Protein Kinase Cε Is a Predictive Biomarker of Aggressive Breast Cancer and a Validated Target for RNA Interference Anticancer Therapy

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Abstract

Tumor metastasis is the major cause of morbidity and mortality in patients with breast cancer. It is critical to identify metastasis enabling genes and understand how they are responsible for inducing specific aspects of the metastatic phenotype to allow for improved clinical detection and management. Protein kinase Cε (PKCε), a member of a family of serine/threonine protein kinases, is a transforming oncogene that has been reported to be involved in cell invasion and motility. In this study, we investigated the role of PKCε in breast cancer development and progression. High-density tissue microarray analysis showed that PKCε protein was detected in 73.6% (106 of 144) of primary tumors from invasive ductal breast cancer patients. Increasing PKCε staining intensity was associated with high histologic grade (P = 0.0206), positive Her2/neu receptor status (P = 0.0419), and negative estrogen (P = 0.0026) and progesterone receptor status (P = 0.0008). Kaplan-Meier analyses showed that PKCε was significantly associated with poorer disease-free and overall survival (log-rank, P = 0.0478 and P = 0.0414, respectively). RNA interference of PKCε in MDA-MB231 cells, an aggressive breast cancer cell line with elevated PKCε levels, resulted in a cell phenotype that was significantly less proliferative, invasive, and motile than the parental or the PKCε-deficient clones were found to have lower RhoC GTPase protein levels and activation. Taken together, these results revealed that PKCε plays a critical and causative role in promoting an aggressive metastatic breast cancer phenotype and as a target for anticancer therapy. (Cancer Res 2005; 65(18): 8366-71)

Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases known to play a critical role in the signal transduction pathways involved in proliferation, differentiation, apoptosis, and migration (1–3). Decades of work on PKCs have clearly shown that PKC isoforms play diverse and complex roles in tumor development and progression. Overexpression of PKCε in rat fibroblasts resulted in transformed cells that exhibited anchorage-independent growth and were able to form tumors in nude mice (4). In contrast, HT29 and SW480 colon cancer cells with PKCε overexpression were less tumorigenic in nude mice resulting in smaller tumors (5, 6). PKCε has been reported to function as a tumor suppressor in rat colonic epithelial cells, but enhances survival and metastatic potential in breast and lung carcinoma cells (7–9). As a whole, these studies provide evidence that different PKC isoforms are involved in tumorigenesis in different tissues and, thus, it is critical to determine the specific PKC isoform that is important for breast cancer development and progression.

PKCε, a novel calcium-independent PKC isoform, has been shown to be a transforming oncogene in fibroblasts and epithelial cells. Overexpression of PKCε in NIH 3T3 fibroblasts and FRC/TEX Cl D rat colonic epithelial cells was shown to increase cell proliferation, enhance anchorage-independent colony formation, and induce a highly tumorigenic in vivo phenotype with tumor incidence of 100% (10, 11). A recent report showed that NIH 3T3 fibroblasts with PKCε overexpression were invasive and displayed a polarized morphology with extended long cellular membrane protrusions (12). FVB transgenic mice with epidermis-specific PKCε expression developed highly malignant and metastatic squamous cell carcinomas (13). Thus, there is increasing evidence in the literature that PKCε specifically promotes a metastatic tumor cell phenotype. However, until now, a direct link between PKCε and breast cancer had not been established. In the present study, our results show that PKCε is crucially involved in establishing an aggressive, invasive, and motile phenotype in breast cancer. Inhibition of PKCε in MDA-MB231 breast carcinoma cells was shown to dramatically decrease tumor growth and metastasis. RhoC GTPase activation was found to be downstream of PKCε signaling and at least partially responsible for PKCε-mediated oncogenesis.

Materials and Methods

Cell lines. MDA-MB231 breast carcinoma cells were generously provided by Dr. Janet Price (the University of Texas M.D. Anderson Cancer Center, Houston, TX) and cultured in MEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Generation of stable siRNA-PKCε MDA-MB231 clones. Double-stranded oligonucleotides (5’-GATCGATCCAAGTCAGCAC-3’) of PKCε were synthesized (Invitrogen, Carlsbad, CA) and cloned into pSilencer2.1-U6 hygro expression vector (Ambion, Austin, TX). A 19 bp scrambled sequence with no significant sequence homology to any known human gene sequences (Silencer negative control 1, Ambion) was cloned into pSilencer2.1-U6 hygro expression vector. Sequencing of small interfering RNA (siRNA)-PKCε and siRNA-control expression vector was done by the University of Michigan DNA Sequencing Core and verified. MDA-MB231 cells
transfected with siRNA-control or siRNA-PKCε using FuGene 6 transfection reagent (Roche-Boehringer Mannheim, Mannheim, Germany). Stable polyclonal and single clone transfectants were established by culturing transfected cells in the described medium supplemented with 100 μg/mL G418 (Life Technologies, Inc., Carlsbad, CA) for 21 days. Protein levels and mRNA expression of PKCε were determined by reverse transcription-PCR (RT-PCR) and Western blot analysis.

Western blot analysis. Whole cell lysates were harvested from cells using radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and 0.3 mg/mL aprotinin; Sigma Chemical Co., St. Louis, MO). Protein lysates (20 μg) were mixed with Laemmli buffer, heat denatured for 3 minutes, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Non-specific binding was blocked by overnight incubation with 2% bovine serum albumin in TBS with 0.05% Tween 20 (Sigma). Immobilized proteins were probed using antibodies specific for PKCε (Upstate Biotechnology, Charlottesville, VA), RhoC GTPase (14), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ).

Cell invasion and random motility assays. Cell invasion was determined as described within the cell invasion assay kit (Chemicon International, Temecula, CA). Cells were harvested and resuspended in serum-free medium. An aliquot (1 × 105 cells) of the prepared cell suspension was added into the chamber and incubated for 48 hours at 37°C in a 10% CO2 tissue culture incubator. Noninvasive cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells were stained and quantified by colorimetric reading at 560 nm. Random cell motility was determined as described from the motility assay kit (Cellomics, Pittsburgh, PA). Cells were harvested, suspended in serum-free medium, and plated on top of a field of microscopic fluorescent beads. After a 48-hour incubation period, cells were fixed and areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified using NIH ScionImager.

Anchorage-independent growth assay. A 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS-supplemented Ham's F-12 medium complete with growth factors, and 1 mL was added to each well of a six-well plate as a base layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 1 × 106 cells in 2.5% FBS-supplemented Ham's F-12/1.5% well./well. A 1 mL layer of medium was maintained on top of the agar to provide nutrients. Colonies 100μm in diameter were counted after 14 days of incubation at 37°C in a 10% CO2, incubator.

Orthotopic animal model of breast cancer. Ten-week-old female athymic nude mice were orthotopically injected with MDA-MB231 cells, siRNA-control clone, or siRNA-PKCε clones (1 × 106 cells) into the upper left mammary fat pad. Cells were trypsinized, washed, and resuspended in HBSS at a density of 1 × 106 cells/200 μL. Mice were anesthetized using 10 mg/mL ketamine, 1 mg/mL xylazine, and 0.01 mg/mL glycopyrrolate, and an incision below the thoracic left mammary fat pad was made. Using a 27-gauge needle, the cell suspension was injected into the exposed mammary gland, suggesting that PKCε levels were increased in normal epithelial cells during the puberty to pregnancy transition of the mammary gland, suggesting that PKCε is involved in the proliferation and differentiation of mammary epithelial cells (17). Moreover, using the pregnancy-dependent mammary tumor GR/A mouse model, progression of pregnancy-dependent mammary tumors to malignant tumors was associated with an increase in PKCε (18). In the present study, the role of PKCε in breast cancer was extensively examined.

Using a high-density tissue microarray, the protein levels of PKCε in 160 consecutive invasive breast carcinomas with long-term follow-up information were determined by immunohistochemistry. Of the 160 invasive carcinomas, 144 cases (n = 406 tissue microarray elements) were available for evaluation in the tissue microarray. PKCε protein was detected in 106 of the 144 (73.6%) primary invasive ductal carcinomas. An increase in PKCε staining intensity was significantly associated with negative estrogen (P = 0.0026) and progesterone receptor status (P = 0.0008), two well-established markers of patient outcome and sensitivities to hormonal therapies, and Her2/neu receptor overexpression (P = 0.0419; Fig. 1). Moreover, high-grade (grade 3) breast tumors were more likely to have higher approval. The tissue microarray contained tissues derived from 160 consecutive patients with invasive carcinomas of the breast, with follow-up information obtained at the University of Michigan from 1987 to 1991. Clinical and pathologic variables were determined following well-established criteria. Estrogen receptor, progesterone receptor, and Her2/neu status were available for most patients. The tissue microarray was constructed as previously described using a tissue arrayer (Beecher Instruments, Silver Spring, MD). Three tissue cores (0.6 mm diameter) were sampled from each block to account for tumor and tissue heterogeneity and transferred to the recipient block. Clinical and treatment information was extracted by chart review done by the surgeon (M.S.S.) with IRB approval.

Immunohistochemistry and scoring of protein kinase Cε protein levels. To test the levels of PKCε in relation to clinical and pathologic features of breast cancer, a 4-μm-thick paraffin-embedded tissue section of the tissue microarray described above was immunostained using a PKCε antibody (dilution 1:100; Upstate Biotechnology). Subsequently, slides were incubated sequentially with biotinylated secondary antibody, avidin-biotin complex, and chromogenic substrate 3,3’-diaminobenzidine. Slides were evaluated for adequacy using a standard bright-field microscope. The majority of array spots contained tissue sufficient for the evaluation. PKCε protein levels were scored by two blinded independent observers using a standard, pathologist-based four-tiered scoring system previously validated as negative (score = 0); weak (score = 1), when there was faint cytoplasmic staining or granular apical staining; moderate (score = 2), when there was diffuse granular cytoplasmic stain; and high (score = 3), when there was diffuse intense cytoplasmic stain (15). Digital images were then acquired using the BLISS Imaging System (Bacus Lab, Lombard, IL).

Statistical analysis. PKCε protein levels were summarized per patient by calculating the median staining score of the patient’s tissue cores. In cases where the median was the midpoint between scoring categories, the higher score was chosen. Associations between the median expression score and clinicopathologic characteristics were determined using the proportional odds model, which correctly models the ordinal expression outcome. Association between the median expression score and overall survival was determined using the product-limit estimator of Kaplan and Meier and the log-rank test statistic. Survival time was constructed from the date of diagnosis until the date of death or last follow-up. Patients known to be alive at their last follow-up were censored. For all analyses, P < 0.05 was considered statistically significant.

Results and Discussion

It is well established that PKCε plays a role in the regulation of proliferation in normal and transformed mammary epithelial cells (16–18). PKCε levels were reported to be increased in normal epithelial cells during the puberty to pregnancy transition of the mammary gland, suggesting that PKCε is involved in the proliferation and differentiation of mammary epithelial cells (17). Moreover, using the pregnancy-dependent mammary tumor GR/A mouse model, progression of pregnancy-dependent mammary tumors to malignant tumors was associated with an increase in PKCε (18). In the present study, the role of PKCε in breast cancer was extensively examined.

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PKC: scores than low-grade (grade 1) breast tumors ($P = 0.0206$). No association was found between PKC: and tumor size ($P = 0.4559$) or nodal status ($P = 0.2680$).

Kaplan-Meier analyses indicated that the disease-free and overall survival rates for patients with tumors expressing moderate/high PKC: (score 2-3) were significantly lower than for patients with tumors with undetectable PKC: (score 0): 33.8% [19.7, 47.9] versus 57% [40.6, 73.4] for disease-free survival (log-rank, $P = 0.0478$) and 36.1% [21.8, 50.4] versus 56.7% [40.2, 73.3] for overall survival (log-rank, $P = 0.0414$), respectively. Moreover, patients with PKC:-positive tumors (score 1-3) had poorer disease-free and overall survival rates than patients with PKC:-negative tumors (score 0); however, these comparisons did not reach statistical significance (log-rank, $P = 0.0874$ for overall survival and $P = 0.1059$ for disease-free survival).

Although PKC: was found to be a significant predictor of patient outcome in bivariate analyses, when adjusting for known prognostic markers such as estrogen or progesterone receptor status using multivariate Cox analyses, PKC: was no longer significantly associated with disease-free and overall survival. This finding may be due, in part, to our small sample size of patients with either moderate/high or undetectable PKC: in our tissue microarray. A larger sample of cases stained for PKC: with clinical outcome information will be needed to definitively assess whether PKC: is an independent marker for overall and disease-free survival.

Our patient outcome data indicate that an elevation of PKC: protein may be sufficient to initiate the transformation of tumor cells to an aggressive phenotype but a protein dosage requirement exists for PKC: to exert its full oncogenic potential. It is intriguing that PKC: was a predictive marker for disease-free survival but was not found to be significantly associated with positive nodes. A number of explanations can be drawn. A possibility is that PKC:-expressing tumors specifically correlate with cells acquiring the ability to preferentially spread hematologically. Alternatively, it is possible that the genetic alteration leading to elevated PKC: levels in the tumor cells is an early event in the metastasis program and that a latency period exists between this event and the physical process of tumor cell metastasis to the lymph nodes. In either of these scenarios, the determination of PKC: levels could potentially aid clinical management decisions as it suggests that patients with tumors that express PKC: but do not have positive nodes might be treated more aggressively as PKC:-positive tumors tend to recur and metastasize. Taken together, our findings indicate that PKC: is associated with worse outcome in breast cancer patients and is a promising biomarker of aggressive breast cancer.

Molecularly targeted therapies have proven to be successful strategies in oncology. Trastuzumab (Herceptin), a Her2/neu inhibitor, in combination with paclitaxel is first-line therapy in Her2/neu positive metastatic breast cancer. Recent approvals of bevacizumab (Avastin), a vascular epidermal growth factor inhibitor, and cetuximab (Erbitux), an epidermal growth factor receptor inhibitor, provide further support that specific targeting of a single gene that is differentially overexpressed in the tumor and responsible for key phenotypic features can be an effective approach for tumor management. In this study, PKC: was found to be elevated in a majority of invasive breast cancer and thus may be a potential target for novel anticancer therapy.

![Figure 1](image)
technology was used to target PKCε in MDA-MB231 breast cancer cells, a cell line that has high endogenous PKCε levels, to determine whether specific inhibition of PKCε will alter the oncogenic phenotype of these cells. RT-PCR showed that mRNA expression of PKCε was suppressed in siRNA-PKCε–transfected MDA-MB231 clones 1 to 3 and polyclonal transfectants (data not shown). Similarly, PKCε protein levels also were significantly lower in these siRNA-PKCε clones than in untransfected or siRNA-control MDA-MB231 cells (Fig. 2). PKCε-deficient MDA-MB231 clones were less proliferative and had decreased capacity to grow in an anchorage-independent manner. Additionally, these PKCε-deficient clones were significantly less invasive and motile than untransfected MDA-MB231 cells or siRNA-control–transfected MDA-MB231 clones. Cell invasion was decreased by 46% to 58% ($P < 0.03, n = 3$) and cell motility was suppressed by 53% to 67% ($P < 0.005, n = 3$) for siRNA-PKCε clones 1 to 3 and polyclonal transfectants ($P < 0.03, n = 3$).

As shown in Fig. 3, the in vivo tumorigenicity and metastatic potential of siRNA-PKCε clone 1 and clone 2 were determined using an orthotopic model of breast cancer. MDA-MB231 and siRNA-control tumor–bearing mice had mean tumor volumes of 1,312 and 1,139 mm$^3$ at 10 weeks posttransplantation, respectively. siRNA-PKCε clone 1 and clone 2 were dramatically less tumorigenic and had mean tumor volumes that were only 10% to 12.5% of siRNA-control ($P < 0.05$). Importantly, incidence of gross lung metastasis was strikingly lower in siRNA-PKCε tumor–bearing mice than in MDA-MB231 or siRNA-control tumor–bearing mice ($P < 0.02$). Only 1 of 20 siRNA-PKCε–bearing mice developed metastatic disease compared with 5 of 7 for MDA-MB231 and 4 of 7 for siRNA control. These observations clearly show that targeted inhibition of PKCε signaling is sufficient to alter the proliferative and metastatic phenotype of MDA-MB231 breast carcinoma cells and, moreover, it results in conditions that are less favorable for the development of highly tumorigenic and metastatic breast carcinomas in vivo.
The downstream signaling pathway used by PKCε to promote a metastatic phenotype is an area of active research. It has been suggested that activated PKCε mediates changes in cell morphology and cytoskeletal remodeling (12). Our study found that RhoC GTPase protein levels were significantly lower whereas mRNA expression remained unchanged in the siRNA-PKCε clone 1 and siRNA-PKCε clone 2 compared with the control. Moreover, these PKCε-deficient clones had a reduction in the amount of activated RhoC GTPase (GTP-bound fraction). These results indicate that PKCε-mediated regulation of RhoC GTPase protein levels was not at the transcriptional level but rather at the translational level.

The Rho GTPase family consists of small, 20 to 30 kDa GTP-binding proteins that are highly conserved throughout evolution in a variety of organisms (22). All aspects of cellular motility and invasion, including cellular polarity, cytoskeletal organization, and transduction of signals from the outside environment, are controlled through interplay between the Rho GTPases (23, 24). Our laboratory has shown that RhoC GTPase is elevated in more than 90% of inflammatory breast cancer, a locally advanced form of breast cancer that is a highly invasive and metastatic tumor specimen compared with 38% of non–inflammatory breast cancer samples (25). Overexpression of RhoC GTPase in immortalized mammary epithelial cells partially recapitulated the inflammatory breast cancer phenotype resulting in cells that are highly angiogenic, invasive, and motile (26, 27). Additionally, RhoC GTPase protein levels were reported to be a potential prognostic marker for small breast tumors (<1 cm) with a propensity to metastasize (14).

Although there is a vast literature on the regulation of the RhoC GTPase during the GDP/GTP cycle, little is known if other regulatory mechanisms are involved in RhoC GTPase activation. Protein phosphorylation, one of the most common types of posttranslational modifications, has been implicated in determining protein stability and function (28–31). PTEN and p21Cip/WAF were shown to be phosphorylated at specific serine/threonine residues resulting in an increase in protein stability (28, 29). ScanProsite analysis of RhoC GTPase amino acid sequence revealed two putative PKC phosphorylation sites, threonine at residue 127 and serine at residue 160. This observation suggests that RhoC GTPase may be a substrate of PKCε resulting in a phosphorylated RhoC GTPase protein that is more stable, leading to more RhoC GTPases being available for activation. A logical extension of this hypothesis is that in PKCε-deficient cells, the protein half-life of RhoC GTPase will not be enhanced because phosphorylation via PKCε will not occur, resulting in cells with lower net amount of RhoC GTPase protein available for activation. This argument is consistent with our observation that steady-state RhoC GTPase protein levels and activation were dramatically lower in the PKCε-deficient cells in comparison with the control cells (Fig. 4). In addition, PKCs have been shown to regulate the activity of Rho guanine nucleotide exchange factors (RhoGEF), Tiam1 and p115RhoGEF, through direct phosphorylation (30, 31). Activation of RhoGEFs leads to the exchange of GDP for GTP, thus activating the Rho GTPases (32). Therefore, an alternative explanation for our results is that the decrease in active GTP-bound RhoC GTPase observed for PKCε-deficient cells may be due to the inability of RhoGEFs to be stimulated via PKCε. Further work will be necessary to examine these possibilities and

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**Figure 3.** RNA interference–mediated inhibition of PKCε reduces in vivo tumor growth and metastasis. MDA-MB231 wild-type, siRNA-control, siRNA-PKCε clone 1, and siRNA-PKCε clone 2 were orthotopically transplanted into the upper left mammary fat pad of 10-week-old female athymic nude mice. Tumors were measured weekly using a microcaliper, and tumor volume was calculated using the formula (length × width²) / 2. *, *P < 0.05. At the end of the experimental protocol, lungs were removed, and gross lung metastasis was counted on the lung surface under ×100 magnification using a dissecting microscope. *P < 0.02.

**Figure 4.** RNA interference–mediated inhibition of PKCε decreases RhoC GTPase protein levels and activation. A, RhoC GTPase mRNA expression. B, RhoC GTPase protein levels. C, RhoC GTPase activation. GST-RBD pull-down assay was used to determine the amount of GTP-bound (active) RhoC. Representative of three independent experiments.
determine the regulatory pathways involved in RhoC GTPase inactivation in PKCα-deficient cells.

In conclusion, PKCε was found to be a predictive biomarker of aggressive breast cancer, and specific disruption of PKCε resulted in a significant inhibition of tumorigenesis and metastasis. Our findings indicate that PKCε may be an excellent target for design of novel anticancer therapies.

References

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