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Loss of PTEN Expression is an Independent Poor Prognostic Factor in Non-small Cell Lung Cancer

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Background: Alterations in the phosphatase and tensin homolog (PTEN) are correlated with tumor progression. Downregulation of PTEN is related to drug resistance of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non-small cell lung cancer (NSCLC). The aim of this study was to evaluate the prognostic significance of PTEN in patients with NSCLC and its correlation with EGFR. **Methods:** Two hundred eighty eight surgically resected NSCLC samples, including 168 adenocarcinomas (ADCs), 99 squamous cell carcinomas (SCCs) and 21 other NSCLCs were analyzed for the PTEN. The results were correlated with other clinicopathological variables including *EGFR* amplification and mutation. **Results:** Loss of PTEN was detected in 42.4% of NSCLCs, specifically 28.6% of ADCs, 66.7% of SCCs, and 38.1% of others. Loss of PTEN was significantly associated with SCC, smoking, male gender, and higher stage. In a multivariate analysis, loss of PTEN was significantly associated with short progression-free survival ($p=0.037$). No association between PTEN and EGFR was observed. **Conclusions:** These results suggest that loss of PTEN results in shorter progression-free survival in patients with NSCLC, and loss of PTEN is more associated with SCC, smoking, male gender, and higher T stage by the 7th tumor, node and metastasis staging system but not EGFR status.

Key Words: PTEN; Carcinoma, non-small-cell; Immunohistochemistry; Prognosis

Lung cancer is a highly aggressive disease and a major cause of cancer deaths worldwide.[1] Although an early diagnosis and surgery are considered the choice treatment, many patients with lung cancer present at the advanced stage. Many studies have been conducted regarding the molecular mechanisms underlying lung cancer to develop new effective therapeutic agents.[2,3] Growth factor receptor signaling pathways constitute an important mediator for tumor growth and proliferation in non-small cell lung cancer (NSCLC). The tumor suppressor phosphatase and tensin homolog (*PTEN*), located at 10q23, encodes a cytoplasmic protein with a protein tyrosine phosphatase domain, has extensive homology to tensin,[4] and is an important negative regulator of cell survival signal transduction along this pathway. The PTEN protein acts by dephosphorylating phosphatidylinositol (3, 4, 5)-triphosphate and negatively regulates the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway.[5] Various alterations including mutations,[6] homozygous deletions,[7] promoter hypermethylation,[8] and microsatellite instability of the *PTEN* gene influence the negative regulation of the PI3K/Akt signaling pathway,[9] a downstream survival signal activated by epidermal growth factor receptor (EGFR).

Signaling pathways activated by EGFRs are important in lung carcinogenesis, and activating mutations in the *EGFR* gene in patients with lung adenocarcinoma (ADC) is associated with an

excellent clinical response to EGFR-tyrosine kinase inhibitors (TKIs).[10] However, patients often experience a tumor recurrence or distant metastasis after treatment with EGFR-TKIs, even when the tumors are initially highly sensitive.[11] Recently, down-regulation of PTEN has been reported as one of the molecular mechanisms underlying resistance to EGFR-TKIs.[12]

In this study, we investigated whether loss of PTEN expression is correlated with clinicopathological parameters or correlated with *EGFR* gene status in patients with surgically resected primary NSCLC.

MATERIALS AND METHODS

Patients and specimens

From May 2003 to December 2006, 288 consecutive lung cancer patients, who underwent a surgical resection at the Seoul National University Bundang Hospital, were compiled. Tumors from patients who died from causes other than lung carcinoma or recurrent or metastatic tumors were excluded. None of the patients enrolled in this study had received preoperative neoadjuvant chemotherapy (CTx) or irradiation. Smoking status was defined as never-smokers (< 100 lifetime cigarettes). Clinical and pathological data were retrieved from the medical records. This study was approved by the Institutional Review Board.

The patients consisted of 206 (71.5%) men and 82 (28.5%) women with a mean age of 62.6 years (range, 21 to 84 years), and the mean tumor size was 3.5 cm (standard deviation, 19.83; range, 0.4 to 16 cm). These patients underwent surgical treatment (wedge resection, n = 12; segmentectomy, n = 5; lobectomy, n = 235; bilobectomy, n = 16; and pneumonectomy, n = 20). Of these, 172 and 84 patients received postoperative adjuvant CTx and postoperative adjuvant radiation therapy, respectively. Among them, 37 patients received additional EGFR-TKIs such as gefitinib or erotinib. *EGFR* mutations and *EGFR* gene copy number data were available in 55 and 278 patients, respectively.[13] Of these, the correlation between *EGFR* gene copy number was assessed by chromogenic *in situ* hybridization (CISH), as previously described.[13]

Hematoxylin and eosin (H&E) stained slides were reviewed in each case to confirm the original diagnosis. Two pathologists (SBY and JHC) independently reviewed these slides to select the most representative sections based on World Health Organization criteria.[14] The tumors consisted of 168 (58.3%) ADCs, 99 (34.4%) squamous cell carcinomas (SCCs), and 21 (7.3%) other NSCLCs.

The cases consisted of 62 stage Ia, 55 stage Ib, 50 stage IIa, 28 stage IIb, 71 stage IIIa, nine stage IIIb, and 13 stage IV tumors according to the 7th edition of the American Joint Committee on Cancer/ International Union Against Cancer (AJCC/ UICC) staging system.[15] There were 62 stage Ia, 65 stage Ib, 14 stage IIa, 49 stage IIb, 55 stage IIIa, 22 stage IIIb, and 21 stage IV tumors according to the 6th edition of the AJCC/UICC staging system. The median follow-up period was 44 months for progression-free survival and 56 months for cancer-specific survival (range, 1 to 84 months).

Construction of a tissue microarray (TMA)

The most representative tumor area was carefully marked on H&E stained slides of sample tissue cores (2 mm in diameter) from formalin fixed and paraffin embedded (FFPE) tissue blocks. A representative core from each tumor was included in the TMA block.

PTEN immunohistochemistry (IHC)

For the IHC analysis, 4 μm thick sections were cut from the TMA blocks and deparaffinized in xylene and rehydrated in a graded alcohol series. The following steps were performed by the Benchmark® XT Slide Autostaining System (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was performed using a microwave in pH 6.0 citrate-phosphate buffer. IHC staining was conducted using a rabbit monoclonal antibody against PTEN (1 : 50, Y184, Epitomics, Burlingame, CA, USA). PTEN immunoreactivity was examined based on cytoplasmic staining using a semi-quantitative scoring method into four categories as follows: 0, negative; 1, 1-25% positive; 2, 26-50% positive; and 3, > 50% positive in tumor cells. When the staining was equal or less than 50% (score 0, 1, or 2), it was considered as loss of PTEN expression.[9] The interpretation of PTEN staining was independently evaluated by two pathologists (SBY and JHC). In the rare instance of a discrepancy in judgment, agreement was reached by discussion at a multihead-microscope.

EGFR mutational analysis

The *EGFR* mutational analysis at exons 18-21 was performed by polymerase chain reaction (PCR) amplification and a direct

DNA sequencing method.[16] The PCR products were analyzed with a Big Dye Terminator Version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA) and sequencing data were obtained with an ABI PRISM 3100 DNA Analyzer (Applied Biosystems).

EGFR CISH

EGFR gene copy number status was examined using the ZytoDotSPEC EGFR Probe Kit protocol (ZytoVision, Bremerhaven, Germany). FFPE tissues were deparaffinized (dewaxed/proteolysis). The next procedures were performed according to the manufacturer's instructions.[13,17] The signals of 200 non-overlapping tumor cells were determined. *EGFR* gene copy number was divided into CISH-negative and CISH-positive groups. Four or more dots per nucleus in ≥40% of tumor cells or the presence of tight gene clusters in ≥10% of tumor cells was confirmed as CISH-positive.[13,17]

Statistical analysis

Fisher's exact test or Pearson's chi-square test were used to evaluate the relationships among the clinicopathological factors, *EGFR* status, and PTEN expression. The Kaplan-Meier method was used to obtain survival curves, and the log-rank test was used for statistical significance. A multivariate survival analysis was performed using the Cox proportional hazard model. Statistical significance was defined as $p<0.05$. All analyses were performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

PTEN protein expression by IHC

PTEN expression was detected in the cytoplasm of normal bronchial epithelial cells, pneumocytes, lymphocytes, and stro-

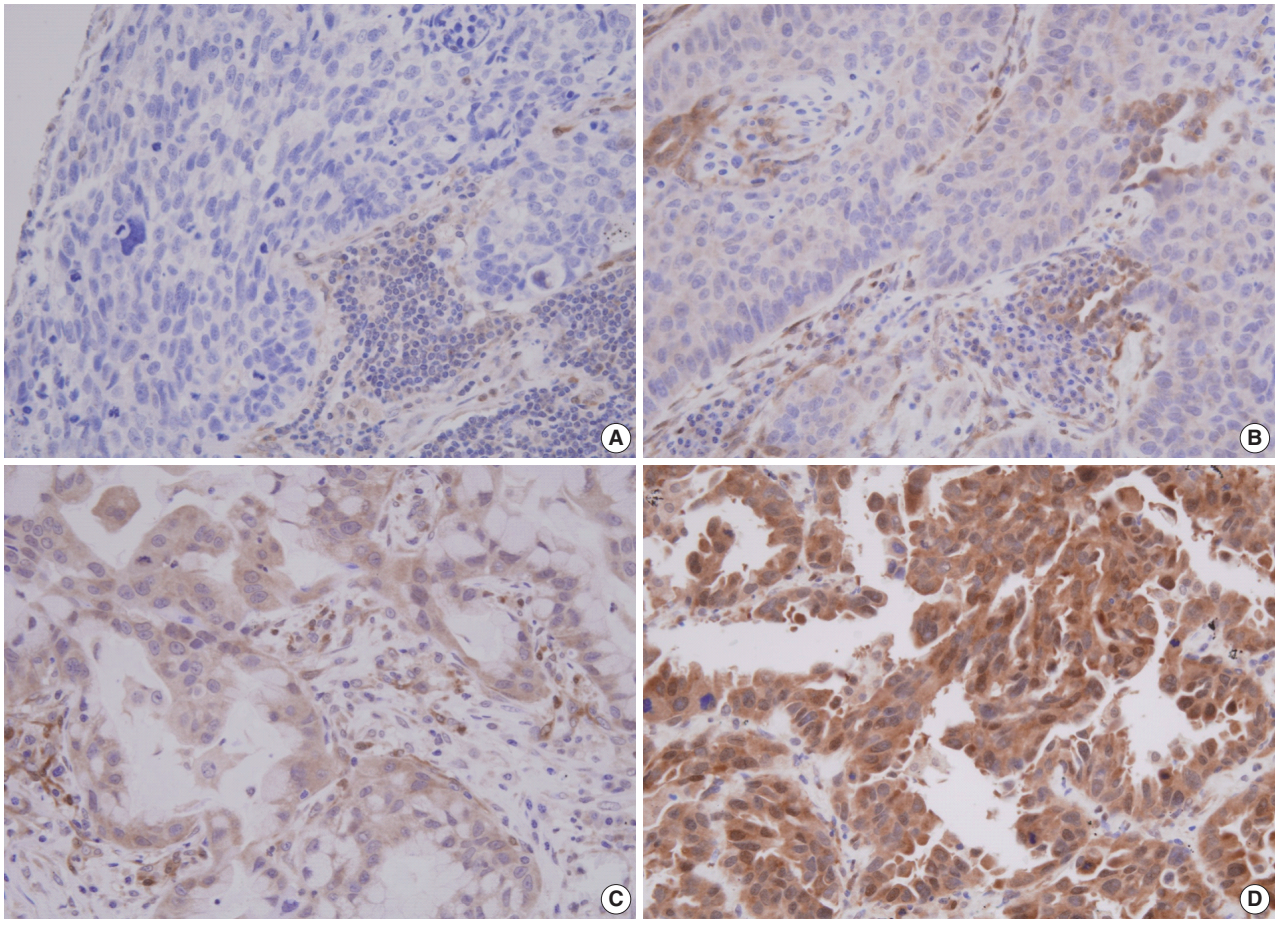

Fig. 1. Immunohistochemical staining patterns of the phosphatase and tensin homolog (PTEN). (A) Negative staining in squamous cell carcinoma, (B) 1+ staining (positive in 1-25%) in squamous cell carcinoma, (C) 2+ staining (positive in 26-50%) in adenocarcinoma, and (D) 3+ staining (positive in >50%) in adenocarcinoma.

mal cells (Fig. 1). The cases were divided into a PTEN loss group (scores 0, 1, or 2; n=122 [42.4%]) and a PTEN intact group (score 3; n=166 [57.6%]) for statistical analysis.[9]

Correlations between PTEN expression and clinicopathological parameters

PTEN expression differed according to histological type, and a loss of PTEN expression was identified in 42.4% (122/288) of NSCLCs. In particular, 28.6% (48/168) of ADCs, 66.7% (66/99) of SCCs, and 38.1% (8/21) of other NSCLCs showed a loss of PTEN expression. Loss of PTEN expression was significantly associated with SCC histology, smoking, male gender, larger tumor size (≥30 mm) and high pathological stage (p-stage) according to the 7th edition of the AJCC/UICC staging system (Table 1). Other clinicopathological variables, including age, performance status, pleural invasion, and lymphovascular invasion showed no correlation with the loss of PTEN expression. No significant association was found between PTEN expression loss and a gain in *EGFR* copy number (amplification or high polysomy) or *EGFR* mutation.

Table 1. Clinicopathological features related to phosphatase and tensin homolog (PTEN) expression in 288 patients with non-small cell lung cancer

	Total (n=288)	PTEN expression		p-value
		Loss group	Intact group	
Sex				
Male	206 (71.5)	100 (48.5)	106 (51.5)	0.001
Female	82 (28.5)	22 (26.8)	60 (73.2)	
Age (yr)				
≥63	174 (60.4)	77 (44.3)	97 (55.7)	0.422
<63	114 (39.6)	45 (39.5)	69 (60.5)	
Smoking				
No	104 (36.1)	27 (26.0)	77 (74.0)	<0.000
Yes	184 (63.9)	95 (51.6)	89 (48.4)	
Tumor size (mm)				
≥30	146 (50.7)	77 (52.7)	69 (47.3)	<0.000
<30	142 (49.3)	45 (31.7)	97 (68.3)	
Pleural invasion				
Absent	174 (60.4)	74 (42.5)	100 (57.5)	0.943
Present	114 (39.6)	48 (42.1)	66 (57.9)	
Vascular invasion				
Absent	245 (85.1)	99 (40.4)	146 (59.6)	0.109
Present	43 (14.9)	23 (53.5)	20 (46.5)	
Lymphatic invasion				
Absent	144 (50.0)	53 (36.8)	91 (63.2)	0.056
Present	144 (50.0)	69 (47.9)	75 (52.1)	
Perineural invasion				
Absent	262 (91.0)	103 (39.3)	159 (60.7)	0.001
Present	26 (9.0)	19 (73.1)	7 (26.9)	
ECOG PS				
1	259 (89.9)	109 (42.1)	150 (57.9)	0.777
2, 3	29 (10.1)	13 (44.8)	16 (55.2)	
Histology				
ADC	168 (58.3)	48 (28.6)	120 (71.4)	<0.000
SCC	99 (34.4)	66 (66.7)	33 (33.3)	
Others	21 (7.3)	8 (38.1)	13 (61.9)	
p-stage (6th)				
I and II	190 (66.0)	78 (41.1)	112 (58.9)	0.531
III and IV	98 (34.0)	44 (44.9)	54 (55.1)	
p-stage (7th)				
I and II	142 (49.3)	45 (31.7)	97 (68.3)	<0.000
III and IV	146 (50.7)	77 (52.7)	69 (47.3)	

Values are presented as number (%).
ECOG PS, Eastern Cooperative Oncology Group Performance Status; ADC, adenocarcinoma; SCC, squamous cell carcinom; p-stage, pathological stage.

Survival analysis

At the time of analysis, the number of cancer-specific deaths was 91 (31.6%). Cancer-specific survival was highly correlated with p-stage (p<0.000). PTEN expression showed no association with cancer-specific survival. However, loss of PTEN expression resulted in significantly shorter progression-free survival than that in the intact PTEN expression group (Fig. 2). A

Table 2. Multivariate analysis of progression-free survival

	p-value	HR	95% CI
p-stage (6th)			
I-II vs III-IV	0.315	0.599	0.220-1.627
p-stage (7th)			
I-II vs III-IV	<0.000	6.506	2.378-17.801
PTEN expression			
Loss group vs Intact group	0.037	1.405	1.020-1.934

HR, hazard ratio; CI, confidence interval; p-stage, pathological stage; PTEN, phosphatase and tensin homolog.

Table 3. Correlation between phosphatase and tensin homolog (PTEN) expression and epidermal growth factor receptor (EGFR) status in patients with non-small cell lung cancer (NSCLC)

	Total	PTEN expression		p-value
		Loss group	Intact group	
EGFR CISH	278 (100)			
Negative	152 (54.7)	68 (44.7)	84 (55.3)	0.396
Positive	126 (45.3)	50 (39.7)	76 (60.3)	
EGFR mutation	55 (100)			
Negative	25 (45.5)	6 (24.0)	19 (76.0)	0.311
Positive	30 (54.5)	11 (36.7)	19 (63.3)	
Exon 18	0 (0)	0 (0)	0 (0)	
Exon 19	18 (60)	7 (38.9)	11 (61.1)	
Exon 20	0 (0)	0 (0)	0 (0)	
Exon 21	12 (40)	4 (33.3)	8 (66.7)	

Values are presented as number (%).
CISH, chromogenic *in situ* hybridization.

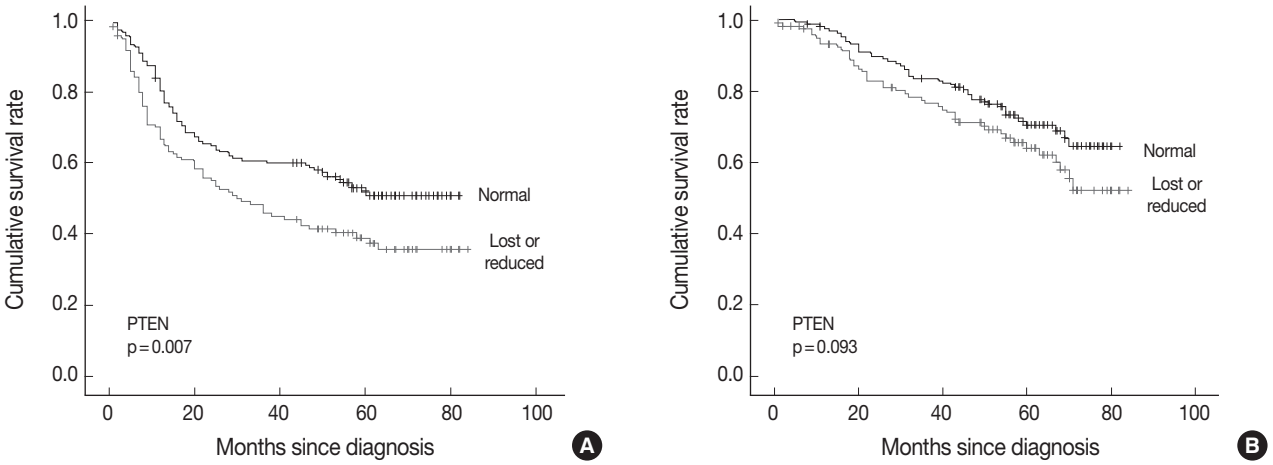

Fig. 2. (A) Loss of phosphatase and tensin homolog (PTEN) expression results in significantly shorter progression-free survival than that in patients expressing intact PTEN. (B) PTEN expression shows no association with cancer-specific survival.

multivariate analysis using the Cox proportional hazard model was performed to evaluate whether PTEN loss was an independent predictor in patients with NSCLC. Table 2 shows, as expected, that p-stage ($p < 0.000$; hazard ratio [HR], 6.506; 95% confidence interval [CI], 2.378 to 17.801) was significantly associated with poor survival. Loss of PTEN expression was associated with progression-free survival ($p = 0.037$; HR, 1.405; 95% CI, 1.020 to 1.934) but not with cancer-specific survival in patients with NSCLC (Table 2).

Correlation among PTEN expression and *EGFR* copy number and *EGFR* gene mutation

The data for *EGFR* copy number and *EGFR* gene mutation were available in 278 and 55 patients, respectively, and the results were correlated with PTEN expression. PTEN expression was not associated with any *EGFR* status. The percentage of intact PTEN tended to be higher than the loss of PTEN in the *EGFR* copy number and *EGFR* mutated groups (60.3% vs 39.7%; 63.3% vs 36.7%); however, the difference was not statistically significant ($p = 0.396$, and $p = 0.311$, respectively) (Table 3).

DISCUSSION

The results of this study demonstrate that loss of PTEN expression was correlated with shorter progression-free survival, suggesting that loss of PTEN expression is an important prognostic predictor of NSCLC.

The loss of PTEN expression is frequent in patients with NSCLC and is associated with shortened survival rates.[18] Jiang and Wang[19] reported that PTEN expression is lower in tumor components compared with that in normal tissue in patients with NSCLC. The lack of PTEN associated with PI3K and Akt upregulation results in tumor development and a poor prognosis in patients with NSCLC.[20,21] Liao *et al.*[22] suggested that low PTEN expression is correlated with tumorigenesis and lymph node metastasis, but no association with pathological type or tumor differentiation was found. These results were similar to previous studies indicating that the loss of PTEN expression is an important event associated with tumor recurrence or progression. Furthermore, loss of PTEN expression was frequently associated with SCC histology, male gender, smoking, larger tumor size, and high p-stage according to the 7th edition of the AJCC/UICC staging system but not the 6th edition of the staging system. These results suggest that the new staging system may reflect prognostic implications of validated biomarker better than the 6th edition staging system.[23] The association between PTEN loss of expression, male gender, and smoking appeared to be affected by frequent PTEN loss in the SCC histology.

PTEN expression was not associated with *EGFR* status, such as *EGFR* copy number or mutation. PTEN is closely involved in the EGFR signaling pathway and its loss of expression in patients with NSCLC is related to the EGFR-TKI drug resistance mechanism and activation of PI3K/Akt/mammalian target of rapamycin signaling.[12,18] It has been proposed that down regulation of PTEN and Akt activation are associated with erlotinib resistance.[24] Although we observed a tendency for more frequent

PTEN protein expression than PTEN loss in the *EGFR* mutation positive cases (64.7% vs 35.3%), no statistical significance was found (p = 0.311). Thus, it is difficult to draw any biological meaning from this result due to the small amount of available *EGFR* mutation data (n = 55). Because the number of patients treated with EGFR-TKIs was also small and because the follow-up duration was short to evaluate the relationship between drug response and PTEN expression loss, it was impossible to investigate the role of PTEN on EGFR-TKI drug responsiveness in this cohort. Further study with a longer follow-up is required to disclose clinical evidence of a relationship between PTEN loss of expression and EGFR-TKIs resistance.

In summary, our study demonstrated that loss of PTEN expression results in shorter progression-free survival in patients with NSCLC, and that loss of PTEN expression was more significantly associated with SCC than ADC, smoking, male gender, and higher T stage but not *EGFR* status. Furthermore, loss of PTEN expression was more correlated with p-stage using the 7th edition tumor, node and metastasis staging system than the 6th edition staging system.

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