Progression of Human Bronchioloalveolar Carcinoma to Invasive Adenocarcinoma Is Modeled in a Transgenic Mouse Model of K-ras–Induced Lung Cancer by Loss of the TGF-β Type II Receptor

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Abstract

Clinical investigations have suggested that repression of the TGF-β type II receptor (Tgfbr2) may be an important step in progression of lung adenocarcinoma from an indolent in situ state to a frank invasive carcinoma. To test this hypothesis, we compared the effects of deleting the murine homolog of this receptor (Tgfbr2) in a mouse model of mutant K-ras–induced lung carcinoma, which normally induces the formation of multifocal tumors of low invasive potential. In this model, loss of Tgfbr2 induced a highly invasive phenotype associated with lymph node metastasis and reduced survival. Tumor-associated stromal cells displayed an immunosuppressive profile marked by increased numbers of B and T cells. Moreover, tumor stromal cell profiling revealed a developmental TGF-β response profile that associated with a collagenized extracellular matrix and increased invasion of TGF-β nonresponsive tumor cells. Together, these results suggest that our KrasTgfbr2−/− mouse model of invasive lung carcinoma mirrors the genomic response and clinical progression of human lung adenocarcinoma, recapitulating changes in lung stromal pathways that occur in the tumor microenvironment during malignant progression in this disease. Cancer Res; 17(21); 6665–75. ©2011 AACR.

Introduction

The extent of the invasive component in lung adenocarcinoma is associated with clinical outcomes. Similar to malignancies in other organs, such as breast and cervix, tumors are defined as noninvasive (in situ carcinoma), minimally invasive (microscopic invasion), or as invasive carcinomas. The World Health Organization has subclassified lung adenocarcinoma on the basis of predominant cell morphology and growth pattern such as bronchioloalveolar carcinoma (BAC), adenocarcinoma with mixed subtypes (AC-mixed), and homogeneously invasive tumors with a variety of histologic patterns (1). BAC tumor cells are cuboidal to columnar, with or without mucin, and grow in a noninvasive, lepidic fashion along alveolar walls. Adenocarcinomas with mixed subtypes frequently contain regions of lepidic pattern tumor at the periphery of invasive tumor. Pure invasive adenocarcinomas are devoid of lepidic morphology.

The clinical importance of lung adenocarcinoma invasion is supported by several recent studies, indicating that the risk of death in nonmucinous BAC is significantly lower than that of pure invasive tumors and in tumors with greater than 0.5 cm of fibrosis or linear invasion (2). These features have been incorporated into a proposed revision of lung adenocarcinoma classification that recognize the indolent nature of pure lepidic nonmucinous tumors as adenocarcinoma in situ (AIS) and of minimally invasive adenocarcinoma (3). Together, these studies suggest that noninvasive lung adenocarcinomas are biologically indolent with 5-year survival after resection approaching 100% and that following the paradigm of other adenocarcinoma in other organs, these in situ tumors may acquire molecular alterations that promote invasion and thus increases the risk of metastatic disease and death.

To identify molecular pathways important for distinguishing adenocarcinoma subclasses and for mediating the acquisition and progression of invasion, we and others (4–8) carried out microarray gene expression profiling of lung adenocarcinomas to identify signatures associated with histology and invasion. The results of unsupervised analyses show lung adenocarcinomas segregate into 3 major branches comprised predominantly of BAC/AIS, AC-mixed subtype, and pure invasive tumors and provide biological plausibility to support the notion that these adenocarcinoma subtypes are distinct. Among the genes differentially expressed in the progression...
from BAC/AIS to invasive tumors was the TGF-β type II receptor (TGFBR2, ref. 4), which was expressed at reduced levels by AC-mixed and solid invasive tumors compared with BAC/AIS. This finding suggests that TβRII repression is important for lung adenocarcinoma progression from BAC/AIS to invasive adenocarcinoma and is supported by in vitro studies showing that loss of TβRII in lung cancer cells increases tumor cell invasion, as well as by constitutive genetic models of TGFBR2–targeted deletion in other organ systems (9–11). This observation is also consistent with data showing that TGF-β signaling in carcinoma cells seems to be a major regulator in the tumor microenvironment. Studies in murine models of oncogene-driven breast cancer in which the TGFBR2 was deleted have shown that loss of response to TGF-β in mammary epithelial cells resulted in increased secretion of the chemokines Cxcl1, Cxcl5, and Cxcl12/Sdf1, followed by increased infiltration with myeloid cells that seemed to alter invasiveness and tumor migration (12). This paradigm has not yet been evaluated in murine models of lung cancer.

The goals of this article were to determine (i) biological significance of our clinical observations relating loss of the TβRII to lung cancer invasiveness by creating a mouse model, in which oncogenesis could be driven in the presence or absence of the TβRII in lung epithelial cells; (ii) whether this model would resemble other mouse models of tumor cell loss of Tgfr2; (iii) whether this new model would more resemble human invasive lung adenocarcinomas than previously described mouse models; and (iv) the downstream signaling events in the tumor microenvironment that are important for lung tumor invasion and progression.

Materials and Methods

Mice

To generate orthotopic lung cancers, we used a model in which an oncogenic mutated Kras gene is expressed ubiquitously under the control of a Lox-stop-Lox promoter (13). The mutated protein is not expressed until the animals are administered an adenoviral vector encoding Cre-recombinase (Ad.Cre). Once the Ad.Cre virus is given intranasally or intratracheally, airway and alveolar epithelial cells begin to produce the mutated Kras product leading to epithelial multifocal tumors that resemble BAC.

Breeding pairs of Lox-stop-Lox (LSL) KrasG12D mice (on a mixed 129Sv/J and C57BL/6 background) were generously provided by Dr. David Tuveson while he was at the University of Pennsylvania (14). Mice were genotyped by PCR amplification of genomic DNA obtained from tail samples (primer sequences available upon request). Breeding pairs of Tgfr2 (Tgfr2lox/lox) mice (on the C57/BL6 background) were generously provided by Dr. Harold Moses of Vanderbilt University (15).

LSL-KrasG12D–positive mice were simultaneously backcrossed to C57/BL6 mice and to the Tgfr2lox/lox mouse to allow comparisons with the same genetic background. Mice were backcrossed 9 generations. Some experiments were conducted at the F5 generation, with similar results to the final F9 generation. Animals used for all experiments were between 6 to 10 weeks old and were housed in the animal facility at Wistar Institute (Philadelphia, PA). All protocols were approved by the Animal Use Committees of the Wistar Institute and University of Pennsylvania and were in compliance with the guide for care and use of animals.

Lung tumor model

To induce tumors, 100 µL of saline containing 109 pfu particles of an adenovirus containing the Cre recombinase (Ad.Cre) was administered to each LSL-KrasG12D mouse intranasally (for details see Wilderman and colleagues; ref. 16). Virus was suspended in serum-free Dulbecco’s modified Eagle’s medium (which contains 125 mg/L of NaH2PO4). Fifteen minutes prior to administration of adenovirus, 0.5 µL of 2 mol/L CaCl2 was added; calcium phosphate coprecipitation has been shown to improve lung gene transfer (14, 17). Animals were closely observed daily for signs of distress. When they appeared lethargic, had ruffled fur or increased breathing rates, the animals were sacrificed. Massive tumor infiltration of the lung was confirmed by histology. At sacrifice, 1 lung was excised and frozen in optimal embedding compound. The other lung and the mediastinum were fixed in formalin and paraffin embedded.

Fluorescence-activated cell sorting

Lungs were removed from euthanized mice and minced into fine pieces in digestion buffer containing 0.1 mg/mL DNase-I and 2.0 mg/mL collagenase type IV (Sigma). Samples were incubated in digestion buffer at 37°C for 30 minutes, filtered through a 70-µm filter, and washed 2 times with serum-free media. All flow cytometry was done using a Becton-Dickinson FACSCalibur flow cytometer. Data analysis was done utilizing FlowJo software. Antibodies were purchased from BD Biosciences Pharmingen.

Genomics (details in Supplementary Methods)

To enrich for tumor and stromal cells, we used the PALM MicroBeam LCM system (Zeiss) system to acquire cells for mRNA extraction using the RNAqueous-Micro Scale RNA Isolation kit (Ambion). From each specimen, approximately 600,000 µL of tissue were dissected, yielding 20 ng RNA for genomics analysis using Affymetrix Mouse 430 2.0 Arrays. We identified genes that were differentially expressed between tumors of KrasTgfr2–/– and those of KrasTgfr2 WT mice using a permuted random variance t test with a 2-fold change cut-off and the significance analysis of microarrays (SAM) method (3). Genes were considered to be statistically significant if their permuted t test P value was less than 0.001 and the estimated false discovery rate (FDR) was less than 0.01 (see Supplementary Table S1). Genomic profiles were compared with human lung adenocarcinoma tumor cells acquired by microdissection in 40 cases (New York Lung Adenocarcinoma). Human studies were approved by the Columbia University Medical Center Institutional Review Board. Gene set Enrichment Analysis (GSEA) was used to determine enrichment of murine tumor gene sets (Supplementary Table S6) in the human New York Lung Adenocarcinoma gene profile and in the Japan Lung Adenocarcinoma (5) gene profiles. Microarray
data have been deposited with the GEO repository, accession number GSE27719.

Immunohistochemistry

Immunohistochemistry (IHC) for cytokeratin 18 (KRT18, polyclonal rabbit antibody, dilution 1:100; Epitomics) was done using Target retrieval buffer pH 9 (Dako), 1 hour primary antibody incubation at room temperature and the Vector Elite Rabbit ABC kit with 3,3′-diaminobenzidine (DAB; Vector Laboratories) for 5 minutes. For mediastinal lymph node dissection, 10 level sections were cut and numbered; and hematoxylin and eosin (H&E) stains were done on the odd sections and IHC for KRT18 was done on the even sections, using a technique similar to sentinel node histologic examination. All foci staining for KRT18 were confirmed by examination of adjacent morphologic tumor on the H&E section before being counted as a node metastasis. Mediastinal lymph node nodules were evaluated in 19 KrasTgfbr2−/− animals and 7 KrasTgfbr2WT animals.

Collagen quantitation

This was done using a modification of the method of Ornberg and colleagues (18). Briefly, 5 fields, each of trichrome-stained tumor at 100×, were captured from KrasTgfbr2−/− lungs (n = 6) and KrasTgfbr2WT lungs (n = 9). Using NIH Image J v1.37c (19) images were converted to grayscale using RGB stack, generating a red, green, and blue grayscale rendition. Using the blue stack, the image threshold was adjusted so that segmented pixels were highlighted, representing areas of the field not containing collagen. Fields did not include blank areas. The area and ratio of blue staining to collagen containing areas were calculated; measurements were averaged by animal and were evaluated using 2-tailed t test.

Statistical analysis

Unless otherwise noted, data comparing differences between groups were assessed using ANOVA with appropriate post hoc testing. Survival studies were assessed using Kaplan–Meier survival curves and analyzed with the Mantel–Cox log-rank test. Differences were considered significant when P value was less than 0.05. Statistics were conducted using SPSS software, version 16.0.

Results

Loss of TβRII leads to increased invasiveness of Kras-induced tumors

To determine the direct role of TβRII repression on adeno- carcinoma invasion in vivo, we generated a genetic model of murine lung adenocarcinoma with targeted deletion of Tgfbr2 (see Methods). In this model, intranasal instillation of Ad.Cre led to both activation of a mutated Kras gene and loss of the Tgfbr2 gene in the same epithelial cells. We wanted to test the hypothesis that in the oncogenic Kras murine model of lung cancer, loss of TβRII would increase tumor invasion and metastasis.

Intranasal instillation of Ad.Cre into the Tgfbr2lox/lox mice did not lead to any discernable tumors over a 10-week period, indicating that loss of TβRII is not sufficient for tumor initiation. When we administered Ad.Cre to mice that were KrasTgfbr2 heterozygous, we did not identify differences in tumor morphology compared with the LSL-KrasTgfbr2WT mice (data not shown). All subsequent breeding was directed toward generation of KrasTgfbr2 KO homozygous mice (KrasTgfbr2−/−). We confirmed recombination of the Tgfbr2 floxed allele and loss of TβRII expression after administration of intranasal Adeno-Cre to KrasTgfbr2lox/lox mice (Supplementary Fig. S1).

In contrast to the heterozygous mice, instillation of Ad.Cre to homozygous animals resulted in striking differences in tumor morphology and tumorigenesis (Fig. 1). Instillation of 105 pfu of Ad.Cre into KrasTgfbr2−/− mice led to the expected development, over a 6- to 10-week period, of noninvasive adenoma and adenocarcinoma characterized by epithelial cell proliferation without destruction of alveolar walls (lepidic growth) or stromal reaction (16). In contrast, lung tumors in KrasTgfbr2−/− were histologically similar to human mixed subtype adenocarcinomas in that lepidic areas were associated with transitions to invasive adenocarcinoma involving vessels, airways, and pleura. Tumor cells were grouped as small glands, small nests, and single cells amidst distinct areas of desmoplastic stroma. IHC for cytokeratin 18 (KRT18) confirmed the epithelial nature of these infiltrating tumor cells. Trichrome stain highlighted the increased collagen in invasive areas in KrasTgfbr2−/− mouse lungs when compared with KrasTgfbr2WT, with significant difference (t test, P < 0.0001) when analyzed quantitatively.

Further examination of the KrasTgfbr2−/− lung tumors showed nuclear TTF-1 (NKH2-1) and cytoplasmic prosurfec- tant protein C immunoreactivity, although nonreactive for CCSP (data not shown), suggesting alveolar type II cell differen- tiation. In addition, the KrasTgfbr2−/− lung tumors showed evidence of inflammatory cell recruitment and tumor micro- environment remodeling with neoangiogenesis that was not seen in the KrasTgfbr2WT tumors (Supplementary Fig. S2).

Vascular invasion and metastasis

In addition to desmoplastic stroma deposition, KrasTgfbr2−/− mouse tumors showed transmural invasion of vessels and pleural invasion (Fig. 2A–D). We also detected regional metastases to mediastinal lymph nodes in 10 of 19 (53%) of KrasTgfbr2−/− mice, whereas no metastases were detected in the KrasTgfbr2WT (n = 7) mice, despite examination of 10 level sections with alternating KRT18 stains (Fisher P = 0.02; Fig. 2E and F). The metastases were comprised of small clusters of tumor cells, detected morphologically and confirmed by KRT18 IHC. No metastases were seen in the liver, spleen, or other extrathoracic sites.

In summary, the morphologic examination indicates that the KrasTgfbr2−/− mouse recapitulates the progression of a pure noninvasive adenocarcinoma to an adenocarcinoma with mixed invasive morphology and metastatic potential.

Loss of TβRII leads to decreased survival in mice with Kras-induced tumors

These changes in morphology led to changes in survival. We studied the effect of intranasal Ad.Cre on survival in the F5 and F9 generation LSL-K-rasG12D-positive mice that were
homozygous for the Tgfr2lox/lox allele versus littermate controls without the floxed Tgfr2 allele (n = 10 in each group). In the F9 mice, the KrasTgfbr2WT mice had a median survival of 79 days, whereas the median survival in the KrasTgfbr2−/− group was reduced to only 43 days (log-rank $P < 0.001$; Fig. 3). The survival data from the F5 mice was similar (data not shown).

**Alterations in gene profiles in KrasTgfbr2−/− versus KrasTgfbr2WT mice**

To determine the key activated molecular signaling pathways in these invasive murine lung adenocarcinomas and to determine whether the murine model transcriptional profiles were similar to those of human adenocarcinoma, we examined gene expression profiles of the microdissected murine tumors using microarray analysis of tumor cell mRNA. We generated a signature of KrasTgfbr2−/− tumors by combining results of a permuted $t$ test, $P = 0.001$ with those from SAMs algorithm, with a FDR of 1%. The intersection of these lists consisted of 243 upregulated and 707 downregulated genes (Supplementary Table S1).

**Comparative genomics**

A primary goal of this work was to determine whether the KrasTgfbr2−/− signature of upregulated genes was enriched in human invasive mixed subtype lung adenocarcinoma tumor cells compared with noninvasive nonmucinous BAC. To accomplish this, we first generated a gene expression dataset of 40 BAC and AC-mixed human tumors from which we acquired mRNA from laser capture microdissected tumor cells (Supplementary Table S2). Unsupervised hierarchical clustering identified 2 main clusters (Fig. 4A). The mixed subtype adenocarcinomas were grouped in cluster 1 (15/23), whereas BACs were grouped in cluster 2 (13/17; Fisher $P = 0.01$. DNA direct sequencing analysis indicated the prevalence of EGFR (epidermal growth factor receptor) and Kras mutations was 38% and 7%, respectively, in these nonsolid adenocarcinomas, which is in the range of those reported by others. Similarly, the clusters of adenocarcinoma histologic subtype classes were independent of EGFR and Kras mutation status (5, 6). Taken together, these results indicate that tumor cell molecular signatures of noninvasive and minimally invasive lung adenocarcinomas are distinct from other mixed subtype invasive adenocarcinoma.

With this human data in hand, we used Gene Set Enrichment Analysis to (i) determine whether the gene signature of KrasTgfbr2−/− tumors was enriched in human AC-mixed tumors compared with BAC tumors and (ii) if the KrasTgfbr2−/− model was more similar to human invasive
lung adenocarcinoma than other previously described mouse lung cancer models. To determine the relative similarity of the KrasTgfbr2<sup>C0/C0</sup> model with other murine lung adenocarcinoma models, we tested gene signatures from (i) the KrasTgfbr2<sup>C0/C0</sup> model (this article); (ii) the noninvasive Kras-LA model (14, 20); (iii) the KrasP53<sup>mut</sup> model (21); (iv) the KrasLkb1<sup>C0/C0</sup>, model (22); and (v) the KrasHif2a models of advanced lung carcinoma (ref. 23; Table 1). For each murine model, we calculated a normalized enrichment score (NES), which indicates the degree to which the gene set is overrepresented at the top or bottom of the ranked list of genes in the human AC expression dataset (New York Lung Adenocarcinoma), normalized across the analyzed gene sets. In human AC invasive tumors, 3 murine gene sets were enriched, with NES of 1.29, 1.03, and 0.87 for the KrasTgfbr2<sup>C0/C0</sup>, KrasLkb1<sup>C0/C0</sup>, and KrasP53<sup>mut</sup> gene sets, respectively; but only the KrasTgfbr2<sup>C0/C0</sup> enrichment was significant with nominal P value less than 0.05 and FDR < 25%. To examine the generalizability of these findings and to exclude laser capture microdissection as a confounding variable for gene set enrichment, we repeated the procedure in a human adenocarcinoma gene expression dataset acquired from 163 lung specimens (Japan invasive AC). We examined gene set enrichment in the adenocarcinomas with terminal respiratory (TRU-a) morphology that is similar to AC mixed tumors in comparison with TRU-B, BAC-like tumors. Again, we detected enrichment of the KrasTgfbr2<sup>C0/C0</sup> gene set with a NES of 1.35 (P = 0.025, FDR = 0.045). The KrasP53<sup>mut</sup> and Kras-LA gene sets were also enriched, but the NES was not significant. Taken together, the genomics results in 2 human lung adenocarcinoma datasets suggest that the murine KrasTgfbr2<sup>C0/C0</sup> model closely recapitulates the gene expression alterations detected in the progression of human BAC to AC mixed subtypes.

**Tumor microenvironment**

The biological process ontology of the KrasTgfbr2<sup>C0/C0</sup> mouse tumor model genes suggested activation of pathways important for immune response and development, including lung branching morphogenesis (Supplementary Table S3). These processes require interactions between epithelial and stromal cells within the tumor microenvironment. To examine the stromal response to Tgfbr2-deficient tumor cells, we compared stromal cell transcriptional profiles from 5-week KrasTgfbr2<sup>C0/C0</sup>...
tumors and 9-week KrasTgfbr2<sup>WT</sup>. We used mice at these time points to allow comparison of the stromal compartment of similarly advanced tumors. However, even with this adjustment, the amount of tumor-associated stroma in advanced 9-week KrasTgfbr2<sup>WT</sup> mice was less than in the 5-week KrasTgfbr2<sup>fl/fl</sup> mice. The gene expression heatmap revealed clusters of genes that were upregulated in stroma and not tumor (indicating successful microdissection), as well as genes differentially expressed in KrasTgfbr2<sup>fl/fl</sup> stroma compared with KrasTgfbr2<sup>WT</sup> stroma (Fig. 4B; Supplementary Table S4). Ontology analysis of KrasTgfbr2<sup>fl/fl</sup> stroma profile showed upregulation of genes with functions important in wound healing response, development, and immune responses (Supplementary Table S5), similar to ontologies of genes acquired from tumor cells.

Tumor microenvironment: lung development and TGF-β

In light of the gene ontologies related to development in both the tumor and stromal analysis (Supplementary Tables S5 and S6) and of prior work suggesting that tumor cell signatures recapitulate key developmental pathways (24), we compared our KrasTgfbr2<sup>fl/fl</sup> gene expression profiles to a published study of lung branching genomics (25). Lu and colleagues showed that expression of TGF-β responsive genes in the distal lung mesenchyme was associated with initiation of branching. Gene set analysis of our KrasTgfbr2<sup>fl/fl</sup> stromal signature showed enrichment in the Lu branching morphogenesis gene expression profile (Efron-Tibshirani GSA P value < 0.005). To determine whether this stromal signature was enriched for TGF-β responsive genes, we carried out gene set analysis for the Gene ontology of cellular response to TGF-β stimulus (GO:00071560) and determined significant enrichment (Efron-Tibshirani GSA P value < 0.005).

As a result of these findings, we examined our microarray data for evidence suggestive of TGF-β effect in the stromal dissected compartment. Despite prior reports that TGF-β levels were increased in mice with tumor lacking TβRII (reviewed in Yang and colleagues; ref. 26), we saw no clear differences in expression levels of Smads or TGF-β isoform mRNA in the stroma microarrays, although TGF-β2 was increased in the tumor microdissection microarrays. We did see a large increase in thrombospondin 1 message (5-fold, P = 3 x 10<sup>-5</sup>), a matrix protein known to be important in activating latent TGF-β. Consistent with evidence of increased TGF-β activity, we saw (i) increases in TGF-β responsive genes in the KrasTgfbr2<sup>fl/fl</sup> tumor stroma including arginase 1 (63-fold, P < 0.0001), TGF-β induced (2.85, P < 0.02), and fibroblast activation protein (4.2-fold, P = 0.045) and (ii) increases in matrix proteins known to be responsive to TGF-β including tenascin C (4.5-fold, P = 0.006), fibronectin 1 (5.88-fold, P = 0.01), collagen type I, alpha 1 (30.3-fold, P = 0.03), collagen type VIII, alpha 1 (2.63-fold, P = 0.018), collagen XV, alpha 1 (3.22-fold, P = 0.045), and cysteine-rich protein 61 (15.2-fold increase, P = 0.02). Thus, our array data suggest a tumor microenvironment characterized by enhanced TGF-β activity in the stromal compartment. We confirmed expression of a number of these TGF-β related genes and calculated the ratio of expression levels of mRNAs by quantitative PCR between needle dissected (tumor plus tumor associated stroma) for KrasTgfbr2<sup>fl/fl</sup> lungs and KrasTgfbr2<sup>WT</sup> lungs. We observed an increase in Arg-1 (2-fold, P = 0.006), fibroblast activation protein (1.8-fold, P = 0.02), and fibronectin 1 (1.9-fold, P = 0.003), all validating the array results.

Tumor microenvironment: immune response

We then compared gene expression between the 2 types of mice with regard to inflammatory and immune modulatory programs (again, comparing the KrasTgfbr2<sup>fl/fl</sup> lungs to the KrasTgfbr2<sup>WT</sup>). Unlike the Yang breast cancer study (12), we did not see significant increases in Cccl5 (RANTES) or Cxcl12 in the KrasTgfbr2<sup>fl/fl</sup> lungs in arrays or using real-time quantitative reverse transcriptase PCR (qRT-PCR). Ccl5 message was increased by 3-fold on the array, but we could not confirm this change using qRT-PCR. The IFN-induced chemokines that bind to Cxcr3 and attract activated CD8 T cells, Cxcl9 (MIG), Cxcl10 (IP10), and Cxcl11 (i-TAC) were all highly downregulated (P < 0.001), TGF-β1 (1.9-fold, P = 0.003), and (ii) increases in matrix proteins known be responsive to TGF-β including tenascin C (4.5-fold, P = 0.006), fibronectin 1 (5.88-fold, P = 0.01), collagen type I, alpha 1 (30.3-fold, P = 0.03), collagen type VIII, alpha 1 (2.63-fold, P = 0.018), collagen XV, alpha 1 (3.22-fold, P = 0.045), and cysteine-rich protein 61 (15.2-fold increase, P = 0.02). Thus, our array data suggest a tumor microenvironment characterized by enhanced TGF-β activity in the stromal compartment. We confirmed expression of a number of these TGF-β related genes and calculated the ratio of expression levels of mRNAs by quantitative PCR between needle dissected (tumor plus tumor associated stroma) for KrasTgfbr2<sup>fl/fl</sup> lungs and KrasTgfbr2<sup>WT</sup> lungs. We observed an increase in Arg-1 (2-fold, P = 0.006), fibroblast activation protein (1.8-fold, P = 0.02), and fibronectin 1 (1.9-fold, P = 0.003), all validating the array results.

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Two important caveats should be noted. First, digestion of lungs preferentially releases leukocytes (defined as CD45$^+$ cells), so the overall percentage of CD45 cells recovered is less informative than analysis of the CD45 subsets. Second, we analyzed the entire lung because we could not microdissect the tumor tissues. Our results thus reflect the admixture of cells within the lung tumors and the non–tumor-bearing lung (Fig. 5).

We noted a number of significant ($P < 0.05$) changes in the lymphocyte populations in the Kras-Tgfbr2$^{WT}$ lungs: (i) the % of CD4 T cells of CD45 cells and the % of B cells of CD45 cells was increased compared with normal lungs and Kras-Tgfbr2$^{WT}$ tumor-bearing lungs, and (ii) the % of CD8 T cells of CD45 cells was increased compared with control lungs. With regard to the CD4 cells, the percentage of CD45 cells that could be considered T-regulatory cells (defined as CD4$^+$/CD25$^+$) was increased (3.2%) in the Kras-Tgfbr2$^{-/-}$ lungs compared with the Kras-Tgfbr2$^{WT}$ lungs (2.3%), but this change did not reach significance.

There were also changes in the myeloid cell populations (defined as CD45$^+$ cells that express the adhesion molecule CD11b). First, the myeloid (CD11b$^+$) cell population was significantly lower in the Kras-Tgfbr2$^{-/-}$ lungs than in the normal or Kras-Tgfbr2$^{WT}$ lungs. The types of myeloid cells were...
given the smaller percentage of myeloid cells in the \textit{KrasTgfbr2} \textsuperscript{−/−} lungs, the total number of lung TAMs would be lower. Finally, there seemed to be a decreased proportion of undifferentiated myeloid cells (M0 cells, defined as CD11b+/F4/80−/Ly6G−) in the \textit{KrasTgfbr2} \textsuperscript{−/−} lungs. In summary, compared with the \textit{KrasTgfbr2} \textsuperscript{WT} lungs, the \textit{KrasTgfbr2} \textsuperscript{−/−} lungs had more T and B cells and less myeloid cells, somewhat different than the results reported in the breast cancer models.

Expression of the TGF-β type II receptor in human lung adenocarcinoma

TβRII protein expression is inversely correlated with extent of invasion in early-stage lung adenocarcinoma (4). We extended this observation by examining the correlation of TβRII protein expression with tumor stage in clinical stage I patients within a larger cohort of 183 lung adenocarcinoma patients (2). Reduced expression (TβRII intensity score 0, 1) was detected in 46% of stage T2 tumors and 22% of stage T1 tumors, \( P < 0.05 \) versus TβRII intensity score 2. We examined the prognostic significance of \textit{TGFBRII} mRNA repression in human lung adenocarcinoma using the Japanese Lung Adenocarcinoma database. Low expression of \textit{TGFBRII} (below median level of expression of probe A_23_P211957) was associated with lymph node metastasis (N1-N2 vs. N0) and with increased risk of death, \( P < 0.001 \) and \( P < 0.05 \), respectively. Taken together, these results confirm the clinicopathologic significance of TGF-β type II receptor expression in human lung adenocarcinoma during malignant progression of this disease.

Discussion

Experience over time shows that human lung adenocarcinoma progresses from a noninvasive lesion, usually manifest as a ground glass opacity, to an invasive carcinoma with capacity for metastasis (27). The purpose of this study is to model this progression in the mouse and to determine the importance of the type II TGF beta receptor in mediating lung adenocarcinoma invasion and metastasis, \textit{in vivo}. We show that loss of

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<th>Gene set</th>
<th>Normalized enrichment score</th>
<th>Nominal ( P )</th>
<th>FDR Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY lung adenocarcinoma</td>
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<td></td>
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</tr>
<tr>
<td>\textit{KrasTgfbr2} \textsuperscript{−/−}</td>
<td>1.29</td>
<td>0.05</td>
<td>0.08</td>
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<td>\textit{KrasLkb1} \textsuperscript{−/−}</td>
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<td>\textit{KrasP53mut}</td>
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<td>\textit{Kras-LA}</td>
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<td>0.10</td>
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<td>\textit{KrasHif2a}</td>
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<td>Japan lung adenocarcinoma</td>
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<td>0.17</td>
</tr>
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</table>

NOTE: For each murine model, we calculated a normalized enrichment score (NES), which indicates the degree to which the gene set is overrepresented at the top or bottom of the ranked list of genes in the human AC expression dataset (New York Lung Adenocarcinoma invasive vs. BAC/AIS and Japan Lung Adenocarcinoma TRU-A vs. TRU-B tumors), normalized across the analyzed gene sets. The significance was indicated by a nominal \( P \) value and by a permuted FDR.

also different. Whereas the % of neutrophils (defined as CD11b+/F4/80−/Ly6G+) of the total myeloid population was significantly \( P < 0.05 \) increased in the \textit{KrasTgfbr2}\textsuperscript{−/−} lungs (58%) versus normal lungs (30%), this increase was not seen in the \textit{KrasTgfbr2} \textsuperscript{−/−} lungs. The percentage of tumor-associated macrophages (TAM, defined as CD11b+/F4/80+/Ly6G−) of total myeloid cells were similar among the 3 groups, however,
Tgfbr2 in lung epithelial cells induces invasive morphology in noninvasive Kras mutant tumors, promotes lymph node metastases, and shortens survival. Furthermore, we show that this murine model recapitulates both the histologic and genomic changes that accompany invasion and metastasis in human lung adenocarcinoma tumors.

TGF-β, the ligand for the TGF-β type II receptor is a pleiotropic cytokine comprised of family members TGF-β1, 2, 3 that regulate tissue homeostasis and prevent tumor initiation by inhibiting cellular proliferation, differentiation, and survival (28). It is secreted as a latent molecule and is activated by cleavage by proteases and other molecules. Signaling primarily occurs through SMAD protein-dependent pathways, whereby ligand binding to TβRII induces phosphorylation and activation of TGF-β type I receptor (TβRI). TGF-β signaling may also proceed via less well understood SMAD independent pathways (29). Depending upon context, TGF-β signaling may alternatively function to suppress tumor growth in early cancers or to promote tumor cell invasion and metastasis in late cancers (30–33). Rojas and colleagues have shown that different levels of repression of the TGF-β receptor are associated with variable activation of the SMAD and mitogen-activated protein kinase pathways, such that at lower levels of TGF-β receptor activation, the protumorigenic non-SMAD signaling pathways dominate (34).

Type II receptor genetic alterations are well characterized in human gastrointestinal tumors, in which 25% of colorectal carcinomas have missense mutations associated with microsatellite instability. Somatic mutations are rare in other human neoplasms. In human lung tumor specimens, type II receptor repression is evident in approximately 40% of lung adenocarcinomas overall and in up to 100% of poorly differentiated adenocarcinomas (4, 35). Potential mechanisms of repression include promoter hypermethylation (36), miRNA (37), microsatellite instability, and frameshift mutations involving the poly(A) tract (38).

In advanced cancers, induction of TGF-β signaling promotes epithelial to mesenchymal transition, a characteristic of invasive and metastatic cells (39, 40), with constitutive activation of TGF-β or TβRI leading to increased metastases in animal models of breast cancer (41–43). Systemic inhibition of TGF-β has been shown to suppress metastasis (44–47) and clinical cancer trials are in progress. In animal models of early tumor progression, TGF-β signaling is tumor suppressive, with animal models of targeted repression of TβRII consistently resulting in tumor cell invasion, progression, and metastasis. However, the signaling pathways that are activated in the setting of TGF-β signaling inhibition are varied. Yang and colleagues showed that targeted deletion of TβRII in the mammary epithelium promoted breast cancer metastases through the Cxcl5/Cxcr2 chemokine axis mediated recruitment of Gr-1+/CD11b+ myeloid-derived suppressor cells. Increased stromal TGF-β levels at the invasive front of tumors was shown to be important for tumor progression and for inhibition of tumor immunosurveillance (12). Our lung model gene expression data support a role for TβRII repression in producing an immunosuppressed tumor microenvironment. Our fluorescence-activated cell sorting data do not show a significant increase of Gr-1+/CD11b+ cells but rather an increase in CD4-positive T cells and B cells that may be important in facilitating tumor progression and invasion in the lung, as has been shown in the prostate and breast (48, 49). The differences in inflammatory cell recruitment and chemokine profile between our inducible model and those reported by Yang and colleagues may be attributable to organ-specific microenvironment influences or to developmental compensation in a constitutive model of Tgfbr2 deficiency.

The identification of developmental gene ontologies and, specifically, the enrichment of genes involved in lung branching morphogenesis in our mouse lung tumor and stroma gene expression profiles support the concept that key events in tumor progression recapitulate fundamental developmental pathways. Similar to the distal branching lung, TGF-β nonresponsiveness in the KrasTgfbr2−/− adenocarcinoma model determines epithelial fate in terms of cell motility, adhesion, and invasion. Unlike normal lung development (25), the tumor model has continuous activation of Kras with TGF-β responsiveness only in the nontumoral stroma, resulting in a TGF-β response-associated stromal matrix profile, but not growth stabilization. The resulting tumor manifestation is disorganized collagenized matrix and invasion, rather than an organized epithelial–mesenchymal structure. Recent work using cell lines with repressed TβRII expression show tumor cell autonomous effects of TβRII repression on tumor invasion (4). Thus, it is unclear whether the tumor invasion and progression phenotype requires an activated TGF-β stromal response or is independent of stromal influence; this will require further testing of systemic and targeted TGF-β signaling blockade.

The oncogenic Kras lung adenocarcinoma mouse has proven to be a reproducible model of noninvasive lung adenocarcinoma that is more similar genomically to human lung adenocarcinoma than the carcinogen-induced models, which also induce noninvasive adenoma lesions. Combination of Kras induction with alterations in Lkb1, p53, and Hif2α has generated lung tumor models with accelerated progression, dedifferentiation, and angiogenesis and provided important insights into mechanisms related to advanced disease. The unique features of our model are the retention of noninvasive morphology in the background of invasive tumor with extensive desmoplastic stromal reaction, the relation of TβRII expression to lymph node metastasis and survival, and the similarity of gene expression profiles in Tgfbr2−/− murine lung tumors to human invasive lung adenocarcinoma. Although not tested yet, it is possible that using smaller amounts of Ad.Cre, would lead to the slower development of tumors and allow for the development of distant metastasis. Of human lung cancer, adenocarcinoma is the most common histology and the incidence is expected to increase with the anticipated increased implementation of lung cancer clinical screening by computed tomography. Screening scans typically detect adenocarcinomas with approximately 25% of tumors of noninvasive BAC/AIS morphology. Thus, the delineation of genomic and molecular alterations that capture the spectrum of noninvasive and invasive adenocarcinoma will have significant impact on this important clinical problem. The model of lung-specific Tgfbr2 loss in the oncogenic Kras mouse faithfully recapitulates
the morphology, genomics, and clinical progression of lung adenocarcinoma and promises to serve as an important tool for the evaluation of diagnostic and therapeutic advances in the management of human lung adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Progression of Human Bronchioloalveolar Carcinoma to Invasive Adenocarcinoma Is Modeled in a Transgenic Mouse Model of K-ras–Induced Lung Cancer by Loss of the TGF-β Type II Receptor

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