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High levels of phosphatase and tensin homolog expression predict favorable prognosis in patients with non-small cell lung cancer

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Abstract. – OBJECTIVE: The prognostic role of phosphatase and tensin homolog (PTEN) in non-small cell lung cancer (NSCLC) has been controversial.

PATIENTS AND METHODS: In this study, levels of PTEN expression were investigated in NSCLC patients and their prognostic value in NSCLC was assessed. PTEN expression in tumor tissues from 68 NSCLC patients was analyzed using immunohistochemistry and confocal microscopy. Survival analysis was performed using the log-rank test and Cox proportional hazards regression analysis.

RESULTS: NSCLC patients classified as expressers of high levels of PTEN (n = 46) had better prognoses than those classified as expressers of low levels (mean survival 17.1 versus 12.9 months, log-rank $p = 0.038$). In patients with adenocarcinoma (AC), high PTEN expression (n = 9) was associated with significantly longer survival than low PTEN expression (mean survival 23.50 versus 15.54 months, log-rank p = 0.043). High levels of PTEN expression resulted in 43% reduction in risk for all NSCLC patients (HR = 0.57, 95% CI: 0.33-0.98, $p = 0.041$). PTEN expression and clinical stage remained significantly associated with survival after adjustment for age, sex and tumor type (HR = 0.56, 95% CI: 0.32-0.99; $p = 0.048$; HR = 0.54, 95% CI: 0.36-0.97; $p = 0.045$). No significant difference in continuous PTEN expression levels was observed among groups with different clinical or pathological characteristics ($p > 0.17$). When levels of PTEN expression were binarized using the optimal cutpoint, higher levels of PTEN expression were observed in patients with T1/T2 than in those with T3/T4 (80% and 58% respectively, $p = 0.049$) and in patients with AC than in those with squamous-cell carcinoma (SCC) (78% and 58% respectively, $p = 0.08$). No significant difference in binarized PTEN expression levels was found among groups with any other clinical/pathologic characteristic ($p > 0.28$).

CONCLUSIONS: Our results suggest that high levels of PTEN expression may be favorable prognostic marker in NSCLC patients.

Key Words:
Phosphatase and tensin homolog, Non-small cell lung cancer, Prognosis, Adenocarcinoma, Squamous-cell carcinoma.

Introduction

The molecular basis of lung cancer is very heterogeneous, and multiple factors, such as genetics, epigenetics, changes in protein expression, may affect lung cancer diagnosis, prognosis, and treatment[1]. Lung cancers development is a multistep process that involves activation of growth-promoting proteins, such as v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), BRAF, MEK-1, HER2, MET, ALK, and rearranged during transfection (RET), and inactivation of tumor suppressor genes, such as P53, phosphatase and tensin homolog (PTEN), and LKB-1[2].

Non-small cell lung cancer (NSCLC) is a common malignancy that accounts for 85-90% of lung cancers and has a 5-year survival rate of less than 20%[3]. The two most common NSCLC histological subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC), are associated with distinct molecular abnormalities[4]. In human NSCLC, activated *KRAS* mutations occur in 25%

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of ACs but are exceedingly rare in SCCs[5]. In contrast, phosphoinositide-3-kinase (PI3K) amplification/mutation and loss of PTEN occur in 35-45% of SCC cases but only 5-10% of AC cases[6,7].

PTEN, located at chromosome 10q23.3, is a tumor suppressor gene[8,9]. Somatic mutation or loss of heterozygosity (LOH) of PTEN has been reported in a variety of malignant neoplasms, such as melanomas, breast, prostate, renal, and endometrial carcinomas[10-15]. Germ-line mutations of *PTEN* have also been found in two hamartoma disorders, the Cowden and Bannayan-Riley-Ruvalcaba syndromes[16,17]. PTEN encodes a lipid and protein phosphatase that inhibits the PI3K/Akt/mTOR signaling pathway by dephosphorylating PI-(3,4,5)-triphosphate[18]. Inactivation of PTEN tumor suppressor gene function leads to unrestricted activation of Akt/protein kinase B that is independent of ligand binding[18]. Mutations of *PTEN* occur in only about 5% of NSCLCs[19]. However, they are more common in SCCs than in ACs, and are associated with a history of smoking. In contrast, reduced PTEN protein expression that has been reported in different tumor types occurs in about 75% of NSCLCs[20].

The potential of PTEN expression levels to serve as a marker to predict survival of NSCLC patients has been assessed by immunohistochemistry. However, recent studies reported contradictory results, demonstrating varying degrees of correlation between PTEN expression and NSCLC outcome[20-24]. Therefore, further insight into the role of PTEN in NSCLC may provide important prognostic and predictive information. In the present study, levels of PTEN expression were investigated in NSCLC patients, and their prognostic impact on a cohort of primary NSCLC patients was assessed.

Patients and Methods

Patients and Clinical Characteristics

Primary NSCLC tumors and normal lung tissues (from the same resection specimen) were obtained from 68 patients (55 men and 13 women with median age of 64 years) who had undergone surgery at Beijing Chest Hospital, Capital Medical University between December 2004 and August 2006. Data regarding stage (as defined by the TNM system), differentiation, and histological type of NSCLC tumors (as defined by the World Health Organization (WHO) classification for NSCLC) are listed in Table I[25]. All patients were treatment-naïve prior to tumor resection (or acquisition of surgical biopsies for stage IV patients). The average follow-up time was 15.8 ± 9.5 months (median 12.5, range 3.6-40.6). All subjects were Han Chinese from northern China. The study protocol was approved by the Institutional Review Board of Beijing Chest Hospital, and written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study.

Immunohistochemistry and Confocal Microscopy

Resected tissues were fixed with acetone, embedded in paraffin and sectioned into 4-6 µm-thick slices. Slides were incubated at 60°C for 60 min to improve fixation, deparaffinized three times with xylene for 15 min, and gradually rehydrated with 100% ethanol for 5 min, 95% ethanol for 5 min, 90% ethanol for 5 min, and 85% ethanol for 5 min. Tissue antigens were retrieved by pressure cooking for 90 s in citric antigen retrieval solution containing 10mM Citric Acid and 0.05% Tween 20, pH 6.0. Slides were blocked with 5% bovine serum albumin (BSA) for 2 h at room temperature and incubated with the rabbit monoclonal PTEN primary antibody (1:150 dilution, Abcam, Cambridge, UK) in 5%BSA overnight at 4°C. This was followed by a 2 h incubation with the FITC-labeled goat anti-rabbit IgG secondary antibody (1:150 dilution, Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China) in 5% BSA. Cell nuclei were visualized by Hoechst 33342 staining (1:150 dilution) in 5% BSA at room temperature for 30 min.

A laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan) was used to measure the fluorescent signal, and the data were analyzed using FV-10-ASW3.0VIEWER software (Olympus). Five microscopic fields were randomly selected for each tissue slide. The final PTEN fluorescence value was calculated by averaging the mean fluorescence measurements of 5 slides.

Statistical Analysis

Unpaired *t*-tests and analysis of variance (ANOVA) were used to compare continuous PTEN expression levels among groups with different clinical and pathological characteristics. Chi-square test or Fisher's exact test was used to compare binarized PTEN expression levels

Table I. Clinical characteristics and PTEN expression in patients with NSCLC.

Characteristic	N (%)	PTEN (mean±SD)	*p* value*	*p* value†
Age				
<65 years	38 (55.9)	77.4 ± 15.8	0.37	0.88
≥65 years	30 (44.1)	80.7 ± 13.3		
Sex				
Male	55 (80.9)	79.1 ± 15.7	0.80	0.52
Female	13 (19.1)	77.9 ± 10.0		
Tumor type				
Adenocarcinoma	32 (47.1)	78.8 ± 10.9	0.98	0.08
Squamous-cell carcinoma	36 (52.9)	78.9 ± 17.6		
Primary tumor stage				
T1-2	30 (44.1)	81.6 ± 12.2	0.17	0.049
T3-4	38 (55.9)	76.7 ± 16.3		
Regional lymph nodes				
N0	28 (41.2)	81.4 ± 12.8	0.23	0.28
N1-3	40 (58.8)	77.1 ± 15.9		
Distant metastasis				
M0	64 (94.1)	79.1 ± 15.0	0.54	0.59
M1	4 (5.9)	74.4 ± 10.1		
Clinical stage				
I-II	19 (27.9)	81.2 ± 13.4	0.43	0.51
III-IV	49 (72.1)	78.0 ± 15.3		
Postoperative stump				
Negative	56 (82.4)	79.2 ± 15.6	0.70	1.00
Positive	12 (17.6)	77.4 ± 10.4		
Involved lymph nodes				
0	28 (41.2)	81.3 ± 12.8	0.36	0.55
1-3	27 (39.7)	75.7 ± 15.9		
4-7	13 (19.1)	80.0 ± 16.2		

SD, standard deviation.
*p value is given for t-test for comparisons of continuous PTEN expression levels between 2 groups and for analysis of variance (ANOVA) for comparison between 3 groups.
†p value is given for chi-squared test or Fisher's exact test for comparisons of binarized PTEN expression levels among groups. The statistically significant p value ($p < 0.05$) is in boldface, and the trending p value is in italics.

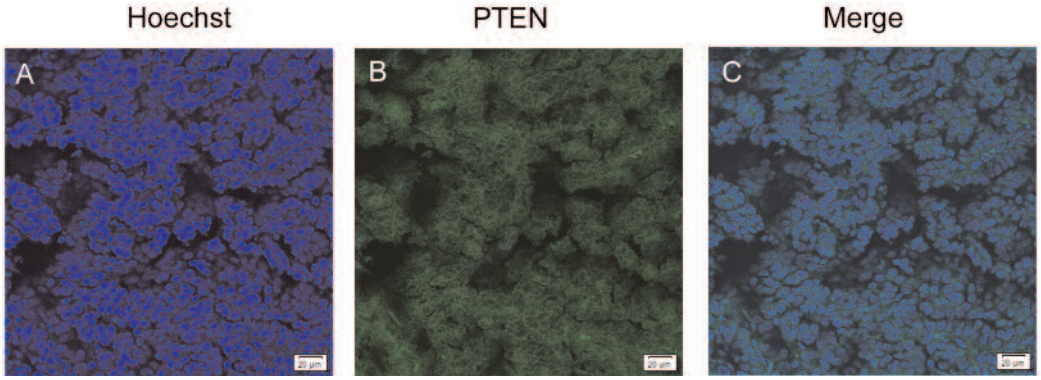
among groups with different clinical and pathological characteristics. The X-tile software was used to determine the optimal PTEN expression cut-point as previously described[26]. Survival curves were constructed using the Kaplan-Meier method and differences in survival were analyzed using the log-rank test. Cox proportional hazards regression analysis was used to determine which independent factors had a combined significant impact on overall survival. In Cox multivariate analysis, age was analyzed as a continuous variable, and sex, tumor type, clinical stage, and PTEN expression were analyzed as categorical variables. All p values were based on two-sided testing and differences were considered significant at $p < 0.05$. All statistical analyses were performed using the SAS software (ver. 9.1.3; SAS, Cary, NC, USA).

Results

PTEN Expression in Normal and NSCLC Lung Cells

First, we evaluated the expression of PTEN in healthy lung tissue and in lung cells of NSCLC patients by immunohistochemistry using anti-PTEN antibody. In normal lung cells we detected low levels of PTEN expression, localized in the cytoplasm (Supplementary Figure 1B), while no PTEN expression was observed in the nuclei as indicated by the merged images (Supplementary Figure 1A,C). As compared to normal lung cells, NSCLC cells exhibited markedly increased expression of PTEN in the cytoplasm (Supplementary Figure 2).

We next evaluated PTEN expression in lung cells of patients with two NSCLC histological

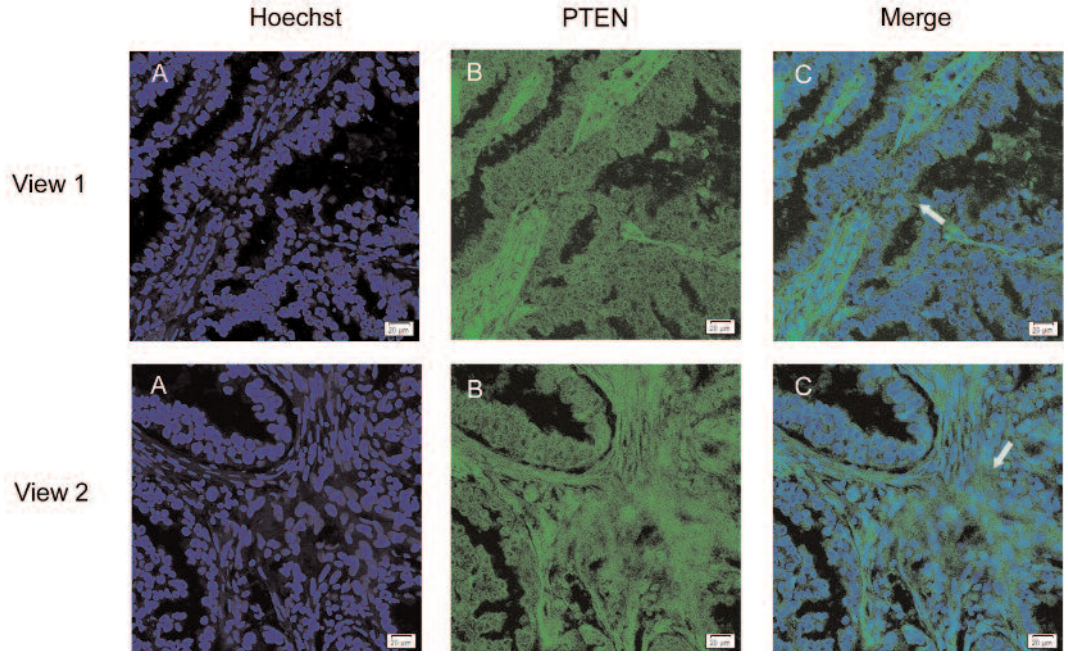


Supplementary Figure 1. Confocal microscopy of PTEN expression in tumor tissues of patients with AC and SCC. PTEN expression levels in paraffin-embedded sections of resected lung tumor tissue were detected by immunohistochemistry. ***A,*** Nuclear staining with Hoechst 33342. ***B,*** FITC-PTEN staining of the cytoplasm of NSCLC cells (bright green). ***C,*** Merged image of nuclear and PTEN staining. Arrows show cytoplasmic localization of PTEN in the cells.

subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC). In agreement with previous observations, PTEN was predominantly expressed in the cytoplasm (Figure 1B), but not in the nuclei of both AC and SCC cells (Figure 1C). As indicated by total fluorescence measurement, NSCLC cells had markedly higher levels of PTEN expression compared to normal lung cells (79.20 ± 1.82 versus 11.34 ± 1.05, $p = 0.004$). Average PTEN expression levels were 85.0 in AC cells and 74.0 in SCC cells (Table I).

Patient Clinical Characteristics and Correlation with PTEN Expression

We next binarized PTEN expression using the optimal cutpoint of 74.0 to further analyze its correlation with clinical/pathologic characteristic of the patients, such as age, sex, tumor type, primary tumor, clinical stage, regional lymph nodes, distant metastasis, postoperative stump, and involved lymph nodes. As summarized in Table I, higher levels of PTEN expression (> 74.0) were observed in patients with T1/T2 than in those



Supplementary Figure 2. Confocal microscopy of PTEN expression in tumor tissue of one patient with AC. ***A***, Nuclear staining using Hoechst 33342. ***B***, Cytoplasm localization of FITC-PTEN (bright green). ***C***, Merged nuclear and PTEN staining. Two fields of view were chosen randomly.

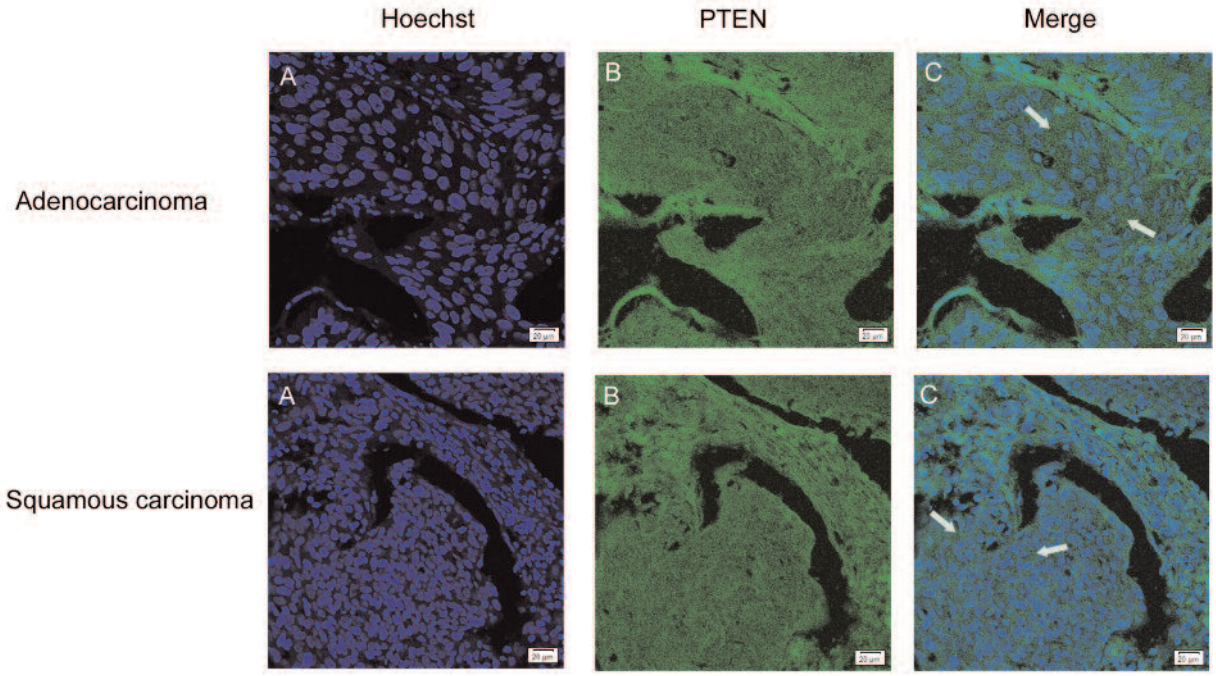


Figure 1. Confocal microscopy of PTEN expression in tumor tissues of patients with AC and SCC. PTEN expression levels in paraffin-embedded sections of resected lung tumor tissue were detected by immunohistochemistry. ***A,*** Nuclear staining with Hoechst 33342. ***B,*** FITC-PTEN staining of the cytoplasm of NSCLC cells (bright green). ***C,*** Merged image of nuclear and PTEN staining. Arrows show cytoplasmic localization of PTEN in the cells.

with T3/T4 (80% versus 58%, $p = 0.049$) and in patients with AC than in those with SCC (78% versus 58%, $p = 0.08$). No significant correlation between PTEN expression and any other clinical/pathologic characteristic were observed among groups ($p > 0.28$; Table I).

Correlation Between PTEN Expression and Survival

Expression levels of PTEN in cells directly correlated with the longer survival of the NSCLC patients as indicated by Kaplan-Meier survival curves of 68 NSCLC patients participating in the study. NSCLC patients who were classified as expressing high levels of PTEN (n = 46) had a better prognosis than those expressing low levels (mean survival of 17.1 versus 12.9 months, log-rank $p = 0.038$; Figure 2). In patients with AC, high PTEN expression (n = 9) was associated with significantly longer survival than low PTEN expression (mean survival of 23.50 versus 15.54 months, log-rank $p = 0.043$) (Figure 3), whereas no such differences were detected in patients with SCC (data not shown). Cox univariate analysis with binarized PTEN expression levels revealed that high levels of PTEN expression were associated with a 43% reduction in risk of mortality for all NSCLC patients (HR = 0.57, 95% CI: 0.33-0.98, $p = 0.041$).

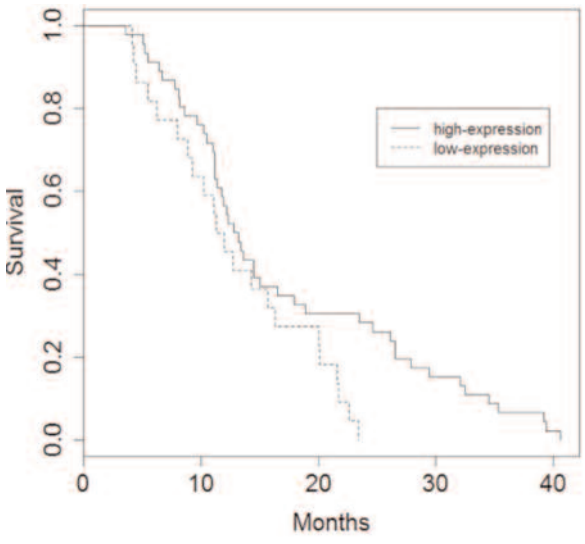


Figure 2. Kaplan-Meier survival curves for 68 NSCLC patients. The optimal PTEN expression cutpoint of 74.0 determined by X-tile software was used to divide the patients into high-expression (PTEN > 74.0) and low-expression (PTEN < 74.0) groups. $p = 0.038$ (obtained from the log-rank test).

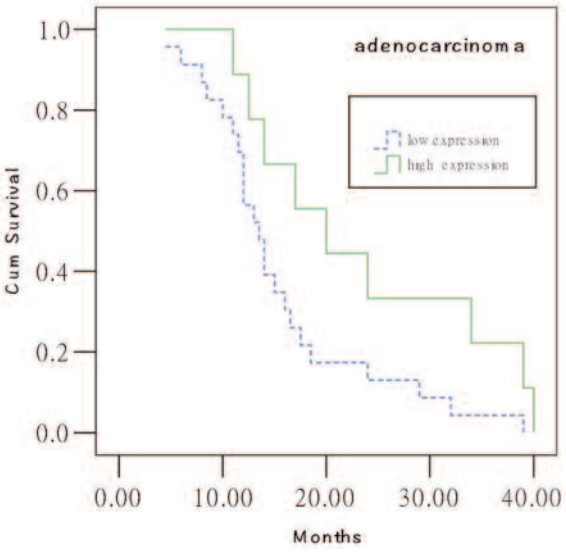


Figure 3. Kaplan-Meier survival curves for 32 AC patients. The patients were divided into high-expression (PTEN > 85.0) and low-expression (PTEN < 85.0) groups. $p = 0.043$ (obtained from the log-rank test).

Assessment of the Independent Potential of PTEN to Predict Survival

Multivariate Cox regression was used to estimate the association between PTEN expression levels and risk of mortality survival in relation to age, sex, tumor type, clinical stage, and PTEN expression (Table II). After adjustment for age, sex, and tumor type, only PTEN expression and clinical stage showed significant correlation with survival ($p = 0.048$ and $p = 0.045$ respectively; Table II). NSCLC patients with high levels of PTEN expression had a 44% reduction in mortality risk (HR= 0.56, 95% CI: 0.32-0.99; Table II).

Discussion

Numerous studies have shown that PI3K/Akt signaling pathway components regulate the proliferation and survival of tumor cells and are frequently altered in human cancers, leading to cell malignant transformation, tumor cell migration and adhesion, angiogenesis and extracellular matrix degradation[27]. As a tumor suppressor gene and an important antagonist of PI3K/Akt pathway, PTEN is able to induce tumor cell apoptosis and impede angiogenesis, thereby, inhibiting tumor growth, invasion and metastasis[28]. There has been some controversy regarding the possible use of PTEN expression as a marker for the survival of cancer patients. A study by Marsit et al[20] that used immunohistochemistry to detect PTEN expression in NSCLC, reported no correlation between PTEN protein expression and survival as indicated by univariate and multivariate analyses. On the other hand, Yanagawa et al[24] reported that in NSCLC patients (predominantly with AC histological subtype, 62% of all NSCLC cases examined) loss of PTEN expression, detected by immunohistochemistry, was associated with significantly worse outcome than in patients with retained PTEN expression. Zheng et al[23] investigated the expression of PTEN protein in early-stage NSCLC (stages IA and IB) using immunofluorescence staining and RT-PCR followed by univariate analysis, and reported no apparent correlation between PTEN expression and survival. Inamura et al[22] assessed PTEN mRNA levels in AC patients using univariate analysis and found that female AC patients with high expression of PTEN mRNA had significantly worse survival compared to patients with normal PTEN expression. While Bepler et al[21] showed longer survival in patients with higher level of tumor PTEN expression by univariate analysis, these findings were not confirmed by multivariate analysis. The controversial results might be due to different treatment during the immunohistochemistry technique used by different groups which were discussed previously[29]. The majority of cases exam-

Table II. Multivariate analysis of overall survival for PTEN expression in patients with lung cancer.

Characteristic	HR (95% CI)	*p* value*
Age	0.99 (0.96-1.03)	0.73
Sex		
Male	1	
Female	0.65 (0.32-1.30)	0.22
Tumor type		
Adenocarcinoma	1	
Squamous-cell carcinoma	0.91 (0.51-1.64)	0.75
Clinical stage		
I-II	1	
III-IV	0.54 (0.36-0.97)	0.045
PTEN		
Low	1	
High	0.56 (0.32-0.99)	0.048

HR, hazard ratio.
*p value is given for Cox multivariate analysis. Statistically significant p values ($p < 0.05$) are in boldface; age was analyzed as a continuous variable and sex, tumor type, clinical stage and PTEN expression as categorical variables.

ined in previous studies were focused on early stage cancers, while only a small percentage of patients in middle and late stage of the disease were investigated. In the present study, we report that high levels of PTEN protein expression were clearly associated with better prognosis in NSCLC patients. Specifically, high levels of PTEN expression correlated with a 43% reduction in mortality risk for all NSCLC patients (HR = 0.57, 95% CI: 0.33-0.98, p = 0.041). Interestingly, the longer survival associated with high PTEN expression was only seen in AC histological subtype of NSCLC in agreement with the previous reports by Bepler et al[21] and Yanagawa et al[24]. Although the decreased PTEN expression was significantly more frequent in SCC patients, it was only prognostic in patients with AC. We may speculate that the conflicting results in regards to association of PTEN expression levels to survival are mainly due to differences in sample size, histological subgroups, immunohistochemical techniques, qualitative scoring systems for PTEN expression, selection of a cutpoint for binarizing PTEN expression levels, and statistical analysis. As compared to previous reports, the present study has several strengths. It has a reasonable sample size and complete survival data (i.e., no censored data) for all NSCLC patients, providing sufficient statistical power to identify a true association. Additionally, the power of the present statistical analysis was improved by using the X-tile software to select an optimal PTEN expression cutpoint[26]. Moreover, the correlation between high PTEN expression and better prognosis in NSCLC patients, which was initially identified by the log-rank test and Cox univariate analysis, was also confirmed by the Cox multivariate analysis after adjustment for age, sex, tumor type, and clinical stage, suggesting that PTEN expression is an independent factor in predicting the NSCLC prognosis.

In the present study, we binarized PTEN expression levels using the optimal cutpoint, and showed that AC patients exhibited overall higher levels of PTEN than SCC patients. These results are consistent with the previous findings in which loss of PTEN expression was observed in SCC but not AC patients[7,24]. One possible explanation for these results is that SCC and AC originate from distinct cell lineages in different regions of the lung[30] and may, therefore, possess different mechanisms of tumorigenesis. In addition, we showed that significantly higher levels of PTEN expression were observed in T1/T2 patients as compared to T3/T4 patients, further indicating that high levels of PTEN may be a favorable prognostic marker.

The present study is a retrospective investigation. However, a prospective prognostic trial of a single marker such PTEN expression level can only rarely be designed outside the setting of a clinical trial. The association between PTEN expression and tumor type and primary tumor stage are nominally significant. A larger sample will be required to confirm these findings.

Conclusions

The levels of PTEN expression and their prognostic impact were investigated in a cohort of primary NSCLC patients. Results suggest that high levels of PTEN protein expression are associated with better prognosis in NSCLC patients. Although a loss of PTEN protein expression occurs more frequently in SCC, longer survival associated with high PTEN expression was only seen in AC patients. Reasons for the observed differences warrant further investigation. In addition, higher levels of PTEN expression were observed in patients with AC than in those with SCC, and in patients with T1/T2 than in those with T3/T4, further indicating that increased PTEN expression may be a favorable prognostic marker.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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