transferred to agar solution to form solid pellets and embedded in paraffin-wax. Pellets were cored and assembled in triplicate using a tissue arrayer (Beecher Instruments), forming a composite pellet array which was sectioned (5 μ m) onto Superfrost slides before assaying as below. Using the clinically defined HScore cut offs to define positivity, sections were assessed for PKC- α and PKC- δ expression as detailed below.

Immunohistochemical examination of clinical samples

Formal-saline (4%) fixed, paraffin-embedded breast cancer tissue was available from 70 primary breast cancer patients, who subsequently received systemic endocrine therapy (primarily using tamoxifen) as detailed previously,²¹ either for locally-advanced primary carcinoma or metastatic disease. Data was available regarding the patients' quality of endocrine response (25 responders (complete, partial and static disease) vs 45 progressive disease) assessed according to UICC criteria at 6 months, duration of endocrine response and survival time on endocrine therapy, ER status (ie, ER⁻, n = 27; ER⁺, n = 43), histological grade (grade 1 or 2, n = 29; grade 3, n = 40), site of disease (locally advanced, n = 27; other sites, n = 42) and menopausal status (premenopausal, n = 21; postmenopausal, n = 48).

Immunostaining of clinical material (and cell pellet arrays) was done by dewaxing paraffin sections (5 μ m) in xylene and rehydration through a decreasing alcohol concentration series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution (5 min) and antigens retrieved by previously optimised procedures (PKC- δ : 0.02% protease E in PBS at 37°C;

Figure 2 Protein kinase C (PKC) α expression assessed by immunocytochemical analysis of paraffin embedded cell pellets. Oestrogen receptor positive (ER⁺), endocrine responsive MCF-7 and T47D cells show low PKC- α staining and therefore a low HScore. Their tamoxifen resistant derivatives (MTR and TTR respectively) show considerably higher levels of PKC- α expression and correspondingly higher HScores. The ER⁻ MDA-MB-231 cells show very high levels of PKC- α staining and thus HScore. Original magnification $\times 40$. Figure representative of three experiments.

PKC- α : microwaving in citrate buffer) followed by water and PBS rinses. Sections were blocked in normal human serum (NHS, 20%, 15 min) and then incubated for 18 h with 16.5 μ g/ml anti-PKC- δ (IgG_{2b} clone 14, Transduction Laboratories, preincubated with NHS for 30 min) or 2 μ g/ml anti-PKC- α (IgG₁ clone 4, Upstate Biotechnology) monoclonal antibody prepared in PBS. Sections were washed in PBS (3 min), then in "DPC"-detergent containing buffer (2×5 min). Biotinylated anti-mouse immunoglobulin (1:40 dilution in PBS containing 1% BSA) was applied for 60 min. After washing, a peroxidase-labelled streptavidin immunodetection system was employed (SuperSensitive Concentrated Detection Kit, Biogenex) using DAB/hydrogen peroxide chromogen (ER-ICA kit, Abbott Laboratories) and methyl green counterstaining.

In the absence of any obvious cut-off point for immunostaining, patients were classified as PKC- α or δ positive using the median of the tumour epithelial HScore immunostaining index for each marker,²⁸ such that PKC- α positive = HScore > 110 and PKC- δ positive = HScore > 120 . Relationships between PKC status and clinicopathological parameters were examined using χ^2 tests. Univariate analysis of survival from initiation of therapy and duration of endocrine response was performed using the Kaplan-Meier method and log rank test. Multivariate analysis was performed using a Cox proportional hazards model, controlling for

Figure 3 Immunocytochemical analysis of protein kinase C (PKC) δ expression in paraffin embedded cell pellets. Oestrogen receptor negative (ER⁻) MDA-MB-231 cells show very low levels of PKC- δ expression and thus HScore. PKC- δ expression is much more abundant in ER⁺, endocrine responsive MCF-7 and T47D cells and thus they have a higher HScore. Their tamoxifen resistant derivatives (MTR and TTR respectively) show a further increase in PKC- δ expression, reflected by further increased HScores. Original magnification $\times 40$. Figure representative of three experiments.

the available clinicopathological features (site of disease, tumour grade, ER status, menopausal status). All p values were two-sided and considered statistically significant if <0.05 .

RESULTS

PKC isoform profile in breast cancer cell lines

We examined PKC isoform expression in tamoxifen responsive ER⁺ (MCF-7 and T47D) cells, acquired tamoxifen resistant sub-lines of MCF-7 cells (MTR) and T47D (TTR) and an ER⁻ (MDA-MB-231) breast cancer cell line using isoform specific antibodies. The ER⁻ cell line expressed significant PKC- α but little PKC- δ , either by western analysis or immunocytochemistry (figs 1, 2 and 3). The ER⁺ endocrine responsive cell lines expressed abundant PKC- δ but relatively low or no PKC- α (figs 1, 2 and 3). Interestingly both acquired tamoxifen resistant cell lines expressed increased levels of both PKC- α and PKC- δ compared to their parental cell lines (figs 1, 2 and 3).

PKC- α expression associates with poor clinical outcome on endocrine therapy

When breast cancer samples from patients with known clinical outcome were examined for PKC- α expression, brown immunostaining was seen within the tumour epithelial cells'

Figure 4 Expression of protein kinase C (PKC) α in human breast cancer samples (positive staining is brown, with methyl green nuclear counterstaining) showing heterogeneity of staining. Representative sections showing (A) strong PKC- α positive staining (HScore = 150) and (B) weaker PKC- α staining (HScore = 100) within oestrogen receptor negative patients. (C) An oestrogen receptor positive section, in this instance showing weak PKC- α (HScore = 10) staining. (D) (i) Negative control section, treated identically to (ii) matched positive staining section except that the control section was incubated with normal human serum-containing diluent instead of primary antibody. Original magnification $\times 40$.

cytoplasm and perinuclear region (fig 4). Staining was heterogeneous, both within samples and between patients, but using a median HScore of >110 (range 0–170) to define substantial PKC- α immunopositivity versus low expression, statistical analysis revealed an association between PKC- α and ER status, with ER⁻ disease showing substantially more PKC- α staining than ER⁺ tissues (table 1 and fig 4). Expression was not significantly related to disease site, menopausal status or grade of tumour but did correlate to quality of response as measured at 6 months' endocrine therapy, with high PKC- α expression related to progressive disease (table 1). Furthermore, univariate analysis showed a significantly shorter duration of endocrine response in those patients whose tumours were PKC- α positive (median duration of response: PKC- α ⁺ 2 months, 95% CI 0.9 to 3.1; PKC- α ⁻ 6 months, 95% CI 2.7 to 9.3; $p = 0.003$). There was also a significant decrease in survival time from initiation of endocrine therapy in PKC- α positive patients (median survival: PKC- α ⁺ 12 months, 95% CI 2.9 to 21.1; PKC- α ⁻ 29 months, 95% CI 22.0 to 36.1; $p = 0.03$). PKC- α status was not however an independent predictor of survival on therapy ($p = 0.86$, HR = 0.95, 95% CI 0.5 to 1.7) or duration of response ($p = 0.70$, HR = 1.13, 95% CI 0.6 to 2.0) by multivariate analysis, controlling for clinicopathological profile (including ER status).

PKC- δ expression predicts for endocrine responsiveness

PKC- δ immunostaining was readily detectable in the tumour epithelial cells' cytoplasm, but again staining was heterogeneous

Table 1 Association between PKC- α expression and clinicopathological variables for the breast cancer patient series

Variable	Patient no	PKC- α positive	PKC- α negative	p-Value
ER status				
Negative	28	21	7	<0.01*
Positive	42	13	29	
Total	70			
Site of disease				0.10
Locally advanced	27	10	17	
Metastatic disease	42	24	18	
Total	69			
Menopausal status				0.06
Premenopausal	21	14	7	
Postmenopausal	48	20	28	
Total	69			
Tumour grade				0.26
Grade 1 or 2	29	12	17	
Grade 3	40	22	18	
Total	69			
Quality of response to therapy at 6 months				0.01*
CR/PR/SD	25	7	18	
PD	45	27	18	
Total	70			

PKC, protein kinase C; ER, oestrogen receptor; CR, complete response; PR, partial response; SD, static disease; PD, progressive disease.
*Statistically significant ($p < 0.05$).

Figure 5 Protein kinase C (PKC) δ expression in a series of breast cancer samples (positive staining is brown, with methyl green nuclear counterstaining) for which patient response to endocrine therapy was known. Representative sections show (A) strong PKC- δ positive staining (HScore = 170) and (B) weak PKC- δ staining (HScore = 110) within oestrogen receptor positive patients. (C) A representative oestrogen receptor negative section showing weak PKC- δ (HScore = 52) staining. (D) Negative control section, treated identically to (C) matched weak positive staining section except that the control section was incubated with normal human serum-containing diluent instead of primary antibody. Original magnification $\times 40$.

both within samples and between patients (fig 5). Using the median HScore (>120 , range 20–190) to define substantial PKC- δ immunopositivity, statistical analysis failed to reveal any association between PKC- δ and ER status (table 2). There was also no association with site of disease, menopausal status or tumour grade. However, a significant relationship was observed between PKC- δ status and quality of response at 6 months on endocrine therapy (table 2). This relationship was retained even after selecting for patients with ER $^{+}$ disease. Univariate analysis confirmed that there was a trend for longer duration of endocrine response to be

Figure 6 Kaplan-Meier curves for (A) duration of endocrine response and (B) survival from initiation of therapy. The phenotype protein kinase C α^{-}/δ^{+} can be seen to have a significantly better response ($p = 0.009$) and survival ($p = 0.066$) than any of the other phenotypes.

Table 2 Association between PKC- δ expression and clinicopathological variables for the breast cancer patient series

Variable	Patient no	PKC- δ positive	PKC- δ negative	p-Value
ER status				
Negative	28	13	15	0.77
Positive	42	21	21	
Total	70			
Site of disease				
Locally advanced	27	12	15	0.65
Metastatic disease	42	21	21	
Total	69			
Menopausal status				
Premenopausal	21	10	11	0.98
Postmenopausal	48	23	25	
Total	69			
Tumour grade				
Grade 1 or 2	29	14	15	0.95
Grade 3	40	19	21	
Total	69			
Quality of response to therapy at 6 months				
CR/PR/SD	25	17	8	0.015*
PD	45	17	28	
Total	70			
Endocrine response in ER ⁺ disease				
CR/PR/SD	24	16	8	0.013*
PD	18	5	13	
Total	42			

PKC, protein kinase C; ER, oestrogen receptor; CR, complete response; PR, partial response; SD, static disease; PD, progressive disease.
statistically significant ($p < 0.05$)

associated with PKC- δ positive tumours (median duration of response: PKC- δ^+ 7 months, 95% CI 2.6 to 11.4; PKC- δ^- 3 months, 95% CI 1.5 to 4.5; $p = 0.08$). PKC- δ status was however poorly associated with survival from initiation of therapy ($p = 0.13$). Controlling for clinicopathological profile, multivariate analysis revealed that PKC- δ status was a significant independent predictor of duration of response, with PKC- δ positive status associated with a reduced risk of relapse of 47% compared to PKC- δ negative patients ($p = 0.045$, HR = 0.53, 95% CI 0.29 to 0.99). On this sample set, however, it was not an effective predictor of survival from initiation of therapy ($p = 0.20$, HR = 0.69, 95% CI 0.4 to 1.2).

Effect of co-isoform expression

Co-expression of PKC- δ and α (PKC- δ^+ /PKC- α^+), paralleling the phenotype seen in the TAM-R cell lines, predicted for a particularly poor clinical outlook, with very short duration of endocrine response and poorer survival on endocrine therapy (fig 6). In contrast, expression of PKC- δ in the absence of PKC- α (PKC- δ^+ /PKC- α^-) appears to be beneficial, with patients exhibiting a longer duration of response and improved survival on endocrine therapy than patients with the other PKC isoform combinations (fig 6; $p = 0.009$ for duration of response and $p = 0.066$ for survival on therapy). Intriguingly, even after selecting for ER⁺ patients, PKC- δ^+ /PKC- α^- patients appeared to have a longer duration of response than PKC- δ^+ /PKC- α^+ patients (median response time 14 months vs 6 months, $p = 0.02$).

DISCUSSION

An association between PKC- α overexpression and the ER⁻ phenotype has previously been established in cell line models of breast cancer.^{13–15} In addition, increases in growth rate,²⁹ ERK2 expression,³⁰ basal AP-1 activity,¹⁵ multidrug resistance,³¹ and morphological changes^{29–32} have been shown to result from PKC- α overexpression. Although a small clinicopathological study (15 pairs of samples)³³ showed PKC- α expression to predict for tamoxifen treatment failure, a 46-sample study

showed a down-regulation of PKC- α expression in advanced tumour samples.¹⁸ This study has not only confirmed the relationship between PKC- α expression and ER negativity in breast cancer cell lines (using ER⁺ MCF-7, T47D and ER⁻ MDA-MB-231 cells) but also shown an association with ER⁻ staining in clinical material, in a larger (70 sample) study.

Cumulatively this study and others,^{14–16} have shown a correlation between ER positivity and PKC- δ expression in a variety of cell line models. Importantly we also show here a good correlation between PKC- δ expression and improved quality and duration of endocrine response in clinical samples. Moreover PKC- δ was shown to be an independent predictor of such response even after correcting for ER status.

Interestingly, over-expression of PKC- δ in cell line and xenograft models of breast cancer has recently been shown to contribute to anti-oestrogen resistance,¹⁷ which would initially appear to conflict with our findings. However, we have shown that TAM-R cell lines have raised levels of both PKC- α and δ versus their responsive parental cells. Careful scrutiny of the published study¹⁷ and other recent studies claiming association between PKC- α expression and tamoxifen resistance,^{34–35} reveals that their tamoxifen resistant cell lines also have raised levels of both PKC- α and δ , in complete agreement with our results. We have looked at the effect of α and δ isoform co-expression on clinical outcome, showing that co-expression of PKC- α and δ predicts for a very short duration of endocrine response and survival on endocrine therapy, in keeping with these being endocrine resistant patients. Although after subdivision our sample size is small, our results are fully in keeping with these previous studies^{17–34} and importantly also add to them, indicating that it is co-expression of PKC- α and δ that is associated with endocrine resistance, both in cell line models and more importantly in clinical samples.

Thus, knowledge of PKC isoform expression could be useful in helping to predict patients' endocrine responsiveness, with expression of PKC- δ in the absence of PKC- α predicting for good endocrine response, PKC- α expression in the absence of PKC- δ associating with ER negativity, and associated endocrine

Take-home messages

- Protein kinase C (PKC) isoform expression is indicative of a patient's likely responsiveness to anti-oestrogen therapy.
- In vitro, oestrogen receptor positive (ER⁺) cell lines express PKC- δ but little PKC- α , ER⁻ cells express PKC- α but little δ , while tamoxifen resistant cells express increased levels of both isoforms.
- These in vitro models emulate the in vivo clinical situation, where high PKC- δ expression correlates with endocrine responsiveness, PKC- α expression correlates with ER negativity, and co-expression of PKC- α and δ correlates with poor endocrine response and short on-therapy survival times.

insensitivity and co-expression of PKC- α with PKC- δ predicting for endocrine resistance.

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Authors' affiliations

J W Assender, CRUK Institute of Cancer Studies, University of Birmingham, Edgbaston, Birmingham, UK

J M W Gee, **I Lewis**, **R I Nicholson**, Tenovus Centre for Cancer Research, Welsh School of Pharmacy, Cardiff University, Cardiff, UK

I O Ellis, Department of Histopathology, City Hospital, Nottingham, UK

J F R Robertson, Professional Unit of Surgery, City Hospital, Nottingham, UK

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