Atypical PKCiota Contributes to Poor Prognosis Through Loss of Apical-basal Polarity and Cyclin E Overexpression in Ovarian Cancer

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Atypical PKC\textsubscript{i} contributes to poor prognosis through loss of apical–basal polarity and Cyclin E overexpression in ovarian cancer

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We show that atypical PKC\textsubscript{i}, which plays a critical role in the establishment and maintenance of epithelial cell polarity, is genomically amplified and overexpressed in serous epithelial ovarian cancers. Furthermore, PKC\textsubscript{i} protein is markedly increased or mislocalized in all serous ovarian cancers. An increased PKC\textsubscript{i} DNA copy number is associated with decreased progression-free survival in serous epithelial ovarian cancers. In a Drosophila in vivo epithelial tissue model, overexpression of persistently active atypical PKC results in defects in apical–basal polarity, increased Cyclin E protein expression, and increased proliferation. Similar to the Drosophila model, increased PKC\textsubscript{i} proteins levels are associated with increased Cyclin E protein expression and proliferation in ovarian cancers. In nonserous ovarian cancers, increased PKC\textsubscript{i} protein levels, particularly in the presence of Cyclin E, are associated with markedly decreased overall survival. These results implicate PKC\textsubscript{i} as a potential oncogene in ovarian cancer regulating epithelial cell polarity and proliferation and suggest that PKC\textsubscript{i} is a novel target for therapy.

epithelial cell polarity | proliferation

Ovarian cancer remains the leading cause of death from gynecological malignancy among women in the U.S. (1). The prognosis for advanced disease has not improved significantly, suggesting that an improved understanding of the genetic aberrations in ovarian cancer is critical to identifying better ways to prevent, diagnose and treat this frequently fatal disease.

Atypical PKC (aPKC) is located at 3q26.2, the most frequent genomic amplicon in ovarian cancer (2), as indicated by array comparative genomic hybridization (3). PKC\textsubscript{i} is the sole catalytic component of the Par3–Par6–aPKC complex, which plays a critical role in the establishment and maintenance of epithelial cell polarity, tight junctions, and adherens junctions (4). In Drosophila, loss of the polarity-determining tumor suppressors Scribble, Discs large, and Lethal giant larvae contributes to tumor formation (5, 6). Importantly, loss of apical–basal cell polarity is required for epithelial–mesenchymal transition (EMT), which is a critical step in cellular motility and invasiveness (7). Loss of polarity also allows several growth factors and receptors, which are normally compartmentalized because of tight junctions in polarized cells, to mediate autocrine cell activation (8, 9). Thus, deregulation of PKC\textsubscript{i}, the key catalytic regulator of the formation and maintenance of polarity and tight junctions, could contribute to the pathophysiology of ovarian cancer.

Materials and Methods

Patients. Primary ovarian cancer patient samples (>80% tumor on histology), normal ovarian epithelium, and information were collected under Institutional Review Board-approved Health Insurance Portability and Accountability Act (HIPAA)-compliant protocols at M. D. Anderson Cancer Center; University of Toronto; Duke University; University of California, San Francisco; and Northwestern University.

Normal ovarian epithelium was obtained by directly scraping ovarian epithelial cells into RNAlater (Ambion, Austin, TX). At least 90% of cells isolated are of epithelial origin, as determined by staining for cytokeratins.

High-Density Array Comparative Genomic Hybridization. Bacterial artificial chromosome (BAC) DNA arrays were prepared and probed as described (3) by using 200 contiguous BAC clones covering 3q26.2q8 centered on 3q26.2 at PKC\textsubscript{i}.

RNA Quantification. Total RNA was extracted from tissue samples by using TRIZol reagent (Invitrogen) according to the manufacturer’s instructions. mRNA levels were determined by TaqMan RT-PCR, using 40 cycles with \(\beta\)-actin as reference.

Tissue Microarray Construction and Immunohistochemical Analysis. Tissue microarrays were generated from paraffin-embedded specimens of 441 cases of epithelial ovarian cancers with outcomes and 85 additional specimens reflecting specific histotypes of tumors at the University of Texas M. D. Anderson Cancer Center. Slides were stained with anti-PKC\textsubscript{i} (1:100, BD Transduction Laboratories), anti-Phospho-PKC\textsubscript{i} (1:300, Abcam), or anti-Cyclin E (HE-12 1:100, Santa Cruz Biotechnology), anti-E Cadherin (1:100, BD Transduction Laboratories), or anti-Ki67 (1:100, DakoCytomation, Carpinteria, CA) antibodies. Staining was detected by streptavidin–biotin–peroxidase and 3,3’-diaminobenzidine. E Cadherin was detected by using FITC-labeled goat anti-mouse antibody (Caltag, Burlingame, CA). Nuclei were stained with DAPI (Sigma). We defined the Ki67 labeling index with >15% as high and ≤15% as low. Cyclin E was judged to be positive when >10% of nuclei stained. Anti-PKC\textsubscript{i} was shown to be specific for PKC\textsubscript{i} by Western blotting of tumor tissue and COS7 cells transfected with plasmids encoding PKC\textsubscript{i} or PKC\textsubscript{\(z\)}. The anti-phospho-PKC\textsubscript{i} antibody crossreacts with phospho-PKC\textsubscript{\(z\)} according to the manufacturer. However, ovarian cancers contain little to no detectable PKC\textsubscript{\(z\)}.

Abbreviations: EMT, epithelial–mesenchymal transition; aPKC, atypical PKC; DapKM, Drosophila atypical protein kinase M; PKC\textsubscript{\(z\)}, persistently active rat PKC\textsubscript{\(z\)}; LMP, low malignant potential; LMW, low molecular weight.

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able PKCζ; thus, the anti-phospho-PKCζ antibody detects primarily phospho-PKCζ.

**Western Blot Analysis.** Western blot analysis was performed as described (10) by using Cyclin E, PKCζ, and Actin monoclonal antibodies (Roche Molecular Biochemicals).

**Fly Stocks.** *Drosophila* atypical protein kinase M (DaPKM) in UAS-DaPKM starts at Met-223 within the hinge region of *Drosophila* PKC (DaPKC) (11). Persistently active rat PKCζ (rPKCζ+) with a 5-aa deletion within the pseudosubstrate domain (residues 117–121) (12) was cloned into the XbaI site of pUAST (13). Eight independent transgenic rPKCζ+ lines gave a similar phenotype. Other stocks were *yw; GMR-GAL4, UAS-GFP* and GMR-GAL4 and GMR-hid-Ala-5 and UAS-p35 and *yw; dpp-GAL4, UAS-GFP/TM6B*.

**Immunohistochemistry and Cell Death Assay of *Drosophila* Imaginal Discs.** Imaginal discs were stained as described (14) with the following antibodies (dilutions): rabbit anti-PKCζ C20 (1:500; Santa Cruz Biotechnology), rat anti-Elav (1:60; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), rabbit anti-Patj (1:400; K. Choi, Baylor College of Medicine, Houston), and mouse anti-BrdUrd (1:50; Becton Dickinson). Donkey Fab fragment secondary antibodies were from Jackson ImmunoResearch. BrdUrd incorporation was for 1 h (14). Apoptosis (TUNEL) was detected by using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis).

**Statistical Analysis.** Experiment results were analyzed with chi-square test of independence, Spearman correlation, Kruskal–Wallis test, Mann–Whitney test, or Wilcoxon rank sum test, as appropriate. Survival rates were calculated by using Kaplan–Meier analysis (15). Differences in survival were analyzed by using the log-rank test and univariate and multivariate Cox proportional hazards models (16). All tests were two-tailed and were considered statistically significant if *P* < 0.05.

**Results**

**Amplification of PKCζ Contributes to Increased PKCζ Expression and Reduced Progression-Free Survival in Ovarian Cancer.** By using a high-density chromosome 3q array comparative genomic hybridization contig, the *PKCζ* gene copy number was increased in >70% of serous epithelial ovarian cancers (Fig. 1a) and was associated with a significantly shorter progression-free survival duration (*P* = 0.0006) (Fig. 1b). Similarly, PKCζ RNA levels were increased in >80% of serous epithelial ovarian cancers, as compared with normal ovarian surface epithelial cells (17, 18), with the magnitude and frequency of PKCζ RNA increases being higher in serous epithelial ovarian cancers than in other histotypes of ovarian cancer and tumor lineages (Fig. 1c). As indicated by TaqMan RT-PCR, PKCζ mRNA levels were markedly increased in advanced (Stage III/IV) ovarian cancers as compared with normal ovarian surface epithelial cells, benign epithelial tumors, or early (Stage I/II) ovarian cancers (Fig. 6a and b, which is published as supporting information on the PNAS web site). Although the magnitude of the RNA increase was consistently greater than the DNA copy number increase, PKCζ DNA and RNA levels were correlated in serous epithelial ovarian cancers (*P* = 0.05, Fig. 6c), indicating that the increase in DNA copy number contributes to the elevated RNA levels.

**Ectopic Expression of Persistently Active aPKC in *Drosophila* Imaginal Eye Discs Results in Loss of Cell Polarity.** We evaluated the potential mechanisms by which increased levels of PKCζ contribute to transformation of epithelial cells by overexpressing two persistently active forms of aPKC in epithelial tissues in the model organism *Drosophila*: (i) DaPKM (11), which produces a naturally occurring active form of DaPKM lacking the Par6-binding site (19) and the aPKC pseudosubstrate site (20), and (ii) rPKCζ+, with a 5-aa deletion within the pseudosubstrate site (12). There is only one aPKC in *Drosophila* (DaPKC), allowing these two constructs to...
represent the effects of PKCs, the aPKC amplified in ovarian cancer. Endogenous DaPKC is an apical cell polarity marker in wild-type eye imaginal discs (21) (Fig. 2 a–c, d–g, and j). Both DaPKM (Fig. 2 h, e, h, and k) and rPKC* (Fig. 2 f, i, and l) eye discs stained for aPKC (red) and Elav (green) are shown. Boxes in a–c indicate areas of magnified views in g–l. Wild-type eye disc (m and p), DaPKM-transgenic eye disc (n and q), and rPKC*-transgenic eye disc (o and r) stained for Pals-associated tight junction protein (Patj) are shown. Lines in planar views (m–o) indicate location of cross-section views in p–r. Anterior is to the left for all discs.

**Fig. 2.** Ectopic expression of persistently active aPKC in Drosophila third-instar larval eye discs causes defects in apical–basal polarity and tissue architecture. Transgenes were expressed in cells posterior to the morphogenetic furrow by using the UAS-GAL4 two-component system (13). Wild-type (a, d, g, and j), DaPKM-transgenic (b, e, h, and k), and rPKC* (c, f, i, and l) eye discs stained for aPKC (red) and Elav (green) are shown. Boxes in a–c indicate areas of magnified views in g–l. Wild-type eye disc (m and p), DaPKM-transgenic eye disc (n and q), and rPKC*-transgenic eye disc (o and r) stained for Pals-associated tight junction protein (Patj) are shown. Lines in planar views (m–o) indicate location of cross-section views in p–r. Anterior is to the left for all discs.

**Persistently Active aPKC Induces Proliferation, Increases in Cyclin E, and Disorganization of Cellular Architecture Without Increasing Apoptosis in Drosophila Epithelial Cells.** In wild-type eye discs, cell proliferation, as indicated by BrdUrd incorporation, was ran-
domly distributed anterior to the morphogenetic furrow, a dorsal–ventral groove marking the boundary of photoreceptor differentiation, arrested in G1 in the furrow (Fig. 3a, arrowhead) and underwent an additional round of cell division referred to as the second mitotic wave posterior to the furrow (Fig. 3a, arrow). Posterior to the second mitotic wave, cells cease proliferation and differentiate into photoreceptor, cone, pigment, and bristle cells (24). Only rare BrdUrd-positive cells were found in the posterior area of wild-type eye discs, where photoreceptor cells express the neuronal marker Elav (25) (Fig. 3 a and g). In contrast to wild-type eye discs, DaPKM- or rPKCζ*-transgenic eye discs showed massive incorporation of BrdUrd posterior to the second mitotic wave (Fig. 3 b and c, asterisk). DaPKM-transgenic (Fig. 3 e and h) and rPKCζ*-transgenic (Fig. 3 f and i) eye discs, in contrast to wild-type eye discs (Fig. 3 d and g), displayed pronounced changes in the spacing, patterning, and size of photoreceptor clusters posterior to the second mitotic wave. In DaPKM-transgenic and rPKCζ*-transgenic eye discs (Fig. 3 k and l), the BrdUrd-positive DNA-synthesizing posterior cells to the second mitotic wave were Elav-negative. Thus, the DNA-synthesizing cells either have lost Elav expression or are nonneural cells. Increased proliferation induced by DaPKM or rPKCζ* was not limited to imaginal eye discs, because there was a dramatic increase in the number of BrdUrd-incorporating cells in transgenic (Fig. 3 q and r), as compared with wild-type (Fig. 3p) wing discs.

In imaginal disc cells, Cyclin E is limiting for S-phase initiation (26). Concurrent with the increase in proliferation, Cyclin E protein levels were dramatically increased in DaPKM-transgenic and rPKCζ*-transgenic eye disc cells posterior to the second mitotic wave (Fig. 3 n and o), as compared with wild-type eye discs (Fig. 3m). Coexpression of the Cyclin E antagonist Dacapo, which is the Drosophila p21CIP/p27kip1 cyclin-dependent kinase inhibitor ortholog, results in amelioration of the DaPKM/rPKCζ* phenotype (data not shown), indicating a critical role of Cyclin E in mediating the DaPKM/rPKCζ* phenotype.

DaPKM-transgenic and rPKCζ*-transgenic eye discs did not show an increase in apoptosis by TUNEL using expression of activated Drosophila proapoptotic Hid as a positive control (Fig. 7, which is published as supporting information on the PNAS web site, and data not shown). Furthermore, expression of p35, a pan-caspase inhibitor, failed to alter the morphological effects of overexpression of DaPKM and rPKCζ* in eye discs (data not presented). Thus, although aPKC increases cell cycle progression, it does not increase apoptosis in Drosophila epithelial tissue.

**PKCζ Protein Is Mislocalized and Overexpressed in Ovarian Cancer.**

Informed by the studies in Drosophila, we assessed whether increased PKCζ DNA and RNA levels in ovarian cancer cells were associated with changes in polarity, Cyclin E expression, and cell proliferation and, furthermore, whether this constellation of effects contributes to the prognosis of epithelial ovarian cancer.

PKCζ was present at the apical membrane and absent from the basal membrane in normal ovarian surface epithelial cells and in benign serous and mucinous cysts (Fig. 4 a, b, and f). In serous low malignant potential (LMP), although PKCζ levels were modestly elevated (Fig. 8, which is published as supporting information on the PNAS web site), membrane localization of PKCζ was lost in >85% (Fig. 4c). As with mRNA levels, PKCζ protein was increased in >85% of low- and high-grade serous epithelial ovarian cancers, as compared with normal ovarian...
surface epithelial cells (Table 1, which is published as supporting information on the PNAS web site). Strikingly, apical membrane location of PKCα was abrogated in all (322) serous epithelial ovarian cancers analyzed (Fig. 4 d and e). Similar to the mRNA data, PKCα protein was increased in a smaller percentage of nonserous ovarian cancers (50%) than serous cancers (Table 1).

In contrast to serous LMP, PKCα was absent from the membrane in only 20% of mucinous LMP tumors. However, PKCα no longer localized to the membrane in 90% of mucinous carcinosomas, 80–90% of clear cell carcinosomas, 60–70% of low-grade endometrioid ovarian carcinosomas, and all high-grade endometrioid ovarian carcinosomas (Fig. 4 f–k). As expected from RNA analysis (Fig. 6 a–c), PKCα protein levels were significantly associated with histotype (P < 0.00001), stage (P < 0.00001), and grade (P = 0.01) (Table 1).

The pattern of localization of the adherens junction marker E-cadherin (27) was concordant with that of PKCα being localized to the apical–lateral membrane domain in serous and mucinous cysts and mucinous LMP, while being predominantly cytoplasmic in serous LMP as well as in low- and high-grade serous and mucinous carcinosomas (Fig. 3, which is published as supporting information on the PNAS web site). This is compatible with the effects of PKCα overexpression in ovarian cancer contributing to aberrant E-cadherin and adherens junction function.

Activated PKCα Is Overexpressed and Mislocalized in the Cytoplasm in Ovarian Cancer. Activated PKCα levels, assessed by using an antibody recognizing the autophosphorylation site of PKCα and thus reflecting PKCα activity, are increased in ovarian carcinosomas as compared with normal ovarian surface epithelial cells and cysts (P = 0.0036) (Fig. 4 l–o). A small group of serous high-grade carcinosomas demonstrated membranous localization of phospho-PKCα (20/376) (Fig. 4o); however, it was mislocalized in all other conditions (Fig. 4 l–n). Similar to total PKCalpha, PKCα activity is an indicator of outcomes with 70/245 (28.6%) patients with low phospho-PKCα protein levels being alive at 5 years vs. 8/58 (13.8%) patients with high phospho-PKCα levels (P = 0.03).

High Levels of PKCα and Cyclin E Protein Contribute to Outcomes in Nonserous Epithelial Ovarian Carcinoma. Based on the effect of the aPKC transgenes on Drosophila epithelia, we assessed the interactions among PKCα, Cyclin E, and Ki67 and their contribution to patient outcomes. Elevated PKCα protein levels were associated with elevated levels of low molecular weight (LMW) forms of Cyclin E (10) protein in 16 of 18 ovarian cancer patient samples (Fig. 5a). In tissue microarrays, PKCα correlated with Cyclin E (using an antibody that recognizes all forms of Cyclin E because antibodies specific to LMW Cyclin E are not available) protein levels (P = 0.01) and proliferation (Ki67 levels, P = 0.02). Ki67 and Cyclin E levels were also highly correlated (P < 0.0001). Four transcriptional profiling data sets comprising a total of 215 ovarian cancer patient samples of mixed histology, grade, and stage demonstrated a direct Spearman correlation [P < 0.001 (in-house data set), P < 0.002 (17), P < 0.05 (28), and P < 0.05 (29)], with a positive linear regression on three of the four data sets [P < 0.01 (in house), and P < 0.05 (28, 29)]. PKCα levels, alone or in combination with Cyclin E levels, were indicative of prognosis in nonserous epithelial ovarian carcinosomas (Fig. 5 b and c). Indeed, nonserous epithelial ovarian carcinosomas with low levels of both Cyclin E and PKCα demonstrated a remarkably good prognosis with almost 90% of patients being alive at 5 years, whereas patients with high levels of both demonstrated a poor prognosis with <20% alive at 5 years. Univariate Cox proportional hazards models (16) showed that patients with nonserous tumors with high PKCα levels had a higher likelihood of death (Table 2, which is published as supporting information on the PNAS web site). This finding is compatible with a previous small study demonstrating an association of PKCα protein levels with outcome (30) and with studies indicating an association of Cyclin E with outcome (10, 31). In a multivariate model that included both PKCα and Cyclin E levels as independent variables, the association between overall survival and PKCα levels remained significant in nonserous epithelial tumors (Table 2). PKCα was either mislocalized or overexpressed in all serous epithelial ovarian carcinosomas, suggesting that the processes normally regulated by PKCα, likely apical–basal polarity, are functionally aberrant in all serous epithelial ovarian carcinosomas. Indeed, supporting this contention, PKCα levels were not predictive of outcomes in serous epithelial ovarian carcinosomas.

Discussion
We show that, in ovarian cancer patients, high PKCα levels correlate with defects in polarity, increased Cyclin E protein expression, and increased proliferation. aPKC levels must apparently be maintained within critical boundaries for the establishment and maintenance of...
epithelial cell polarity, because both increase and loss of aPKC result in defects in apical–basal polarity in Drosophila (our data and refs. 32 and 33). Although the tumor suppressors Discs large, Lethal giant larvae, and Scribble regulate apical–basal polarity, cell survival, and cellular proliferation (34, 35), loss of polarity is not sufficient to induce cellular proliferation, at least in part because of altered cell survival (32, 36). In contrast, overexpression of activated aPKC was sufficient to induce cellular proliferation in Drosophila epithelial tissues, potentially because of a failure of overexpressed aPKC to induce apoptosis.

Many receptors are located in different compartments and are separated by tight junctions or specifically localized to and activated at junctional complexes (8, 9). Under conditions such as wounding, where polarity and junctional complexes are abrogated, an autoimmune reaction between growth factors and receptors contributes to wound healing. In ovarian cancer, the disruption of polarity as a consequence of overexpression and activation of PKCζ could result in aberrant autocrine signaling. Furthermore, polarity defects could cause mislocalization of intracellular signal transduction components (37). Thus, a loss of polarity due to overexpression of PKCζ could directly lead to increased proliferation contributing to tumorigenesis. Loss of E-cadherin, which plays a pivotal role in epithelial organization and suppresses aberrant proliferation (7, 38), from adherens junctions because of aberrant PKCζ activity and subsequent loss of polarity could also contribute to increased proliferation. Indeed, E-cadherin is mislocalized and associated with outcomes in ovarian cancer (39, 40). The tumor suppressor Disseopathies in Drosophila immediately basement membrane attachment of ovarian epithelial cells, thus ensuring correct positioning, emphasizing the critical importance of maintenance of polarity (41).

The Drosophila in vivo epithelial model system informed subsequent human studies demonstrating an interaction between PKCζ and Cyclin E levels and patient outcome. Because overexpression of aPKC is sufficient to increase Cyclin E protein in Drosophila, up-regulation of PKCζ may play a causal role in Cyclin E deregulation in ovarian cancer. Strikingly, LMW forms of Cyclin E and PKCζ were coordinately up-regulated in ovarian cancers. Because the LMW forms of Cyclin E are hyperactive, associated with resistance to p21 and p27 and with genomic instability (10, 42, 43), the interaction between PKCζ and LMW Cyclin E may play a role in the initiation and progression of ovarian cancer as well as in patient outcomes. Although increased Cyclin E levels had been shown to be associated with a worsened outcome in ovarian cancers (10, 31), concurrent analysis of Cyclin E and PKCζ levels provides a superior predictor of outcome in nonseerous ovarian cancers than either alone, indicating an interaction among these two determinants. Cyclin E levels are increased in a number of ovarian cancers without elevated PKCζ, suggesting that additional mechanisms must regulate Cyclin E protein levels. Once again, a convergence in studies in Drosophila and human ovarian cancer may be informative, because Archipelago, which has been demonstrated to regulate Cyclin E degradation in Drosophila, is mutationally inactivated in a fraction of ovarian cancers (44).

PKCζ protein levels and the incidence of PKCζ mislocalization increase with stage and grade, suggesting that PKCζ plays a role in tumor progression. PKCζ contributes to tumor aggressiveness, because high PKCζ protein levels are associated with reduced survival. Taken together, it appears that PKCζ plays a role in the pathophysiology of ovarian cancer contributing to tumor progression and aggressiveness. Thus, PKCζ should be explored as a marker of prognosis, in particular aggressiveness of ovarian cancers, and should be evaluated as a potential therapeutic target.

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