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/PI3KCA 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 $1 \times 10^8$...
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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. Antibody 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). cDNA were generated using random hexamer priming and Super-script 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 C_v 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) 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 C_v 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.

Hemizygous inactivation of Pten accelerates murine SCLC

Inactivation of Rbl/p53 led to morbidity from lung tumors arising with long latency. Mice in the Rbl/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 Rbl/p53-deficient tumors (Supplementary Fig. S1). Inactivation of one allele of Pten in the Rbl+/p53+/− background significantly accelerated tumorogenesis. Here, mice became moribund at an average ± SD of 242 ± 59 days (Fig. 1A) and lung tumors exhibited histologic features similar to the Rbl+/p53+/− model (Fig. 1C and Supplementary Fig. S2). Heterogeneity in CGRP and synaptophysin staining was seen within and between tumors in the Rbl+/p53+/−; Pten+/− group. As in the Rbl+/p53+/− model, the major tumor component of the Rbl+/p53+/−; Pten+/− mice was SCLC, with a minor component of adenocarcinoma. PCR analysis showed that both fixed alleles of Rbl and p53 were recombined in six of six Rbl+/p53+/−; Pten+/− 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 Pt en heterozygous background reveals that Pt en is a critical cooperating tumor suppressor gene in SCLC.

Homozygous inactivation of Pt en

Adenoviral Cre delivered to Rb<sup>lox/lox</sup>;p53<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> 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<sup>lox/lox</sup>;p53<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> 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<sup>lox/lox</sup>;p53<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> 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<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> versus Rb<sup>lox/lox</sup>;p53<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> models, Krt19 levels were significantly increased in the Rb<sup>lox/lox</sup>;p53<sup>lox/lox</sup>;Pt en<sup>lox/lox</sup> adenocarcinoma model (Supplementary Fig. S4). Thus, despite common expression of neuroendocrine markers, the Rb<sup>lox/lox</sup>;
p53lox/lox;Ptenlox/lox;Rblox/lox;Ptenlox/lox SCLC and Rblox/lox;p53lox/lox;Ptenlox/lox adenocarcinoma models could be distinguished by histologic features and by CK19/Krt19 positivity. In contrast with the Rb/p53 and Rb/p53/Pten heterozygous models, liver metastasis was not found in the Pten homozygote AdCre model. We did not perform long-term aging studies on Ptenlox/lox mice in the context of wild-type Rb and p53. However, sacrifice of 5 nonmoribund Ptenlox/lox animals (wild-type for Rb and p53) at 5-months after AdCre did not reveal evidence of lung neoplasia (data not shown). Overall, these data indicate that homozygous Pten inactivation synergizes with Rb and p53 loss to promote lung adenocarcinomas with neuroendocrine 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/+; p53lox/+; Ptenlox/lox: eight tumors from 3 animals, Rblox/lox; p53lox/lox; Ptenlox/lox: 13 tumors from 6 animals, Rblox/lox; p53lox/lox; Ptenlox/lox: six tumors from 3 animals. C, patterns of transitions and transversions in primary murine SCLC.
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/A>T 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/PI3K3CA** 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 Rbfl/fl;p53fl/fl-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 inactivating both Pten alleles in the Rbfl/fl-p53 floxed background led to a shift in tumor spectrum. Multifocal adenocarcinomas, with the major component exhibiting neuroendocrine differentiation, led to rapid lethality. Interestingly, a subset of human adenocarcinomas that acquired resistance to targeted therapy acquired neuroendocrine characteristics and transformed into SCLC (18). In one patient, transformation to SCLC was associated with a newly acquired activating PI3K mutation present in the SCLC but not in the original adenocarcinoma (18). It will be interesting to investigate whether the RB, p53, and/or PTEN pathway alteration can be linked to the acquisition of neuroendocrine properties in a non-neuroendocrine cell of origin. Application of neuroendocrine promoter-driven adenoviral vectors specifically to the lung (19) will enable late-stage SCLC modeling with homozygous Pten inactivation.

We found that murine SCLCs exhibited a lower number of protein-altering mutations than human SCLCs. In the AdCre Rbfl/fl;p53fl/fl model, we found 15.8 protein-altering mutations per tumor, a number much reduced in comparison with human SCLC. This difference is at least, in part, due to the fact that human SCLC typically arises in heavy smokers, leading to a high smoking-induced mutational burden. Hemizygosity for Pten in this model led to reduced somatic point mutations. The Pten hemizygous model will be particularly useful for studies of metastasis, as metastatic SCLC arises frequently and rapidly. Matched comparisons between primary and metastatic murine SCLCs may shed light on the genetic determinants of metastasis. The lower number of mutations in mouse SCLC models may facilitate study of individual SCLC-mutated genes. The high mutational burden in human SCLC is likely to lead to increased noise in similar analyses of human SCLC. Assessment of vulnerabilities to therapies associated with a specific mutation will be particularly informative using murine SCLC models given the reduced mutational complexity.

We demonstrated the critical role for the PTEN/PI3K pathway in SCLC. This finding has important implications for using targeted therapies directed toward this pathway to treat the most aggressive form of lung cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Cui, M. Rongione, K. Conkrite, D. MacPherson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Cui, A. Augert, A.Y. Nikitin, N. Ingolia, D. MacPherson

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