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Astrid M. Eder  
*University of Texas M.D. Anderson Cancer Center*

Xiaomei Sui  
*University of Texas M.D. Anderson Cancer Center*

Daniel G. Rosen  
*University of Texas M.D. Anderson Cancer Center*

Laura K. Nolden  
*University of Texas M.D. Anderson Cancer Center*

Kwai Wa Cheng  
*University of Texas M.D. Anderson Cancer Center*

See next page for additional authors

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Atypical PKC\(\alpha\) contributes to poor prognosis through loss of apical–basal polarity and Cyclin E overexpression in ovarian cancer

Astrid M. Eder\(^1\), Xiaomei Sui\(^1\), Daniel G. Rosen\(^2\), Laura K. Nolden\(^1\), Kwai Wa Cheng\(^1\), John P. Lahad\(^3\), Madhuri Kango-Singh\(^3\), Karen H. Lu\(^4\), Carla L. Warneke\(^5\), Edward N. Atkinson\(^1\), Isabelle Bedrosian\(^1\), Khandan Keyomarsi\(^1\), Wen-lin Kuo\(^1\), Joe W. Gray\(^1\), Jerry C. P. Yin\(^1\), Jinsong Liu\(^1\), Georg Halder\(^3\), and Gordon B. Mills\(^1\)\(^*\)

Departments of \(^1\)Molecular Therapeutics, \(^2\)Pathology, \(^3\)Biochemistry and Molecular Biology, \(^4\)Gynecologic Oncology, \(^5\)Biostatistics and Applied Mathematics, and \(^1\)Experimental Radiation Oncology, M. D. Anderson Cancer Center, University of Texas, 1515 Holcombe Boulevard, Houston, TX 77030; \(^1\)Lawrence Berkeley National Laboratory, 84 One Cyclotron Road, Berkeley, CA 94720; and \(^3\)Departments of Genetics and Psychiatry, University of Wisconsin, 425 Henry Mall, Madison, WI 53706

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We show that atypical PKC\(\alpha\), 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\(\alpha\) protein is markedly increased or mislocalized in all serous ovarian cancers. An increased PKC\(\alpha\) DNA copy number is associated with decreased progression-free survival in serous epithelial ovarian cancers. In a \textit{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 \textit{Drosophila} model, increased PKC\(\alpha\) protein levels are associated with increased Cyclin E protein expression and proliferation in ovarian cancers. In nonserous ovarian cancers, increased PKC\(\alpha\) protein levels, particularly in the presence of Cyclin E, are associated with markedly decreased overall survival. These results implicate PKC\(\alpha\) as a potential oncogene in ovarian cancer regulating epithelial cell polarity and proliferation and suggest that PKC\(\alpha\) 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\(\alpha\) 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 \textit{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\(\alpha\), 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 ~28 Mbp of 3q26-q28 centered on 3q26.2 at PKC\(\alpha\).

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\(\alpha\) (1:100, BD Transduction Laboratories), anti-phospho-PKC\(\alpha\) (1:300, Abcam, Cambridge, MA), anti-Cyclin E (HE-12 1:100, Santa Cruz Biotechnology), and anti-E cadherin (1:100, BD Transduction Laboratories), or anti-Ki67 (1:100, DakoCytoimmun, Carpintia, 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 \(\leq 15\%\) as low. Cyclin E was judged to be positive when >10% of nuclei stained. Anti-PKC\(\alpha\) was shown to be specific for PKC\(\alpha\) by Western blotting of tumor tissue and COS7 cells transfected with plasmids encoding PKC\(\alpha\) or PKC\(\alpha\). The anti-phospho-PKC\(\alpha\) antibody crossreacts with phosphorylated PKC\(\alpha\) according to the manufacturer. However, ovarian cancers contain little to no detect-

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

\(^*\)To whom correspondence should be addressed. E-mail: gmills@mdanderson.org.

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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 (1:100; K. Choi, Baylor College of Medicine, Houston), and mouse anti-BrdUrd (1:50; Becton Dickinson). Donkey Fab segmentary 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 χ² 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 DaPKC 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 PKC, the aPKC amplified in ovarian cancer. Endogenous DaPKC is an apical cell polarity marker in wild-type eye imaginal discs (21) (Fig. 2a–c, d, g, i, and j). DaPKM-transgenic (b, e, h, and k) and rPKCz*-transgenic (c, f, i, and l) eye discs stained for aPKC/aPKM (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 rPKCz*-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 rPKCz*-transgenic (c, f, i, and l) eye discs stained for aPKC/aPKM (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 rPKCz*-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
dermatination, 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 BrdUr-d-positive cells were found in the
posterior area of wild-type eye discs, where photoreceptor cells
express the neuronal marker Elav (25) (Fig. 3b). Only rare BrdUr-d-positive cells were found in the
posterior area of wild-type eye discs, where photoreceptor cells
express the neuronal marker Elav (25) (Fig. 3b, arrow). In contrast to wild-type eye discs, DaPKM-
or rPKCζ-transgenic eye discs showed massive incorporation of BrdUr-d posterior to the
second mitotic wave (Fig. 3b and c, asterisk). DaPKM-
transgenic (Fig. 3e and h) and rPKCζ-transgenic (Fig. 3f and i) eye discs, in contrast to wild-type eye discs (Fig. 3d 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. 3k and l), the BrdUr-d-positive DNA-synthesizing cells
posterior 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 BrdUr-d-incorporating
cells in transgenic (Fig. 3q 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. 3n 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 progres-
sion, 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 constella-
tion 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. 4a, 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 PKC\(\text{e}\) was abrogated in all (322) serous epithelial ovarian cancers analyzed (Fig. 4 d and e). Similar to the mRNA data, PKC\(\text{e}\) protein was increased in a smaller percentage of nonserous ovarian cancers (50%) than serous cancers (Table 1).

In contrast to serous LMP, PKC\(\text{e}\) was absent from the membrane in only 20% of mucinous LMP tumors. However, PKC\(\text{e}\) no longer localized to the membrane in 90% of mucinous carcinomas, 80–90% of clear cell carcinomas, 60–70% of low-grade endometrioid ovarian carcinomas, and all high-grade endometrioid ovarian carcinomas (Fig. 4 f–k). As expected from RNA analysis (Fig. 6 a–c), PKC\(\text{e}\) 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 PKC\(\text{e}\) 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 carcinomas (Fig. 9, which is published as supporting information on the PNAS web site). This is compatible with the effects of PKC\(\text{e}\) overexpression in ovarian cancer contributing to aberrant E-cadherin and adherens junction function.

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

High Levels of PKC\(\text{e}\) and Cyclin E Protein Contribute to Outcomes in Nonserous Epithelial Ovarian Cancer. Based on the effect of the aPKC transgenes on Drosophila epithelia, we assessed the interactions among PKC\(\text{e}\), Cyclin E, and Ki67 and their contribution to patient outcomes. Elevated PKC\(\text{e}\) 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, PKC\(\text{e}\) 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 (\(P < 0.00001\)). 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)]. PKC\(\text{e}\) levels, alone or in combination with Cyclin E levels, were indicative of prognosis in nonserous epithelial ovarian cancers (Fig. 5 b and c). Indeed, nonserous epithelial ovarian cancers with low levels of both Cyclin E and PKC\(\text{e}\) 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 PKC\(\text{e}\) 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 PKC\(\text{e}\) 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 PKC\(\text{e}\) and Cyclin E levels as independent variables, the association between overall survival and PKC\(\text{e}\) levels remained significant in nonserous epithelial tumors (Table 2). PKC\(\text{e}\) was either mislocalized or overexpressed in all serous epithelial ovarian cancers, suggesting that the processes normally regulated by PKC\(\text{e}\), likely apical–basal polarity, are functionally aberrant in all serous epithelial ovarian cancers. Indeed, supporting this contention, PKC\(\text{e}\) levels were not predictive of outcomes in serous epithelial ovarian cancers.

Discussion

We show that, in ovarian cancer patients, high PKC\(\text{e}\) 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 autocrine interaction between growth factors and receptors contributes to wound healing. In ovarian cancer, the disruption of polarity as a consequence of overexpression and activation of PKCα 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 PKCα 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 PKC 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 Displasmatin in Drosophila immediately basememembrane 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 PKCα and Cyclin E levels and patient outcome. Because overexpression of aPKC is sufficient to increase Cyclin E protein in Drosophila, up-regulation of PKCα may play a causal role in Cyclin E deregulation in ovarian cancer. Strikingly, LMW forms of Cyclin E and PKCα 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 PKCα 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 PKCα levels provides a superior predictor of outcome in nonserous ovarian cancers than either alone, indicating an interaction between these two determinants. Cyclin E levels are increased in a number of ovarian cancers without elevated PKCα, suggesting that additional mechanisms should regulate Cyclin E protein levels. Once again, a convergence of studies in Drosophila and human ovarian cancer may be informative, because Archipelago, which has been demonstrated to regulate Cyclin E degradation in Drosophila, is mutational inactivated in a fraction of ovarian cancers (44).

PKCα protein levels and the incidence of PKCα mislocalization increase with stage and grade, suggesting that PKCα plays a role in tumor progression. PKCα contributes to tumor aggressiveness, because high PKCα protein levels are associated with reduced survival. Taken together, it appears that PKCα plays a role in the pathophysiology of ovarian cancer contributing to tumor progression and aggressiveness. Thus, PKCα 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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