PTEN Is a Potent Suppressor of Small Cell Lung Cancer

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Abstract

Small cell lung carcinoma (SCLC) is a highly metastatic tumor type with neuroendocrine features and a dismal prognosis. PTEN mutations and PIK3CA activating mutations have been reported in SCLC but the functional relevance of this pathway is unknown. The PTEN/PI3K pathway was interrogated using an AdenoCre-driven mouse model of SCLC harboring inactivated Rb and p53. Inactivation of one allele of PTEN in Rb/p53-deleted mice led to accelerated SCLC with frequent metastasis to the liver. In contrast with the high mutation burden reported in human SCLC, exome analyses revealed a low number of protein-altering mutations in mouse SCLC. Inactivation of both alleles of PTEN in the Rb/p53-deleted system led to nonmetastatic adenocarcinoma with neuroendocrine differentiation. This study reveals a critical role for the PTEN/PI3K pathway in both SCLC and lung adenocarcinoma and provides an ideal system to test the phosphoinositide 3-kinase (PI3K) pathway inhibitors as targeted therapy for subsets of patients with SCLC.

Implications: The ability of PTEN inactivation to accelerate SCLC in a genetic mouse model suggests that targeting the PTEN pathway is a therapeutic option for a subset of human patients with SCLC.

Visual Overview: http://mcr.aacrjournals.org/content/early/2014/04/28/1541-7786.MCR-13-0554/F1.large.jpg. Mol Cancer Res; 12(5); 654–9. ©2014 AACR.

Introduction

Small cell lung carcinoma (SCLC) is a highly metastatic neuroendocrine tumor that results in the deaths of >20,000 people per year in the United States alone. It has been known that the p53 and RB tumor suppressor genes are mutated in the majority of SCLCs, and that MYC family members are frequently amplified (1, 2). Alterations in the PTEN pathway have also been reported in SCLC, through direct PTEN mutation/deletion (3, 4) or through PIK3CA activation (5). PIK3CA and/or PTEN mutations were more recently found in two recent next-generation sequencing studies of SCLC (6, 7). The huge number of somatic mutations in human SCLC (6–8) necessitates the functional evaluation of key SCLC-mutated genes. As inhibition of phosphoinositide 3-kinase (PI3K) or the downstream effectors AKT and mTOR can be achieved using targeted therapies, the importance of the PTEN pathway in SCLC is particularly critical to elucidate. Murine models for SCLC have been generated that accurately recapitulate the cardinal features of human SCLC, including recapitulating key secondary alterations (9–12). In this study, we use a mouse model to interrogate PTEN as a potential SCLC driver.

Materials and Methods

Mice

Rblox mice were obtained from Tyler Jacks (MIT). p53lox mice were generated by Anton Berns (Netherlands Cancer Institute; Amsterdam, the Netherlands) and obtained from the Mouse Models of Human Cancer Consortium. Ptenlox mice were generated by Hong Wu (University of California, Los Angeles; Los Angeles, CA) and obtained from Jackson Laboratories. All mice were maintained on a mixed genetic background. Mouse experiments were approved by the Animal Use and Care Committees at the Carnegie Institution (Baltimore, MD) and Fred Hutchinson Cancer Research Center (Seattle, WA).

AdenoCre SCLC model. After breeding the Ptenlox allele into the Rblox;p53lox background, Rblox, p53lox, Ptenlox mice were intercrossed to obtain littermate controls that differed in Pten status. Mice were infected with $1 \times 10^8$
pfu AdenoCre driven by the cytomegalovirus promoter (University of Iowa Gene Transfer Core; Iowa City, IA) in 75 μL using intratracheal intubation as described (13). Mice were aged until moribund and the lungs were fixed in 4% paraformaldehyde or Bouin solution for histologic analyses. The following antibodies were used for immunohistochemistry: calcitonin gene-related peptide (CGRP; 1/2,000, Sigma), synaptophysin (1/33, Invitrogen), and CK19 (1/250, Abcam ab52625). CGRP immunostaining was performed on Bouin fixed tissue. Antigen retrieval was performed using boiling sodium citrate (pH 6.0) and samples were incubated overnight with primary antibody. We used the Vectastain ABC Kit (Vector Laboratories) for biotin-mediated signal amplification, and horseradish peroxidase-based detection was with 3, 3′-diaminobenzidine (Vector Laboratories).

**Real-time PCR.** Total RNA from lung tumors was extracted using TRIzol reagent (Life Technologies). cDNAs were generated using random hexamer priming and SuperScript III reverse transcriptase (Life Technologies). Real-time PCR was performed with Sybr Select master mix (Life Technologies) in 384-well format using an ABI 7900HT Real-Time PCR System. Pten copy number was examined by designing primers to exon 5 of the murine Pten gene and comparing Ct values relative to a control gene, Actb across phenol-chloroform isolated genomic DNA from tumors. All primer sequences used for real-time PCR are shown in Supplementary Table S1.

**Next-generation sequencing to identify SCLC mutations.** Mouse tumor or tail DNA was isolated following proteinase K digest and phenol-chloroform extraction. The SureSelectXT Mouse All Exon platform (Agilent Technologies) was used for exon capture and library preparation. Samples were sequenced using an Illumina HiSeq2000 with generation of 75bp paired end reads. The Burrows Wheeler Aligner (14) was used to align reads to the mouse mm9 reference genome. SAMtools was used to remove duplicates arising from PCR and was also used to detect variants (mismatches, and small insertions and deletions; ref. 15). Variant positions were identified from matched pairs of tumor samples and normal tissue or cell line controls using SAMtools with computation of genotype likelihood in each sample (options -u, -D, and -S). Variant genotypes were called using bcftools (15) with Bayesian inference, per-sample (options -u, -D, and -S). Variant genotypes were called using bcftools (15) with Bayesian inference, per-sample (options -u, -D, and -S). Variant genotypes were called using bcftools (15) with Bayesian inference, per-sample (options -u, -D, and -S).

**Western blotting.** Cell lysates were prepared in ice-cold radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate) supplemented with protease inhibitor tablets (Roche). The following primary antibodies were used: anti-Pten (9559, Cell Signaling Technology), anti-Phospho Akt (Ser-473; #4060, Cell Signaling Technology), anti-Akt (9691, Cell Signaling Technology) and anti-actin (sc-1615, Santa Cruz Biotechnology).

**Results**

Despite previous reports of Pten deletions (3, 4, 17) and PIK3CA activating mutations (5) in SCLC, the overall importance of the Pten/PIK3CA pathway for this cancer remains unclear. Tumor dependence on mutations in the Pten/PI3K pathway may provide an avenue for SCLC treatment through therapies that target this pathway. Thus, we explored the functional importance of this pathway for SCLC. To assess the importance of the Pten/PI3K pathway for SCLC, we used the Berns SCLC mouse model (9). This model uses adenoviral Cre (AdCre) to drive Rb and p53 deletion. Resulting lung tumors arise with long latency and mimic critical features of human SCLC, including neuroendocrine characteristics and metastatic spread (9). We infected Rbflox/lox;p53flox/+, Ptenflx/lox;Rbflx/flx;+p53flx/flx;Ptenflx/+ and Rbflx/flx;p53flx/flx;Ptenflx/flx cohorts with AdCre delivered using intratracheal intubation. Cohorts were aged and followed until the mice were moribund.

**Hemizygous inactivation of Pten accelerates murine SCLC**

Inactivation of Rb/p53 led to moribidity from lung tumors arising with long latency. Mice in the Rb+/p53 cohort became moribund with lung tumor burden at an average ± SD of 387 ± 57 days (Fig. 1A). Tumors in the model were overall histologically similar to the SCLC tumors previously described (9). Most tumors exhibited neuroendocrine features, staining positively for neuroendocrine markers CGRP (Fig. 1B, inset) and synaptophysin (Supplementary Fig. S1), although variability in staining was observed. The SCLCs were aggressive with invasion into vessels and local lymph nodes (Fig. 1B and Supplementary Fig. S1). We noted a minor component of acinar adenocarcinoma with neuroendocrine differentiation in some Rb+/p53-deficient tumors (Supplementary Fig. S1). Inactivation of one allele of Pten in the Rbflx/flx;p53flx/flx background significantly accelerated tumorigenesis. Here, mice became moribund at an average ± SD of 242 ± 59 days (Fig. 1A) and lung tumors exhibited histologic features similar to the Rbflx/flx;p53flx/flx model (Fig. 1C and Supplementary Fig. S2). Heterogeneity in CGRP and synaptophysin staining was seen within and between tumors in the Rbflx/flx;p53flx/flx;Ptenflx/+ group. As in the Rbflx/flx;p53flx/flx;Ptenflx/+ model, the major tumor component of the Rbflx/flx;p53flx/flx;Ptenflx/+ SCLCs examined (Supplementary Fig. S3A and S3B), and real-time PCR analysis of Pten copy number was consistent with loss of Pten heterozygosity in each case (Supplementary Fig. S3C). Necropsy
analysis revealed gross liver metastasis in 16 of 25 of mice examined (64%) and histologic analyses of liver metastases showed exclusively SCLC. The strong acceleration of SCLC in a $Pten^{+/-}$ heterozygous background reveals that $Pten$ is a critical cooperating tumor suppressor gene in SCLC.

Homozygous inactivation of $Pten$

Adenoviral Cre delivered to $Rb^{lox/lox}, p53^{lox/lox}, Pten^{lox/lox}$ animals resulted in a distinct phenotype. Here, lung tumors arose extremely rapidly (average ± SD of 123 ± 30 days; Fig. 1A) with each lobe of the lung filled with tumors at the time of morbidity (Fig. 2A). The major component of the $Rb^{lox/lox}, p53^{lox/lox}, Pten^{lox/lox}$ tumors was acinar and mixed adenocarcinoma with neuroendocrine differentiation revealed by CGRP and synaptophysin immunohistochemistry (Fig. 2A–C). The tumors had acinar and papillary patterns of growth (Fig. 2B). We also observed dysplastic and hyperplastic neuroendocrine lesions in the airways (Fig. 2D), likely precursor lesions to SCLC. The adenocarcinomas, including those with neuroendocrine features, stained positively for cytokeratin 19 (CK19; Fig. 2C), whereas the hyperplastic neuroendocrine lesions along the airways were negative for this marker (Fig. 2D). We note that SCLC tumors that arose in the $Rb^{lox/lox}, p53^{lox/lox}, Pten^{lox/lox}$ model did not stain positively for CK19 (Supplementary Fig. S2). Also, although Krt7 and Krt18 mRNA expression was not significantly different between lung tumors in the $Rb^{lox/lox}, Pten^{lox/lox}$ versus $Rb^{lox/lox}, p53^{lox/lox}, Pten^{lox/lox}$ models, Krt19 levels were significantly increased in the $Rb^{lox/lox}, Pten^{lox/lox}$ adenocarcinoma model (Supplementary Fig. S4). Thus, despite common expression of neuroendocrine markers, the $Rb^{lox/lox}$;
p53lox/lox;Ptenlox/lox;Ptenlox/lox

differentiation. The rapid lethality from many independent adenocarcinomas likely impaired development of advanced SCLC with liver metastasis in the Pten homozygous model.

Molecular analyses of lung tumors

Western blot analysis of SCLCs from the Pten heterozygous model revealed complete loss of PTEN protein in four of four tumors examined (Fig. 3A). This is consistent with inactivation of the remaining wild-type Pten allele (Supplementary Fig. S3C). We were unable to control for the normal level of phospho-AKT in pulmonary

![Figure 2. Homozygous Pten inactivation in Rb/p53-mutant lung. A, hematoxylin and eosin (H&E) stain showing tumor-filled lung from Rblox/lox; p53lox/lox; Ptenlox/lox mouse 3 months 20 days after AdCre (left). CGRP immunostaining of adjacent section showing neuroendocrine character of many tumor nodules (right). Boxed region shows magnified view of adenocarcinoma with neuroendocrine differentiation (Ac-NE) along with adenocarcinoma negative for CGRP (*). B, high-magnification (>100) view of adenocarcinoma histology. C, synaptophysin (SYP) and CK19 immunostaining of adenocarcinoma. Adjacent lesions both exhibit CK19 positivity, but only tumor area to left is synaptophysin positive. D, synaptophysin (SYP) positivity and absence of CK19 immunostaining in hyperplastic neuroendocrine cells along the airway (NEC-HP). Scale bars for A (top), 2 mm; A (bottom), 400 microns; B, 13 microns; C and D, 80 microns.

![Figure 3. Analyses of murine lung neuroendocrine tumors. A, Western blot analyses of normal lung and SCLCs from the indicated genotypes showing PTEN, Phospho Akt S473, pan-AKT, and actin loading control. B, number of protein-altering mutations in murine lung neuroendocrine tumors of the indicated genotypes. Metastatic samples are indicated (*). Rblox/lox; p53lox/lox; Ptenlox/lox; six tumors from 3 animals, Rblox/lox; p53lox/lox; Ptenlox/+; 13 tumors from 6 animals, Rblox/lox; p53lox/lox; Ptenlox/lox; 13 tumors from 6 animals, Rblox/lox; p53lox/lox; Ptenlox/lox; eight tumors from 3 animals, Rblox/lox; p53lox/lox; Ptenlox/lox; six tumors from 3 animals. C, patterns of transitions and transversions in primary murine SCLCs.

![Image](image-url)
neuroendocrine cells in these Western blot analyses, as such cells are extremely rare in the lung. However, compared with Pten wild-type mouse SCLC, Pten hemizygous and homozygous lung tumors showed increased phosphorylation of AKT at Ser 473, indicative of pathway activation (Fig. 3A).

Secondary alterations in lung tumors

Human SCLC is a smoking-associated cancer with high mutational load (6–8). In one study, an average of 175 protein-altering mutations per SCLC tumor were reported (7). To compare the somatic mutational load in murine SCLC with human SCLC, we performed whole-exome studies. In contrast with human SCLCs, the murine SCLC exome showed few protein-altering somatic mutations. We found an average of 15.8 protein altering mutations per murine SCLC in the Rbfl/fl; p53fl/fl model (Fig. 3B). Pten heterozygote tumors exhibited a variable and intermediate number of mutations (average 8 protein-altering mutations). There were no recurrent mutations or mutations in known cancer genes in this small sample set. We also characterized exonic mutations in the Rbfl/fl; p53fl/fl; Ptenfl/fl lung adenocarcinomas; here, we found a near absence of selection for protein-altering mutations (average 0.7 mutations/tumor exome). In our murine exome analyses, on average, 92% of the mouse tumor exome was sequenced to 10× coverage whereas 82% was sequenced to 20× coverage (Supplementary Table S2). In contrast with human smoking-associated SCLC (6–8), C:G to T:A transitions in murine SCLC were infrequent (Fig. 3C). Thus, murine SCLC does not exhibit high numbers of point mutations typical of human smoking-associated SCLC.

Discussion

PTEN/PI3KCA mutations have been described in SCLC; however, the overall importance of this pathway for SCLC is not clear. Thus, we tested the importance of this pathway using mouse genetics. We inactivated Pten in an Rbl/p53-deleted mouse model of SCLC that recapitulates human SCLC in metastatic pattern and in neuroendocrine features (9). When even a single allele of Pten was inactivated, SCLC occurred with much faster kinetics. Moreover, the tumors in the Pten heterozygous model metastasized to the liver.

These data definitively show that Pten is a critical tumor suppressor in a genetic mouse model of SCLC. As there are no targeted therapies for SCLC, these data may provide incentive to treat human patients with SCLC with PI3K or Akt inhibitors. Murine SCLC models will be ideal for in vivo testing of such strategies. Additionally, the mouse tumor exome was sequenced to 10× coverage (6–8). In one study, an average of 175 protein-altering mutations per SCLC tumor were reported (7). To compare the somatic mutational load in murine SCLC with human SCLC, we performed whole-exome studies. In contrast with human SCLCs, the murine SCLC exome showed few protein-altering somatic mutations. We found an average of 15.8 protein altering mutations per murine SCLC in the Rbfl/fl; p53fl/fl model (Fig. 3B). Pten heterozygote tumors exhibited a variable and intermediate number of mutations (average 8 protein-altering mutations). There were no recurrent mutations or mutations in known cancer genes in this small sample set. We also characterized exonic mutations in the Rbfl/fl; p53fl/fl; Ptenfl/fl lung adenocarcinomas; here, we found a near absence of selection for protein-altering mutations (average 0.7 mutations/tumor exome). In our murine exome analyses, on average, 92% of the mouse tumor exome was sequenced to 10× coverage whereas 82% was sequenced to 20× coverage (Supplementary Table S2). In contrast with human smoking-associated SCLC (6–8), C:G to T:A transitions in murine SCLC were infrequent (Fig. 3C). Thus, murine SCLC does not exhibit high numbers of point mutations typical of human smoking-associated SCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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