

## Identification of a Subset of Human Non–Small Cell Lung Cancer Patients with High PI3K $\beta$ and Low PTEN Expression, More Prevalent in Squamous Cell Carcinoma

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### Abstract

**Purpose:** The phosphoinositide 3-kinase (PI3K) pathway is a major oncogenic signaling pathway and an attractive target for therapeutic intervention. Signaling through the PI3K pathway is moderated by the tumor suppressor PTEN, which is deficient or mutated in many human cancers. Molecular characterization of the PI3K signaling network has not been well defined in lung cancer; in particular, the role of PI3K $\beta$  and its relation to PTEN in non–small cell lung cancer NSCLC remain unclear.

**Experimental Design:** Antibodies directed against PI3K $\beta$  and PTEN were validated and used to examine, by immunohistochemistry, expression in 240 NSCLC resection tissues [tissue microarray (TMA) set 1]. Preliminary observations were extended to an independent set of tissues (TMA set 2) comprising 820 NSCLC patient samples analyzed in a separate laboratory applying the same validated antibodies and staining protocols. The staining intensities for PI3K $\beta$  and PTEN were explored and colocalization of these markers in individual tumor cores were correlated.

**Results:** PI3K $\beta$  expression was elevated significantly in squamous cell carcinomas (SCC) compared with adenocarcinomas. In contrast, PTEN loss was greater in SCC than in adenocarcinoma. Detailed correlative analyses of individual patient samples revealed a significantly greater proportion of SCC in TMA set 1 with higher PI3K $\beta$  and lower PTEN expression when compared with adenocarcinoma. These findings were reinforced following independent analyses of TMA set 2.

**Conclusions:** We identify for the first time a subset of NSCLC more prevalent in SCC, with elevated expression of PI3K $\beta$  accompanied by a reduction/loss of PTEN, for whom selective PI3K $\beta$  inhibitors may be predicted to achieve greater clinical benefit. *Clin Cancer Res*; 20(3); 595–603. ©2013 AACR.

### Introduction

More people die as a consequence of lung cancer than any other form of cancer (1, 2). There are two major histologic types of non–small cell lung cancer (NSCLC)—adenocarcinoma and squamous cell carcinoma (SCC)—and the prevalence and incidence of these two histologies varies on a global geographic basis. Currently, on a global

basis, SCC represents approximately one third of the NSCLC burden and, until very recently, the molecular pathology of SCC was poorly understood. There are currently no approved therapies for SCC beyond the standard of care of doublet or singlet chemotherapy. Recent efforts to identify the molecular "drivers" of SCC (e.g., The Cancer Genome Atlas Research Network; ref. 3) have revealed significantly altered pathways in SCC including *CDKN2A* and *RB1*, *NFE2L2* and *KEAP1*, and phosphatidylinositol-3-OH kinase pathway genes, in addition to *DDR2* mutations (4) and focal amplifications of *FGFR1* (5). These advances offer new potential targets for future therapeutic intervention in SCC (6, 7); however, approximately 50% of the disease "drivers" in SCC remain to be characterized and may be more subtle than the mutations, deletions, inversions, or translocations of genetic material seen in adenocarcinoma (8–10).

The phosphoinositide 3-kinase (PI3K) pathway is frequently deregulated in cancer in a variety of ways, both epigenetic and genetic (11), and it plays a critical role in cell growth, proliferation, motility, and survival. The two most common genetic mutations are the somatic activating

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### Translational Relevance

The phosphoinositide 3-kinase (PI3K) pathway is deregulated in multiple ways in non-small cell lung cancer (NSCLC). As a member of the PI3K family, PI3K $\beta$  is considered to be activated primarily via receptor tyrosine kinases and G protein-coupled receptor signaling. However, relatively little is known about the expression of PI3K $\beta$  in NSCLC and the concurrent loss of PTEN, a negative regulator of the PI3K pathway. Via an immunohistochemistry approach utilizing two independent patient cohorts, we have demonstrated that PI3K $\beta$  protein expression level is significantly higher in NSCLC with squamous histology and this higher expression is significantly inversely correlated with the expression of PTEN. A subset of patients with NSCLC with relatively high PI3K $\beta$  and relatively low PTEN protein has hereby been identified. Squamous NSCLC, unlike adenocarcinoma, currently has no approved targeted therapies, and these results may help to direct future studies using inhibitors of the PI3K pathway.

mutations of p110 $\alpha$  (*PIK3CA*) and loss of the tumor suppressor PTEN. However, lung cancer lacks genetic loss of PTEN or the incidence of *PIK3CA* mutations seen in some other cancers (12). The lipid kinase PI3K $\beta$  is a member of the class I PI3K family of enzymes, which comprise p110 $\alpha$ , p110 $\beta$ , p110 $\delta$  (class I), and p110 $\gamma$  (class IB); proteins that are activated to varying extents by receptor tyrosine kinases and G protein-coupled receptors (13). The *PIK3CA* and *PIK3CB* genes (which encode for p110 $\alpha$  and - $\beta$ , respectively) are positioned on chromosome 3q (3q25-27), a region often amplified in NSCLC (14). The molecular characterization of the PI3K pathway in lung cancer is not as well defined as in other tumor types. A number of preclinical studies suggest that the PI3K pathway is key to lung cancer cell growth and survival (15–17) and the deregulation of this pathway has been linked to resistance to Epidermal Growth Factor Receptor (EGFR) therapy for example (18). To further our understanding of the molecular pathology of lung cancer, we have investigated the expression of PI3K $\beta$  and PTEN by immunohistochemistry (IHC) across 39 tissue microarrays (TMA) comprising a total of 1,060 human lung tumors acquired from two independent centers. In this study, we identify a subset of patients with NSCLC more prevalent in SCC, with relatively high PI3K $\beta$  expression accompanied by a reduction/loss of PTEN that may benefit from targeted inhibitors of the PI3K pathway.

### Materials and Methods

#### Human lung TMAs

Formalin-fixed, paraffin-embedded (FFPE) human lung cancer resection tissues from primary tumors (TMA set 1,  $n = 240$ ; 47.5% SCC and 52.5% adenocarcinoma) were sourced by AstraZeneca under approved legal contract from three commercial tissue suppliers (Asterand Plc,

Indivumed GmbH, and GCI/GE Healthcare) and a hospital tissue bank (Liverpool Heart and Chest Hospital NHS Trust). Cores (0.6 to 1 mm diameter, 3–4/patient) were taken for TMA construction. In addition, a commercial NSCLC TMA (1.5 mm diameter cores, 1 core/patient) was obtained from Tristar Technology Group. A larger cohort of NSCLC resection tissues from primary tumors (TMA set 2,  $n = 820$ ; 35.4% SCC and 64.6% adenocarcinoma) was examined at The University of Texas MD Anderson Cancer Center (MDACC; Texas) following construction of TMAs (1 mm cores, 3 cores/patient) from tissues obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Bank (MDACC). At each site, the suitability of samples for immunohistochemical analysis was determined by a pathologist following histologic examination of H&E stained specimens. Detailed clinicopathologic characteristics of patients are summarized in Supplementary Tables S1 and S2. All investigations were conducted with appropriate written informed consent and ethical approval.

#### Antibodies

A custom rabbit polyclonal antibody was generated in-house by immunizing with a C-terminus peptide of human PI3K $\beta$  (AGG4888; AstraZeneca), raising an antibody specific for total PI3K $\beta$ . Specificity of AGG4888 for PI3K $\beta$  was confirmed by ELISA and Western blot using recombinant p110 isoforms ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ; Supplementary Fig. S1). Two commercially available PI3K $\beta$  antibodies (Cell Signaling Technology Inc., clone C33D4; Santa Cruz Biotechnology Inc., clone S-19) were rejected due to cross reactivity with other p110 isoforms (Supplementary Fig. S1) and another antibody (Serotec; clone 4H2) was rejected due to poor recognition in Western blots of a band at the correct molecular weight, plus additional bands in tumor cell lysates (data not shown). A rabbit monoclonal antibody directed against human PTEN (Cell Signaling Technology; clone 138G6) was selected for use following successful validation by IHC across a series of known PTEN-expressing (A549, Du145, MCF7, HT29, HCT116, Colo205, Ovar5, Skov3, and SW620) and PTEN-null cell lines (PC3, U118MG, and U87MG) using an AstraZeneca-constructed xenograft TMA (Supplementary Fig. S2); this clone having undergone thorough validation also by the supplier and by Lotan and colleagues (19–22).

#### IHC and analysis

IHC methods for detection of PI3K $\beta$  and PTEN were validated before assessment of FFPE NSCLC TMAs. For staining TMAs, FFPE tissues were sectioned at 4  $\mu$ m onto slides, dewaxed, and rehydrated. Antigen retrieval was performed in a RHS microwave vacuum processor (Milestone) at 110°C in either pH9 retrieval buffer (S2367; Dako, 2 minutes) for PI3K $\beta$  or pH6 retrieval buffer (S1699; Dako, 5 minutes) for PTEN. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, non-specific binding of avidin/biotin reagents (PTEN protocol) was blocked using an avidin/biotin blocking kit (SP-2001;

Vector Laboratories) and nonspecific binding sites were blocked with either serum-free protein block (X0909; Dako, PI3K $\beta$  protocol) or Vectastain Elite protein block (PK-6101; Vector Laboratories, PTEN protocol) for 20 minutes. Sections were incubated for 1 hour in primary antibodies (PI3K $\beta$ ; 5.6  $\mu$ g/mL or PTEN; 0.13  $\mu$ g/mL) diluted in Tris-buffered saline containing 0.05% Tween (TBS-T). Staining was visualized using either rabbit Envision HRP-linked polymer (K4003; Dako, PI3K $\beta$ ) or Vectastain Elite secondary antibody/ABC (PK-6101; Vector Laboratories, PTEN) according to the manufacturer's instructions, followed by incubation for 10 minutes in diaminobenzidine (K3466; Dako). Counterstaining was conducted using Carazzi's hematoxylin. All washes were performed in TBS-T and all incubations were at room temperature. No staining was observed in samples incubated with appropriate isotype control antibodies.

Stained TMAs were scored independently at each site by trained pathologists (AstraZeneca, C. Womack and D. Morosini; MDACC, I.I. Wistuba and X. Tang) using a 4-point scoring system (0+, negative; 1+, weak; 2+, moderate; and 3+, strong) for localization of PI3K $\beta$  and PTEN expression in tumor cytoplasm, membrane, and nucleus separately, as appropriate. Where multiple cores were present (3–4 for some cases), all were scored and the highest score recorded as representative of the potential expression of the marker for the whole tumor. Although semiquantitative, the scoring system selected combines intensity and distribution enabling experienced pathologists to provide a subjective assessment in a timely fashion across multiple cores from large numbers of patients. A 10% quality control of MDACC scores was conducted by pathologists from AstraZeneca and agreement of >90% was reported.

#### Mutation analysis

Matching mutation analyses were conducted for a total of 75 of the NSCLC samples (32 SCC and 43 adenocarcinoma) acquired by AstraZeneca from the Liverpool Heart and Chest Hospital NHS Trust. Mutation screening was performed using Sanger sequencing (*EGFR*, *p53*, and *LKB1*) and pyrosequencing (*KRAS*) methodologies (23).

#### FISH and analysis

FISH assay was used to detect *PIK3CB* and *PTEN* gene copy number changes across the NSCLC TMA from Tristar comprising 49 NSCLC cases.

The *PIK3CB* and *PTEN* FISH probes were generated internally by directly labeling bacterial artificial chromosome (RP11-112O24 for *PIK3CB* and RP11-765C10 and CTD3007P15 for *PTEN*) DNA with Spectrum Green (ENZO; 02N32-050) for *PIK3CB* or Spectrum Red (ENZO; 02N34-050) for *PTEN*. The CEP3-Spectrum Orange (Vysis; 32-110003) and CEP10-Spectrum Aqua (Vysis; 32-111010) were used as internal controls for *PIK3CB* and *PTEN*, respectively. The FISH assay was performed as reported previously (24). Enumeration of the *PIK3CB* or *PTEN* gene, and chromosome 3 or 10, was conducted by microscopic examination of 50 tumor nuclei, which yielded a ratio of

*PIK3CB/CEP3* and *PTEN/CEP10*, and average copy number of *PIK3CB* or *PTEN* gene. Tumors with *PIK3CB/CEP3* ratio  $\geq 2$  were defined as amplified, and average *PTEN* copy number  $\leq 1.5$  was defined as deletion.

#### Statistical analyses

This exploratory investigation descriptively summarizes the subjects' clinicopathologic features with frequency and percentages by SCC and adenocarcinoma histologic subtypes for each of the two cohorts (TMA sets 1 and 2).

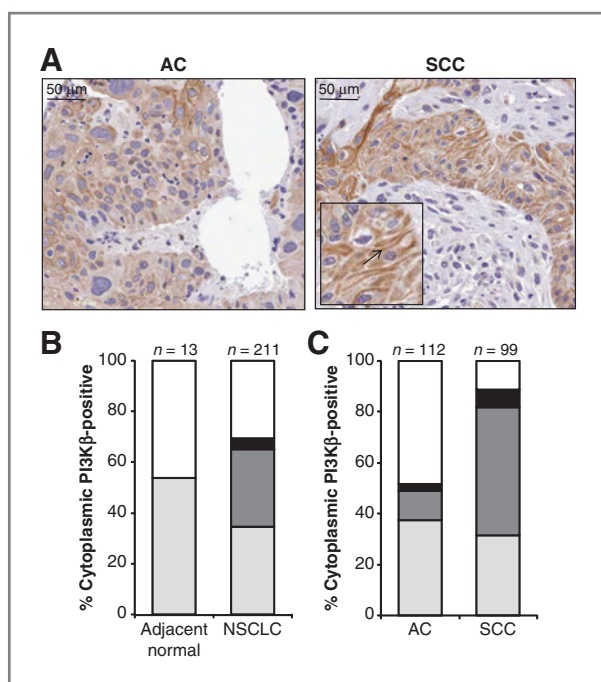
The object of these analyses was to evaluate the association between histologic subtype (adenocarcinoma and SCC) and tumor tissue expression level of PI3K $\beta$ , expression of PTEN protein, and expression of PI3K $\beta$  high (as defined by a tumor score 2 and greater) with PTEN low (as defined by a tumor score of 0 or 1) in the cytoplasm, membrane, and nucleus. The global Cochran–Mantel–Haenszel test was applied to TMA set 1 to control for variation due to different sources of tumor tissue. The Zelens exact test for common odds ratio was applied instead when cell counts were fewer than 5. The  $\chi^2$  test was applied to TMA set 2. When cell counts were fewer than 5, the Fisher exact test was performed instead. Mutation status (categorized as either mutant or wild type, WT) of *KRAS*, *EGFR*, *p53*, and *LKB1* was descriptively summarized with frequencies and percentages by SCC and adenocarcinoma histology diagnosis, PI3K $\beta$  high, and PTEN low.

## Results

### Elevated expression of PI3K $\beta$ in SCC compared with adenocarcinoma

Expression of PI3K $\beta$  was investigated in the first instance across TMA set 1, comprising 240 NSCLC resection tissues (47.5% SCC and 52.5% adenocarcinoma) representing largely early stage disease (stage I, 43.8%; stage II, 17.1%; stage III, 15%; stage IV, 2.5%; unknown, 21.6%; Supplementary Table S1). Localization of PI3K $\beta$  by IHC across these NSCLC cases was found to be largely cytoplasmic (Fig. 1A), with expression accumulating toward the plasma membrane in some areas of tumor (Fig. 1A, inset). Where tumors were positive for PI3K $\beta$ , heterogeneity in the level of expression was apparent, ranging from cores in which all tumor cells were similarly positive to those with varying levels of expression across the tissue core. In addition to tumor cells, some stromal cells, inflammatory infiltrates, and vessels were also positive for PI3K $\beta$  (data not shown).

Pathology scores for cytoplasmic PI3K $\beta$  expression in tumor are summarized in Fig. 1B and C. Of the 240 lung tumor samples present in TMA set 1, 211 were evaluable for PI3K $\beta$  expression and adjacent normal lung tissue was available for 141 samples, although normal bronchial epithelium was present for scoring in only 13 of these (Fig. 1B). Compared with adjacent normal epithelium, which exhibited low-level expression of PI3K $\beta$  (1+) for 53.8% (7 of 13) of patients with the remainder negative, PI3K $\beta$  was expressed in 69.2% (146 of 211) of all lung tumors (Fig. 1B).



**Figure 1.** Elevated expression of PI3K $\beta$  in SCC compared with adenocarcinoma (AC) in TMA set 1. A, representative images showing cytoplasmic localization of PI3K $\beta$  in NSCLC samples (inset: the arrow indicates accumulation of PI3K $\beta$  at plasma membrane). B, cytoplasmic PI3K $\beta$  expression in NSCLC compared with adjacent normal lung tissue. C, elevated expression of cytoplasmic PI3K $\beta$  in SCC compared with adenocarcinoma. Scoring key: □, 0+; ■, 1+; ■, 2+; ■, 3+ ( $n$  = number of patients).

A more detailed analysis of tumors for histologic subtype revealed that PI3K $\beta$  was overexpressed significantly ( $P < 0.0001$ ) in SCC compared with adenocarcinoma (Fig. 1C). Thus, 88.9% of SCC was positive for PI3K $\beta$  compared with 48.2% of adenocarcinoma, with a greater proportion of SCC exhibiting a pathology score of 2+ (50.5%) compared with adenocarcinoma (11.6%). Some tumors also displayed localization of PI3K $\beta$  that was focused toward the plasma membrane (Fig. 1A, inset). Pathologist scores revealed that membrane localization was elevated significantly ( $P < 0.0001$ ) in SCC (33.3% positive) compared with adenocarcinoma (16% positive; data not shown).

Elevated expression of PI3K $\beta$  in tumor was not associated with any other clinicopathologic features available for this patient cohort, such as stage or grade of disease. Furthermore, expression and distribution of PI3K $\beta$  in other tumor compartments (stroma, inflammatory cells and vessels) did not differ between SCC and adenocarcinoma or any of the other patient features summarized in Supplementary Table S1.

### Reduced PTEN expression in SCC compared with adenocarcinoma

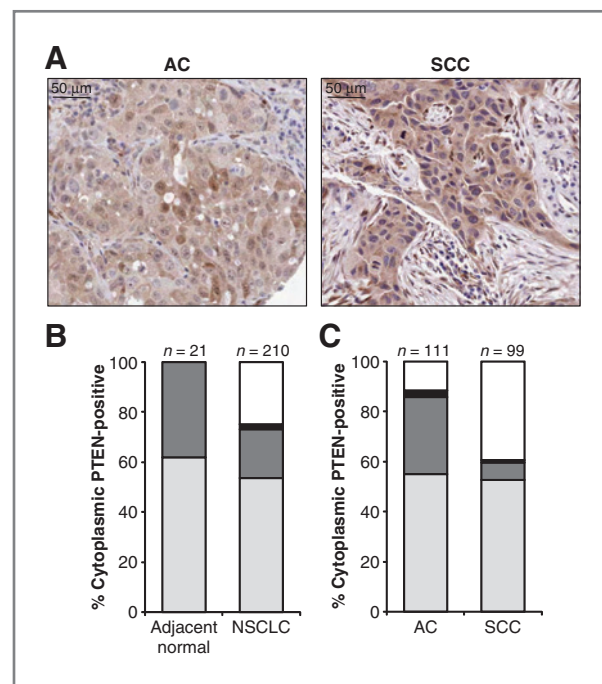
Consecutive sections of set 1 TMAs were analyzed also by IHC for PTEN expression. PTEN protein was found to be distributed throughout stromal cells, inflammatory infil-

trates and vessels in all tumor samples. Where tumor expression of PTEN was evident, two patterns of expression were observed: cytoplasmic staining and nuclear staining (Fig. 2A). This pattern of distribution for PTEN is in accordance with that reported previously (19).

Less heterogeneity of expression within and between cores was observed for PTEN compared with PI3K $\beta$ . Thus, for approximately 80% of cases in which multiple cores were present, a consistent positive or negative response was observed between cores. For the remaining cases, positive and negative cores were present, the higher score being the result recorded. Variable expression (positivity and negativity) within a core was observed occasionally, but for the majority a positive or negative result for a core was consistent.

The bronchial epithelium of all (100%) adjacent normal lung samples, where evaluable ( $n = 21$ ), expressed PTEN protein; however, only 75.2% of specimens in TMA set 1 exhibited tumor staining for PTEN (Fig. 2B). In samples in which PTEN staining in tumor was absent (24.8%), surrounding stromal cells, inflammatory infiltrates and vessels remained positive.

Examination of histologic subtype revealed that loss of cytoplasmic expression of PTEN protein in tumor was significantly greater ( $P < 0.0001$ ) in SCC (39.4% PTEN-negative) compared with adenocarcinoma (11.7% PTEN-negative; Fig. 2C), with moderate (2+) staining for PTEN



**Figure 2.** Reduced PTEN expression in SCC compared with adenocarcinoma (AC) in TMA set 1. A, representative images showing cytoplasmic and nuclear localization of PTEN in SCC and adenocarcinoma. B, cytoplasmic PTEN expression in NSCLC compared with adjacent normal lung tissue. C, reduced expression of cytoplasmic PTEN in SCC compared with adenocarcinoma. Scoring key: □, 0+; ■, 1+; ■, 2+; ■, 3+ ( $n$  = number of patients).

being reduced predominantly in SCC versus adenocarcinoma. Nuclear PTEN exhibited a similar pattern of loss to that observed for cytoplasmic PTEN (Supplementary Fig. S3A and S3B), with significantly greater loss ( $P < 0.0001$ ) of nuclear PTEN observed for SCC (54.5% PTEN-negative) compared with adenocarcinoma (25.2% PTEN-negative).

No difference between SCC and adenocarcinoma was evident for PTEN expression in stromal cells, inflammatory infiltrates or vessels (data not shown), or for PTEN staining in tumor in relation to other clinicopathologic features available for this patient cohort.

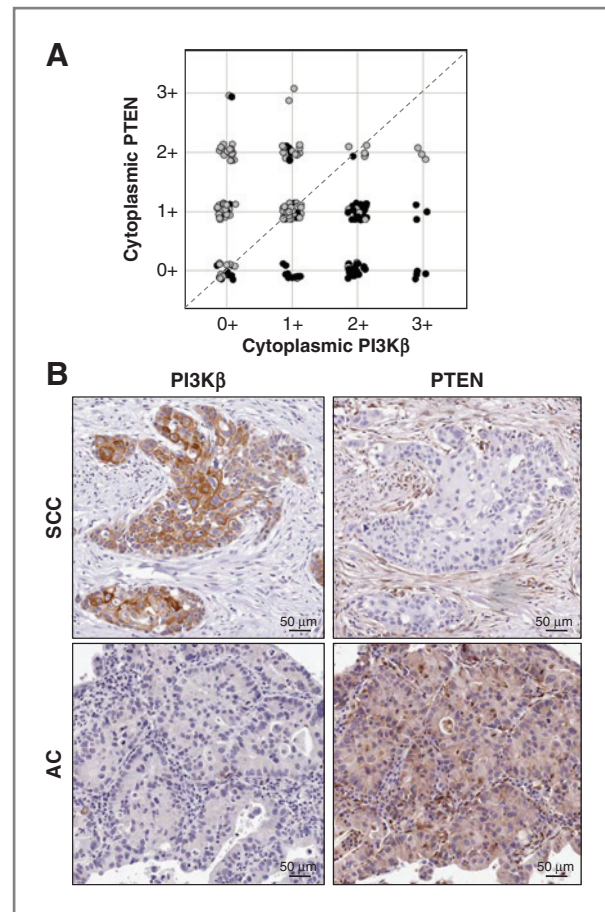
### Identification of a subset of NSCLC with high PI3K $\beta$ and low PTEN more prevalent in SCC

The data described in Figs. 1 and 2 indicate that PI3K $\beta$  is overexpressed and PTEN expression is decreased in patients with NSCLC with SCC compared with those with adenocarcinoma. To investigate whether tumors overexpressing PI3K $\beta$  are those also exhibiting reduced PTEN, a more detailed analysis was conducted to examine colocalization of PI3K $\beta$  and PTEN expression in individual tumor cores (Fig. 3).

A proportion of NSCLC samples (31.6%) exhibited a direct relationship between cytoplasmic PI3K $\beta$  and cytoplasmic PTEN expression (those samples falling on the diagonal line in Fig. 3A) and these tumor samples comprised both SCC and adenocarcinoma to a similar extent. However, for the remaining NSCLC samples it was clear that an inverse relationship existed between SCC and adenocarcinoma with respect to their expression patterns for PI3K $\beta$  and PTEN. Thus, a significantly ( $P < 0.0001$ ) higher proportion of SCC (66.3%; 65 of 98) displayed an expression pattern skewed toward higher PI3K $\beta$  and lower PTEN (those samples to the right-hand side of the diagonal line in Fig. 3A) than adenocarcinoma (11.1%; 12 of 108). Furthermore, 33.0% of SCC (32 of 98) displayed tumors that were positive for cytoplasmic PI3K $\beta$  but which lacked completely any staining for PTEN in tumor, although other features such as stroma, inflammatory cells and vessels retained PTEN positivity (Fig. 3B; SCC, top). In contrast, only 2.8% of adenocarcinoma (3 of 108) exhibited this pattern of expression (higher PI3K $\beta$  and negative PTEN). Conversely, 50.0% of adenocarcinoma (54 of 108) exhibited the opposite pattern of expression (low PI3K $\beta$  and higher levels of PTEN; samples to the left-hand side of the diagonal line in Fig. 3A and bottom of Fig. 3B, adenocarcinoma), with only 10.2% of SCC (10 of 98) showing this relationship (low/negative PI3K $\beta$  and higher PTEN).

A similar significant correlation ( $P < 0.0001$ ) to that described above was drawn for tumor expression of cytoplasmic PI3K $\beta$  and nuclear PTEN (Supplementary Fig. S3C). Interrogation of the data revealed no further significant relationships between the level of expression of PI3K $\beta$  and PTEN and clinicopathologic features of this patient cohort.

For the samples acquired through Liverpool Heart and Chest Hospital to construct TMA set 1, mutational analyses were conducted in which sufficient tissue was available



**Figure 3.** Identification of a subset of NSCLC more prevalent in SCC, with high PI3K $\beta$  and low PTEN (TMA set 1). A, correlation between expression of cytoplasmic PI3K $\beta$  and cytoplasmic PTEN within individual SCC (●) and adenocarcinoma (AC; ●) NSCLC cases. Each circle represents an individual tumor core ( $n = 206$  patients with NSCLC comprising  $n = 98$  SCC and  $n = 108$  adenocarcinoma). The diagonal line highlights patients with direct relationship between PI3K $\beta$  and PTEN. B, consecutive sections showing the converse relationship between the expression of PI3K $\beta$  and PTEN in SCC (top) and adenocarcinoma (bottom).

( $n = 32$  SCC and  $n = 43$  adenocarcinoma; Supplementary Table S3). All SCC were EGFR WT (adenocarcinoma, 7% EGFR mutant), whereas 3 of 32 SCC (9.4%) harbored KRAS mutations (adenocarcinoma, 48.8% KRAS mutant). Both SCC and adenocarcinoma exhibited a low frequency of LKB1 mutation (SCC, 12.5%; adenocarcinoma, 20.9%), with a higher proportion of both histologic subtypes possessing p53 mutations (SCC, 62.5%; adenocarcinoma, 37.2%). Analyses of PI3K $\beta$  and PTEN expression for these samples revealed no correlations between higher expression of PI3K $\beta$  and/or PTEN loss and any of the mutations examined.

To further investigate the explanation for elevated PI3K $\beta$  and reduced PTEN expression in this subset of NSCLC, FISH analyses for *PIK3CB* amplification and *PTEN* gene copy deletion were conducted across a small number of NSCLC cases (49; 36 SCC and 13 adenocarcinoma) from the Tristar NSCLC TMA that contributed to the data for TMA set 1. Of

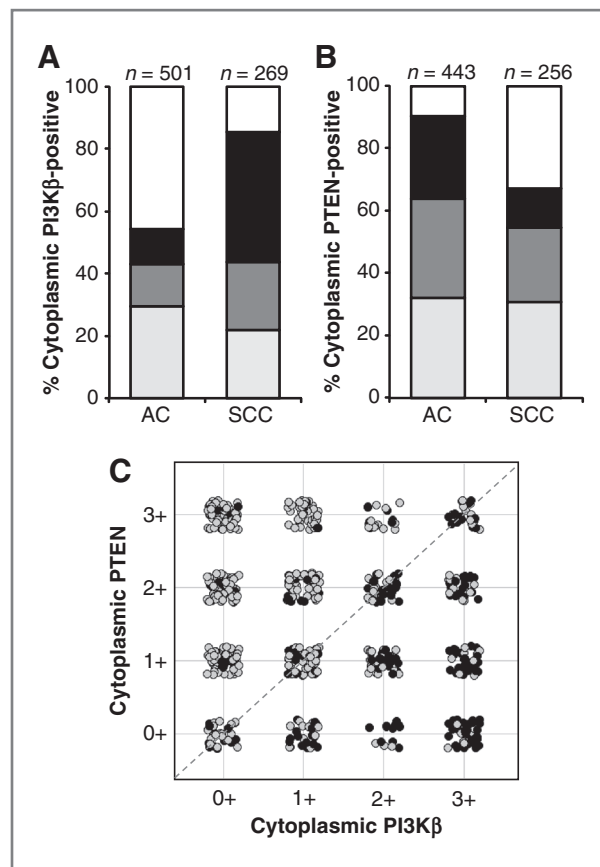
the 49 NSCLC cases on this TMA, 15 (14 SCC and 1 adenocarcinoma) exhibited elevated PI3K $\beta$  and reduced PTEN expression by IHC. Only two cases were amplified for *PIK3CB*, one SCC (IHC scores, PI3K $\beta$  3+/PTEN 1+) and one adenocarcinoma (IHC scores, PI3K $\beta$  1+/PTEN1+). Three cases showed *PTEN* gene copy deletion (one copy loss), all of which were SCC (IHC scores, 2 of 3 PI3K $\beta$  3+/PTEN 0+ and 1 of 3 PI3K $\beta$  2+/PTEN 1+). Although a limited sample set, these preliminary data suggest that protein expression in these cases is not related directly to *PIK3CB* amplification or *PTEN* gene copy deletion.

#### Confirmation in an independent patient cohort (TMA set 2) of a subset of NSCLC with high PI3K $\beta$ and low PTEN more frequent in SCC

An independent set of tissues (TMA set 2) comprising 820 NSCLC patient samples (35.4% SCC and 64.6% adenocarcinoma; stage I, 58.2%; stage II, 17.9%; stage III, 19.3%; stage IV, 4.6%; Supplementary Table S2) were examined for PI3K $\beta$  and PTEN expression in a separate laboratory using the same validated antibodies and IHC protocols. Of the 820 samples incorporated into TMA set 2, 770 and 699 were evaluable for PI3K $\beta$  and PTEN respectively, 663 tumor cores being suitable for correlative analyses of both markers. Although differences were apparent in staining intensity between TMA sets 1 and 2, with more intense staining evident for TMA set 2 tumor tissues, localization patterns for both markers were identical between both sets of TMAs.

Thus, PI3K $\beta$  was found to be overexpressed significantly ( $P < 0.0001$ ) in the cytoplasm of 85.5% of SCC compared with 54.3% of adenocarcinomas (Fig. 4A), with the proportion of tumor cores displaying a pathology score of 3+ being greater for SCC than adenocarcinoma. Consistent with analyses of the TMA set 1 patient cohort, the proportion of tumors exhibiting membrane localization of PI3K $\beta$  was also elevated significantly ( $P < 0.0001$ ) in SCC (38.4% positive) compared with adenocarcinoma (12.6% positive; data not shown). Similarly, loss of cytoplasmic PTEN was significantly greater ( $P < 0.0001$ ) in SCC (32.8% negative) than adenocarcinoma (9.5% negative; Fig. 4B).

Importantly, correlative analyses of this independent patient cohort reinforced the observation that a significantly ( $P < 0.0001$ ) higher proportion of SCC (57.0%; 138 of 242) displayed an expression pattern that was skewed toward higher cytoplasmic PI3K $\beta$  and lower cytoplasmic PTEN (those samples that fall to the right-hand side of the diagonal line in Fig. 4C). Furthermore, 27.7% of TMA set 2 SCC (67 of 242) depicted tumor cells that were positive for PI3K $\beta$  but which lacked PTEN completely, whereas only 5.1% of adenocarcinomas (21 of 415) exhibited this pattern of expression. In contrast, only 18.2% of TMA set 2 SCC (44 of 242) exhibited the converse pattern of expression (lower PI3K $\beta$  accompanied by higher PTEN), compared with 63.9% of TMA set 2 adenocarcinomas (265 of 415). As observed for TMA set 1, a proportion of NSCLC samples (22.0%) exhibited a direct relationship between PI3K $\beta$  and PTEN expression, divided approximately equally between SCC and adenocarcinoma. Correlative analyses of cyto-



**Figure 4.** Confirmation in an independent patient cohort (TMA set 2) of a subset of NSCLC more prevalent in SCC, with high PI3K $\beta$  and low PTEN. A, elevated expression of cytoplasmic PI3K $\beta$  in SCC compared with adenocarcinoma (AC). Scoring key: □, 0+; ▒, 1+; ▓, 2+; ■, 3+ ( $n$  = number of patients). B, reduced cytoplasmic PTEN expression in SCC compared with adenocarcinoma. C, correlation between expression of cytoplasmic PI3K $\beta$  and cytoplasmic PTEN within individual SCC (●) and adenocarcinoma (●) NSCLC tumor cores. Each circle represents an individual sample ( $n$  = 657 patients with NSCLC comprising  $n$  = 242 SCC and  $n$  = 415 adenocarcinoma). The diagonal line highlights patients with direct relationship between PI3K $\beta$  and PTEN.

plasmic PI3K $\beta$  with nuclear PTEN expression revealed a similar output ( $P < 0.0001$ ) to that observed for cytoplasmic PI3K $\beta$  and cytoplasmic PTEN (Supplementary Fig. S4). Elevated PI3K $\beta$  expression, in the presence or absence of PTEN, failed to correlate significantly with any other clinicopathologic features of this patient cohort including stage or grade of disease, or patient survival.

Thus, correlative analyses of both independent patient cohorts reveal that elevated cytoplasmic PI3K $\beta$  expression together with low/negative cytoplasmic PTEN expression is a feature of histologic subtype in NSCLC, more prevalent in SCC than adenocarcinoma.

#### Discussion

In this study, we compared IHC analyses of TMAs constructed from NSCLC specimens acquired across two independent centers, each comprising both squamous and

adenocarcinoma histologic subtypes representing primarily early stage disease (I–III). The purpose of these analyses was to investigate the potential co-occurrence of PI3K $\beta$  protein overexpression and reduced expression of PTEN protein in NSCLC. Our findings demonstrate a statistically significant association of PI3K $\beta$  protein expression with squamous histology, and a statistically significant inverse correlation of PI3K $\beta$  protein with PTEN protein expression in squamous NSCLC. To the best of our knowledge, this is the first extensive report of PI3K $\beta$  protein expression levels in NSCLC.

Activation of the PI3K/AKT signaling network via mutation and amplification in NSCLC has already been reported (12, 24–26) and perturbations in this pathway are more prevalent in squamous histology compared with adenocarcinoma (3, 9, 17). Until recently, these histologic subtypes have been treated as a single disease entity with treatment strategies based on disease stage; however, emerging therapies showing greater benefit in adenocarcinoma reveal the need for different therapeutic strategies for these histologic subtypes (27). Evidence of unique biology has emerged with the discovery of new SCC-associated genetic changes, including alterations in the PI3K pathway, suggesting new areas for targeted therapy for these patients. In fact, that PI3K pathway alteration is more prevalent in SCC and that these alterations predict for responsiveness in preclinical models of lung cancer have been reported recently (28). However, the explicit role of PI3K $\beta$  and its relationship to PTEN protein expression in clinical samples of adenocarcinoma and SCC have not been explored to date.

It is possible that elevated PI3K $\beta$  protein expression, setting the basal level of PI3K pathway signaling, in combination with reduced expression of PTEN could provide a sufficient independent proliferative drive in NSCLC. Certainly, loss of PTEN activity is known to lead to hyperactivation of the PI3K–AKT pathway, and specifically a crucial role for *PIK3CB* in cell growth, metabolism, and tumorigenesis of PTEN-deficient tumors has been reported (29, 30). Furthermore, a selective p110 $\beta$  inhibitor has been shown to inhibit tumor growth in PTEN-deficient tumor models (31), whereas conversely PI3K inhibitors with insufficient activity against the p110 $\beta$  isoform were less effective at inhibiting the PI3K pathway in PTEN-null tumor models (32). These data, taken together with the identification of a subset of patients with NSCLC with elevated PI3K $\beta$  and reduced PTEN described here, suggest that these patients may represent a population for whom selective inhibitors of PI3K $\beta$  may be beneficial.

Of considerable interest is the genetic basis for the subset of patients with NSCLC with elevated PI3K $\beta$  and reduced PTEN, and whether these expression levels are reflected by alterations in *PIK3CB* and *PTEN* genes. Although only a limited number of cases were explored by FISH, our data suggest for these cases at least, that *PIK3CB* amplification is not a clear driver of elevated PI3K $\beta$  expression, and that *PTEN* deletion is not the cause of PTEN protein loss for all cases. In support of this conclusion, it is reported widely in the literature that although PTEN protein loss is a relatively

frequent event in NSCLC, genetic mutations and deletions of this gene are uncommon (33, 3, 34, 35). Furthermore, although epigenetic regulation through promoter hypermethylation might offer an alternative explanation for regulation of PTEN protein expression, reports suggest that promoter methylation may also not account for all cases of PTEN protein loss in NSCLC (36–38). In addition, whereas *PIK3CA* mutation and amplification are also relatively frequent in NSCLC, reports of similar changes for *PIK3CB* are scarce. The overall conclusion drawn is that genetic analyses of *PIK3CB* amplification or *PTEN* mutation/deletion/methylation in NSCLC will not define fully the subset of patients identified here by IHC analysis of protein expression, and that multiple mechanisms may contribute to expression of PI3K $\beta$  and PTEN in NSCLC.

The explanation for elevated PI3K $\beta$  expression in SCC compared with adenocarcinoma is unknown, although we and others have observed greater loss of the tumor suppressor PTEN measured by IHC in SCC (17, 28). Given that SCC and adenocarcinoma develop from distinct cell lineages in different regions of the lung (39), and display differing genetic mutations, it is logical to assume that these histologic subtypes may assume different mechanisms for tumorigenesis. Of interest is whether the downstream effectors of PI3K $\beta$ , such as AKT, mTOR, FOXO, and S6 protein levels are also activated in tissue samples in which high levels of PI3K $\beta$  and/or PTEN deficiency are observed. Increased pAKT staining has been shown to correlate with more advanced stage of NSCLC disease (17, 24–26); however, no correlation between AKT activation and PTEN expression has been demonstrated in NSCLC to date (17, 40). Despite this, evidence is available to suggest that overexpression of phospho-AKT and loss of PTEN may represent independent indicators of poor prognosis in NSCLC and adenocarcinoma, respectively (15, 35). The expression level of PI3K pathway markers in samples with high PI3K $\beta$  and low PTEN should form the basis of future studies.

In mammals, class I PI3Ks are present in all cell types (41) and PI3K $\beta$  displays a broad tissue distribution as assessed by Northern and Western blotting, with signaling through this pathway activated in cancers due to loss of PTEN or the presence of activating mutations or amplifications in genes encoding key components of the pathway, such as *PIK3CA* or *AKT1*. Elevated expression of PI3K $\beta$  as described here for SCC might also relate to activation of the PI3K pathway, especially where membrane localization of PI3K $\beta$  is coincident with PTEN loss indicating recruitment of PI3K $\beta$  to substrate PIP<sub>2</sub>, which resides at the plasma membrane (42). With respect to PTEN, early studies proposed that PTEN expression was localized exclusively to the cytoplasm in which it functioned to inhibit PI3K–AKT signaling through dephosphorylation of PIP<sub>3</sub> to PIP<sub>2</sub>. However, more recently a role for nuclear PTEN in tumor suppression has also been revealed with highly proliferating advanced tumors exhibiting lower nuclear PTEN (43). We, and others (19), have described the presence of both cytoplasmic and nuclear PTEN in tumors, and in the present data set the

correlation between high PI3K $\beta$  expression and reduced PTEN in SCC was evident for both cytoplasmic and nuclear localization of PTEN.

Knowledge of the coincidence of other molecular drivers will also be required to establish whether other oncogenic drivers may be involved. The preliminary data presented in this publication (Supplementary Table S3) and others indicate no particular bias toward co-occurrence with mutations in the genes for EGFR, KRAS, p53, or LKB1, other than EGFR gene mutations occurring only in adenocarcinoma, although with substantial variability observed between studies (23). The dataset described in this study is insufficient in size to attach statistical significance to each molecular subset of the separate histologies and a larger patient sample set analysis should be the subject of future studies. Likewise, a larger patient sample set may be required to fully exclude any statistically significant relationship to tumor stage/grade and outcome. A molecular analysis of the etiology of squamous NSCLC would help to determine the impact of PI3K $\beta$  and PTEN expression alongside relatively early genetic changes such as the mutation of gene for p53.

All of the tissues used in this study are from surgical resections, with just 6 adenocarcinoma tissue samples being from stage IV disease (Supplementary Tables S1 and S2). The role of PI3K $\beta$  protein expression in late-stage metastatic NSCLC cannot therefore be deduced and likewise the possibility of clonal selection in synchronous and asynchronous metastases will also require further studies. The tissues are also limited to North American and European patients and wider studies would be required to determine geographic differences in prevalence as have been seen for driver mutations such as EGFR and KRAS (22, 33).

Our results indicate that using IHC pathology score inclusion thresholds of  $\geq 2+$  for cytoplasmic PI3K $\beta$  and  $\leq 1+$  for PTEN, a predominantly squamous patient popu-

lation can be delineated (representing 27% and 15% of the overall study population for TMA set 1 and TMA set 2 data sets, respectively).

Preclinical and clinical studies will be required to establish the validity of the hypothesis that these patients represent a subset of NSCLC for whom inhibitors of PI3K $\beta$  may be predicted to achieve the greatest clinical benefit.

#### Disclosure of Potential Conflicts of Interest

G. Beran and S.P. Dearden have ownership interest (including patents) in AstraZeneca. I.I. Wistuba received commercial research grant from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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# Clinical Cancer Research

## Identification of a Subset of Human Non–Small Cell Lung Cancer Patients with High PI3K $\beta$ and Low PTEN Expression, More Prevalent in Squamous Cell Carcinoma

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