Published in final edited form as:
Lung Cancer. 2011 October ; 74(1): 12–24. doi:10.1016/j.lungcan.2011.02.006.

Pleiomorphic Adenoma Gene-like 2 Expression Is Associated with the Development of Lung Adenocarcinoma and Emphysema

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Abstract

Previous study of transgenic mice with long-term expression of pleiomorphic adenoma gene-like 2 (PLAGL2), a surfactant protein C (SP-C) transactivator, in type II cells showed the manifestation of centrilobular emphysema in vivo. Since emphysema is an independent risk factor for bronchogenic carcinoma, we hypothesized that the mouse lungs with induced PLAGL2-expression had increased incidences in developing lung adenocarcinoma. To test the hypothesis, mouse lungs were examined for the presence of tumors. Male mice with induced PLAGL2-expression in the lungs were more vulnerable to tumorigenesis than female mice ($p < 0.05$). Epithelial cells expressing pro-SP-C and Clara cell secretory protein (CCSP) at the terminal bronchioles and the bronchoalveolar duct junction (BADJ) were increased in the induced transgenic mice, suggesting a role of PLAGL2 in expanding SP-C expression cells. Co-expression of TTF-1, pro-SP-C and CD133 (a stem-cell marker) in cancer and distal airway epithelial cells indicated that both cells were derived from common progenitors. This result supported a common-cell-origin mechanism for the comorbid diseases - emphysema and lung cancer. Furthermore, a public lung cancer gene expression profiling database was examined to determine the relevance of PLAGL2 expression and lung adenocarcinoma in humans. Patients with high PLAGL2 expression in lung tumors were readily found. Female patients (N=218) with low PLAGL2 expression (the lowest quartile of total patients) at the early-stage of disease had better prognosis in survival. Male patients, on the other hand, had no such correlation. Generally, their survival rate was significantly poorer than of female patients. Taken together, our data suggested a pathological role of PLAGL2 in lung adenocarcinoma development and a preferable prognosis of low PLAGL2 expression in female patients.

Keywords

PLAGL2; Comorbidity; Lung Adenocarcinoma; COPD/Emphysema; Bronchogenic carcinoma; Gene Expression

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Conflict of Interest statement: None to declare

Introduction

Lung cancer and chronic obstructive pulmonary disease (COPD) represent the second and fourth leading causes of death in the United States [1]. Although only 10 to 15% of smokers develop lung cancer and 20 to 40% of them have COPD, accumulating evidence indicates emphysema and airway obstruction contribute to the risk of lung cancer separately from the direct impact of cigarette smoke [2;3]. Emphysema alone was sufficient to increase a patient's risk of lung cancer; indeed, quantitative airflow obstruction alone does not correlate with an increased risk [2;3]. Adenocarcinoma is the most common subtype of lung cancer associated with emphysema [2]. In non-smokers, increased lung cancer risk is also associated with the presence of emphysema, but not with chronic bronchitis [4]. Moreover, emphysema is associated with a poor prognosis in non-small cell lung cancer (NSCLC) [5]. In summary, pulmonary emphysema is an independent clinical risk factor for lung cancer, independent of airflow obstruction or smoking history. Gender also is an independent prognostic factor for patients with emphysema and lung cancer. The association between these two diseases is stronger in men than in women [4]. In one study, about 10% of patients (all males) with severe emphysema were found to have concomitant peripheral lung cancer [6]. Similarly, the prevalence of emphysema in individuals screened for lung cancer is significantly higher in Japanese males (5.0%) than females (0.5%) [7].

Pulmonary emphysema and lung cancer share some common etiologies. For example, both diseases are associated with smoking, oxidative stress, inflammation, and "cells-of-origin", namely bronchoalveolar stem cells (BASCs) at the bronchoalveolar duct junction (BADJ) (review in [8]). Pulmonary emphysema is characterized by cell death/injury leading to reduced cell numbers, while lung cancer results from uncontrolled cell proliferation. Although lung remodeling may increase cancer incidence, the underlying mechanism is unclear.

Pleomorphic Adenoma Gene-like 2 (PLAGL2) belongs to a small zinc-finger protein family that includes PLAGL1 and PLAG1 [9;10]. Both PLAGL2 and PLAG1 can transform NIH3T3 cells [11;12]; and PLAGL2 was a suspected candidate of cancer gene in colorectal cancer [13]. The chromosome location of PLAGL2 was a high-recurrence-focal locus for gene amplification in the colon cancer [13]. A recent report further suggest a role of PLAGL2 in interfering neural stem cell differentiation and lead to oncogenesis [14]. Paradoxically, like PLAGL1 (Zac1/Lot1)[11;12], PLAGL2 can also provoke cell apoptosis in response to hypoxia or a low-serum medium [15]. Increased PLAGL2 in cells under hypoxic stress can stimulate expression of bNip3 (a pro-apoptotic protein dimerized with Bcl-2) and subsequently promote HIF-1 associated cell death [16]. BNip3 is also a known cancer gene [13] when its expression is dysregulated [17]. Besides bNip3, the surfactant protein-C (SP-C) promoter is another downstream target of PLAGL2. The promoter can be transactivated by PLAGL2, in addition to TTF-1, in cells [18]. When H441 cells (lung adenocarcinoma, a Clara-type cell) responded to hypoxia by up-regulating PLAGL2 expression, PLAGL2 occupancy of the SP-C promoter was increased [19;20].

In the lungs of induced PLAGL2 transgenic mice, increased cell death and endoplasmic reticulum (ER) stress were observed in the epithelial cells of the terminal bronchioles particularly at the BADJ, where PLAGL2 was over expressed [21]. Given that PLAGL2 transforms NIH3T3 fibroblasts [10], is associated with acute myeloid leukemia (AML)[22], and promotes renewal of neural stem cells [14], elevated PLAGL2 expression can be oncogenic in various tissues. The current study demonstrates a link between emphysema and bronchogenic carcinoma in a transgenic animal model, further supporting a role for PLAGL2 in the pathogenesis of both diseases.

Materials and Methods

Animals and statistical analyses

The generation of PLAGL2 transgenic mice, the doxycycline (Dox) induction of PLAGL2 expression condition, animal husbandry, lung fixation, and histology were performed as described in a previous publication [21]. In brief, a DNA fragment containing full-length human PLAGL2 cDNA with an intron was used to produce the $(TetO)_7$CMV-PLAGL2 transgenic mice [21]. Hemizygous mice were selected for studies to avoid potential lethal mutations. Four founders of PLAGL2 transgenic mice (P1 ~ P4 with various copy numbers from estimated 4 to 180) were obtained and tested for gene expression. Each founder was bred with SP-C-rtTA$^{+/-}$ mice to generate PLAGL2$^{+/-}$/SP-C-rtTA$^{+/-}$ double-transgenic (DT) mice for PLAGL2 expression. DT mice were identified by genotyping of tail DNA using PCR as previously described [21]. PLAGL2 transgene expression in the DT mice was induced by Doxycycline (Dox, 1 mg/ml in H_2O in amber bottles) [23]. Those mice fed with Dox water were started at 6-week old and were continuously treated until sacrificed (up to 10-month old). Dox H_2O was replenished three times a week. Mice were maintained in a barrier facility with a 12-h light-dark cycle, housed in sterilized cages and received sterilized food and water ad libitum. Animal studies were performed with protocols approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center. All mice with lung tumors were histologically identified and verified under microscope by examining hematoxylin & eosin stained lung tissue sections. All animals, males and females, included in this study were young and fertile when sacrificed. The odds ratio, relative risk, and z-test analyses of mice with tumors were performed by Sigmaplot software (version 11.0; Systat Software, Inc. San Jose, CA).

Quantitative PCR analysis of PLAGL2 expression in lung cancer

Human lung cancer tissue cDNA arrays were obtained from OriGene (Rockville, MD). Quantitative PCR was employed by real-time PCR to measure the level of PLAGL2 in samples. Total of 5 control and 22 adenocarcinoma lung tumor samples were analyzed. The PCR primers used for PLAGL2 analysis and real-time PCR condition were the same as previously described [18;21]. Duplicate samples were measured and averaged cycle threshold (C_T) values were applied for further analysis.

Immunohistochemistry and antibodies

For SP-B, proSP-C (Chemicon/Millipore, Temecula, CA), Clara cell secretory protein (CCSP)(Seven Hills Bioreagents, Cincinnati, OH), CD133, and aldehyde dehydrogenase 1 (ALDH1)(Abcam, Cambridge, MA) detection, sections were directly treated with blocking and probing procedures after deparaffinization and rehydration. For TTF-1 and PLAGL2 staining, antigen retrieval was performed prior to the blocking step in 0.01 M citrate buffer (pH 6.0). Treated slide sections then were incubated with antibodies to TTF-1 (Upstate/Millipore, Lake Placid, NY) and PLAGL2 (Sigma, St. Louis, MO) overnight at 4 °C. Immunodetection was achieved using Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA), then counterstained with hematoxylin as described previously [21]. Human lung sections containing normal tissue, adenocarcinoma and bronchoalveolar carcinoma (BAC) were obtained from OriGene.

Lung adenocarcinoma gene expression database and survival rate analyses

The published gene expression profiling of the lung adenocarcinoma database [24] was employed to explore the correlation between PLAGL2 expression and patient survival time. The normalized data obtained from the NIH database was further analyzed to determine the level of PLAGL2 expression in samples. The relative level of PLAGL2 expression was

calculated by dividing its value with the averaged expression of total genes (22283 entries) in each patient sample [PLAGL2 (202924_at)/the mean of total genes expression] to minimize variations introduced during sample collection, preparation, and evaluation. Lung cancer patients (N = 406) were grouped in quartiles from low to high PLAGL2 expression, according to the calculated relative value. The differences of patient survival time between the compared groups were evaluated with a Kaplan-Meier log rank analysis to calculate the mean survival time (in months) and to plot the survival curve. A Cox regression study was used to determine the hazard ratio significance. These analyses were performed using the SigmaPlot software.

The gene profiling data and lung sample sections used in the human studies were either already available in the public database (NIH) or purchased commercially (OriGene). There was no need to acquire institutional review board approval for the human studies.

Results

Lung adenocarcinoma in PLAGL2 transgenic mice

The lungs of the DT mice with induced PLAGL2 expression developed adenocarcinoma along with airway destruction (Figure 1). Various forms of adenoma-carcinoma tumors (Figure 2) including epithelial hyperplasia [atypical adenomatous hyperplasia (AAH, Figures 2A and B) and bronchioalveolar hyperplasia (or bronchioalveolar carcinoma (BAC), Figures 2C and D)], solid adenoma (Figures 2E and F), and papillary adenocarcinoma (Figures 2G and H) were observed in the lungs of these mice. Those tumors were of the adenoma-carcinoma subtype and located in the peripheral region of the lungs. Almost all lung sections with histological evidence of tumors/cancerous cells also had lesions with various extent of enlarged airways indicating the presence of comorbid diseases in those mouse lungs.

The incidence of lung tumors was not sporadic. Tumors were detected in two lines of Dox-induced PLAGL2 DT mice before they were 11 months old. The rates of developing tumors in these two lines were similar, 13 and 19%. The control mice (male, single or double transgenes) without induced PLAGL2 expression did not have tumors at the same age (Table I). The overall incidence of tumors was significantly higher in the induced male DT mice (P3+P4, 17%, N = 41) than in the control group (male, P4+P3 no induction; < 2.5%, $p < 0.05$, N = 45; Table I). The odds ratio (9.06, 95% CI: 1.06 – 77.19), relative risk (7.68, 95% CI: 0.99 – 59.81), and proportions of sample population (0.15±0.06, 95% CI: 0.03 – 0.27) between these two groups were statistically different. In addition, sex was another independent factor in developing adenocarcinoma. The differences in odds ratio (9.88, 95% CI: 1.16 – 84.07), relative risk (8.37, 95% CI: 1.07 – 65.23), and proportions of sample population (0.15±0.06, 95% CI: 0.03 – 0.27) between the male and female mice with induced PLAGL2 expression were statistically significant. It is noteworthy that the manifestation of tumors and emphysema occurred in the group of mice bearing double transgenes under a Dox-induced PLAGL2-expression condition. Tumors identified in these mouse lungs were commonly in or accompanied with lesions of destructed distal airways (Figure 1).

PLAGL2 expression in adenocarcinoma tumors

In the induced DT mouse lungs, PLAGL2 was detected in various subtypes of lung adenocarcinoma (Figure 3), indicating a correlation between PLAGL2 expression and tumor development. The coexpression of TTF-1 suggested that the tumors were originated from the epithelial cells (Figures 4A and B). They could be type II, Clara or airway epithelial cells. Type II cell markers SP-B and SP-C (Figures 4C-F) were either absent (SP-B, Figure

4E) or minimally expressed (SP-C, Figure 4F) in tumor cells, but were abundant in the epithelial cells surrounding the tumors. No CCSP expression was detected in the tumor cells (data not shown).

To determine whether human PLAGL2 expression and lung adenocarcinoma had pathophysiological relevance similar to that in the animal study, patient lung sections were examined for PLAGL2 (Figures 5A, C, and E) and TTF-1 (Figures 5B, D, and F) expression. In the alveolar septa of normal lung tissues, epithelial cells with TTF-1 expression were diffusely distributed (Figure 5B) and only few of them were PLAGL2 positive (Figure 5A). Cancer cells (e.g. bronchioalveolar carcinoma [BAC] and adenocarcinoma), on the other hand, had high levels of expression of both proteins (Figures 5C - F). Both animal and human data indicated that tumors were originated from TTF-1-expressing epithelial cells in alveoli or in distal airways.

CD133 expression in lung adenocarcinoma and distal airway epithelial cells

Epithelial cells at the terminal bronchioles including the BADJ are frequently Clara cells, which express CCSP. In normal lungs, few of these cells coexpress SP-C. Those CCSP and SP-C double positive cells were rare and were characterized as BASC (< 3 cells per BADJ) [25]. Interestingly, the number of cells with proSP-C expression at this location in the induced DT mouse lungs was increased (> 3 per BADJ)(Figure 6), as was PLAGL2 expression [21], suggesting an expansion of proSP-C expression progenitor cells.

Although some cancer cells were positive in pro-SP-C expression, two progenitor cell markers CD133 [26] and ALDH1 [27;28] were examined to verify that cancer cells were derived from these progenitor cells at the distal airways. The results were shown in Figures 7–9. In cancer cells, CD133 was ubiquitously expressed and distributed in the cytoplasm (Figures 7A, C and E;8D and E). In contrast, no ALDH1 expression was detected in tumors (Figures 7B and D;Figures 9D and E). Both genes were widely expressed in airway epithelial cells (bronchi, bronchioles, terminal bronchioles and the BADJ)(Figures 7,8B and C, and 9B and C), but not in type II and alveolar duct epithelial cells (Figures 7A and B,8A, and 9A). Some ciliated airway epithelial cells had dense CD133 staining at the apical region of cells (Figures 7E,8B and C insets) as previously reported [29], however, the majority of cells had a weak, diffused distribution in the cytoplasm. ALDH1 also had broad distribution in the cytoplasm of airway epithelial cells (Figures 7B, D and F;9B and C). Overall, the similarity of TTF-1 ([21], and Figure 4) and CD133 expression in cancer and distal airway epithelial cells suggested that both cells were derived from the same progenitors.

PLAGL2 expression in early-stage lung adenocarcinoma in humans

A cDNA array analysis of lung adenocarcinoma patient samples revealed an association between PLAGL2 expression and the development of lung adenocarcinoma (Figure 10). To address its pathological relevance in human, we analyzed the published lung adenocarcinoma gene profiling database [24] to determine the correlation between PLAGL2 expression and disease prognosis. While female patients generally had a better survival rate than male patients (hazard ratio for women, 0.748; mean survival time [month]: female = 99.0±7.5 (N = 218), male = 78.4±5.5 (N = 188), p = 0.037; Figure 11A), the level of PLAGL2 expression in tumors contributed an additional effect to disease prognosis (Figure 11B- D). The female patients with the lowest quartile of PLAGL2 expression (≤25%; relative ratio ≤0.23 [PLAGL2/mean expression of total genes]) had significantly better survival than the female patients with higher PLAGL2 expression (> 25% [2^{nd} to 4^{th} quartiles], Figure 11B) (survival time (mon) = 125.5±14.1, N=56 vs. survival time (mon) = 78.9±6.1, N=162, p = 0.008). In contrast, low PLAGL2 expression in men did not have the same prediction of better survival prognosis (survival time (mon) = 64.5±9.9, N = 45,

p<0.001; Figure 11C) as in women. The disease is generally more severe in men than in women (Figure 11A) given the poor survival rate in male patients regardless the level of PLAGL2 expression. This result was concord with the animal data that male mice were more accessible to the tumor development (Table I). The possible reasons that cancers were less frequently detected in female mice will be discussed later. Furthermore, the significance of PLAGL2 expression in survival prediction was mainly in female patients diagnosed at the early stage of disease (N^0T^1 and/or N^0T^2)(PLAGL2 level: low = 152.3±15.4, N = 43; high = 94.0±8.0, N = 102, p = 0.026; Figure 11D), not in those with the advanced cancers (N^0T^3 or later, data not shown). In summary, our data suggested that PLAGL2 expression was elevated in the early stage of lung cancer and the low level of expression had preferable survival prognosis in women at the early stage of disease.

Discussion

Since COPD/emphysema is a known risk factor for lung adenocarcinoma [30] and increased PLAGL2 expression aggravates emphysema [21], we hypothesized that PLAGL2 might increase the rate of tumorigenesis in PLAGL2 transgenic mice. Indeed, induced PLAGL2 DT mice developed lung adenocarcinoma (Figures 1 and 2). Enlarged alveolar spaces and degraded alveolar wall fragments are the features of distal airway destruction. They were commonly found in the emphysematous lungs characterized by morphometrical, histological and functional analyses [21], or in the lungs with cancer (Figures 1A-C).

With a wide-range study of the genome copy number changes in lung adenocarcinoma, TTF-1, a key factor for lung function and development, was identified as a proto-oncogene involved in oncogenesis [31]. Many other genes with copy-number gain or loss associated with this subtype of lung cancer, including EGFR, KRAS, ERBB2, CDKN2A/CDKN2B, PTEN, and RB1, have also been reported [31]. Some of them have known cellular functions relevant to carcinogenesis such as tumor suppression or cell cycle regulation; many others are still undetermined. In this study, we identified PLAGL2 as a potential proto-oncogene during the oncogenesis of lung adenocarcinoma. It is also noteworthy that duplication of chromosome 20 in which PLAGL2 gene is located (20q11) occurs in all of the immortalized lung epithelial cell lines [32]. This further echoes PLAGL2's oncogenic activity observed in other tumors [14].

Although only a fraction of lifetime smokers develop COPD/emphysema (<20%)[33], there is no doubt that exposure to smoke plays a major role in the disease's manifestation. Since COPD/emphysema patients with reduced pulmonary function have increased incidences of lung cancer [30], an animal model is greatly needed to study the comorbidity *in vivo*. Given that PLAGL2 is oncogenic in transforming fibroblasts *in vitro* and promoting AML carcinogenesis in association with *Cbfb-MYH11* fusion protein [10;22], it is reasonable to anticipate that tumors would develop in animal lungs with PLAGL2 over expression. Indeed, the lungs of Dox-induced DT mice developed tumor lesions including AAH, BAC, solid adenoma and adenocarcinoma (Figure 2) in addition to emphysema (Figure 1). Expression of TTF-1 in tumor cells (Figure 4) suggests that the tumors are originated from epithelial cells, not endothelial cells or cells metastasized from other organs. Two different transgenic mouse founders have similar rates of tumor development (Table I), excluding the contribution of irrelevant gene(s) at the transgene integration site(s) to oncogenesis.

CD133 and ALDH1 are known stem cell markers in normal and cancer cells [27;34;35]. While the stem cell population is expected to be small, these two cell markers are commonly expressed in the epithelial cells of conductive airways in humans (Figures 8 and 9) and mice (Figure 7, including trachea, bronchi, bronchioles, and terminal bronchioles). The result is consistent with a wide expression of CD133 in differentiated luminal duct epithelial cells of

various organs such as the pancreas and colon [34;36]. Omnipresent expression of CD133 is seen in primary adenocarcinoma of colon and pancreatic cancers [34;36] as well. Thus, the broad expression of CD133 in lung airway epithelial cells and in lung adenocarcinoma (Figures 7 and 8) is not an artifact of staining result. Rather, it suggests a common-cell-origin for both types of cells. The discrepancy between being a marker of rare stem cells and ubiquitous expression in airway epithelial cells could be caused by subcellular localization differences. The apical distribution of CD133 in cells, including cancer and normal epithelial cells (see insets in Figures 7E and 8B - D), indicates the molecule transmembrane integration in microvilli [29]. These cells may bear stem cell properties and thus can be separated by sorters [26]. In contrast, cells with cytosolic CD133 can-not be detected or isolated by sorter regardless of their phenotypes.

No ALDH1 expression is detected in cancer cells. It is expressed in airway epithelial cells (Figures 7 and 9). The data is different from other reports that ALDH1 is a marker of lung cancer [28]. The following reasons could explain the discrepancy: 1) the differences in antibody and IHC protocol employed may contribute to the variations. 2) The tumors analyzed in mouse and human samples were at the early stage of tumorigenesis. ALDH1 expression is known to be associated with aggressive NSCLC behavior in later stages [27]. More than 75% of stage I NSCLC do not express ALDH1 [27]. 3) Unlike airway epithelial cells, cancer cells may down-regulate ALDH1 expression during oncogenesis. Thus, tumor and airway epithelial cells are likely originated from the same CD133- and ALDH1-positive progenitor cells. Whether the expansion of local progenitors such as BASC population (CCSP and SP-C positive cells) contributes explicitly to the tumorigenesis requires further investigation.

COPD/emphysema and lung cancer are two disorders which co-migrate in a manner only partially explained by smoking. One potential role of PLAGL2 in the development of both diseases is to modulate the fate of cells in the common-origin. Cells located at the distal airways including the BADJ with stem cell markers, CCSP and SP-C, are increased in the lungs of our DT mice (Figure 6). Given that PLAGL2 can promote neural stem cells renewal capability and inhibit their differentiation [14], PLAGL2 is likely to expand the progenitor cells at the terminal bronchioles. Although these cells are probably the same population undergoing cell injury/death activities in the emphysematous lungs [21], some of them may bypass the programmed cell death signals and become oncogenic. Reports have shown that PLAGL2 is a oncogene [11;12;14] and the dysregulation of its downstream target gene bNip3 activity via an abnormal modification of the gene can block apoptosis in pancreatic and hematopoietic tumors [17;37]. Thus, PLAGL2 oncogenicity and/or its capability in epigenetically modifying the bNip3 gene may program cell growth and contribute to the development of lung adenocarcinoma in the emphysematous lungs. These possibilities remain to be further explored. Nonetheless, our data support a pathogenic role for PLAGL2 in provoking COPD/emphysema and adenocarcinoma in the lungs.

In a gene expression profiling study of lung adenocarcinoma deposited in the NIH public database [24], female patients with relatively low PLAGL2 expression had a better survival rate (Figures 11). This finding suggests a clinical correlation between PLAGL2 expression and the disease prognosis in women. However, it is unclear why the same low level of PLAGL2 expression in male patients does not have a favorable prediction as that for the female patients (Figure 11C). It is possible that sex hormone may have a role in modulating the effect of PLAGL2 in lung adenocarcinoma development. Reports have shown that estrogen treatment can influence the risk of lung cancer in postmenopausal women and estrogen receptor-β negative male lung cancer patients have the worst survival rate [38;39]. The sex hormone influence in estrogen receptors cellular location and activity changes in cells [40] could contribute to the differential gene responses between genders. Thus, the low

incidence of tumorigenesis in the induced female mice could be caused of hormone. Tumorigenesis rate may raise in the induced female mice when they are older or are ovariectomized with reduced sex hormone. Nonetheless, upregulated PLAGL2 expression raises cancer incidence in young mice supporting an unprecedented role of PLAGL2 in lung oncogenesis.

Ras expression was detected in mouse tumors (supplementary data), suggesting a smoke-related signaling pathway [41] in PLAGL2-conducted tumorigenesis. Since PLAGL2 is up-regulated in cells under hypoxic stress [15;20], its expression could be induced in lung cells responding to smoke-exposure. This animal model could provide a useful tool for the molecular and biochemical studies of the smoke-exposure related lung comorbidities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Arun Alex and the Molecular Pathology Core at University of Texas Southwestern Medical Center for technical assistance and Dr. Jingsheng Yan for statistical analysis consultations.

Sources of Support: Supported by funding from the Will Rogers Institute and the James M. Collins Center for Biomedical Research (JCW) and partially (the emphysema study) by NHLBI Grant R03-HL-095407 (Y.-S. Yang).

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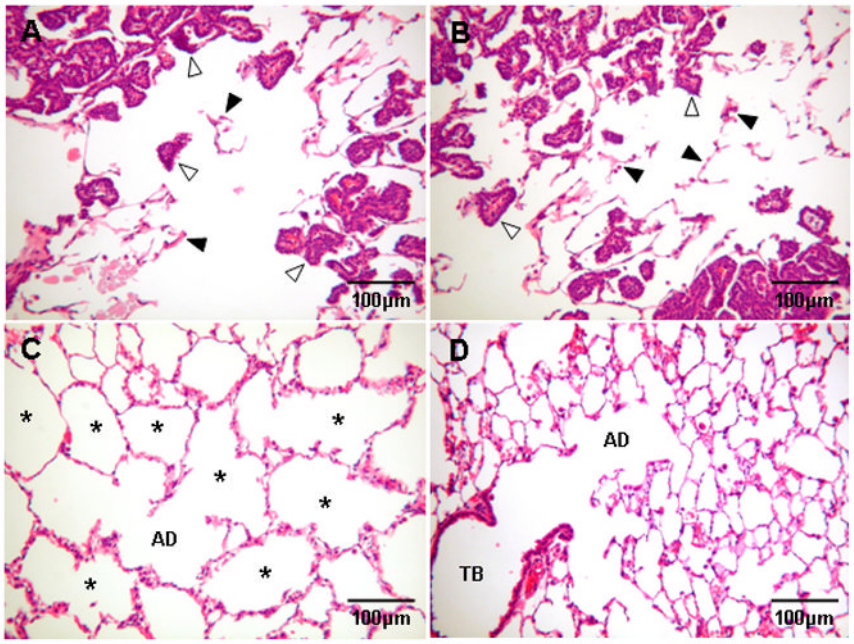


Figure 1.

Bronchioloalveolar carcinoma (BAC) and emphysema coexisted in the lungs of mice induced with PLAGL2-expression. (A and B) A subtype of adenocarcinoma BAC (open triangles) was present in the lungs of Dox-induced transgenic mice (male, 8-mon old) along with degraded airway fragments (closed triangles). Centrilobular emphysema lesions occurred in the lung parenchyma without tumor (C). (D) The normal lung structure from the same mouse lung without lesions of either disease. *: enlarged alveolar space.

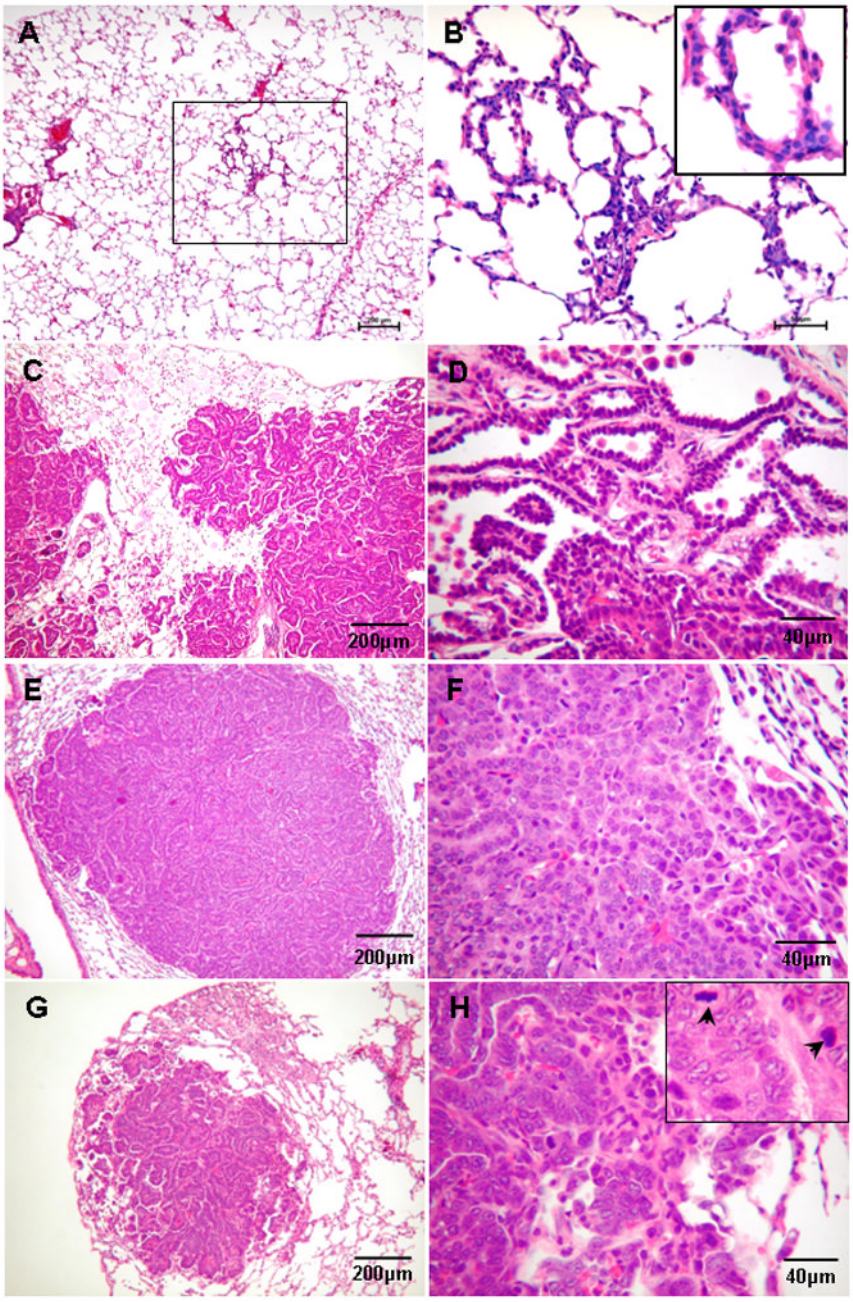


Figure 2.

Different subtypes of adenocarcinoma in the lungs of induced DT mice. (A and B) Low grade atypical adenomatous hyperplasia (AAH). Boxed section in A and B were enlarged to show a focal proliferative epithelial lesion in the periphery of the lung. Cubical epithelial cells covered the preexisting alveolar framework. (C and D) AAH of severe atypia and bronchioloalveolar carcinoma (BAC). Tumor cells lined the alveolar walls, but there was no stromal invasion. (E and F) A well-differentiated solid adenoma with a low degree of nuclear atypia. (G and H) Well-differentiated adenocarcinoma in the lung periphery. Cells with active division (mitotic nuclei, arrow heads in the inset of H) were frequently found in adenocarcinoma tumors (H). Magnification: A, C, E, and G: 4 X; B, D, F, and H: 20 X. H & E staining.

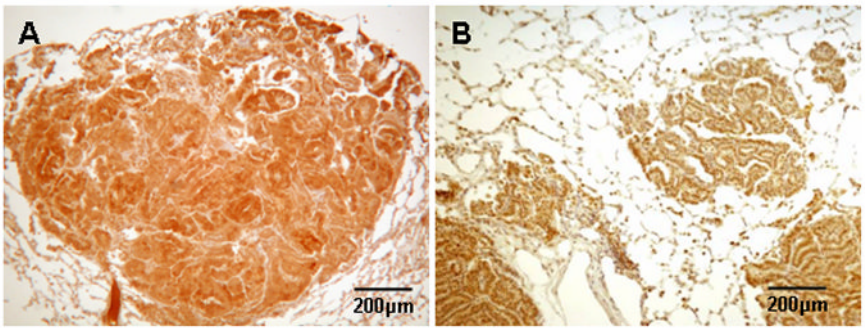


Figure 3.
PLAGL2 expression in adenoma-carcinoma tumor cells in the lungs of the induced DT mice. Lung sections from adenocarcinoma (A) or AAH of severe atypia/BAC (B) were probed with PLAGL2 antibody and developed with DAB (brown color). Original magnification: 4 X.

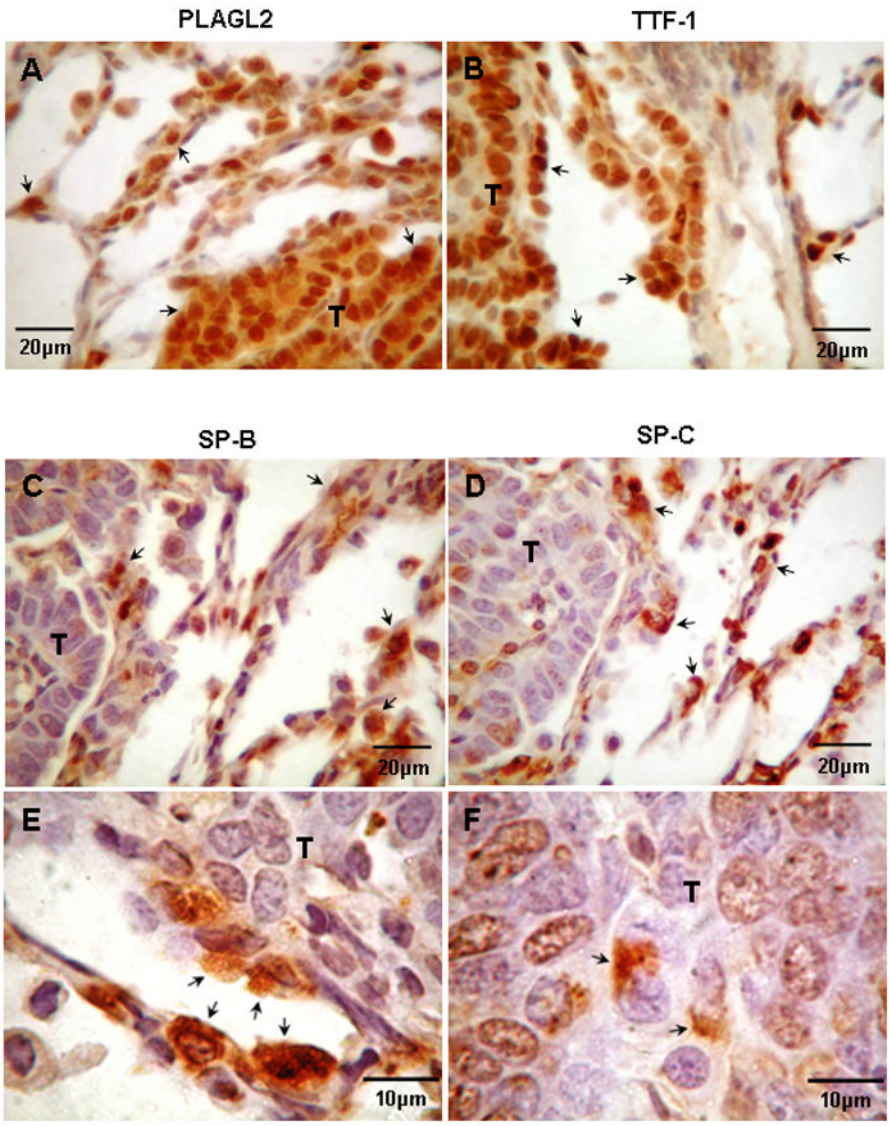


Figure 4.

Expression of genes in adenocarcinoma and the epithelial cells surrounding the tumors of the induced DT mice. Lung sections from adenocarcinoma were probed with antibodies to PLAGL2 (A), TTF-1 (B), SP-B (C and E), and SP-C (D and F), then hematoxylin counterstained. Arrows denote examples of positively stained cells (brown color) with the antibody as indicated. T: tumor; Magnification: A – D, 40 X; E and F, 100 X.

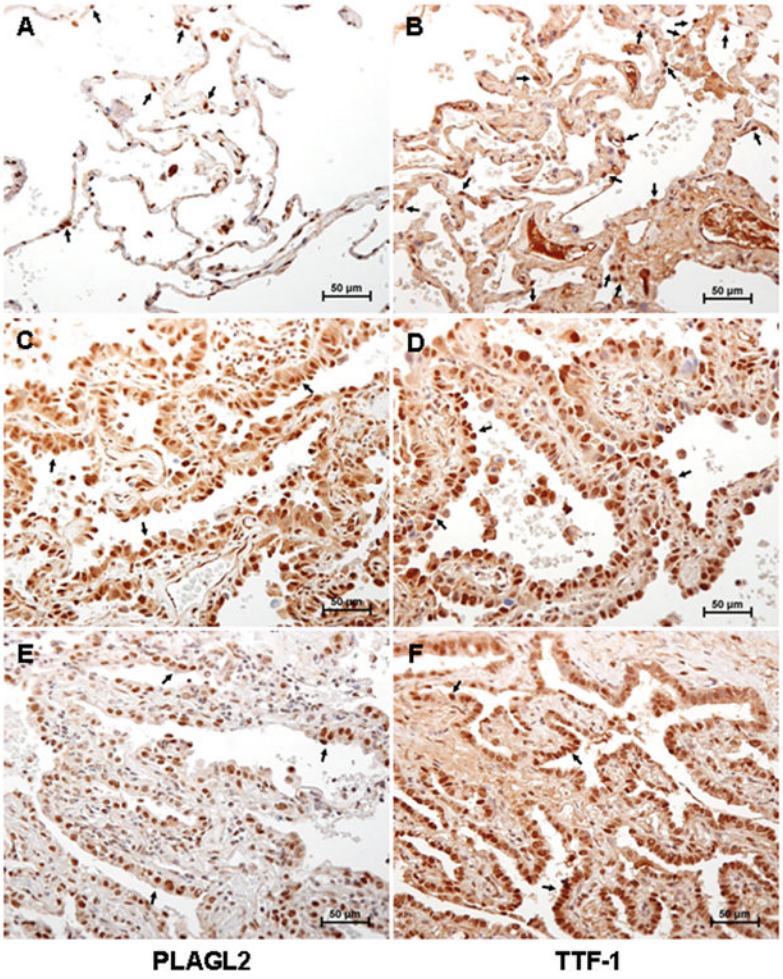


Figure 5.

PLAGL2 expression in human lungs and adenocarcinoma cancer cells. A and B, normal lung tissue; C and D, BAC; E and F, adenocarcinoma. Both PLAGL2 (A, C, and E) and TTF-1 (B, D, and F) expression were detected in normal lung parenchyma epithelial cells and in BAC and adenocarcinoma. Arrows mark the cells stained with PLAGL2 or TTF-1 in A and B, respectively; and few examples in cancer cells (C – F). Original magnification: 20X

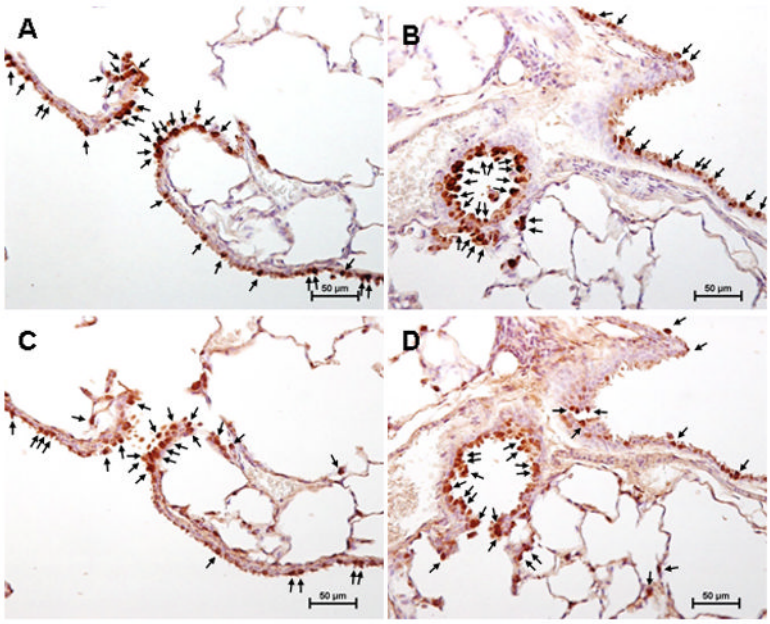


Figure 6.

CCSP and SP-C expression in epithelial cells at the BADJ of the induced DT mice. Continuous serial lung sections from the induced DT mice were probed with antibodies to CCSP (A and B) and proSP-C (C and D). Positively stained epithelial cells (brown) along the bronchiolar wall and in alveoli were marked by arrows. Original magnification, 20X

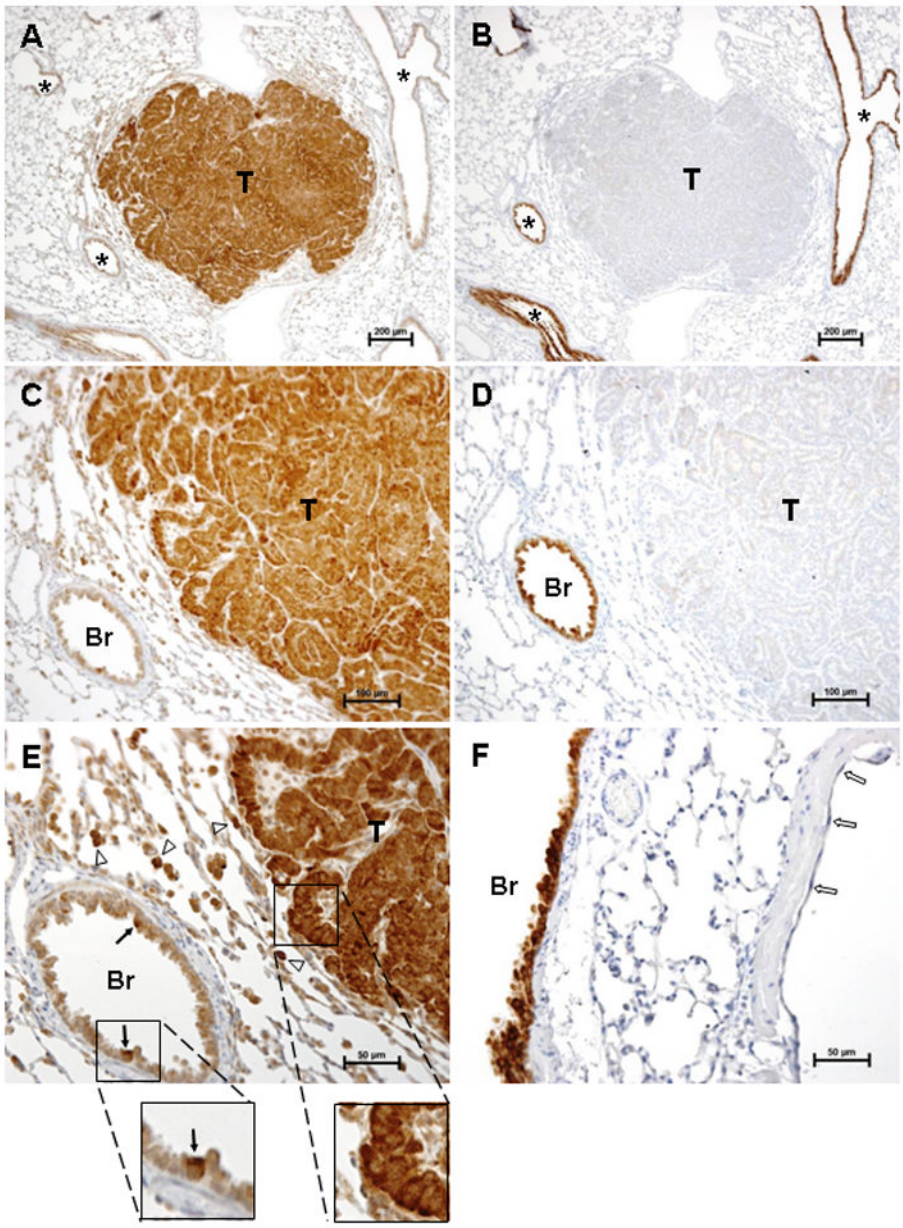


Figure 7.

CD133 and ALDH1 expression in the lungs of induced DT mice. Mouse lung sections with adenocarcinoma were probed with antibodies to CD133 (A, C, and E) or ALDH1 (B, D, and F). Epithelial cells in bronchioles and cancer cells stained with CD133 were enlarged to show the subcellular compartmentalization in E (inset). Arrows mark the apical location of CD133 in cells. Br, bronchioles; open triangle, positively stained alveolar macrophage; boxed arrow, endothelial cells of blood vessel; *, bronchus or bronchioles; T: tumor; original magnification: A and B, 4X; C and D, 10X; E and F, 20X

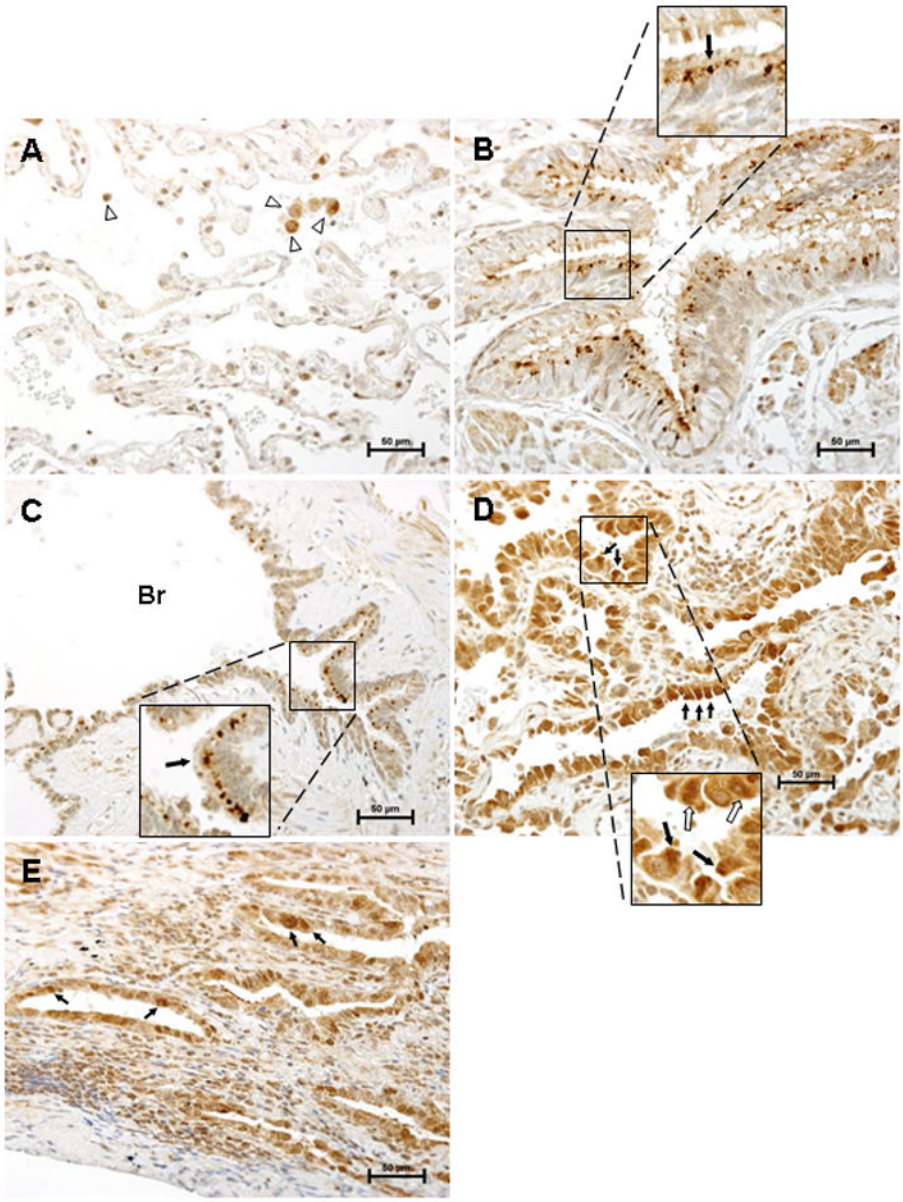


Figure 8.

CD133 expression in human lung sections with normal tissue and tumors. A and B are alveolar septa and the epithelium of the bronchus from a normal human lung tissue section. C and D are the epithelium of bronchioles and BAC from a cancer patient. E is lung adenocarcinoma. CD133 positive cells were stained brown. Insets in panels B, C, and D are enlarged area to show the subcellular location of CD133 expression. Arrows mark the apical location of CD133 in cells and open arrows point to a diffused cytosolic distribution of CD133. Br., bronchioles; open triangle, positively stained alveolar macrophage; original amplification: 20X

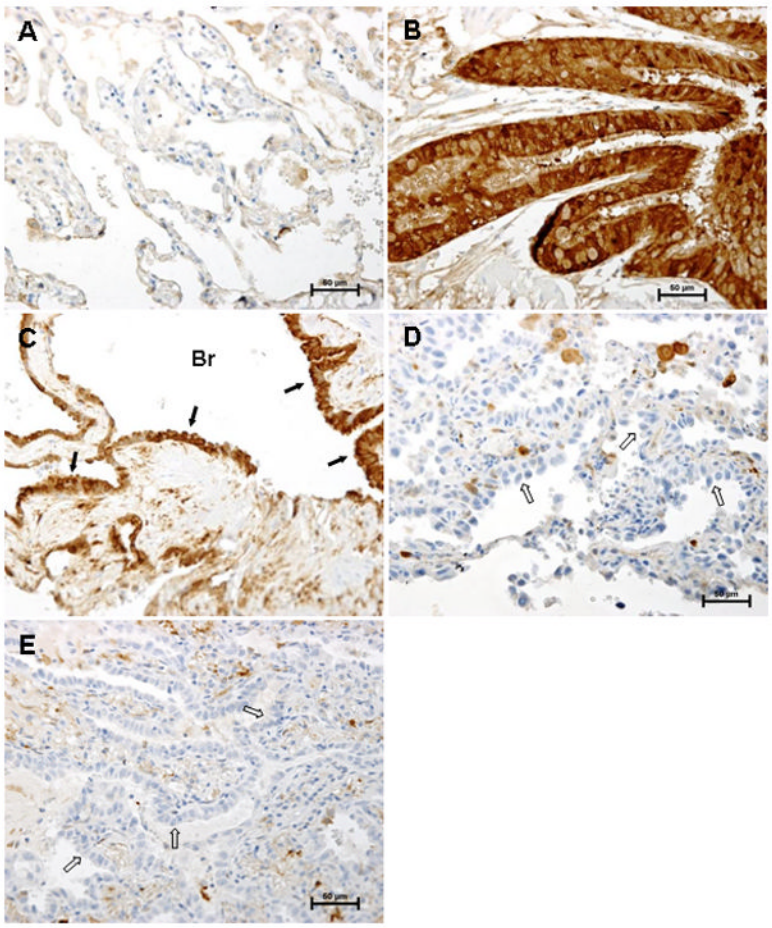


Figure 9.

ALDH1 expression in the human lung sections of normal tissue and tumors. Pictures of the lung sections are the same as described in Figure 8. Arrows mark the positively stained ciliated epithelial cells, and open arrows depict the negative expression of ALDH1 in cancer cells. Br., bronchioles; original amplification: 20X

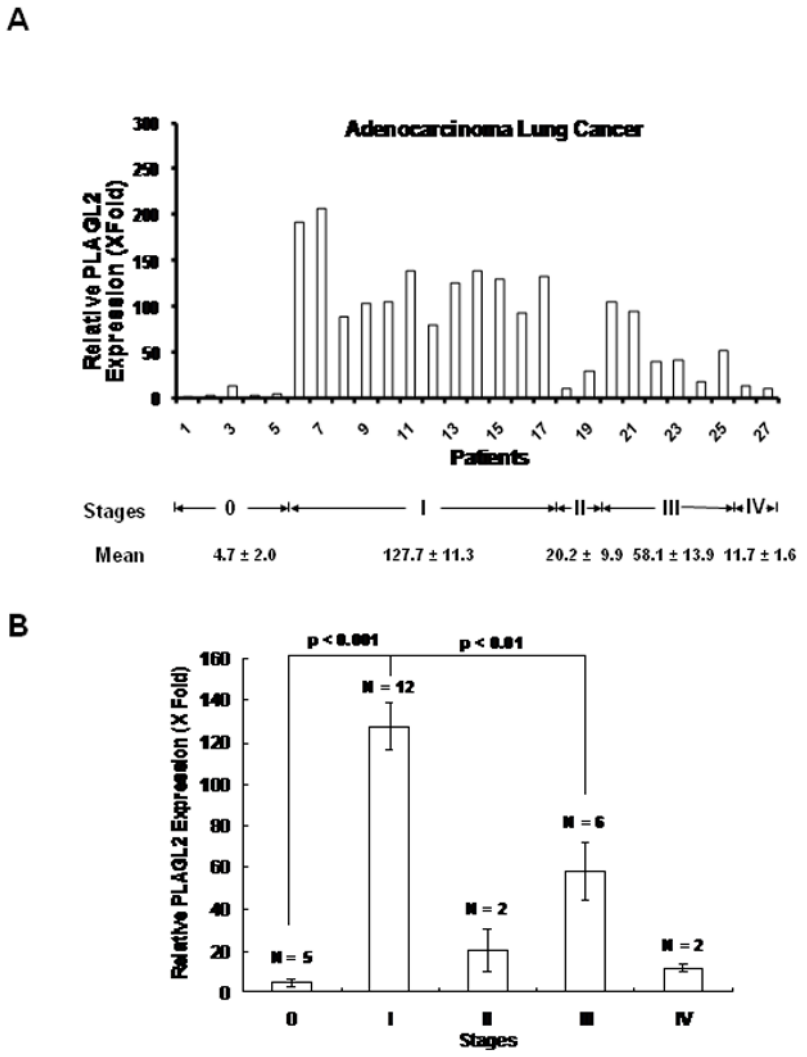


Figure 10.

PLAGL2 expression is increased at the early stage of lung adenocarcinoma. A cDNA array containing lung tissues from 22 adenocarcinoma lung cancer patients and 5 normal lungs (Origene Inc.) was examined for PLAGL2 expression using quantitative RT-PCR analysis. (A) The relative changes in fold differences in PLAGL2 messages within each sample were calculated by comparison with the sample with the lowest C_T value, which was set to 1. The patients' disease stages are summarized below, and the mean value with standard deviation of the fold changes within each category is shown below. (B) The increased PLAGL2 expression at the early stage of disease is significantly different from the control in normal lung tissue ($p<0.001$). The ANOVA single factor analysis shows that PLAGL2 expression in stage 1 is significantly different from that of the later stages (II, III, and IV; $p=0.002$). There is no difference in PLAGL2 expression levels among stages II, III and IV.

Figure 11A

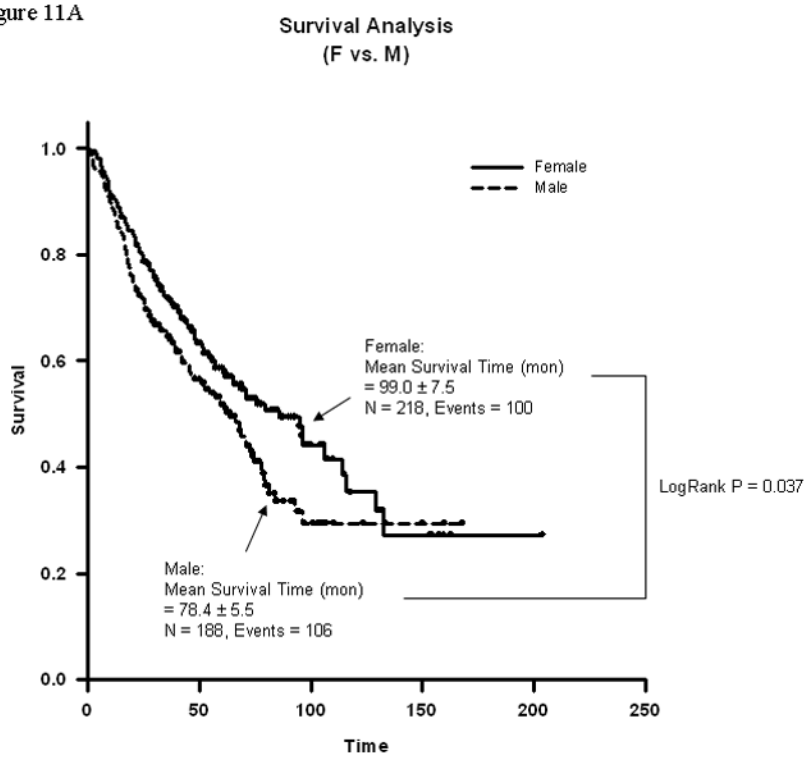
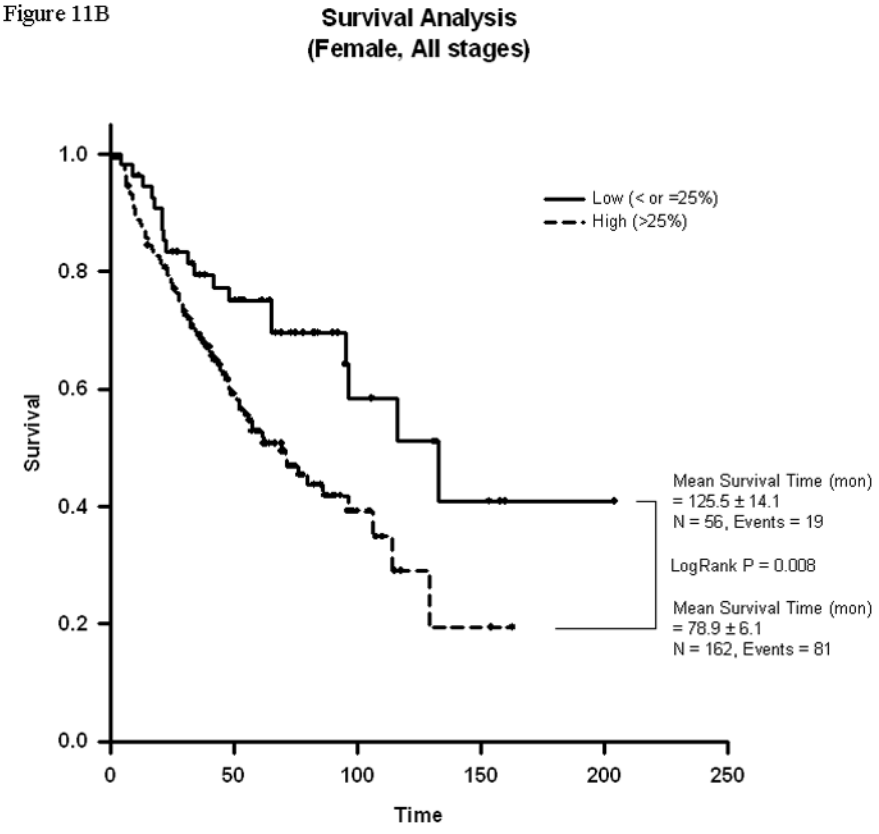


Figure 11B



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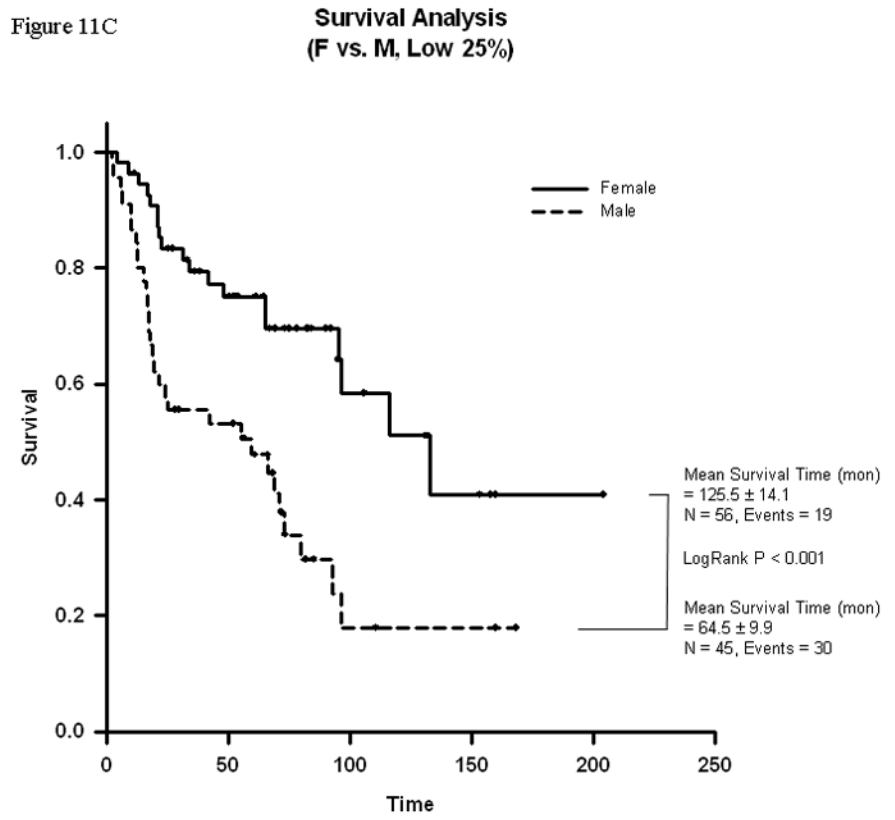

Figure 11D

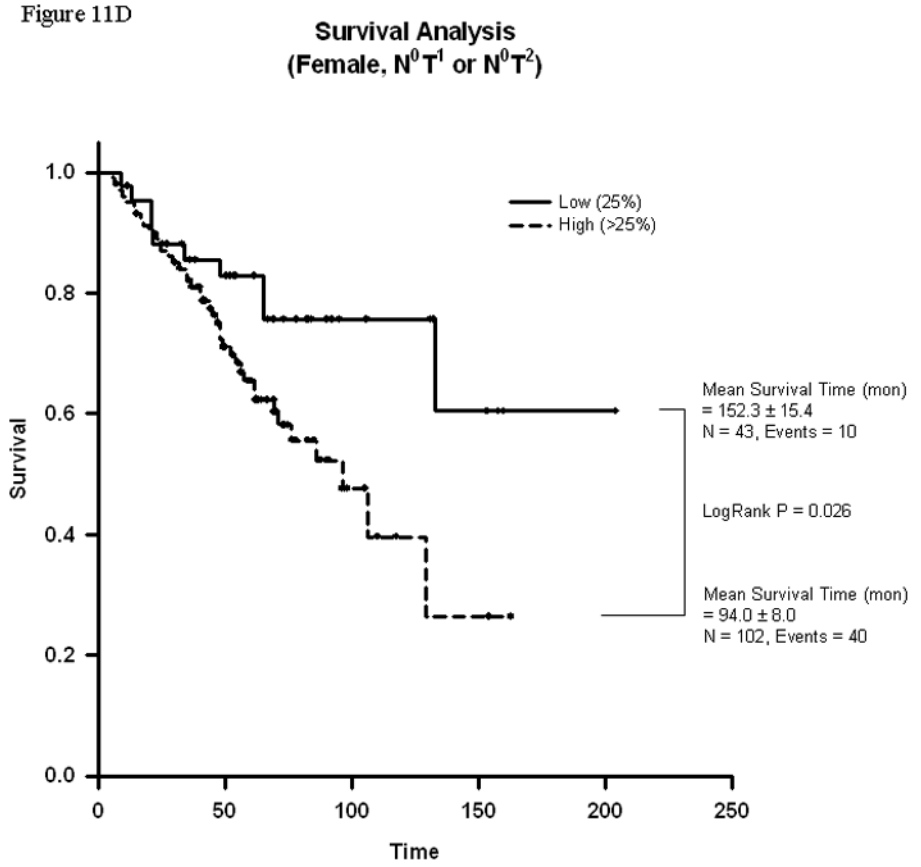

Figure 11.

Kaplan-Meier survival estimates for adenocarcinoma patients. (A) Survival estimates for the cancer patients according to gender. (B) Survival estimates for women (all stages) subgrouped into low (≤25%) and high (>25%) PLAGL2 expression groups. (C) Survival estimates for men and women (all stages) with low PLAGL2 expression. (D) Survival estimates for women at the early stage of disease (N^0T^1 or N^0T^2) with either low or high PLAGL2 expression. P-values and the mean survival time (months) were obtained with a log rank test. Dots indicate patients whose data were censored by the time of the last clinical-assessment. Events = death.

Table I

Gender preference in developing adenoma-carcinoma in the lungs of induced DT mice.

Mouse strain (Gene copies)		Induced DT mice	Control
P4 (180)	male	5/27 (18.5%)	0/22 (0%)
	female	1/26 (3.7%)	0/24 (0%)
P3 (26)	male	2/14 (12.5%)	1/23 (4.3%)
	female	0/23 (0%)	0/20 (0%)

The number of mice that developed tumors is presented as a ratio to total mice examined in each category. The percentage is shown in parenthesis. Control mice had single PLAGL2 transgene littermates with or without Dox induction and DT mice in the absence of PLAGL2 expression.