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Presence of a putative tumor-initiating progenitor cell population predicts poor prognosis in smokers with non-small cell lung cancer

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Abstract

Smoking is the most important known risk factor for the development of lung cancer. Tobacco exposure results in chronic inflammation, tissue injury and repair. A recent hypothesis argues for a stem/progenitor cell involved in airway epithelial repair that may be a tumor-initiating cell in lung cancer, and which may be associated with recurrence and metastasis. We used immunostaining, quantitative real-time PCR, Western blots and lung cancer tissue microarrays to identify

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subpopulations of airway epithelial stem/progenitor cells under steady state conditions, normal repair, aberrant repair with premalignant lesions and lung cancer and their correlation with injury and prognosis. We identified a population of keratin 14 (K14)-expressing progenitor epithelial cells that was involved in repair after injury. Dysregulated repair resulted in persistence of K14+ cells in the airway epithelium in premalignant lesions. The presence of K14+ cells in non-small cell lung cancer (NSCLC) samples predicted poorer outcomes. This was especially true in smokers where the presence of K14+ cells in NSCLC was predictive of metastasis. The presence of K14+ progenitor airway epithelial cells in NSCLC predicted a poor prognosis and this predictive value was strongest in smokers, where it also correlated with metastasis. This suggests that reparative K14+ progenitor cells may be tumor-initiating cells in this subgroup of smokers with NSCLC.

Keywords

Lung carcinogenesis; dysregulated repair; injury

Introduction

Lung cancer has the highest mortality rate of all cancers and patients with the diagnosis of metastatic NSCLC have a median survival of just 4–5 months ([1]). Lung cancer is a heterogeneous disease and the natural history is still not well understood. The classic exponential growth model of tumor metastasis may not be relevant in some tumors, where *biology* of disease may impact prognosis more than the time and size of growth of the tumor ([2]). For example, as many as 40% of patients with completely resected stage I NSCLC will experience a recurrence of their disease, which suggests that a subpopulation of cells in these tumors is more prone to micrometastatic behavior ([2]).

The cancer stem cell (CSC) model of tumor development and progression refers to the presence of a population of rare cells in a tumor that have stem cell properties, namely they are capable of self-renewal and differentiation to their progeny. In this model, the self-renewal capacity of the CSCs are responsible for maintaining tumor growth indefinitely and the other cells that make up most of the tumor are actively proliferating and differentiating and therefore susceptible to current conventional cancer therapies ([3–10]). Consistent with this model, CSCs would be considered to be tumor-initiating cells ([3–10]). Recently, it has been found that CSCs may not necessarily represent rare cells in a tumor and that the tumor-initiating cell in a cancer reflects a cell with the property of indefinite self-renewal and that this could be a rare stem cell, a progenitor cell or a differentiated cell that has developed the ability to self-renew ([11]). These tumor-initiating cells are thought to arise from cells that have dysregulated repair resulting in indefinite self-renewal and are associated with relapse and recurrence of cancers and a poor prognosis, presumably due to resistance to chemotherapy and radiotherapy ([3, 5–10]). This model of CSCs leading to tumor resistance fits well with the natural history of lung cancer, with its high incidence of recurrence and metastasis. In lung cancer, no population of dysregulated self-renewing cells has previously been found that correlates with poor prognosis.

Our understanding of stem and progenitor cells in the proximal airway epithelium is limited, but some populations have been identified with self-renewing and differentiation properties ([12–15]). Keratin 5 (K5)-expressing basal cells are considered to be progenitor cells in the adult large airways at steady state and during airway epithelial repair([12–15]). In humans, unlike mice, K5-expressing basal cells have been found throughout the tracheobronchial tree ([12]). It was previously thought that K14 is the obligate intermediate filament-binding partner of K5 in the basal cells of the airway epithelium ([12, 16]). However, although K14+ progenitor epithelial cells in the airway are important for repair, K14+ cells are rarely found

in the airway epithelium under homeostatic conditions while K5+ cells are relatively abundant ([12, 16]). We show here for the first time that K14-expressing cells are a unique subpopulation of airway epithelial cells that are almost exclusively present in the submucosal glands in the steady state ([17]). While K14+ progenitor epithelial cells in the airway are important for normal repair ([12, 16]), persistence of K14 expression is found in aberrant repair with premalignant lesions and in a subset of NSCLCs associated with injury from smoking. The primary objective of this study was to determine whether K14-expressing reparative progenitor airway epithelial cells within primary NSCLCs correlated with smoking injury, poor prognosis and metastasis.

Materials and Methods

Human and Mouse Tissue

Sections were obtained from uninjured C57BL/6 mouse tracheas as well as from C57BL/6 mouse syngeneic tracheal transplants. We used a well-established, reproducible model of tracheal epithelial regeneration using syngeneic subcutaneous tracheal transplants from wild-type C57Bl/6 mice into wild-type C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) [21,22]. For this model, donor wild-type C57Bl/6 mice were euthanized and the tracheas dissected out, removing the blood supply to the tracheas and causing an hypoxic-ischemic injury. Recipient wild-type C57Bl/6 mice were sedated with ketamine and an incision was made in the skin of the back of the mice. The donor tracheas were placed heterotopically under the skin of the recipient mice. Mice were euthanized at 7, 14 and 21 days after transplantation and the tracheal transplants were harvested for fixation in formalin and then paraffin embedding. Animal use for these studies was approved by the Department of Laboratory Animal Medicine, David Geffen School of Medicine at UCLA. Tissue sections were obtained from human lung cancer specimens archived in the UCLA Lung Cancer SPORE tissue bank (IRB#02-07-011). The research protocol was approved by the UCLA Institutional Review Board and all human participants gave written informed consent.

Dual Immunofluorescence and Immunohistochemistry

Dual immunofluorescence was performed as described ([18]). Briefly, tracheal tissue was fixed in 4% paraformaldehyde for 18–24 hours and then embedded in paraffin and sectioned. Sections (4 μm) were deparaffinized in xylenes and rehydrated in graded ethanols and boiled in 10 mM sodium citrate buffer for 10 min. Blocking was performed with serum-free protein block (Dakocytomation). The primary antibodies used were rabbit anti-mouse K5 (dilution 1:500; Abcam, Cambridge, MA), mouse anti-K14 (dilution 1:20; Abcam) and rabbit polyclonal anti-PCNA (Dilution 1:50; Abcam),

For calculating the proportion of proliferating (PCNA+), K5+, and K14+ cells in the epithelia, premalignant lesions, or tumors, tissue immunofluorescence images were obtained using a Zeiss AxioImager microscope (Carl Zeiss, Germany). For mouse samples, cross sections through the same level of each trachea were selected for measurement. Cells were manually counted at 20X magnification. Total K14+K5+ cells and K14-K5+ cells in an epithelium or lesion were counted to determine the percentage of K14+ cells within all the K5-expressing cells. K14+PCNA+ cells and K14+PCNA- cells in a premalignant lesion or tumor lesion were counted to determine the percentage of K14-expressing cells that were proliferating.

Immunohistochemical analysis of human lung tissue was performed as described ([19]) with the K5 and K14 antibodies described above. The lung TMAs were sectioned just prior to use, and serial sections were stained for K14 or K5 using a two-step immunohistochemical protocol.

Histological definitions: Reserve cell hyperplasia was defined as a continuous and double layer of basal cells. Squamous metaplasia requires development of horizontally oriented squamous cells with intercellular bridges. Dysplasia was diagnosed in the setting of epithelial thickening with nuclear pleomorphism and partial loss of normal maturation from the basal to luminal surface. Carcinoma *in situ* has marked nuclear pleomorphism and coarse chromatin with no maturation from basal to luminal surface and the absence of frank invasion.

Lung cancer tissue microarray (TMA)

The TMAs were constructed under appropriate IRB and HIPAA regulations using formalin-fixed, paraffin-embedded archival lung samples from the UCLA Department of Pathology and Laboratory Medicine and the lung cancer Specialized Program of Research Excellence (SPORE) tissue bank at The University of Texas M. D. Anderson Cancer Center (Houston, TX) ([19]). The characteristics of these TMAs have been previously described in detail ([19]). The TMA was scored in a semi-quantitative fashion by a pathologist (MA), and spot-checked by a second pathologist (VM); both of whom were blinded to clinical and outcomes information. K5 and K14 cytoplasmic staining was quantified based on the intensity and frequency of cell staining, similar to previously described methods ([19]). A total of 399 patients from the UCLA TMA and 505 patients from the M.D. Anderson TMA were used in these studies.

Statistical Analysis

Analyses were performed using the open source R software (http://www.R-project.org) including survival, Design and Hmisc packages. Pooling criteria were similar to those previously described ([19]). K5 and K14 expression differences among various subgroups were determined using the Wilcoxon signed rank test or Kruskal-Wallis rank sum test. For dichotomized (positive versus negative staining for K5 and K14) expression, the Fisher exact test was used for analysis with categorical variables such as stage, grade, smoking history and presence of metastasis. Survival curves were calculated using the Kaplan-Meier method and comparisons were made using the log-rank test. The Cox proportional hazards model (univariate and multivariate) was used to determine the significance of various factors related to survival. LogRank and Fisher exact P-values were two-sided and a $P < 0.05$ was considered significant.

Methods used for the experiments found in the Supplemental Data are in the Supplemental Data Methods section.

Results

Identification of K14+K5+, K14-K5+cell populations in the steady state airway epithelium and submucosal glands

Dual immunofluorescent staining of the steady state proximal airway epithelium and submucosal glands and submucosal gland ducts demonstrated the presence of K14+K5+ cells throughout the submucosal glands and submucosal gland ducts. Dual K5 and K14 expression was found in only 10.7 ± 3.4% of basal cells of the mouse pseudostratified columnar epithelium (Figure 1A, Supplemental Data Table 3) and 1.3% ± 0.8% of basal cells in the human pseudostratified columnar epithelium (Figure 1B, Supplemental Data Table 3). K5+K14- cells comprised the remainder of the basal cells of the pseudostratified columnar epithelium.

K14+K5+ cells are reparative in the context of airway epithelial injury

We further examined the relative abundance and location of K14+K5+ cells in a model of airway epithelial injury. To do this, we performed heterotopic, syngeneic tracheal transplants in mice and examined the repairing tracheal airways for K14 and K5 expression after hypoxic-ischemic injury ([20, 21]). We found K14+K5+ cells in the submucosal glands, submucosal gland ducts, as well as in cells on the basement membrane repairing the surface airway epithelium. These K14+K5+ cells persisted in the airway epithelium during all stages of repair and represented 85.6% ± 5.3% of all cells of the mouse repairing surface airway epithelium (Figure 2A, Supplemental Data Table 1). In the repaired pseudostratified columnar epithelium only K5 expression was present in the basal cells (Figure 2Aiii, iv).

K14+K5+ cells populate pre-neoplastic and neoplastic lesions

We further explored the expression of K14+K5+ cells in human disease representing chronic injury and repair after smoking. For this we performed dual immunofluorescent staining of airway tissue from patients with chronic obstructive pulmonary disease (COPD). As in the mouse airway injury model, we observed a persistence of K14+K5+ cells in repairing areas of reserve cell hyperplasia (Figure 2B). We also observed a predominance of K14+K5+ cells in potentially pre-neoplastic lesions represented by squamous metaplasia, dysplasia and carcinoma *in situ* (Figure 2B and 2C). We found K14+K5+ cells in all premalignant lesions from all patients examined to date and in the premalignant lesions K14+K5+ cells represented 75.3% ± 3.4% of cells in the lesions (Figure 2B, C, Supplemental Data Table 1).

The presence of K14+ cells in NSCLC tumor samples confers a worse prognosis

Based on the over-representation of K5+K14+ cells in pre-neoplastic lesions, we further assessed whether the presence of K14 in primary NSCLC tumors was associated with lung cancer development and/or progression. To do this, we examined protein expression on a population basis using high-density lung TMAs. We first examined 399 patients from the UCLA TMA (adenocarcinoma 237, adenosquamous 19, squamous cell carcinoma 100, neuroendocrine 7, large cell carcinoma 32, other 4). Levels of K14 and K5 were found to be similar in all NSCLC with the notable exception of tumors with squamous differentiation. In squamous cell carcinoma 90% of cells were K5 positive and 60% were K14 positive compared with 57% and 18% of cells in adenocarcinomas respectively (positivity defined by 5% cut point, Supplemental Data, Figure 1). These results were verified by quantitative real-time PCR) (Supplemental Data, Figure 2A), review of publicly available lung cancer microarray expression data sets (Supplemental Data, Figure 2B, Supplemental Data Table 2), and Western blot analysis on frozen adenocarcinoma and squamous lung cancer samples (Supplemental Data, Figure 2C) ([22, 23]). The percentage of K14+ or K5+ cells in NSCLC did not correlate with stage, and although lower grade tumors tended to have somewhat higher percentages of K5 and/or K14 positive cells, a significant association was only seen for K14 in squamous carcinomas (data not shown). Tumor samples from male subjects had slightly higher percentages of both K5+ and K14+ cells than did samples from female subjects (Supplemental Data, Table 3).

We further examined whether tumors expressing K14 represented a more aggressive substratum of tumors. Consistent with this, patients with NSCLC that expressed K14 were found to have a significantly worse prognosis than patients with NSCLC in which K14 was below the level of detection (P=0.004, hazard ratio = 1.58)(Figure 3A). We also validated this TMA data with an independent TMA obtained from the M.D. Anderson Cancer Center. We found identical results to those found on the UCLA TMA: patients with K14-expressing tumors had a worse prognosis (p=0.003, hazard ration = 1.60)(Figure 3B).

The presence of K14+ cells in NSCLC tumor samples confers a worse prognosis in smokers and is associated with metastasis

It is generally accepted that cigarette smoking has a causal relationship with lung cancer. Smoking results in chronic airway epithelial injury and dysfunctional repair is commonly seen ([24]). We hypothesized that the presence of dysregulated K14+ reparative cells that predict poor prognosis might have resulted from chronic smoking injury. Consistent with this hypothesis, we found a striking increase in the predictive value of K14 expressing NSCLC tumors in individuals who were current or former smokers (P=0.001, hazard ratio = 1.77) (Figure 4A). In all smokers, K14 positivity (>5%) was an independent predictor of poor prognosis (P=0.027) in a multivariate Cox proportional hazards model, which also included stage, grade, and age (Supplemental Data, Table 4). When separating current from former smokers, the predictive value of K14 positivity was more pronounced in current smokers (P=0.01, hazard ratio = 2.11, Figure 4B). Current smokers were defined as those patients who were currently smoking or who had quit within one year of when their tissue sample was collected. However, K14 positivity was still predictive of poor prognosis in former smokers as well (P=0.04; hazard ratio = 1.68; Figure 4C). Former smokers were defined as those patients who had quit more than a year before their tissue sample was collected. This was true for individuals with either squamous cell carcinoma or adenocarcinomas. In never smokers, the presence of K14+ cells had no predictive value for outcome (Figure 4D). Never smokers were defined as having smoked less than 100 cigarettes over their lifetime.

Validation of these results was performed on an independent TMA from the M.D. Anderson Cancer Center and smoking was again associated with poor prognosis (P=0.004, hazard ration = 1.59) (Supplemental Data Figure 4A), but again there was no association between K14 expression and prognosis in non-smokers (P=0.356, hazard ratio = 2.51)(Supplemental Data Figure 4B).

We found that the presence of K14+ cells in the primary tumors of current smokers was associated with metastatic disease (P=0.02)(Table 1a). We further found that non-adenocarcinoma primary NSCLCs from smokers with metastases had a higher percentage of K14+ cells. (P=0.004)(Table 1b).

Examination of K14 expression in distant metastatic sites revealed a significant increase in the number of K14+ cells in metastases compared to the primary sites in squamous lung cancer (P<0.001), but not in other histologic subtypes (Supplemental Data, Figure 5).

K14-expression is not a marker of proliferation

We next assessed whether K14 expression was prognostic merely because it might be a surrogate marker for cell proliferation. Therefore, in order to determine whether the poor prognosis in K14-expressing tumors was related to increased proliferation in these tumors, we performed dual immunostaining for K14 and PCNA to assess the percentage of K14-expressing cells that are also proliferating in premalignant lesions and NSCLC. In premalignant lesions we found that 57.8% ± 5.1% of K14+ cells also expressed PCNA (Figure 5i, ii). In squamous lung cancer patient samples, we found that 67.3% ± 7.3% of K14+ cells also expressed PCNA (Figure 5iii, iv). We also found many other cell populations, which were K14 negative that expressed PCNA. There was also clearly a subpopulation of K14+ cells that were not proliferating (Figure 5). K14-expressing cells are therefore not a unique marker of proliferating cells, as many other cell populations are proliferating in lung cancer. This is consistent with the point that K14 is a marker of poor prognosis but may not functionally be important for proliferation.

In addition, we performed K14 knockdown studies in BEAS2B immortalized normal human bronchial epithelial cells. We used siRNA technology to reduce expression of K14 in BEAS2B cells by 90% at 5 days post-transfection compared to control siRNA transfected cells. The MTS proliferation assay showed no effect on cell proliferation in the K14 siRNA transfected cells compared to the control siRNA transfected cells and there was also no effect on cell morphology. Similarly, PCNA expression in transfected cells was found to be equivalent by western blot analysis in K14 siRNA and control siRNA transfected cells (Supplemental Data Figure 6).

Discussion

Cigarette smoking causes cycles of injury and repair of the airway and is a known cause of lung cancer ([24]). We, and others, have shown that K14+ progenitor cells are a reparative cell population and contribute to repair of the epithelium of the cartilaginous airways and in the more distant bronchioles after injury, such as hypoxic-ischemic injury, naphthalene injection and sulfur dioxide inhalation ([12, 16]). Here we propose that in the context of injury, K5+K14+ cells originate from the submucosal gland K5+K14+ cells and/or from the K5+K14- basal cells that then acquire K14 expression on the repairing surface airway epithelium. However, once normal repair is completed, K14 expression is no longer seen in the mature basal cells of the pseudostratified columnar epithelium. This implies that K14 expression is tightly regulated at steady state and the persistence of K5+K14+ cells on the surface airway epithelium after injury represents self-renewing cells that do not differentiate to mature airway epithelial cell types and represent dysregulated repair. Our data are, therefore, consistent with the development of dysregulated repair after injury leading to a self-renewing K14+ progenitor cell population in premalignant lesions. These cells could therefore potentially survive long enough to accumulate the genetic and epigenetic mutations that are thought to be necessary to develop a tumor ([3]). We found that the presence of dysregulated K14+ progenitor cells in NSCLC after chronic smoking injury was associated with increased mortality from lung cancer. This implies that there could be a novel putative tumor-initiating cell population in a subset of smoking-related NSCLCs with a poor prognosis.

A self-renewing tumor-initiating cell population associated with poor prognosis in human NSCLC has not yet been described. Kim *et al* isolated a putative lung stem cell termed the bronchoalveolar stem cell or BASC, which expressed markers of both Clara cells (CCSP) and type II pneumocytes (SP-C), proliferated for repair and were seen in the earliest cancerous lesions and increased as the tumors advanced ([25]). However, these studies were performed in mice and it is not clear what the equivalent human cell surface markers are that would enable the purification and propagation of these cells in xenograft models to determine whether these are truly CSCs in lung cancer patients. In addition, the heterogeneity of lung cancers suggests that there are likely to be multiple tumor-initiating cell populations for different lung cancer histologic subtypes and locations. K14-expressing cells have been found for repair in the distal bronchioles[16] and we found K14 mRNA and protein expression in adenocarcinomas as well as squamous cell cancers. In addition, K14 expression correlated with poor prognosis in all NSCLC histologic subtypes, although it only correlated with metastases in non-adenocarcinoma histologies.

Classical validation of a CSC tumor-initiating cell population involves reconstituting the human tumor in an immunodeficient mouse, followed by the indefinite serial xenotransplantation of these CSCs. Eramo *et al* found CD133 expression in both small cell and non-small cell lung tumors. High numbers of CD133+epCAm+ cells isolated from fresh lung tumor specimens were capable of generating tumor xenografts upon subcutaneous injection. However, the self-renewal capacity of CD133+ cells was not evident and CD133

expression was found not to be prognostic in NSCLC although it did correlate with expression of chemotherapy resistance genes ([26]). In order to demonstrate the tumor-initiating potential of K14-expressing cells in NSCLC, by current definitions, the development and serial transplantation of NSCLC in immunodeficient mice is required ([27]). However, no surface markers have as yet been identified to allow the isolation of live K14-expressing cells from tumors. It is therefore not currently possible to evaluate the tumor-initiating ability of K14-expressing cells in NSCLC in a serial xenotransplantation model. We are therefore not functionally able to test the tumor-initiating potential of K14-expressing cells.

Precursor lesions of squamous lung cancer are known to have high levels of K14 expression, from basal/reserve cell hyperplasia to squamous metaplasia and dysplasia to carcinoma *in situ* as well as invasive carcinoma itself ([28]). Our data suggest that K14-expressing cells in the airway epithelium in premalignant lesions may represent self-renewing, reparative progenitor cells, that may have the potential to be tumor-initiating cells. We also believe that K14 expression alone is not sufficient to generate a malignancy and that subsequent genetic and epigenetic changes are needed to develop NSCLC. This is illustrated by work from Dakir *et al* who used a mouse Clara cell specific 10kDa protein promoter (CC10) to constitutively express human K14 in bronchial epithelium. The CC10-hK14 overexpressing transgenic mouse developed a squamous differentiation program in the mouse lung, but failed to promote squamous maturation with rare squamous metaplastic lesions and squamous carcinomas in old age mice ([28]). This supports the idea that K14 expression in airway epithelial cells is a marker of a self-renewing progenitor cell, and is a putative tumor-initiating cell, which requires genetic and/or epigenetic changes in order to be sufficient for carcinogenesis. While we found no difference in the proliferative capacity of K14-expressing cells compared to non-K14-expressing cells in premalignant lesions and in NSCLC, it is possible that the K14+ cells are an important subset of tumor cells as the keratin14 cytoskeletal protein may allow for changes in cell shape and motility with an increased potential for cell migration.

In summary, the presence of K14+ cells in NSCLC is a biomarker of tumors with a worse prognosis. This was especially predictive in smokers, and furthermore these patients had an increased likelihood of metastases. K14 expression in NSCLC in smokers may therefore be useful as a biomarker of poor prognosis and of metastases in squamous lung cancer. Furthermore, identifying the genetic and epigenetic changes that occur in the K14+ cell population that lead to dysregulated repair may result in the discovery of novel biomarkers and therapeutic targets for chemoprevention in smokers([29]).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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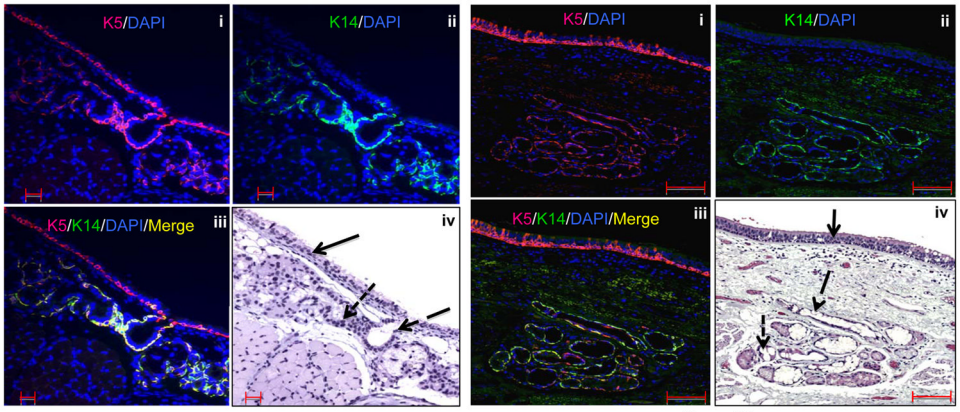

Figure 1. K14 and K5-expressing progenitor cell populations in the airway epithelium at steady state and during repair

A, B. Representative sections of immunofluorescent staining identifies cells in the submucosal glands and submucosal gland duct that express K14 (Alexa fluor 488, green) and K5 (Cy3, red). Basal cells of the pseudostratified columnar airway epithelium express K5 but do not express K14. A) is representative of immunostaining seen in mice (scale bar = 20μm), B) is representative of staining in humans (scale bar = 100μm). H&E stained representative sections are included to demonstrate the anatomy of the pseudostratified columnar airway epithelium (arrow), the submucosal glands (dotted arrow) and submucosal gland ducts (dashed arrow).

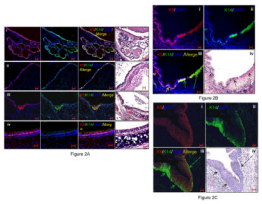

Figure 2.

Figure 2A. Representative sections of immunofluorescent staining of K14 (Alexa fluor 488, green) and K5 (Cy3, red) expressing cells in the mouse tracheal airway epithelium after hypoxic-ischemic injury from tracheal transplantation.

i. K14 and K5-expressing cells are seen in the submucosal glands, submucosal gland ducts and repairing surface airway epithelium. ii. K14 and K5-expressing cells are seen on the repairing surface airway epithelium. iii. K14 and K5-expressing cells are seen in a hyperplastic area of repairing surface airway epithelium but in areas of pseudostratified columnar epithelium K5 expression is present in the basal cells but K14 expression is absent. iv. Repaired pseudostratified columnar epithelium with K5 expression in the basal cells and absence of K14 expression.

Corresponding H&E sections are included to demonstrate the histopathology of the repairing airway.

2B. Representative sections of immunofluorescent staining of K14 (Alexa fluor 488, green) and K5 (Cy3, red) expressing cells in repairing airway epithelial human tissue from smokers with reserve cell hyperplasia and squamous metaplasia. K5+K14- basal cells are seen in normal airway epithelium (red arrow). A few K14+K5+ few basal cells are also present (yellow arrow). K14+K5+ cells are seen in an area of reserve cell hyperplasia and in squamous metaplasia (green arrows). H&E staining of the section demonstrates the areas of normal pseudostratified columnar epithelium (arrows), reserve cell hyperplasia (dotted arrow), and squamous metaplasia (dashed arrow), (scale bar = 20μm).

2C. Representative sections of immunofluorescent staining of K14 (Alexa fluor 488, green) and K5 (Cy3, red) expressing cells in repairing airway epithelial human tissue from smokers with dysplasia and carcinoma *in situ* lesions.

K14+K5+ cells are seen in areas of moderate dysplasia (green arrows) and carcinoma *in situ* (severe dysplasia)(green dashed arrow). H&E staining of the section demonstrates the areas of moderate dysplasia (arrows) and carcinoma *in situ* (severe dysplasia) (dashed arrow), (scale bar = 20μm).

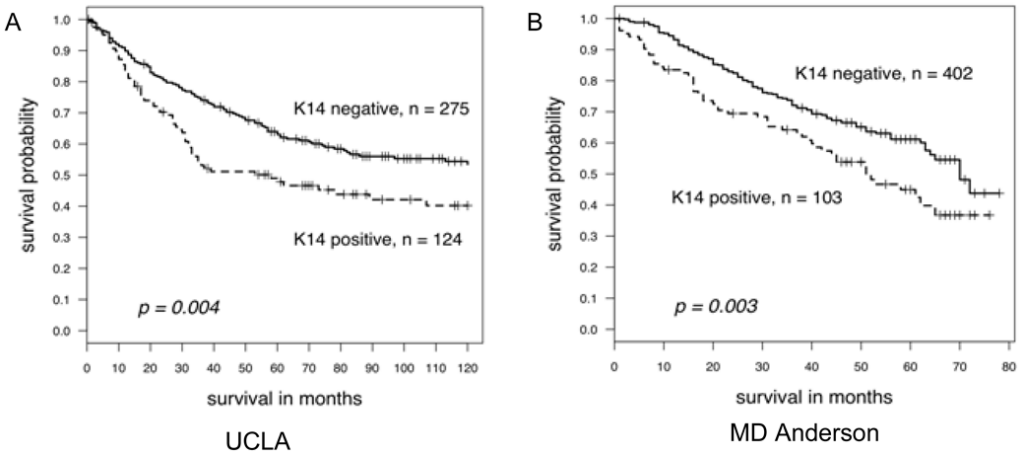

Figure 3. Kaplan Meier Survival curves showing that K14 expression in NSCLC correlates with poor prognosis

A. Analysis of the UCLA TMA revealed that patients with NSCLC that expressed K14 had a significantly worse prognosis than patients with NSCLC in which K14 was below the level of detection (P=0.004, hazard ratio = 1.58).

B. Analysis of the M.D. Anderson TMA also showed that patients with NSCLC that expressed K14 had a worse prognosis than patients with NSCLC in which K14 was below the level of detection (P=0.003, hazard ration = 1.60).

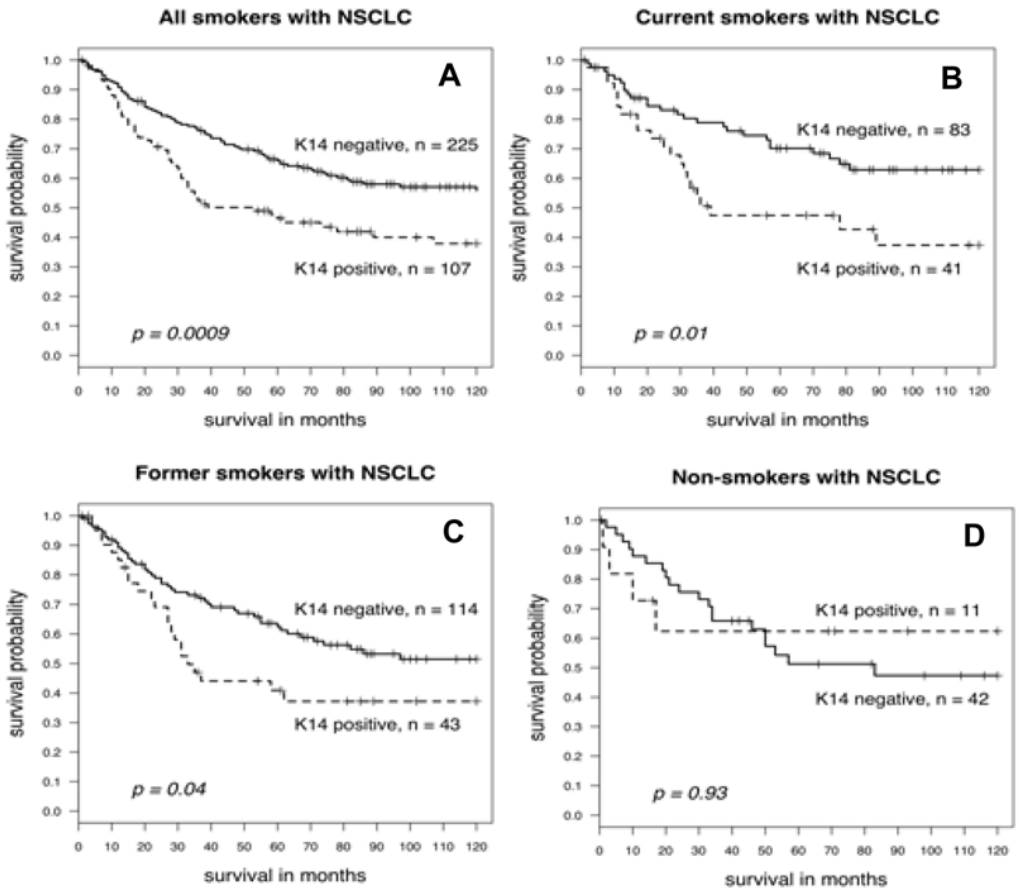

Figure 4. Kaplan Meier Survival curves from the UCLA TMA showing that the poor prognosis related to K14-expressing NSCLC tumors correlated with smoking

A. In all smokers (current and former) K14 positivity in NSCLC tumors had the highest predictive value of death from NSCLC (P=0.0009, hazard ratio = 1.77, n = 332).

B. The predictive value of K14 expressing NSCLC tumors in individuals who were current smokers (P=0.01, hazard ratio = 2.11, n = 124).

C. K14 positivity was still somewhat predictive of death due to disease in former smokers as well (P=0.04, hazard ratio = 1.68, n = 157).

D. In never smokers, the presence of K14+ cells had no predictive value for outcome (P=0.93, hazard ratio = 0.95, n=53).

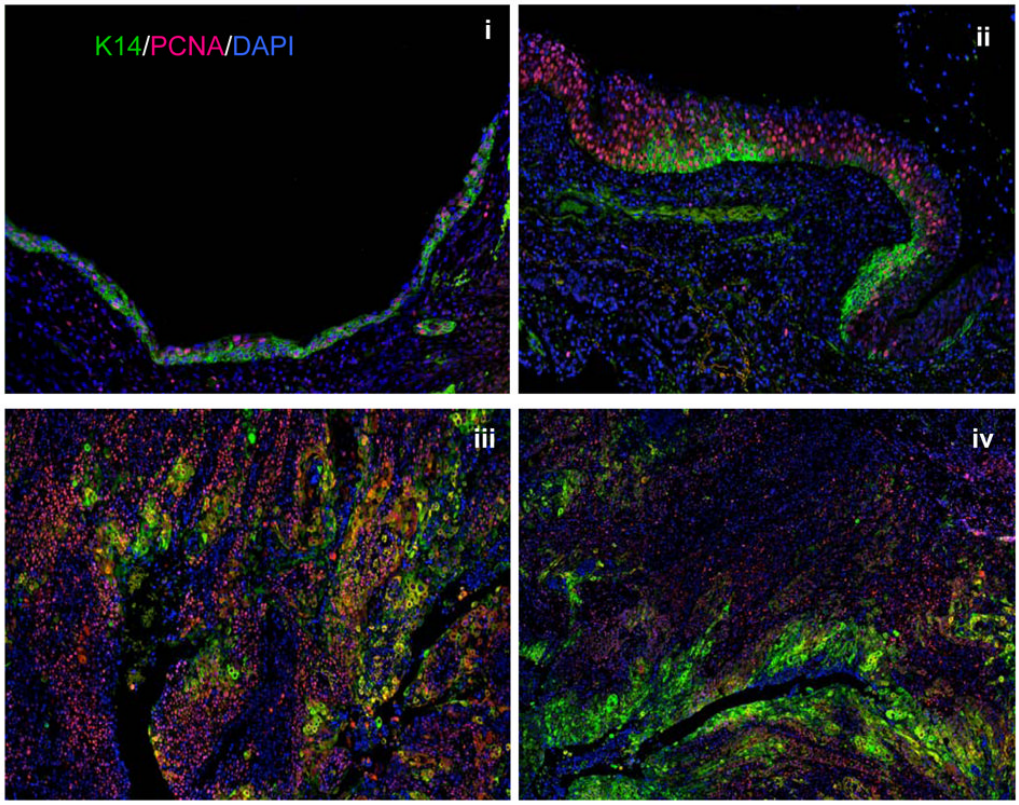

Figure 5. Dual immunofluorescent staining of human premalignant lesions and tumors to assess populations of proliferating cells that also express K14

i. and ii. Dual immunofluorescent staining of premalignant lesions for K14 and PCNA. In premalignant lesions we found that 57.8% ± 5.1% of K14+ cells also expressed PCNA.

iii and iv. Dual immunofluorescent staining of tissue from SCC for K14 and PCNA. In SCC we found that 67.3% ± 7.3% of K14+ cells also expressed PCNA

Table 1a

Primary Tumor K14 Presence/Absence in Current Smokers: No Metastases vs. Any Metastases

The presence of K14+ cells in primary NSCLCs in current smokers was associated with metastatic disease (P=0.02). This correlation was particularly significant in non-adenocarcinoma primary NSCLCs (P=0.004).

Histology (n)	Fisher P-value
All histologies (124)	**0.02**
Adenocarcinoma (61)	0.71
Squamous carcinoma (39)	**0.05**
Large cell carcinoma (14)	0.09
Not adenocarcinoma (63)	**0.004**

Table 1b
Mean Percentage K14+ Cells in Current Smokers: No Metastases vs. Any Metastases

Primary NSCLCs from current smokers with metastases had a higher percentage of K14+ cells than non-metastatic NSCLCs (P=0.033). This correlation was particularly significant in non-adenocarcinoma histology NSCLCs (P=0.004).

Histology	Mean percentage K14+ cells, no mets (n)	Mean percentage K14+ cells, any mets (n)	Mann-Whitney P-value
All histologies	12.7 (84)	26.9 (40)	**0.033**
Adenocarcinoma	7.7 (44)	4.5 (17)	0.424
Squamous carcinoma	21.8 (24)	53.4 (15)	**0.023**
Large cell carcinoma	11.6 (9)	31.5 (5)	0.061
Not adenocarcinoma	18.2 (40)	43.4 (23)	**0.004**