PKCη Is a Novel Prognostic Marker in Non-small Cell Lung Cancer

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Abstract. *Background: Novel biomarkers which may serve as therapeutic targets are essential for lung cancer treatment. Here we investigated the prognostic significance of protein kinase Cη (PKCη), a cell cycle regulator involved in tumorigenesis and chemotherapy resistance, in patients diagnosed with non-small cell lung cancer (NSCLC). Patients and Methods: Sixty-three chemotherapy-naïve patients were examined for PKCη by immunohistochemistry and divided into PKCη H-Score tertiles (low, intermediate and high). Time until event (relapse or mortality) within one year was determined using Cochran-Armitage test and Cox proportional hazards regression model. Results: The distribution of patients according to clinical stage 1-4 was: 27%, 5%, 26% and 42%, respectively. PKCη overexpression was associated with advanced stage (p=0.03) and the risk for an event (p=0.045). Patients of the lowest tertile were less likely to experience an event. Conclusion: PKCη is a novel prognostic marker in NSCLC that may predict poor prognosis. The use of PKCη-specific inhibitors in NSCLC may prove valuable.*

Lung cancer is the world's most common and deadliest type of cancer. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of total lung cancer malignancies. Treatment is often ineffective because of late diagnosis and the emergence of radio- or chemotherapy resistance. Tumor node metastasis (TNM) staging and histological grading are useful, but not satisfactory classification systems to predict prognosis.

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Lung tumorigenesis is a multistep process that involves genetic and epigenetic alterations of oncogenes and tumor-suppressor genes. It affects different signal transduction pathways and alters regulation of major cellular functions, such as cell proliferation, differentiation, cell cycle control, survival and apoptosis (1, 2). The protein kinase C (PKC) family of serine/threonine kinases plays an important role in regulation of key cellular processes (3, 4). Alterations in PKC isoform activation and expression have been found in human lung carcinomas (5-7). Moreover, PKC-dependent mechanisms were shown to be involved in lung cell transformation and resistance to chemotherapy (8, 9). The PKC family is divided into three major groups according to structure, activation requirements, tissue distribution and subcellular localization: classical PKCs (α, β1, β2 and γ), novel PKCs (δ, ε, η and θ) and atypical PKCs (ζ and ι) (10). Several PKC isozymes have been identified as being therapeutic targets, and a number of isozyme-selective PKC inhibitors have been developed for clinical use (11, 12). Two PKC isoforms were indicated as prognostic biomarkers in NSCLC: Patients with high PKCι expression were found to be at a higher risk of dying from cancer, independently of stage (13). In addition, PKCε overexpression was detected in more than 90% of NSCLC specimens and its inhibition in NSCLC cells diminished the aggressive phenotype *in vitro*: cells treated with dominant-negative PKCε exhibited lower cell proliferation and anchorage-independent growth (14, 15).

PKCη, a member of the novel PKCs, is primarily expressed in epithelial cells (16). It is implicated in diverse cellular functions, including a role in terminal differentiation (17-21) and in tumor proliferation of transformed cells (22, 23). Recent studies suggested a special role for PKCη in drug resistance and regulation of apoptosis. In several types of tumor, its expression was reported to correlate with drug resistance-associated genes, such as multidrug resistance receptors. It was associated with drug-resistance in patients with breast cancer, ovarian cancer and acute myeloid

Table I. *Patients' demographical, clinical and histological data according to event within one year of diagnosis.*

Variable	All patients n=63	Event n=38	No event n=25	*p*-Value
Male, n (%)	50 (79.4)	33 (86.8)	17 (68.0)	0.07
Age at diagnosis (mean±SD), years	64.94±9.87	64.65±10.84	65.36±8.38	0.79
Ethnicity, n (%)				
Ashkenazi	23 (36.5)	11 (28.9)	12 (48.0)	0.29
Sephardic	26 (41.3)	17 (44.7)	9 (36.0)	
Other	14 (22.2%)	10 (26.3)	4 (16.0)	
Smoking, n (%)	49 (77.8)	31 (81.6)	18 (72.0)	0.37
Histological type, n (%)				
Adenocarcinoma	26 (41.3)	14 (36.8)	12 (48.0)	
Squamous cell	22 (34.9)	15 (39.5)	7 (28.0)	
Large cell	12 (19.0)	9 (23.7)	3 (12.0)	
Bronchoalveolar	3 (4.8)	0 (0.0)	3 (4.8)	
Adenocarcinoma histological grade, n (%)				
Well-differentiated	15 (60)	6 (46.2)	9 (75.0)	0.23
Poorly differentiated	10 (40)	7 (53.8)	3 (25.0)	
Stage, n (%)				
1	17 (27.4)	2 (5.3)	15 (62.5)	
2	3 (4.8)	0 (0.0)	3 (12.5)	
3	16 (25.8)	16 (42.1)	0 (0.0)	
4	26 (41.9)	10 (52.6)	6 (25.0)	
Surgery, n (%)	23 (36.5)	4 (10.5)	19 (76.0)	<0.001
Chemotherapy, n (%)	29 (46.0)	19 (50.0)	10 (40.0)	0.44
Radiotherapy, n (%)	31 (49.2)	24 (63.2)	7 (28.0)	0.01
PKCη tertiles, n (%)				
≤90	21 (33.3)	10 (26.3)	11 (44.0)	
91-150	23 (36.5)	13 (34.2)	10 (40.0)	
≥151	19 (30.2)	15 (39.5)	4 (16.0)	0.12

leukemia blasts (24-26). In A549 lung cancer cells, down-regulation of PKCη resulted in a significant increase in caspase-3 activity and sensitization of these cells to vincristine and paclitaxel (27). In addition, PKCη was implicated in antagonizing the apoptotic response in a number of cancer lines; however, the molecular mechanisms are largely unknown (22, 28-34). We have recently shown that PKCη contributes to the resistance of breast adenocarcinoma MCF-7 cells by inhibiting c-Jun N-terminal kinase (JNK) activity (33).

In the present study, we examined PKCη expression in biopsies from NSCLC patients.

Materials and Methods

Patients. Sixty-three patients with histological diagnosis of NSCLC were included in this study. All patients were diagnosed and treated at the Soroka University Medical Center (SUMC) during 1998-2008. Demographical data, including gender, age and ethnicity was obtained for all patients. Medical records were also reviewed for clinical data, including patient smoking status, types of treatment, and outcome. Pathological data included stage of disease and histological type of cancer. The study was conducted with the approval of the Ethical Review Board of the SUMC.

Histological examination. Hematoxylin and eosin stained slides of our patient's biopsies were reviewed for confirmation of histopathological diagnosis and for selection of adequate specimens for analysis as described previously (35).

Immunohistochemistry. Paraffin-embedded human lung tissue specimens of pre-treatment core biopsies from 63 patients of our study group were stained with PKCη-specific antibody (Santa Cruz Biotechnology, Inc., CA, USA) was performed using the avidin-biotin peroxidase complex method with the Vectastain kit of Vector Laboratories (Burlingame, CA, USA), as described previously (35).

PKCη expression score. The immunohistichemical expression of PKCη was determined by applying a semiquantitative method, incorporating both the intensity and the distribution of specific staining (35, 36). All tumor cell areas in each slide were evaluated for their intensity of staining with PKCη-specific antibody, according to the following four category scale: 0=no staining, 1=weak staining, 2=moderate staining and 3=intense staining. The percentage of tumor cells stained within each category of intensity was then determined. For each tissue, a value designating the H-Score was derived by summing the percentages of cells stained at each intensity (Pi) multiplied by the weighted intensity of staining; H-Score=Σ Pi(i+1), where: i=1, 2, 3 and Pi ranges from 0 to 100%. For statistical purposes, PKCη H-Score values were divided into tertiles: the first tertile included patients with low PKCη staining

Table II. *Patients' demographic, clinical and histological data according to PKCη expression within one year of diagnosis.*

Variable	Tertiles			*p*-Value
	1st	2nd	3rd	
Male, n (%)	16 (76.2)	20 (87.0)	14 (73.7)	0.52
Age at diagnosis (mean±SD), years	63.62±10.88	66.87±9.44	64.06±9.34	0.50
Ethnicity, n (%)				
Ashkenazi	8 (38.1)	11 (47.8)	4 (21.1)	0.21
Sephardic	7 (33.3)	9 (39.1)	10 (52.6)	
Other	6 (28.6)	3 (13.0)	5 (26.3)	
Smoking, n (%)	14 (66.7)	19 (82.6)	16 (84.2)	0.33
Histological type, n (%)				
Adenocarcinoma	8 (38.1)	7 (30.4)	11 (57.9)	0.22
Squamous cell	6 (28.6)	11 (47.8)	5 (26.3)	
Large cell	5 (23.8)	4 (17.4)	3 (15.8)	
Bronchoalveolar	2 (9.5)	1 (4.3)	0 (0.0)	
Adenocarcinoma histological grade, n (%)				
Well-differentiated	6 (75.0)	3 (42.9)	6 (60.0)	0.46
Poorly differentiated	2 (25.0)	4 (57.1)	4 (40.0)	
Stage, n (%)				
1	7 (33.3)	8 (34.8)	2 (11.1)	0.03
2	0 (0.0)	3 (13.0)	0 (0.0)	
3	4 (19.0)	7 (30.4)	5 (27.8)	
4	10 (47.6)	5 (21.7)	11 (61.1)	
Surgery, n (%)	7 (33.3)	11 (47.8)	5 (26.3)	0.34
Chemotherapy, n (%)	12 (57.1)	9 (39.1)	8 (42.1)	0.45
Radiotherapy, n (%)	10 (47.6)	12 (52.2)	9 (47.4)	0.94
Event in one year, n (%)	10 (47.6)	13 (56.5)	15 (78.9)	0.12
Death in one year, n (%)	10 (47.6)	10 (43.5)	12 (66.7)	0.31

with H-Score values in the range of 0-90; the second tertile included patients with intermediate PKCη staining, with H-Score values in the range of 91-150; the third tertile included patients with high PKCη staining, with H-Scores above 150.

Statistical analysis. An event was defined as death of a patient or lung cancer relapse within twelve months of diagnosis; all patients with stage 4 disease were regarded as relapsed disease to begin with, so that an event in this group of patients was defined as death. Patients alive at the end of the follow-up period or those who died throughout it of any cause other than lung cancer were censored from the cumulative survival calculation. Quantitative and qualitative variables are reported with the use of descriptive statistics. The SPSS statistical program, version 16.01 (SPSS Inc, Chicago, IL, USA) was used for data extraction and analysis. Student's *t*-test and the Wilcoxon rank sum test were used to formally test normally and non-normally distributed continuous variables, respectively. The Cochran-Armitage test for trend was used to compare risk ratios for primary outcome. The Kruskal-Wallis test was used to compare groups of non-parametrical variables. The Cox-proportional-regression model was used to compare groups of different PKCη H-Score values (in tertiles) and events. The model included age, stage, histology and PKCη H-Score values. Stepwise forward regression models with a stay criterion of 0.10 were used. Kaplan–Meier analysis with the log-rank test was applied to compare survival between the groups. A *p*-value of ≤0.05 (two-sided) was considered significant.

Results

Patients' characteristics. Table I describes the characteristics of the 63 patients included in the present study, with regard to relapse or death within one year of diagnosis. The study group consisted of 20 (32%) patients diagnosed at early clinical stages 1+2, 16 (26%) diagnosed at clinical stage 3 and 26 (42%) diagnosed at clinical stage 4. Primary outcome of relapse or mortality within one year of diagnosis was observed in 38 (60%). Table II describes demographic and clinical data of patients according to PKCη H-Score tertiles. The distribution of patients according to tertiles was: 21 (33%) in the first tertile, 23 (37%) in the second tertile and 19 (30%) in the third tertile.

Immunohistochemistry of patient's biopsies. The staining intensity of PKCη was assessed using the H-Score method. Most of the staining was in the cytoplasm, while the plasma membrane and nuclear envelope exhibited almost no staining. Figure 1 shows tumors with different degrees of staining for PKCη, demonstrating tumors with negative, low, intermediate and high staining.

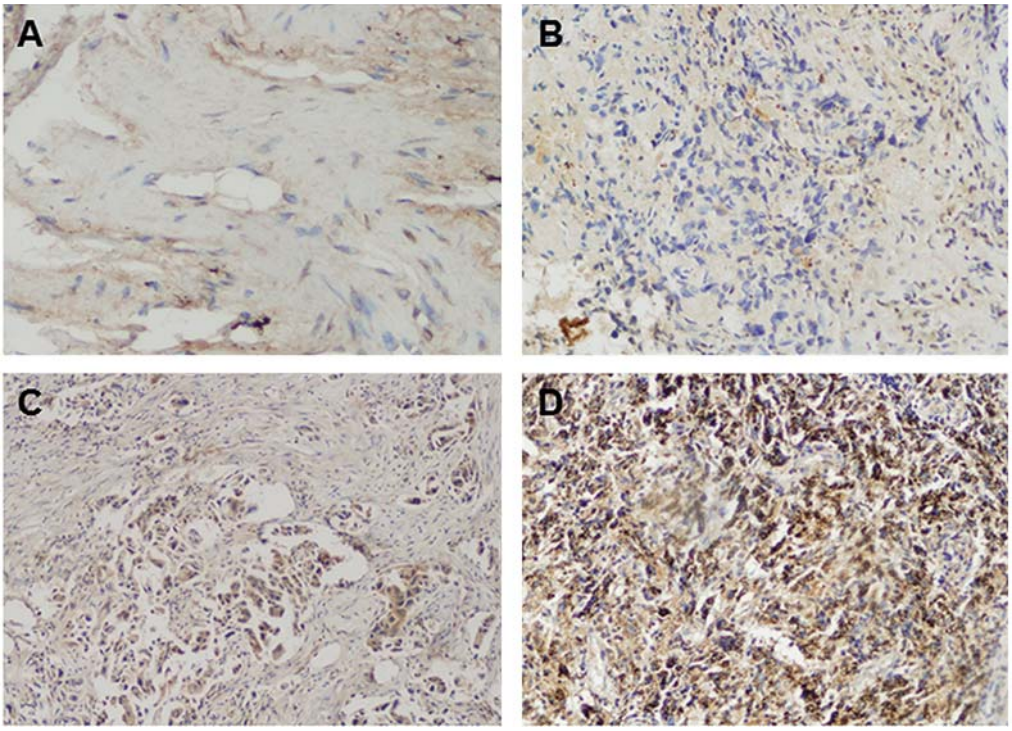

Figure 1. *Protein kinase Cη (PKCη) staining in non-small cell lung cancer (NSCLC) biopsies according to H-Score values. Core biopsies were stained by the labeled avidin-biotin technique using an anti-PKCη specific antibody. Cytoplasmic protein variation in tumors was assessed by a semiquantitative method (H-Score) based on stained cell number and intensity. A: H-Score=0 (no staining), ×20. B: H-Score=110 (intermediate staining), ×20. C: H-Score=190 (high-intermediate staining) ×20. D: H-Score=240 (highest staining in the study group) ×40.*

PKCη distribution according to stage at diagnosis. As shown in Figure 2, lung cancer patients with clinical stage 4 disease had higher PKCη H-Score values compared to patients with disease at earlier stages. The Kruskal-Wallis test showed that PKCη expression increased with disease progression (p=0.03). Stages 1 and 2 were merged into one group for purposes of statistical convenience.

Risk for an event within one year of diagnosis according PKCη expression. The risk for an event in one year according to PKCη H-Score values was evaluated using the Cochran-Armitage test for trend (Figure 3). Patients exhibiting high PKCη expression were found to be at a greater risk for an event compared to patients with low expression (p=0.045).

Multivariate survival analysis of the primary event. The variables included in the multivariate model for prediction of an event within one year of diagnosis were stage (hazard ratio=2.36, p<0.001) and PKCη tertiles (hazard ratio=1.45, p=0.06). Patients with low H-Scores (first tertile) were less likely to experience an event within one year compared to patients with intermediate and high H-Scores (second and third tertiles) (Figure 4).

Discussion

In the present study, we show that the expression of PKCη in patients with NSCLC correlates with poor prognosis within one year of diagnosis. High H-Score values were in correlation with the stage of disease progression. Patients with high PKCη expression exhibited greater risk for an event compared to patients with intermediate and low PKCη expression. Accordingly, survival rates were better for patients with low PKCη H-Scores. Thus, PKCη expression may be of prognostic value and identifies groups of patients with tumors of a more aggressive biology.

PKCη is primarily expressed in epithelial tissues including skin, heart, and lung (16). It was also found to be activated by cigarette smoke extracts (37). Here we show that PKCη overexpression in lung cancer could be a biomarker for clinical outcome. Its expression was in positive correlation with disease/stage progression and with a tendency for increased relapse or death of NSCLC patients within one year of diagnosis. Moreover, PKCη overexpression was of significant predictive value for risk of an event. Although our study group was not large enough, PKCη expression was in correlation with poor survival within one year of diagnosis, irrespective of clinical stage; patients with low PKCη

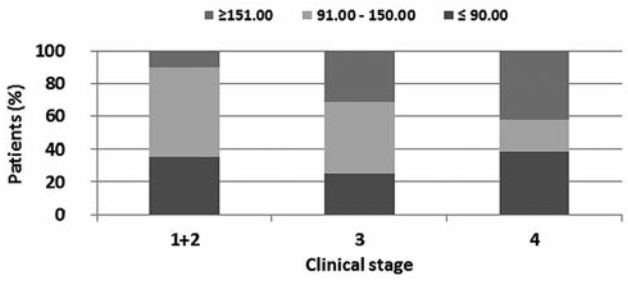

Figure 2. *Expression of Protein kinase Cη (PKCη) increases with disease progression. Patients were divided according to the stage of the disease at diagnosis and the percentage of patients in each tertile was determined as described in the Materials and Methods (p=0.03). Clinical stages 1 and 2 were merged due to small study group in stage 2.*

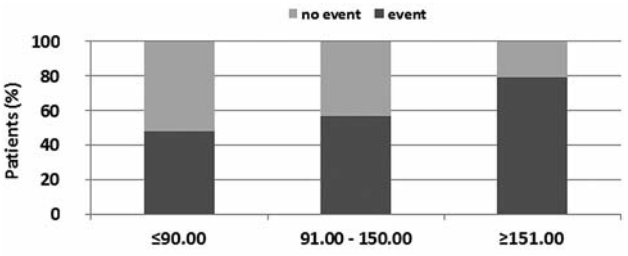

Figure 3. *The risk for an event within one year of diagnosis according to Protein kinase Cη (PKCη) expression. A significant positive correlation was found between PKCη expression and the risk for an event within one year of diagnosis using the Cochran-Armitage test (p=0.045).*

expression had better prognosis compared to patients with intermediate and high PKCη expression. The fact that PKCη expression is associated with tumor aggressiveness in lung cancer patients is in agreement with its reported role in promoting cell proliferation; in MCF-7 adenocarcinoma and glioblastoma cells, its expression correlated with enhanced growth rates (23, 39). In some of these studies, its mechanism of action appeared to be through modulation of cell-cycle components (21, 40) and association with the complex of cyclin E and the cell cycle-dependent kinase 2 (CDK2) (41). PKCη elevated expression was also found to be associated with tumor progression of renal cell carcinoma (42) and with hyperplasia in prostate carcinoma (43). In breast cancer, positive correlation was shown between PKCη overexpression and invasion to lymph nodes (38). Our results suggest that PKCη plays an important role in tumor and metastasis progression, although the underlying molecular mechanism is currently unknown. Indeed, here we show that PKCη expression increases with stage progression and predicts lower survival rates. This suggests its role during late clinical stages. This is unlike PKCι which was reported to predict risk of relapse in early stages (13). It is therefore possible that different PKC isoforms affect different stages of NSCLC.

Previous studies have shown that PKCη confers protection against DNA damage induced by chemotherapeutic agents (33, 34, 44). Moreover, we have recently shown that increased expression and localization of PKCη in cellular membranes correlated with poor clinical outcome in patients with locally advanced breast cancer undergoing neoadjuvant chemotherapy (consisting of cyclophosphamide, doxorubicin and fluorouracil, CAF protocol) (45). It is therefore possible that the increased risk for an event seen in the present study for patients with high H-Scores resulted from the resistance to chemotherapy conferred by PKCη. Accordingly, the better prognosis during the first 12 months after diagnosis, seen in

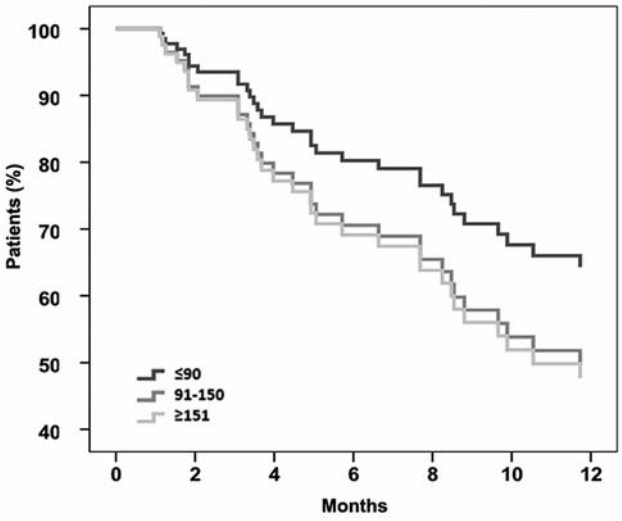

Figure 4. *The risk for an event decreases in patients with tumors exhibiting low PKCη expression. The variables included in the multivariate model for prediction of an event within one year (relapse or death) were stage (hazard ratio=2.36, p<0.001) and PKCη expression (hazard ratio=1.45, p=0.06), as described in the Materials and Methods.*

patients exhibiting low PKCη expression, could be related to their greater response to chemotherapy. Thus, our findings suggest that PKCη may serve as a predictive factor for patients who will benefit from chemotherapy, especially in the setting of patients with early lung cancer and adjuvant chemotherapy.

Our study suggests that PKCη could be a novel prognostic biomarker in patients with NSCLC. Its expression correlates with the stage of the disease. Once diagnosed with lung cancer, PKCη may predict outcome; patients of the third tertile were at greater risk for an event within one year of diagnosis. When adjusted for stage, patients with PKCη

overexpression tended to exhibit an increased risk of death within the first year after diagnosis. A larger study group is needed in order to confirm this observation. Due to the biological importance of PKCη for tumor progression and resistance, the use of specific kinase inhibitors may potentially benefit patients with NSCLC.

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