SPANDIDOS PUBLICATIONS

PTEN and phosphorylated AKT expression and prognosis in early- and late-stage non-small cell lung cancer

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Abstract. Non-small cell lung cancer (NSCLC) is the commonest cause of cancer mortality worldwide. Growth factor receptor signalling pathways constitute an important mediator for tumor growth and proliferation. PTEN and pAKT play important roles in regulating signal transduction along this pathway. Separate cohorts of stage I (n=25) and stage IV (n=34) NSCLC were examined by immunohistochemistry for PTEN and pAKT expression. There was no correlation between PTEN expression and pAKT expression and neither were associated with age, sex or smoking status. Patients with stage IV disease who overexpressed pAKT (at least 2+) or were PTEN-null had poorer overall survival and progression-free survival. This suggests that PTEN-null or pAKT-positive tumors constitute more aggressive tumors whose clinical course is not altered by therapy. There was no difference in the clinical outcome for stage I disease by PTEN or pAKT expression. A greater proportion of the stage IV patients had PTEN-null disease compared to the stage I cohort, suggesting that loss of PTEN is important in the tumor biology of advanced disease. Loss of PTEN or overexpression of pAKT predicts for an aggressive subset of lung tumors that have a poor prognosis. This will allow identification of a poor prognosis subset that can be targeted with novel treatments that either restore PTEN function or target activated AKT, mTOR and other downstream signal transduction molecules.

Introduction

Cancer is driven by many mechanisms of growth (1). Of these, the epidermal growth factor receptor (EGFR) driven pathway in non-small cell lung cancer is recognized as important for tumor proliferation. Targeting this pathway has become a subject of intense interest and frustration for clinical investigators. Unlike in breast cancer where careful selection of patients for overexpression of Her2 by fluorescent *in situ* hybridization (FISH), predicts for benefit both in the metastatic and adjuvant setting (2-4), overexpression of EGFR does not successfully predict for treatment advantage with targeted therapeutics in NSCLC (5). With non-small cell lung cancer (NSCLC), the search for a similar marker along the EGFR pathway of sufficient impact is ongoing. The advent of EGFR tyrosine kinase inhibitors (TK-I) and the hope generated in the responses seen was increased further by the description of somatic mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase binding domain (5,6) that associated strongly with response. However, there are several caveats that potentially limit its usefulness as a pharmacogenetic test. These include low population prevalence in certain ethnicities (Caucasians, African Americans) (7), unpredictable genotype-response relationships, difficulties in access to tissue and facilities for mutation profiling, and resected NSCLC with specific mutations (e.g., L858R, Exon 19 deletion) appear to have a better or worse survival independent of any TK-inhibitor treatment (7) compared to wild-type EGFR suggesting that these mutations affect prognosis as well as predict for treatment efficacy.

It is therefore of interest to develop in patients with NSCLC other markers that predict for prognosis and also treatment efficacy with EGFR TK-inhibitors. One way is to identify phosphorylated downstream signal molecules along the EGFR pathway that indicate potentially active pathways that may be inhibited by small molecule TK-inhibitors. AKT (protein kinase B) is a serine-threonine kinase that is activated by phosphatidylinositol-3 kinase and sits along the EGFR signal cascade. In turn it phosphorylates a host of other kinases including the mammalian target of rapamycin (mTOR). One convenient way to identify the presence of activated AKT is by immunohistochemistry for the phosphorylated protein. Some investigators have associated phosphorylation of AKT on immunohistochemistry with gefitinib efficacy. There was significant association of pAKT expression with non-smoking status, adenocarcinoma and female sex (8,9). Of significance, one of the pathways by which AKT is negatively regulated is through PTEN.

PTEN is a tumor suppressor gene located on chromosome 10. However loss of function mutations are of very low frequency in NSCLC in comparison to its loss of expression,

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suggesting that other genetic and epigenetic phenomenon, e.g. methylation, play a role in inactivating PTEN as well. Loss of PTEN expression is frequent in prostate (10) and cervical cancer (11). PTEN down-regulates the AKT and PI3K (phosphatidylinositol-3 kinase) pathways (12,13). PTEN-null tumors have therefore greater proliferative activity, growth and survival. Putatively PTEN inactivation results in active AKT and other downstream molecules that could therefore be used as biomarkers of TK-inhibitor response.

We conducted an exploratory analysis of a cohort of resected stage I non-small cell lung cancer patients and late-stage (stage IV) patients, to determine if differential expression of pAKT and its negative regulator PTEN in tumors had any impact on prognosis in a cohort of early- and late-stage patients with NSCLC.

Materials and methods

Patients and tissue. Two cohorts of patients were selected. An early-stage cohort with stage I NSCLC resected at Barnes-Jewish Hospital and a late-stage cohort consisting of patients with stage IV NSCLC treated with gefitinib in Siteman Cancer Center were identified. Clinical data was retrieved from case records available at the Barnes-Jewish Hospital and the Siteman Cancer Center. In the stage I cohort, the clinical end-points used were time to relapse of disease and overall survival. Patients were included only if they had complete follow-up information. For the stage IV cohort, clinical end-points included response as defined by RECIST (14) and duration of both overall survival and progression-free survival. There were 69 patients with stage I disease resected between 1998 and 2000 where 5-year follow-up data was available, and 270 patients with stage IV disease treated with gefitinib between 2000 and 2004. Only 130 patients had available formalin-fixed and paraffin-embedded tissue in the Surgical Pathology Tissue Bank for the study. Paraffin embedded tissue on slides was reviewed by two independent surgical pathologists (CRM, WHZ) unaware of the clinical outcome.

Of these 130 tissue samples, only 49 patients with stage IV disease and 34 patients with stage I disease had tissue sufficient for IHC scoring. Patients were further excluded from clinical outcome analysis if there was incomplete follow-up information. This resulted in attrition of patient numbers to 25 patients in the late-stage cohort and 34 patients in the early-stage cohort. The patient characteristics of each cohort are shown in Table I.

Immunohistochemistry. Polyclonal rabbit anti-PTEN antibody (PAD:PN37) was purchased from ZYMED laboratories (San Francisco, CA). Phospho-AKT (Ser473) antibodies were from Cell Signaling (Beverly, MA). Reagents for immunohistochemistry were purchased from BioGenex. Immunohistochemistry was performed using the streptavidin biotin complex method on a BioGenex i6000™ automated staining system based on protocols provided by the manufacturer (BioGenex, San Ramon, CA). The primary antibodies were diluted 1:50. The secondary antibody was purchased from BioGenex and ready to use without dilution. Slides were scanned with Nikon Eclips (E800) using a MetaMorph Imaging System.

Table I. Patient characteristics.

	Stage I cohort (n=34)	Stage IV cohort (n=25)
Median age (range)	67 years (53-83)	67 years (45-83)
Sex		
Male	21	12
Female	13	13
Histology		
AC/BAC	13	15
SCC/LC/NOS	21	10
Smoker status		
Current or former smoker	15	22
Never smoker	15	3
Unknown	4	0

AC, adenocarcinoma; SCC, squamous cell carcinoma; LC, large cell; NOS, neuroendocrine.

A semi-quantitative assessment of protein expression was used to score PTEN and pAKT in lung tissue (15). The intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%), according to the percentage of cells stained positive for each protein. The sum of the intensity and extent scores was used as the final score (0-7). Tissues having a final score >2 were considered positive. Final scores of 2-3 were considered +, 4-5, ++; and 6-7, +++. Phosphorylated AKT was considered overexpressed if the final score was at least ++, and PTEN was considered expressed if at least +.

Statistical analysis. Results were analyzed using SPSS version 12 (Chicago, IL). χ^2 tests and Fisher's exact test were used to determine significant differences between populations. Survival analyses were determined using Kaplan-Meier methods.

Results

Expression of PTEN and pAKT. There were no significant differences in expression of pAKT or PTEN in relation to smoking status, gender or histological subtypes (Table II). However PTEN was present in a greater percentage of patients in the stage I cohort compared with the stage IV cohort (85% vs. 68%; p=0.12). pAKT was also overexpressed in only 8% of the stage IV cohort as compared to 26% of the early-stage cohort (p=0.18). No relationship between pAKT and PTEN expression was observed.

PTEN/pAKT and clinical end-points. The two cohorts were analyzed for any association between clinical outcomes and PTEN and pAKT expression. In the late-stage cohort, the single patient with a partial response was a non-smoking

Immunohistochemistry and patient characteristics.

	Stage I cohort (n=34)								Stage IV cohort (n=25)							
	pAKT				PTEN				pAKT				PTEN			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Histology																
Adenocarcinoma	3	5	1	1	3	5	2	0	2	11	1	0	10	15	4	1
BAC	0	2	0	1	0	1	1	1	0	1	0	0	1	1	0	0
Large cell/ neuroendocrine	0	0	1	0	0	1	0	0	0	4	0	0	2	3	1	0
Squamous	3	12	2	3	2	12	4	2	1	4	1	0	2	5	2	1
Male	3	7	1	2	2	7	3	1	1	11	0	0	4	6	2	0
Female	3	12	3	3	3	12	4	2	2	9	2	0	4	7	2	0
Smoker	4	8	4	2	3	6	4	2	3	17	2	0	7	11	4	0
Never smoker	5	13	2	2	2	11	0	2	0	3	0	0	1	2	0	0

female with bronchioloalveolar carcinoma. She had 1+ pAKT and 1+ PTEN staining. Six patients had stable disease, of whom only one had 2+ staining for pAKT and all others had weak or no staining, however 5/6 of them stained for PTEN. A number of tumors that had no pAKT staining achieved stable disease (Table III). Patients who overexpressed pAKT of at least 2+ or had PTEN-null tumors on IHC demonstrated significantly worse disease-free and overall survival from the point of diagnosis of cancer (Fig. 1). In the stage IV cohort, the median survival of the PTEN-null group was 1.2 years as compared with 3.0 years for the PTEN-positive group. The median survival for the group with pAKT overexpression was 0.5 months as opposed to 3 years where the pAKT expression was <2+. In the early-stage cohort, there was no significant difference in overall survival in relation to the expression of PTEN or pAKT expression, however there was a trend to better overall survival in the PTEN-positive group (6.0 vs. 1.7 years; p=0.40). Lack of PTEN expression did not impact the time to relapse (p=0.74).

Table III. Immunohistochemistry and best response to gefitinib in the stage IV cohort (n=25).

	pAKT				PTEN			
	-	+	++	+++	-	+	++	+++
PR	0	1	0	0	0	1	0	0
PD	0	17	1	0	7	8	3	0
SD	3	2	1	0	1	4	1	0

PR, partial response; SD, stable disease.

Discussion

This study was aimed at exploring the differential impact of PTEN or pAKT on prognosis in early- and late-stage non-small cell lung cancer. Stage IV tumors that overexpressed pAKT or were PTEN-null had poorer overall survival. PTEN-null tumors also had a shorter progression-free survival compared to PTEN expressing tumors. This suggests that PTEN-null tumors constitute a more aggressive tumor, whose course is not altered by gefitinib therapy. This resistant phenotype may yet be overcome by restoring PTEN function. Indeed PTEN-null cells have demonstrated resistance to EGFR TK-inhibitors (16) and conversely sensitivity to gefitinib has been restored in PTEN-null cells by reconstitution of PTEN (17,18). In the current study, a smaller percentage of tumors were PTEN-null compared to stage IV tumors. This suggests that loss of PTEN is likely a late event in the development of NSCLC. Patients with early-stage disease and loss of PTEN did not have a shorter time to relapse. Loss of PTEN similarly carried no prognostic significance in early-stage disease in previous studies (19). Contrast this to Bepler *et al*, in whose study using univariate analysis high RRM1 and PTEN RNA expression by real-time polymerase chain reaction predicted for better overall and disease-free survival in patients with resectable (majority stage IA/B) NSCLC (20). Apart from the different method used to quantify PTEN expression, in the multivariate analysis, only high expression of RRM1 predicted for good prognosis. The poor prognostic value of pAKT overexpression and PTEN loss is further suggested by a recent study of NSCLC (21) which demonstrated similar findings to ours of an increasing loss of PTEN as stage advances. However, that study also demonstrated a clear inverse relationship between pAKT and PTEN expression which was not demonstrated in the current study, probably because the sample size was small and also because in sporadic cancers there are other upstream perturbations by which phosphorylation of AKT can occur other than via repression of the PTEN-PI3K pathway (22-24). Together with these studies, our results suggest that PTEN and its function is more commonly lost in advanced disease and predicts for an aggressive phenotype in the late stages of NSCLC.

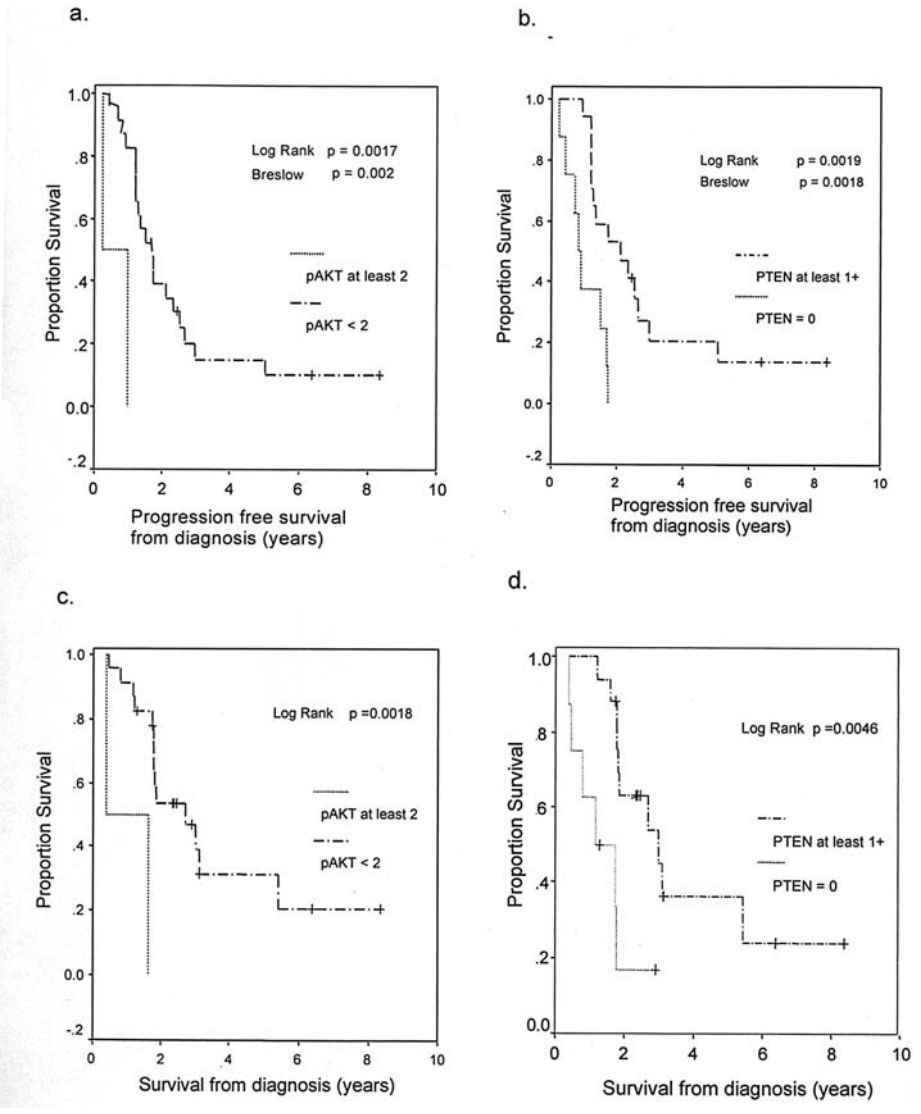

Figure 1. Clinical outcome for stage IV cohort by pAKT or PTEN expression (n=25). (a) PFS AKT, (b) PFS PTEN, (c) OS AKT, (d) OS PTEN.

A number of patients in the stage IV cohort of patients achieved disease stabilization in the absence of pAKT over-expression on IHC. This suggests that gefitinib may yet function in ways independent of an activated pAKT pathway and patients cannot be selected for therapy based on pAKT by IHC alone. Indeed, pAKT-positive tumors, in the absence of somatic mutations in the EGFR TK binding domain, do not fare well when treated with gefitinib (25). Taken together, pAKT alone is not a sufficiently good predictive marker of gefitinib response.

We acknowledge that the sample size in this study was small and its retrospective nature did not take into account clinical prognostic factors such as performance status and weight loss. However, the present results add to the growing evidence that PTEN and pAKT are important determinants of prognosis in patients with advanced non-small cell lung cancer. Loss of PTEN or overexpression of pAKT predicts for an aggressive subset of lung tumors that have a poor prognosis. Identifying this subset using IHC is easy to do, and reproducible. This will allow identification of a poor prognosis subset that can be targeted with novel treatments that either restore PTEN function or target activated AKT, mTOR and other downstream signal transduction molecules. So far the lung cancer patient populations where PTEN and pAKT have been studied are small (<100). Larger patient populations with advanced lung cancer stratified for uniform stage and therapy need to be examined for these biomarkers to validate their importance as a prognostic factor in NSCLC.

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