Review Article

Protein Kinase C Alpha in Breast Cancer: A Focus on Endocrine Resistant and Triple Negative Breast Cancer

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Abstract

Breast cancer remains the most common cancer in women worldwide and the second most common cancer among women in the U.S. The optimal treatment for patients is based on the expression of the estrogen receptor α (ER), progesterone receptor (PR) and HER2 as assessed by immunohistochemical methods. Patients with tumors that express ER are treated with endocrine therapy including tamoxifen or aromatase inhibitors administered in the adjuvant or neo-adjuvant setting. Unfortunately a significant percentage of patients experience endocrine resistance and disease recurrence. Such targeted therapies do not exist for patients with triple negative breast cancer (TNBC), who are currently principally treated with cytotoxic chemotherapy. Endocrine resistance in patients of ER+ subtype and the aggressive nature of TNBC subtype continue to be significant roadblocks to successful therapeutic management. Protein kinase C alpha (PKCα) is a serine-threonine kinase implicated in numerous physiological and pathological processes, including breast cancer. PKCα participates in a number of oncogenic pathways, making it a very attractive target for breast cancer therapy. Many of these pathways are functionally relevant in both ER+ and TNBC patients. In addition, in ER+ patients, expression of PKCα is a promising biomarker for poor response to endocrine therapy. This review will summarize the current understanding of PKCα in both ER+ and TNBC subtypes.

ABBREVIATIONS

PKC: Protein Kinase C; ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor 2; EGFR: Epidermal Growth Factor Receptor; TNBC: Triple Negative Breast Cancer; TCGA: The Cancer Genome Atlas; CSC: Cancer Stem Cells; SERM: Selective Estrogen Receptor Modulator; SEM: Selective Estrogen Mimics; Sherpa: Selective Estrogen Receptor Partial Agonists

INTRODUCTION

Breast cancer is the one of the most commonly diagnosed cancers among American women. Treatment success depends on multiple factors, including stage at diagnosis and the inherent features of the tumors. Over the last decade, major advances in microarray analysis have reproducibly established that breast cancers encompass several distinct disease entities, referred to as the intrinsic subtypes of breast cancer [1-4]. Among these, tumors of luminal subtype usually express estrogen receptor alpha (ER) and account for about 70% of breast cancer cases [5]. Basal-like breast cancer, which occurs in about 15-20% of breast cancer patients, is commonly known as triple negative breast cancer (TNBC) because the majority of cases lack expression of ER, progesterone receptor (PR), and HER2/neu amplification [6,7]. However, it is important to note that not all basal-like breast cancers are TNBC and not all TNBC are basal-like breast cancer. Basal-like phenotype is determined by microarray profiles whereas TNBC is based on immunohistochemical assays for ER, PR, and HER2 only. The reconciliation between molecular subtype and clinical subtype has been of great research interest, and several immunohistochemical markers have been proposed to define molecular subtypes. It is generally accepted that luminal tumors are ER+ and express many epithelial luminal genes including luminal cell keratin such as 8/18 [1,3,4]. However, the definition of basal breast cancer using immunohistochemical markers is more controversial. Several different panels have been proposed, the most often described is the expression of one or more high-molecular-weight/basal cytokeratins (CK5/6, CK14, and CK17) and/or EGFR in addition to the lack of ER, PR, and HER2 [6,8-10]. An insightful study by Prat et al compared clinical phenotype (immunohistochemistry-based) and molecular phenotype (microarray-based) and determined that

approximately 78% of TNBC tumors are of the basal molecular subtype, and the rest are a mixture of HER2, luminal, and normal-like [7]. Therefore, even though the two terms should not be used interchangeably, there is a remarkable overlap between TNBC and basal breast cancer.

Treatment options for ER+ and TNBC are different, but relapse following therapy resistance is a common clinical challenge. ER+ patients are routinely treated with endocrine therapies, which disrupt the ER-mediated signaling pathway either by blocking estrogen synthesis (aromatase inhibitors), promoting ER degradation (selective estrogen receptor downregulators-SERDs), or competing with estrogen for ER (selective estrogen receptor modulator- SERMs). Tamoxifen is the most commonly prescribed SERM in the last 20 years both for the treatment of early and advanced breast cancer [11]. Unfortunately, a large proportion of women will relapse with acquired resistance [12]. TNBC patients, on the other hand, do not benefit from molecular-targeted therapy and often present an aggressive neoplasia with a strong association with distant recurrence and visceral metastases [13]. Patients of acquired resistance to hormonal therapies and of TNBC subtype present persistent challenges in the successful management of the disease. Ongoing efforts are focused on identifying and characterizing novel molecular targets that can serve as biomarkers for treatment response and/or targets to overcome resistance.

Compared to healthy breast tissues, breast cancers display elevated expression and activity of protein kinase C (PKC) proteins [14-16]. Consisting of twelve isozymes, this family of serine-threonine protein kinases participates in a variety of cellular functions [17-20] (Figure 1). PKCα belongs to the classical subgroup that requires diacylglycerol, calcium, and phospholipid for its activity, and can support proliferation, differentiation, motility, and cell cycle progression in a tissue and cell-specific manner [18,21,22]. A functional role of PKCα has been implicated in a number of pathological conditions including cancers [18,21,23-25]. In this review, we will summarize the current understanding of PKCα in breast cancer, specifically for endocrine-resistant and TNBC.

Expression of PKCα in breast cancer

PKCα is ubiquitously expressed in various mammalian tissues. Expression of PKCα was found to be upregulated in some cancers (prostate, bladder, endometrial) and down-regulated in others (colorectal, malignant renal cell carcinoma) [26-30]. The role of this isozyme in breast cancer is complex, since there are conflicting reports on PKCα expression levels in breast tumors compared to normal healthy breast tissues. Several immunohistochemical studies, including our own, found an overexpression of PKCα in breast tumors compared to non-malignant breast tissues [31-33] whereas two independent research groups found the opposite [34,35]. Overall, these studies reported that expression of PKCα in breast tumors ranges from 12% to 78% [31-33,35,36] (Table 1). In studies that report PKCα overexpression in breast tumors, expression of PKCα is significantly associated with adverse features in breast cancer patients, including shorter time to relapse and resistance to antiestrogen therapy [31,32,37]. We recently re-evaluated our own data using composite scores calculated from both staining intensity and frequency to identify the upper quartile of PKCα expression levels. Using this more stringent scoring criteria we estimated PKCα expression to be 20% (Table 2), representing a significant portion of patients whose tumors are predicted to express PKCα. The significance of the level of PKCα expression is as yet unclear.

Figure 1 Schematic representation of the primary structure of conventional, novel, and atypical PKCs. All PKCs consists of a regulatory domain and a catalytic domain connected together by a hinge region. The presence of the pseudosubstrate keeps the enzyme inactive and gets released once the enzyme translocates to and interacts with the membrane. The conventional isozymes are activated by diacylglycerol and Ca2+, novel by diacylglycerol alone, and atypical can be activated in the absence of both diacylglycerol and Ca2+.
The underlying mechanism for the induction of PKCα protein remains elusive. One study found that, compared to normal breast tissues, breast cancers do not display elevated levels of PKCα transcripts [38]. Among The Cancer Genome Atlas (TCGA) samples with high PKCα protein levels, we found that only 36% have correspondingly high levels of the transcript [39-41]. Interestingly, when only tumors that are ER-negative were considered (N=174), almost 70% of patients with high PKCα protein expression also show elevated transcripts. This finding implies that the regulatory mechanism of PKCα protein is not straightforward and may vary according to different cellular contexts. In contrast, only 8% of patients display concomitant gain of copy number and high expression of PKCα [39-41], suggesting that gene amplification is not a major determinant of PKCα expression at least in the TCGA cohort. It is, however, important to note that since these microarray data present relative instead of absolute expression levels, it is not a simple task to reconcile mRNA and immunohistochemical data. Discrepancies between the available information on PKC α expression at the protein and mRN A levels, measured by microarray, has been noted for PKC α [42].

Alternatively, elevated expression of PKCα protein may be a result of post-translational modifications that alter protein half-life. For example, syndecan-4, a transmembrane heparan sulfate proteoglycan, directly interacts with PKCα and enhances PKCα stability without affecting its mRNA stability or transcription level [43-46]. As a key adhesion molecule, syndecan-4 is ubiquitously expressed but often at low levels in normal tissues [47,48]. An immunohistochemical study found that syndecan-4 is expressed in about 70% of breast tumors [49], yet few studies have examined in depth the role of syndecan-4 in breast malignancy. TCGA breast cancer samples with high PKCα protein expression do not have higher mRNA level of syndecan-4 (SDC4) compared to samples with lower PKCα protein expression [TCGA, Nature 2012] [40,41]. A correlation analysis between PKCα and syndecan-4 at the protein level would be more informative. However, there are limited comprehensive cancer proteome databases that allowed us to perform this analysis. The Cancer Proteomics Atlas (TCPA) provides a user-friendly portal on protein expression data over a large number of cell lines and tumors including those from TCGA [50,51]. As of now, however, information on syndecan-4 is not yet publicly available.

A recent study by Antal et al systematically characterized a number of cancer-associated PKC mutations in the TCGA dataset, and concluded that they are all loss of function and may act in a dominant-negative manner [52]. It appeared that only one PRKCA mutation was identified in breast (A444V), suggesting that mutations of this gene are not a common event in breast cancer. In fact, the authors noted that PKC isoforms are more often mutated in certain cancers (melanomas, colorectal, and lung squamous cell carcinoma) than in others including breast. In agreement with this, we found the frequency of PRKCA mutations among TCGA breast cancer samples to be only 1% [39-41]. The clinical significance of these mutations is unknown.

**PKCα in endocrine-resistant, ER+ breast cancer**

Expression of PKCα was first reported by our laboratory to confer tamoxifen resistance in an ER- breast cancer model [53]. The prognostic value of PKCα in predicting endocrine resistance, response duration, breast cancer specific survival, and overall survival was thereafter demonstrated [31,32,37]. PKCα can transduce a multitude of signaling pathways, some of which have been implicated in the development of endocrine...
resistance. One of these pathways may involve alteration of the ER and ER-mediated signaling pathway. Expression of ERα and PKCα are inversely correlated in human breast cancer cell lines as well as clinical samples [14,31,53,54]. We and others have demonstrated that forced expression of PKCα in ER+ breast cancer cells results in either a reduction of ERα mRNA levels or down-regulation of ERα function [53-55], which contributes to the estrogen-independent and tamoxifen-resistant phenotype [53,56]. In clinical samples, up to 30% of patients with acquired resistance lose expression of ERα [57-59]. The exact mechanism by which PKCα down-regulates expression and function of ERα requires more comprehensive studies, but one proposed hypothesis is the ability of PKCα to inhibit c-Jun phosphorylation, a regulator of ERα transcriptional activity [54,60]. Elevated expression of EGFR/HER2 and its downstream pathways such as MAPK/ERK and AKT have been reported to accompany tamoxifen resistance [61-69]. Identified as a downstream effector of HER2, PKCα expression and activity were deemed critical for breast cancer cell invasion [70]. In turn, PKCα increases the level of recycled HER2 protein at the cell membrane [71]. Several lines of evidence clearly show that expression of HER2 can be amplified over the course of disease progression [58,72]. It is hence an intriguing hypothesis that expression of PKCα could be modulated over time and contributes to the emergence of therapy resistance. These findings suggest that co-targeting PKCα and EGFR/HER2 pathway may offer additional clinical benefits in patients with aggressive disease. For example, dual inhibition of PKCα and ERK resulted in apoptosis of tamoxifen-resistant MCF-7 cells [73]; and dual inhibition of PKCα and Src had therapeutic implication in breast cancer metastasis [70]. Of significant interest, PKCα was recently shown to delay HER2 therapy resistance due to its ability to block Notch-1 signaling in BT474 and MDA-MB-453 cell lines [74]. The authors suggest PKCα is a biomarker for good prognosis in HER2+ patients, which is opposite to the well-established role of PKCα in ER+ patients. This discrepancy may primarily be attributed to the intrinsic differences between tumor subtypes, as the functions of PKCα are known to be very pleiotropic and cell type-dependent.

Over the years, therapy resistance has been strongly linked to the activity of breast cancer stem cells (CSCs) [75-78]. In that regard, Notch signaling has been implicated in the regulation of CSCs in both ductal carcinoma in situ and invasive carcinoma of the breast [79-81]. Indeed, inhibition of Notch-4 signaling was found to reverse the endocrine-resistance phenotype [82,83]. PKCα was identified as a positive regulator of Notch-4 and its downstream effects in endocrine-resistant breast cancer [83]. It is reasonable to speculate that PKCα promotes resistance to endocrine therapy via promoting Notch-induced stem cell activity. In agreement with this, our laboratory showed that ectopic expression of PKCα in T47D breast cancer cells led to a significant increase in the number of CD44+/CD24−/low cells [84], indicative of stem cells [85].

A number of alternative signaling transducers and pathways have been suggested to be co-targeted for the clinical management of tamoxifen-resistant breast cancer [86-88]. The studies presented so far strongly point at PKCα as a promising therapeutic target for these patients. Our laboratory was the first to show that expression of PKCα in primary breast tumors is predictive of recurrence on tamoxifen treatment [37]. These PKCα-expressing tumors regressed upon treatment with both 17β-estradiol (E2) and the SERM raloxifene, although upon raloxifene withdrawal, tumor growth resumes [89]. Raloxifene has a favorable antiestrogenic profile in the uterus and has proven safety over 15 years of clinical use in postmenopausal osteoporosis and breast cancer chemoprevention. This observation led us to design and investigate the therapeutic efficacy of selective estrogen mimics (SEM) and selective estrogen receptor partial agonists (ShERPA), based on the benzothiophene scaffold of raloxifene [90,91]. In contrast to E2, SEM and ShERPA compounds induce tumor regression without uterine proliferation; a known unwanted side effect of both tamoxifen and E2 [90,91]. The anti-tumorigenic effect exerted by E2 and raloxifene is multifaceted, including repression of the pro-survival, anti-apoptotic Akt pathway, activation of the Fas/FasL apoptotic pathway, and extranuclear translocation of the ERα receptor [89,92].

PKCα in TNBC

Compared to endocrine-resistant breast cancer, reports on the functions of PKCα in TNBC have been relatively few. Our laboratory recently reported TNBCs exhibit significantly higher levels of PKCα compared to tumors of other subtypes [33]. Interestingly, TNBCs display significantly higher levels of PKCα transcripts compared to other subtypes (Figure 2). In fact, several microarray studies reveal that PKCα expression ranges from 60% to 80% in TNBC compared to only 20-40% in non-TNBC patients, indicating a potential function of this kinase in TNBC (Oncomine™). Accordingly, inhibition of PKCα expression and activity in TNBC cells significantly reduces their cellular migration in vitro and metastasis in vivo [93, 94]. Several downstream effectors of PKCα have been identified, including CXCR4 and Rac1, expression of which can promote directional migration and metastatic potential of breast cancer cells [93-98]. It appears that PKCα modulates CXCR4 at both the transcript and post-transcript level [94]. Transcriptional regulation of CXCR4 is dynamic and involves a wide variety of signaling molecules, including NF-kB [99]. Further investigation on how PKCα interacts with these molecules will provide a better understanding of CXCR4 regulation by PKCα in breast cancer.

Similar to ER+ breast cancers, the involvement of PKCα in breast cancer stem cells has been demonstrated in TNBC [100,101]. It is thought-provoking that PKCα was shown to have a supportive role only in the breast cancer stem cell but not in the non-stem cell population; therefore, targeting PKCα was more efficient at killing the former [100,101]. In both studies, PKCα expression and activity were crucial for the survival and tumor-initiating capacity of TNBC compared to non-TNBC cells, implying that this particular subtype may rely more heavily on...
the stem cell population for tumor initiation and maintenance. These results highlight the well-known inter- as well as intra-tumor heterogeneity of breast cancer. As a result, co-targeting cancer stem and non-stem cell population will likely offer superior therapeutic effects for the treatment of the disease.

In addition, the study by Tam et al identified PDGFR as the principle pathway downstream of PKCα [101]. This finding, in companion with the known crosstalk between PKCα and EGFR/HER2 in ER+ breast cancers underscores the functional diversity of PKCα in various cell types. Notably, the FOXC2 transcription factor was recently reported to be an upstream regulator of PDGFRβ in TNBC [102]. These independent reports are suggestive of a functional link between PKCα and FOXC2 in TNBC and TNBC stem cells. Our data demonstrate that in both TNBC and ER-, endocrine resistant cell lines, PKCα is a positive regulator of FOXC2, and this regulation likely involves transcriptional modulation of FOXC2, leading to its overexpression [103]. Indeed, several independent studies have shown that TNBC tumors display elevated expression of FOXC2 compared to non-TNBC tumors [102-104]. Our finding therefore provides a novel underlying mechanism for the induction of FOXC2 expression in TNBC tumors. Lastly, co-expression of PKCα and FOXC2 is significantly associated with poorer relapse-free survival in breast cancer patients of ER-negative, basal intrinsic subtype, as determined by Kaplan-Meier analysis (Figure 3A) [105]. The predictive value of PKCα and FOXC2 co-expression, on the other hand, was not as obvious in ER+ patients (Figure 3B) [105]. When patients were stratified based on treatment received (endocrine versus chemotherapy), the co-expression had no significant value at predicting their relapse free survival.

Similar to what was observed in endocrine-resistant, ER+ breast cancer, expression of Notch receptors, such as Notch-4, and their ligands are elevated in TNBC [106-109]. Inhibition of Notch signaling reduces colony formation and xenograft growth of TNBC cells [107,110]. It will be of great interest to determine whether there is a functional relationship between PKCα and Notch, as observed in ER+ breast cancer cells, also holds true in the TNBC subtype. Therefore, a growing body of evidence highlights PKCα as a therapeutic target in TNBC.

PKCα as a common therapeutic target for ER+, endocrine resistant and TNBC

Hormone resistance remains a significant roadblock to the successful treatment of luminal tumors. One possible mechanism of resistance is the existence of the CSC population [75-78]. Originally identified as cells having CD24+/CD44+/low marker profile, CSCs have the ability to initiate tumors in immunocompromised or syngeneic mice, self-renewal capacity measured by tumor formation in secondary mice, and the capacity to differentiate into the non-self-renewing cells, which constitute the tumor bulk [85]. They do not only possess higher inherent resistance to therapies, but also reportedly thrive with these treatments [75,82,85]. A subpopulation of ER+/PR+/CD44+/cytokeratin (CK) 5+ cells that share the properties of CSCs has been identified in ER+/PR+ breast cancer xenografts [111]. Treatment with tamoxifen or fulvestrant led to selective enrichment of these cells, whereas the population of ER+/PR- cells was decreased [112]. Importantly, Haughian et al revealed that this subpopulation of ER+/PR+/CD44+/CK5+ cells in luminal tumors displays a gene expression profile similar to that of TNBC/ basal tumors, and therefore was named as luminobasal [113]. Intriguingly, ER+/PR+/CD44+/CK5+ cells also depend on Notch signaling for survival and proliferation. These findings suggest that different breast cancer subtypes may rely, albeit to different extents, on similar pathways for their oncogenic behaviors. Here we have described evidence to suggest that PKCα is a promising target for both ER+, endocrine-resistant and basal/TNBC. With the ability to crosstalk to a number of oncogenic pathways, PKCα presents itself as a novel therapeutic target that is functionally important in multiple cell lineages and can be exploited for the effective management of breast cancer. A simplified summary of this crosstalk is depicted in Figure 4.

Several approaches are used to target PKC isozymes therapeutically. These inhibitors either compete for PKCα substrates, ATP, or interfere with the binding of activated PKCα to the membrane [23,114-116]. In general, these compounds do not have selective specificity for PKCα due to a high degree of structural conservation with other PKC isozymes. Past clinical trials with PKC modulators, either by themselves or with other
Figure 3 Patients whose tumors co-express high levels of PKCα and FOXC2 experience significant shorter relapse free survival compared to those whose tumors do not. A. Patients of ER-negative, basal subtype, N=339. B. Patients of ER-positive subtype, N=1802.

Figure 4 Crosstalk between PKCα and several key signaling pathways. References are indicated in brackets.

Anti-cancer modalities were disappointing due to their lack of therapeutic efficacy [23,114-116]. Despite promising preliminary results with the antisense oligonucleotide Aprinocarsen (ISI 3521, LY900003) specifically targeting human PKCα mRNA, no clinical benefit was observed in a small phase II with metastatic breast cancer patients [117,118]. This outcome is multifaceted, and could in part be due to the lack of prior screening for PKCα expression in patients receiving treatment. Further investigation for aprinocarsen was discontinued after a few more unsuccessful trials [119-124].

A relatively more recent attempt to develop selective PKC inhibitors focuses on disrupting the interactions between PKCs and anchoring proteins, which play a critical role in positioning activated PKCs to various cellular compartments, including the cell membrane. Many of these interactions map to the C2, V2, V3 and V5 regions [125] (Figure 1). Pioneer work done by Mochly-Rosen's lab demonstrates that several of them are isozyme-specific and, therefore, can be targeted to achieve selective inhibition [116]. αV5-3 was designed from the V5 region of PKCα based on this rational approach [94]. In vivo administration of this agent completely abrogated metastasis of breast cancer cells to lung [94]. In addition, no signs of toxicity were observed in the animals, making αV5-3 a promising agent for the management of mammary tumor metastasis. Mechanistic studies found that reduction of MMP9 and NF-κB activity was a downstream effect of PKCα inhibition by αV5-3 and contributed to the peptide’s anti-metastasis property [94]. Of interest, another research group found that curcumin and decursin can also inhibit PKCα translocation, and reduce MMP9 and NF-κB activity in breast cancer [126,127]. Curcumin, which is extracted from the plant Curcuma longa, is recognized for its chemopreventive and antitumoral activities against various cancers, including breast [128,129]. Similarly, studies that demonstrate the efficacy of decursin, a pyranocoumarin, have been reported for both TNBC [130,131] and ER+ breast cancer [131]. Whereas curcumin is able to inhibit PKC activation by competing with both phosphatidylserine and calcium [132,133], the exact mechanism...
whereby decorin inhibits PKCα translocation remains to be studied in depth.

Of note, several miRNAs were discovered that target PKCα transcripts [93,134,135]. Among these, decreased miR-200b expression in TNBC was observed, likely contributing to elevated PKCα expression. Ectopic expression of miR-200b drastically reduced TNBC cell migration and inhibited tumor metastasis in an orthotopic mouse mammary xenograft tumor model [93]. Similarly, reduced miR-200b expression was demonstrated to contribute to endocrine resistance in breast cancer cells [136]. Over expression of miR-200b sensitized resistant cells to growth inhibition by tamoxifen [136]. Recent advancement in the field of in vivo miRNA delivery will allow us to therapeutically evaluate and compare the magnitude of the effect mediated by miR-200b in ER+ versus TNBC patients.

CONCLUSION

Clearly, there is a need for a more stringent and clinically relevant evaluation of the significance and prevalence of PKCα expression in breast cancer patients. Regardless, PKCα is a promising therapeutic target for the treatment of various breast cancer subtypes. We are evaluating the expression of PKCα as a biomarker for endocrine therapy response in ER+ breast cancer patients in companion with novel SEM and ShERPA compounds that could offer therapeutic benefit to tamoxifen resistant patients. Specific targeting of PKCα in a cell type- and tissue-specific manner remains a challenge as the high conservation of domain structures among PKC members prevent selective inhibitors of PKCα to be effectively transitioned to the clinic.

REFERENCES

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