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Role of PTEN in basal cell derived lung carcinogenesis

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Abstract

Lung adenocarcinoma (AdC) and lung squamous cell carcinoma (SCC) are the most common non-small cell lung cancer (NSCLC) subtypes, however, most genetic mouse models of lung cancer produce predominantly, if not exclusively, AdC. Whether this is secondary to targeting mutations to the distal airway cells or to the use of activating Kras mutations that drive AdC formation is unknown. We previously showed that targeting $Kras^{G12D}$ activation and transforming growth factor β receptor type II (TGFβRII) deletion to airway basal cells via a keratin promoter induced formation of both lung AdC and SCC. In this study we assessed if targeting phosphatase and tensin homologue (PTEN) deletion to airway basal cells could initiate lung tumor formation or increase lung SCC formation. We found that PTEN deletion is capable of initiating both lung AdC and SCC formation when targeted to basal cells and although PTEN deletion is a weaker tumor initiator than $Kras^{G12D}$ with low tumor multiplicity and long latency, tumors initiated by PTEN deletion were larger and displayed more malignant conversion than $Kras^{G12D}$ initiated tumors. That PTEN deletion did not increase lung SCC formation compared to $Kras^{G12D}$ activation, suggests that the initiating genetic event does not dictate tumor histology when genetic alterations are targeted to a specific cell. These studies also confirm that basal cells of the conducting airway are capable of giving rise to multiple NSCLC tumor types.

Keywords

non-small cell lung cancer; adenocarcinoma; squamous cell carcinoma; Kras

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Introduction

Non-small cell lung cancer (NSCLC) is a common and deadly malignancy with over 175,000 cases per year in the US and a five year survival rate of less than 20% [1]. The most common NSCLC histologic subtypes, lung adenocarcinoma (AdC) and lung squamous cell carcinoma (SCC), are associated with distinct molecular abnormalities and are thought to have distinct cells of origin [2, 3]. It is hypothesized that lung AdCs arise from distal airway epithelial cells [4, 5] while lung SCCs arise from the basal cell population of the upper airway. In human NSCLC, activating Kras mutations occur in 25% of AdCs but are exceedingly rare in lung SCCs [6]. In contrast, phosphoinositide-3-kinase (PI3K) amplification/mutation or phosphatase and tensin homologue (PTEN) loss occur in 35–45% of SCCs but only 5–10% of AdCs [7, 8]. However, it is unknown whether specific oncogenic events influence tumor histology.

Genetic NSCLC mouse models produce mainly adenomas and AdCs [9], although some models also produce a limited number of SCCs [10, 11]. Whether this is secondary to strategies targeting distal airway epithelial cells or to the use of oncogenic Kras as an initiator is unknown. Some data suggest it is a function of targeting distal airway cells since models employing Clara cell secretory protein (CCSP) or surfactant protein C (SPC) promoters produce exclusively adenomas and AdCs regardless of whether the initiating event is Kras activation [12, 13], mutant PI3K expression [14], or PTEN deletion [15]. In contrast, models employing adenoviral Cre recombinase (AdCre) targeting produce can exclusively AdC [16, 17], a mixture of AdC, SCC, and large cell carcinomas [10], or exclusively small cell lung cancers (SCLC) [18, 19], depending on the genetic alterations. This suggests that the initiating event influences tumor histology or, alternatively, that specific genetic events allow the outgrowth of tumors arising from different progenitors.

Keratin positive basal cells are a multi-potent progenitor of the upper airway that can self-renew as well as differentiate into Clara cells and ciliated epithelial cells [20, 21]. Basal cells may contain the lung SCC cell of origin. We previously found that targeting $Kras^{G12D}$ activation and transforming growth factor receptor type II (TGFβRII) deletion to airway basal cells resulted in NSCLC formation [11]. Despite targeting with a keratin 5 promoter, the tumors produced in this model were predominantly adenomas and AdCs; SCCs were only rarely observed [11]. In the current study, we tested whether PTEN deletion could initiate tumor formation when targeted to airway basal cells and whether replacing $Kras^{G12D}$ activation with PTEN deletion would drive lung SCC formation.

Materials and Methods

NSCLC mouse models

All animal studies were IACUC approved and performed in a C57BL/6 background. The TGFβRII conditional deletion allele, PTEN conditional deletion allele, lox-stop-lox-(LSL)$Kras^{G12D}$ knock-in allele, K5CrePR* transgene, and K14CrePR1 transgene have been previously described [22–27]. Mice with the indicated genotypes were produced by appropriate breeding strategies. Only heterozygotes harboring the K5CrePR* and K14CrePR1 transgenes and the LSL-$Kras^{G12D}$ allele (hereafter referred to as $Kras^{G12D}$)

were used. Mice were treated with tracheal RU486 (500μg in 25μl 10% acetone/90% sesame oil) or oral RU486 (1,000 μg total in two divided doses) between 4–6 weeks of age. Mice were euthanized between 12–18 months of age, if they lost >15% of their body weight, or exhibited dyspnea. At euthanasia, tumors were enumerated and samples collected as previously described [11]. Differences in tumor frequency were analyzed by Fisher's exact test with Prism5 (Graph Pad, La Jolla, CA).

Tumor characterization and immunostaining

Lesions were classified as adenomas, AdC, or SCC by a pathologist (DM) using previously described consensus criteria [28]. All tumors initiated by PTEN deletion were scored; for each $Kras^{G12D}$ genotype, 6–8 tumor containing lung sections from different animals were scored. Confirmatory immunostaining was performed as previously described [11] with antibodies against keratin 5 (K5, 1:500, Covance PRB-160P), thyroid transcription factor (TTF, 1:1000, Abcam #40880), PTEN (1:100, Abcam #32199), pAKT (1:50, Cell Signaling #3787), and pERK 1/2 (1:100, Cell Signaling #9101). Images were acquired on a Nikon Eclipse 80i with Nikon Elements Software.

Results and Discussion

PTEN deletion initiates lung tumor formation when targeted to airway basal cells

Previously we showed that targeting $Kras^{G12D}$ to airway basal cells resulted in 2–3 tumors per animal and that deletion of one or both TGFβRII alleles increased both tumor number and tumor size [11]. To address whether PTEN deletion initiates lung tumor formation when targeted to airway basal cells and whether TGFβRII deletion similarly promotes the development of lung tumors initiated by PTEN deletion, we created mice in which PTEN and TGFβRII could be deleted via a keratin 5 (K5) or keratin 14 (K14) promoter targeted, RU486-inducible, Cre recombinase-progesterone receptor (PR) fusion protein [25–27]. We previously showed that both K14CrePR1 and K5CrePR* are induced in airway basal cells after tracheal RU486 [29] and that K5CrePR* can be used to direct NSCLC tumor formation [11]. Using this system, we compared the effects of PTEN deletion and $Kras^{G12D}$ activation in different TGFβRII genetic backgrounds (Table 1).

Deletion of one PTEN allele resulted in lung tumor formation in 8/41 (20%) animals while deletion of both PTEN alleles resulted in lung tumor formation in 2/23 (9%) animals (Table 1). In contrast $Kras^{G12D}$ activation initiated lung tumors in 23/40 (58%) animals ($p<0.001$ compared to PTEN+/− or PTEN−/−). PTEN deletion was confirmed by reduced PTEN immunostaining and increased pAKT immunostaining while $Kras^{G12D}$ activation was confirmed indirectly by increased pERK immunostaining and (Fig. 1A). Although more lung tumors were observed with deletion of a single PTEN allele than after deletion of both alleles, this trend did not reach statistical significance (8/41 vs 2/23, $p=0.47$). Nonetheless, this is the opposite of what was observed when PTEN deletion is broadly targeted to prostate epithelial cells and homozygous deletion markedly increase tumor formation [30]. While this may reflect tissue specific differences in the role of PTEN, it is also possible that targeting a facultative progenitor cell is responsible for this observation, as PTEN is required for stem cell maintenance in both the nervous and immune systems [31] and homozygous

PTEN deletion in embryonic fibroblasts promotes senescence in the presence of wild type p53 [32].

Tumors initiated by PTEN deletion also exhibited longer latency than $Kras^{G12D}$ initiated tumors; all tumors initiated by PTEN deletion were observed in animals over 52 weeks of age and 50% (6/12) of tumors occurred in animals older than 80 weeks. The average age at euthanasia of animals with Kras-initiated lung tumors was 50.4 ± 1.3 weeks (mean ± SEM) compared to 71.0 ± 3.8 weeks in animals with tumors initiated by PTEN deletion ($p<0.001$). In addition, mice with Kras-initiated tumors had 22.1 ± 7.7 tumors per animal (mean ± SEM) while mice with tumors initiated by PTEN deletion had 1.2 ± 0.13 tumors per animal ($p=0.01$). These data demonstrate that although PTEN deletion can initiate lung tumor formation when targeted to airway basal cells, it is a less robust initiator than $Kras^{G12D}$ activation. This is similar to what was seen in models targeting distal airway cells where Kras activation caused multifocal lung tumors resulting in death within 24 weeks [5, 33] but PTEN deletion did not cause lung tumor formation out to one year [33].

Compared to $Kras^{G12D}$ initiated tumors, PTEN+/− and PTEN−/− tumors were significantly larger and displayed more frequent malignant conversion as evidenced by a larger percentage of AdCs (Table 1). This suggests PTEN loss may more efficiently promote tumor growth in established tumors, though it is possible that this observation is attributable to the increased time these tumors were allowed to progress. Though all animals were subject to the same monitoring criteria, $Kras^{G12D}$ animals were typically euthanized earlier then PTEN animals secondary to their larger tumor burden. Although TGFβRII deletion increased both the number and size of $Kras^{G12D}$ initiated tumors (Table 1), too few tumors were generated by PTEN deletion for a robust statistical analysis of the effect of TGFβRII deletion on tumor size or number. Interestingly, TGFβRII deletion did not significantly increase tumor incidence regardless of the initiating event.

PTEN deletion does not drive lung SCC formation

In NSCLC, Kras mutations are almost exclusively seen in AdC while PTEN loss and PI3K amplifications are far more common in SCC [6–8]. We previously found that targeting $Kras^{G12D}$ activation and TGFβRII deletion with K5CrePR* resulted in predominantly adenomas and AdCs [11]. To determine whether replacing oncogenic Kras activation with PTEN deletion increased SCC formation, we classified lung tumors using consensus criteria [28] and further confirmed tumor histology by immunostaining. As shown in Fig. 1B, AdCs display glandular morphology and stain positive for thyroid transcription factor (TTF) while SCCs have keratinization and intercellular bridges and stain positive for K5. We found that 8% (1/12) of lung tumors initiated by PTEN deletion were lung SCCs; this was not significantly different than the 2% (4/176) of lung SCCs observed with $Kras^{G12D}$ initiation (Table 1), suggesting that the initiating oncogenic event does not dictate tumor histology when tissue or cell specific promoters are used to target oncogenic mutations to the lung. This is in marked contrast to AdCre based targeting strategies where the initiating events clearly impact tumor histology. For example, simultaneous Kras activation and p53 deletion leads exclusively to AdCs but simultaneous Kras activation and LKB1 deletion produces a mixture of AdCs and SCCs [10] while simultaneous p53 and Rb deletion produces

exclusively SCLCs [18]. This suggests that certain genetic alterations can facilitate the growth of tumors arising from specific progenitor cell populations. This notion is further supported by the finding that while simultaneous p53 and Rb deletion targeted by AdCre results in SCLC formation [18] targeting the same deletions with an adenovirus that expresses Cre recombinase under the control of the SPC promoter causes AdCs [19].

Only a subset of K5 positive upper airway basal cells also express K14 [29, 34] and the lung SCC progenitor may arise from this subpopulation [35]. When we separately analyzed tumor formation in K14CrePR1 animals, we found that 23% (3/13) of the animals developed lung tumors; this was comparable to the tumor formation observed in animals with PTEN deletion targeted by K5CrePR*. We were unable to analyze lung tumor formation in K14CrePR1.Kras^{G12D} mice, as these animals develop life-limiting oral and anal tumors prior to lung tumor development, likely related to leakiness of K14CrePR1 in combination with a potent tumor initiator like Kras^{G12D}. Interestingly, the single SCC that developed with keratin directed PTEN deletion occurred with the K14CrePR1 transgene, suggesting a multipotent tumor initiating cell resides within the K14/K5 double positive population. Although keratin positive basal cells have been best characterized in the conducting airways, K5 positive cells can be found more distally in the lung parenchyma during repair after influenza injury [36]. Our observation that both lung SCC and lung AdC are produced by targeting oncogenic mutations to keratin positive cells might be explained by this facultative progenitor cell population.

In conclusion, we show that targeting PTEN deletion to airway basal cells can initiate lung tumor formation, but with a low tumor incidence and long latency. PTEN deletion does not increase lung SCC formation suggesting that, at least in a keratin promoter driven model, the initiating genetic event does not significantly influence tumor histology. Finally, our findings confirm that basal cells of the conducting airway can give rise to both lung SCC and AdC.

Acknowledgments

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Abbreviations

NSCLC	non-small cell lung cancer
SCLC	small cell lung cancer
AdC	adenocarcinoma
SCC	squamous cell carcinoma
CCSP	Clara cell secretory protein
SPC	surfactant protein C
K5	keratin 5
K14	keratin 14

TTF	thyroid transcription factor
PI3K	phosphoinositide-3-kinase
PTEN	phosphatase and tensin homologue
TGFβRII	transforming growth factor receptor type II
Rb	retinoblastoma
AdCre	adenovirus Cre recombinase
PR	progesterone receptor
SEM	standard error of the mean

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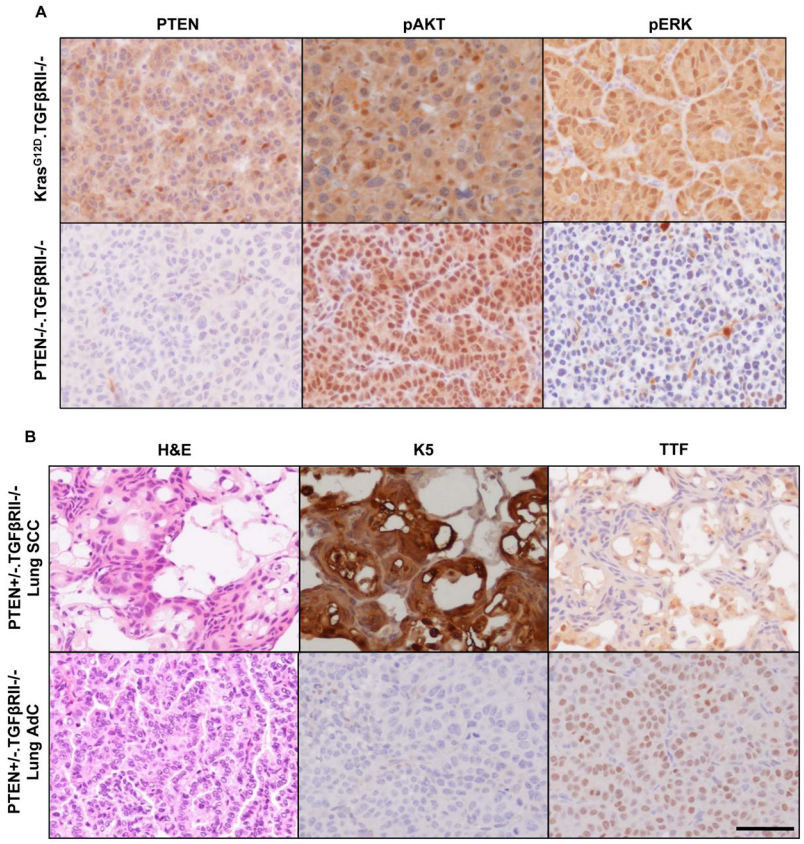

Figure 1. Confirmation of Kras activation, PTEN deletion, and tumor histology

(A) PTEN deletion was validated by reduced PTEN immunostaining and increased pAKT immunostaining in PTEN −/−.TGFβRII−/− tumors, while Kras activation in $Kras^{G12D}$.TGFβRII−/− tumors was confirmed by increased pERK staining. (B) Tumor histology was analyzed by H&E staining and confirmed by immunostaining. SCCs stained positive for keratin 5 (K5) while AdCs stained positive for thyroid transcription factor (TTF). Scale bar is 50μm.

Table 1

Comparison of PTEN deletion and Kras^{G12D} activation lung tumor initiators

Animals were treated with oral (n=12) or tracheal (n=92) RU486 and monitored as described in methods. No tumors were observed in animals without a Cre recombinase transgene (n=6) or in vehicle treated animals (n=7). Genetic manipulations were targeted with either the K5CrePR* transgene (n=91) or the K14CrePR1 transgene (n=13). Tumor incidence includes all animals with grossly visible lung tumors. Tumor number, size, age, and histology data reflect only tumor bearing animals. Tumor number and size are expressed as mean ± SEM; age indicates when animals were euthanized. Tumor histology was scored according to consensus criteria [28]. The number of tumors analyzed for histology exceeds the number of tumor bearing animals since many animals had multiple tumors; percentages do not total 100% because of benign lesions (adenomas). Heterozygous PTEN deletion initiated tumor formation in 20% of animals; homozygous PTEN deletion initiated tumor formation in 9% of animals; Kras^{G12D} activation initiated lung tumor formation in 58% of animals († $p<0.001$ vs. PTEN+/− or PTEN−/−). Compared to Kras^{G12D} initiated tumors, tumors initiate by PTEN deletion were larger (‡ $p<0.001$ vs Kras^{G12D} with the same TGFβRII status) and exhibited a higher percentage of AdCs (§ $p<0.05$ vs. Kras^{G12D} with the same TGFβRII status). Both Kras activation and PTEN deletion also initiated a low level of lung SCC formation. Although TGFβRII deletion increased tumor size and number of Kras initiated tumors (* $p<0.05$ vs Kras^{G12D}), insufficient tumors were observed with PTEN deletion to analyze the effect of TGFβRII deletion on these variables. Two lymphomas were also observed (one in a PTEN−/−.TGFβRII+/− animal and one in a PTEN−/−.TGFβRII−/− animal); this may be related to keratin expression in the thymus [37]. One oral SCC was seen in a PTEN+/−.TGFβRII−/− animal, likely secondary to oral keratin expression. Abbreviations used in table: TGFβRII, transforming growth factor beta receptor type II; PTEN, phosphatase and tensin homologue; AdC, adenocarcinoma; SCC, squamous cell carcinoma; NA, not applicable.

Genotype		Tumor Incidence	Tumor Number	Tumor Size (mm)	Age (wk)	% AdC	% SCC
PTEN+/−	TGFβRII+/+	18% (2/11)	1.5 ± 0.5	3.1 ± 0.9 ‡	60–75	100% § (3/3)	0% (0/3)
	TGFβRII+/−	13% (2/15)	1	3.3 ± 0.6 ‡	52–80	100% § (2/2)	0% (0/2)
	TGFβRII−/−	27% (4/15)	1.3 ± 0.3	1.2 ± 0.2	72–88	40% (2/5)	20% (1/5)
Total PTEN+/−		20% (8/41)				70% § (7/10)	10% (1/10)
PTEN−/−	TGFβRII+/+	0% (0/6)	NA	NA	NA	NA	NA
	TGFβRII+/−	0% (0/5)	NA	NA	NA	NA	NA
	TGFβRII−/−	17% (2/12)	1	4.3 ± 2.7 ‡	80–81	50% (1/2)	0% (0/2)
Total PTEN−/−		9% (2/23)				50% (1/2)	0% (0/2)
Kras^{G12D}	TGFβRII+/+	53% (9/17)	3.4 ± 1.3	0.9 ± 0.1	43–62	7% (1/15)	13% (2/15)
	TGFβRII+/−	56% (5/9)	25.4 ± 5.3 *	1.3 ± 0.2 *	31–56	16% (11/67)	0% (0/67)
	TGFβRII−/−	64% (9/14)	27.1 ± 14.3 *	1.2 ± 0.1 *	40–59	11% (10/92)	2% (2/92)
Total Kras^{G12D}		58% (23/40) †				13% (22/176)	2% (4/176)