Lung Adenocarcinoma Global Profiling Identifies Type II Transforming Growth Factor-β Receptor as a Repressor of Invasiveness

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Rationale: **Lung adenocarcinoma histology and clinical outcome are heterogeneous and associated with tumor invasiveness.** *Objectives:* **We hypothesized that invasiveness is associated with a distinct molecular signature and that genes differentially expressed in tumor or adjacent stroma will identify cell surface signal transduction and matrix remodeling pathways associated with the acquisition of invasiveness in lung adenocarcinoma.** *Main Results:* **Microarray analysis of microdissected noninvasive bronchioloalveolar carcinoma (BAC) and invasive adenocarcinoma and adenocarcinoma-mixed type with BAC features identified transcriptional profiles of lung adenocarcinoma invasiveness. Among the signature set that was lower in adenocarcinoma-mixed compared with BAC was the type II transforming growth factor β (TGF-β) receptor, suggesting downregulation of *TGFβRII* is an early event in lung adenocarcinoma metastasis. Immunostaining in independently acquired specimens demonstrated a correlation between TβRII expression and length of tumor invasion. Repression of *TGFβRII* in lung cancer cells increased tumor cell invasiveness and activated p38 mitogen-activated protein kinases. Microarray analysis of invasive cells identified potential downstream mediators of *TGFβRII* with differential expression in lung adenocarcinomas.** *Conclusions:* **The repression of type II TGF-β receptor may act as a significant determinant of lung adenocarcinoma invasiveness, an early step in tumor progression toward metastasis.**

Keywords: adenocarcinoma; lung cancer; microarray analysis; neoplasm invasiveness; transforming growth factor-β

Lung cancer, the leading cause of cancer death in the United States, with 163,000 deaths expected in 2005, is also the leading cause of cancer death worldwide, with 1.1 million annual deaths (1). In contrast to other common neoplasms in breast, colon, and prostate, which are predominantly adenocarcinoma, only 40% of lung cancers are adenocarcinomas. Within the subtype of non–small cell lung carcinoma, all tumors are treated similarly without regard to biological heterogeneity, which may be associated with histologic subclassification. The poor outcome of lung cancer compared with other common cancers (15 vs. 62–97% average 5-year survival) is partly attributable to the current limited ability to distinguish fundamental differences in tumor biological predisposition to metastasis that may be associated with histologic heterogeneity.

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Lung cancer metastasis is frequent; approximately 40% of patients have distant metastases at the time of diagnosis. Furthermore, among patients who present with localized resectable disease, approximately 30% will develop metastases and succumb to their disease within 5 years. Lung cancer metastasis to lymphatics and visceral organ beds via the systemic circulation is the result of several well-characterized, sequentially acquired properties of tumor cells. These steps include the following: enzyme-mediated invasion of organ stroma, circulation in lymphatic or vascular channels, and extravasation and proliferation in distant organ beds (2). Using high-throughput genomic strategies, the molecular programs driving the tumor–stromal interactions that lead to metastases are becoming well characterized, with delineation of roles for transcription factors (3, 4), proteinases, such as matrix metalloproteinase-11 and cathepsin L2 (5), and chemokines, such as CXCL12 and CXCL14 (6).

We and other researchers have previously reported molecular signatures discriminative of non–small cell lung carcinoma differentiation and prognosis (7–10). Gene signatures of lung carcinoma prognosis often contain gene classifiers of metastasis in other tumor systems. A potential limitation of molecular signatures of prognosis derived from resected tumors is that associations of gene expression with survival may be confounded by tumor biological heterogeneity and non–tumor-related properties, such as patient performance status, comorbid disease, and non–cancer-related causes of death. To address this limitation, we have focused on identifying signatures of invasiveness, an intrinsic biological tumor attribute directly related to the clinically relevant endpoint of metastasis.

Within lung adenocarcinoma, histology is heterogeneous and associated with tissue invasion and clinical outcomes. The World Health Organization has subclassified adenocarcinoma on the basis of predominant cell morphology and growth pattern (11), such as bronchioloalveolar carcinoma (BAC), adenocarcinoma with mixed subtypes, and homogenously invasive tumors with a variety of histologic patterns. BAC cells are cuboidal to columnar, with or without mucin, and grow in a noninvasive fashion along alveolar walls. The histologic distinction between BAC and other adenocarcinoma subclassifications is tissue invasion, the first step of the metastasis process, in which epithelial cells lose cell–cell adhesion, gain motility, and invade adjacent stroma (2). Adenocarcinomas with mixed subtypes frequently contain regions of noninvasive tumor at the periphery of invasive tumor. Pure invasive adenocarcinomas are devoid of bronchioloalveolar morphology.

The spectrum of intratumoral histologic heterogeneity in adenocarcinoma suggests invasiveness represents a continuum of disease, from noninvasive BAC to adenocarcinoma-mixed subtype with BAC component to pure invasive adenocarcinoma. The molecular events essential to this transition in the lung are presently unknown. This study focuses on invasion in lung adenocarcinoma, a significant biological and morphologic characteristic of cancer. We hypothesize that adenocarcinoma invasiveness will

be distinguished by a unique molecular signature and that genes differentially expressed in tumor or in adjacent stroma will identify cell surface signal transduction and matrix remodeling pathways associated with the acquisition of invasiveness in lung adenocarcinomas. We used DNA microarray gene expression profiling to identify signatures of invasiveness. Among the genes in the acquisition of invasiveness classifiers was the type II transforming growth factor β (TGF-β) receptor (*TGFβRII*), which was expressed at lower levels in invasive tumors. We examined the role of TβRII, showing that repression is directly associated with increased invasiveness, and we identified potential effectors of TGF-β–mediated invasion in lung adenocarcinoma. Some of the results have been reported previously in the form of an abstract.

METHODS

DNA Microarray Analysis

Methods for RNA extraction, labeling, and hybridization for DNA microarray analysis of lung tumor specimens have been described previously (10). Some of the microarray data have been reported previously (12). Probe level analysis was performed using the Robust Multiarray Algorithm (13). Gene expression data were normalized to a baseline value obtained from nonmalignant lung tissues. The gene list was filtered to include only genes with a log ratio range greater than 1 and those present in at least two specimens. Thus, 2,194 genes remained for analysis. Hierarchic clustering was performed with Pearson correlation using GeneSpring version 7.0 (Silicon Genetics, Redwood City, CA). Specimen acquisition procedures were approved by the Columbia University Medical Center Institutional Review Board.

For the cell line data, RNA was extracted from H23 and SK-LU (obtained from American Type Culture Collection [ATCC]) snap-frozen cell pellets acquired 48 hours after administration of siRNA constructs in experiments performed in duplicate. cRNA were hybridized to the Affymetrix U133 Plus 2.0 array (Redwood City, CA). Probe level analysis was performed using Robust Multiarray Algorithm normalization without a baseline. The gene list was filtered to include 14,035 genes with a log ratio range greater than 1. To determine expression of U133 Plus 2.0 array genes in lung tumor specimens analyzed with U95Av2 arrays, homologous probes were identified using the following website source: http://www.affymetrix.com/analysis/index.affx.

Quantitative Real-Time Polymerase Chain Reaction in Tumors

Tumor RNA was isolated from frozen sections with the RNeasy Kit (Qiagen, Valencia, CA) and converted into cDNA using SuperScriptIII (Invitrogen, Basel, Switzerland).

Immunostaining

TβRII tumor immunostaining was performed in formalin-fixed paraffin-embedded sections (antibody source, R&D Systems, Minneapolis, MN: AF-241-NA). The antibody was diluted 1:50. Pituitary adenoma was used as a positive control. The staining of TβRII was recorded as negative (score 0), low (faint multifocal or diffuse staining, score 1), or high (strong multifocal or diffuse staining, score 2). All slides were reviewed blinded to the results of the analysis of greatest linear dimension of histologic invasiveness, which was performed before immunostaining.

For immunofluoresence, cells transfected with siRNA or negative control were grown on glass cover slips for 48 hours until fixation. Immunofluoresence was performed using primary antibody diluted 1:100 and visualized on the Nikon Eclipse E600 (Melville, NY) using Alexa Green conjugated antigoat antibody. Images were obtained through the SPOT analysis program (Diagnostic Instruments, Sterling Heights, MI).

RNA Interference

Predesigned annealed siRNA (sense 5′-GGUCGCUUUGCUGAGGUC Utt-3′, antisense 5′-AGACCUCAGCAAAGCGACCtt-3′) against human *TGFβRII* and control siRNA, which has no significant homology to any known gene sequences, were purchased from Ambion (Austin, TX; catalog no. 16704 and 4611). Cells were seeded in 6-well plates at a concentration of 200,000 cells/well for 24 hours before transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen) using 100 nM annealed siRNA as directed. Transfection efficiency was measured using the Silencer B-actin siRNA control system (catalog no. 4607; Ambion).

Transwell Migration

TGFβRII knock-down and control cells were harvested 48 hours after transfection and placed into serum-free media. A total of 50,000 cells were loaded into the top chamber of the BD Biocoat Matrigel Invasion Chamber (BD Biosciences, San Diego, CA), with fetal bovine serum 25% in the lower chamber, and were incubated for 22 hours. Noninvading cells adherent to the top surface were removed by scrubbing, and invasive cells were fixed and stained with Diff-Quik (Dade Behring, Deerfield, IL). Five representative fields (5×) were counted.

Western Analysis

Cells were treated with TGF-β (R&D Systems) 1 ng/ml after maintenance in serum-free media for 1 hour. Whole cell protein extracts from cells were prepared using radio immunoprecipitation assay (RIPA) buffer (0.15 mM NaCl/0.05 mM Tris HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate) and from tissues using TNT lysis buffer (20 mm Tris HCl, pH 8.0/150 mm NaCl/1% Triton X-100). Immunoblots were incubated with the indicated antibody and detected using a BM chemiluminescence kit (Roche). Intensity (densitometric units) was obtained using Image J v1.33 (http://rsb.info.nih.gov/ij/). Sources of antibody were as follows: Cell Signaling (Beverly, MA) (total p38, phospho-p38, phospho-Smad2, total Akt, phospho-Akt); BD Transduction Laboratories (San Diego, CA) (total-Smad2), Sigma (St. Louis, MO) (β-actin), and Santa Cruz (Santa Cruz, CA) (TβRII, no. 17792).

RESULTS

We examined gene expression signatures associated with invasiveness in lung adenocarcinoma represented by the subclasses: BAC (n = 5), invasive carcinoma (n = 10), and adenocarcinoma with both BAC and invasive components (adenocarcinoma-mixed, n = 10; Figure 1). The complete gene expression dataset of the microdissected lung adenocarcinoma specimens is available at http://hora.cpmc.columbia.edu/dept/pulmonary/5ResearchPages/Laboratories/Powell%20Lab.htm. Demographic attributes for the patients in the study are provided in Table 1. Unsupervised hierarchic clustering identified three subgroups of specimens that were associated with invasiveness. Specimens derived from invasive adenocarcinomas clustered separately from other tumors, and 13 of 15 BAC and adenocarcinoma-mixed segregated according to histologic subtype class (Figure 2). Two other tumors (A20 and B1) displayed a transcriptional profile distinct from other specimens with similar histologic subtype. Because morphologic characteristics or clinical parameters did not account for clustering of these two specimens, it is possible the gene signature was affected by tissue heterogeneity that persisted despite needle microdissection or by other tumor properties not macroscopically obvious. Overall, the dendrogram indicates that adenocarcinoma histology subclassification and invasiveness are associated with global differences in gene expression.

We performed supervised analysis using an F test within BRB array tools (developed by Richard Simon and Amy Peng Lam; http://linus.nci.nih.gov/BRB-ArrayTools.html) to identify genes associated with histologic subtype. A total of 319 genes were differentially expressed among the three subclasses ($p < 0.01$; Figure 3, *left panel*, and Table E1 in the online supplement). As expected, the gene expression signature of pure invasive tumors was distinct from signatures of other adenocarcinomas. Functional annotation of the invasive genes suggests that, when tumor cells acquire an invasive phenotype, they also acquire a predomi-

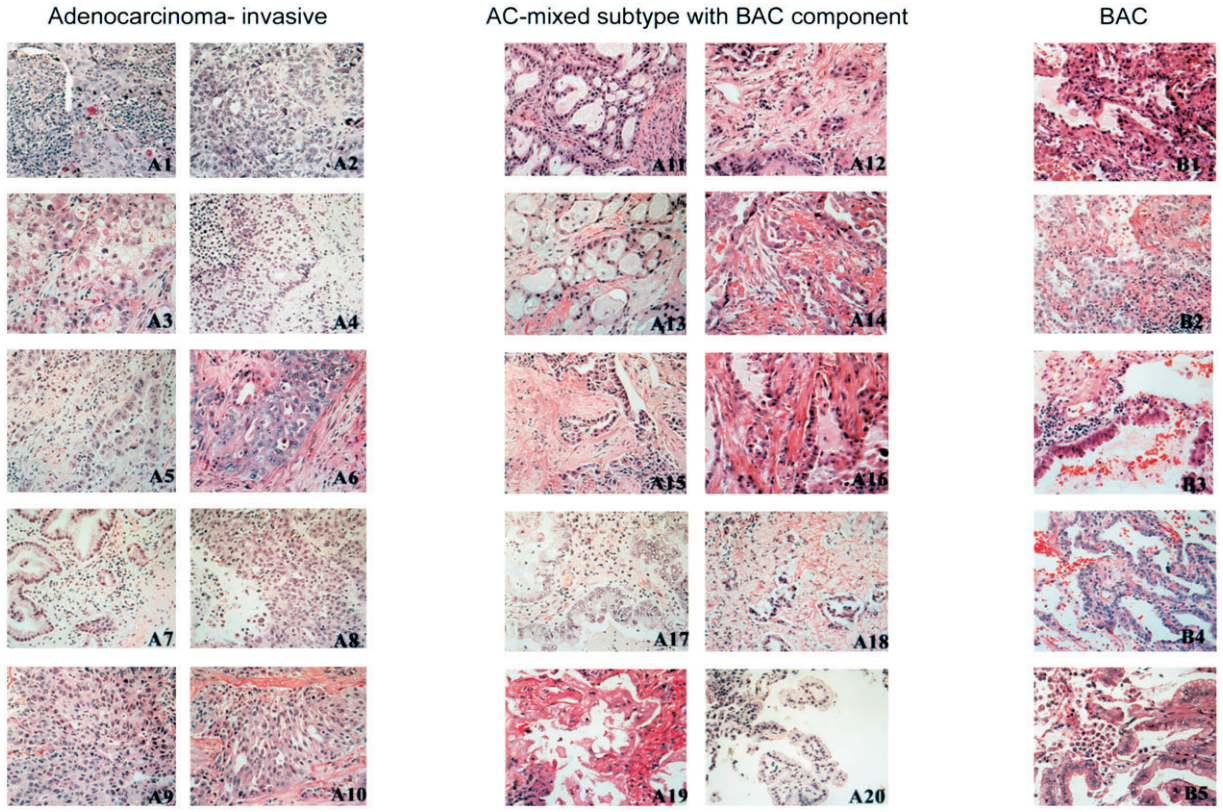


Figure 1. Lung adenocarcinoma histologic subtypes. Photomicrographs of representative permanent sections of regions microdissected from adenocarcinoma tumors for gene profiling. The histopathology patterns of adenocarcinoma correspond to three major groups, using World Health Organization classification. The tumors in the *left panel* are invasive adenocarcinomas without a bronchioloalveolar component. The tumors in the *center panel* are mixed subtype adenocarcinomas (AC), showing a bronchioloalveolar carcinoma (BAC) component that ranged from 25 to 80% of the entire tumor. The tumors in the *right panel* are BAC. Hematoxylin–eosin stain; original magnification, ×100.

TABLE 1. CHARACTERISTICS OF PATIENTS' ADENOCARCINOMA TUMORS

ID	Age	Sex	Histology	Size (*cm*)	Stage
20009	61	M	Invasive	3	T1N0
22003	59	F	Invasive	4	T2N0
21006	77	F	Invasive	1.5	T1N0
22005	68	M	Invasive	2	T1N0
99034	79	F	Invasive	4.5	T2N0
21012	68	F	Invasive	5	T2N0
99015	66	F	Invasive	2.6	T1N0
99035	70	F	Invasive	3.5	T2N1
21014	51	F	Invasive	1.3	T1N0
23005	55	M	Invasive	6	T3N1
20014	73	F	AC-mixed	5	T2N0
21002	39	F	AC-mixed	12	T2N1
20033	62	M	AC-mixed	3.5	T2N2
21001	63	M	AC-mixed	1.2	T1N0
21011	56	F	AC-mixed	1.5	T1N1
99043	56	F	AC-mixed	1.8	T1N0
22037	70	M	AC-mixed	3	T2N0
22051	80	F	AC-mixed	2.5	T2N0
22048	77	M	AC-mixed	3.5	T2N0
21013	67	M	AC-mixed	1.4	T1N0
21028	74	M	BAC	2	T1N0
22057	65	F	BAC	2.5	T1N0
22056	77	F	BAC	3	T1N0
22058	68	F	BAC	4	T2N0
22011	56	F	BAC	2.6	T1N0

Definition of abbreviations: AC = adenocarcinoma; BAC = bronchioloalveolar carcinoma; F = female; M = male.

nant gene expression signature corresponding to functions acquired by advanced malignant cells, such as cell cycle regulation, cell proliferation, and DNA replication and repair. The supervised and unsupervised clustering analyses indicate that gene expression profiles of BAC and pure invasive tumors are starkly distinct. The pattern of adenocarcinoma-mixed subtype gene expression suggests it may represent an intermediate phenotype in terms of gene expression and morphology.

We were interested in identifying signaling pathways that might induce the acquisition of invasiveness in adenocarcinoma-mixed type lung adenocarcinoma. We therefore directed our attention to the BAC and adenocarcinoma-mixed subtype tumors that were similar in terms of differentiation and morphology, with the exception of invasion. We used a *t* test within BRB array tools to identify the subset of 30 genes that were differentially expressed in BAC versus adenocarcinoma-mixed subtype tumors ($p < 0.01$). The genes with the most significant differential expression between the two tumor subtypes are as ranked in Table 2 and Figure 3 (*right panel*). Among the invasion signature genes are several transcription factors, enzymes, and signaling agonists and antagonists. Consistent with their role in tumorigenesis, SIX1 and CTSL2 were highly expressed in adenocarcinoma-mixed type tumors compared with BAC tumors. SIX1 is a homeodomain containing transcription factor important in the development of eye, muscle, and brain, and is implicated in the pathogenesis of cancer (14). SIX1 transcriptionally regulates cyclinA1-mediated tumor cell proliferation (15) and, more relevant to our focus, promotes tumor cell invasiveness (16). CTSL2 encodes cathepsin L, a lysosomal C1A family

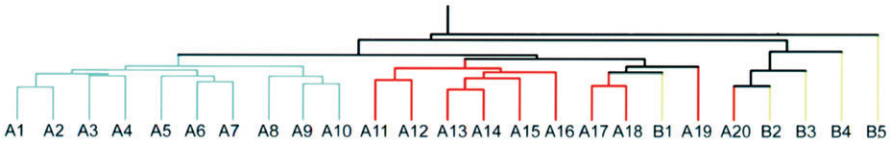


Figure 2. Hierarchical clustering of adenocarcinoma histologic subtypes. Lung adenocarcinoma tumor specimens representing BAC, pure invasive, and AC-mixed subtype were subjected to microarray analysis using the Affymetrix U95Av2 array. Unsupervised hierarchical clustering diagram indicates that invasive adenocarcinomas (*blue*) cluster separately from other subclassifications. Specimens are labeled to match order of panels displayed in Figure 1. The clustering procedures were performed using randomly assigned study identification numbers. With two exceptions, BAC tumors (*yellow*) cluster together separately from AC-mixed with BAC features (*red*).

cysteine proteinase that degrades intracellular and extracellular proteins. Cathepsin L mediates tumor cell invasion in a matrix metalloproteinase–independent fashion (17). We examined the list of probe sets lower in adenocarcinoma-mixed tumors compared with BAC to identify potential lung adenocarcinoma invasiveness suppressors. Among this list were two probes representing the tumor suppressor *TGF*β*RII*, which encodes a transmembrane receptor that modulates TGF-β signaling. Importantly, in colorectal carcinoma, loss of TGF-β responsiveness attributable to the mutation of *TGF*β*RII* is associated with acquisition of invasiveness in adenomatous polyps (18). Our results suggested that, analogous to its role in colorectal carcinoma, repression of TβRII in lung adenocarcinoma promotes the acquisition of invasiveness.

The microarray data were confirmed by examination of type II TGF-β receptor transcription and translation in lung adenocarcinoma. We examined *TGF*β*RII* expression with quantitative real-time polymerase chain reaction in tumor specimens and observed that expression measured by DNA microarray correlated (Pearson correlation coefficient $r = 0.59$, $p < .004$) with expression measured by polymerase chain reaction. Western analysis of tumor homogenates similarly confirmed the microarray results (Figure E1). To determine if this result was generalizable, we examined protein localization and expression in 55 independently acquired primary lung adenocarcinoma specimens. TβRII expression was detectable in nonmalignant bronchial epithelial cells and in noninvasive BAC cells (Figure 4, *top panel*). In contrast, expression was absent or diminished in 61 and 70% of the adenocarcinoma-mixed and invasive tumors, respectively (Table 3). To determine the correlation of protein expression with invasion, we examined the association of TβRII immunostaining with the measured maximal length of invasion in each tumor. The maximal invasion length was inversely correlated with immunostaining intensity, with a Spearman correlation coefficient $r = -0.36$, $p = 0.007$ (Figure 4, *bottom panel*). These results confirm the microarray data indicating that *TGF*β*RII* expression was lower in invasive adenocarcinomas compared with BAC tumors and was negatively correlated with tumor invasiveness.

We hypothesized repression of TβRII was directly associated with increased invasiveness in lung adenocarcinoma. To test this hypothesis, we examined tumor cell invasive mobility in two TGF-β–responsive lung cell lines, H23 and SK-LU, in which we knocked down expression of the type II TGF-β receptor with siRNA. Expression was reduced 70 to 80% after transfection with siTGFβRII, as detected quantitatively by real-time polymerase chain reaction and qualitatively by immunofluoresence (Figure E2). Tumor cell mobility and invasion were investigated using the transwell matrigel migration assay. We observed that repression of *TGF*β*RII* was associated with a three- to fourfold increase in the number of invasive cells ($p < 0.001$; Figure 5), indicating an inverse correlation of *TGF*β*RII* expression with invasiveness in lung adenocarcinoma. Interestingly, there was no difference in cell number between control and knock-down cells in uncoated control wells, suggesting TGF-β signaling via proliferation control pathways did not account for the observed differences.

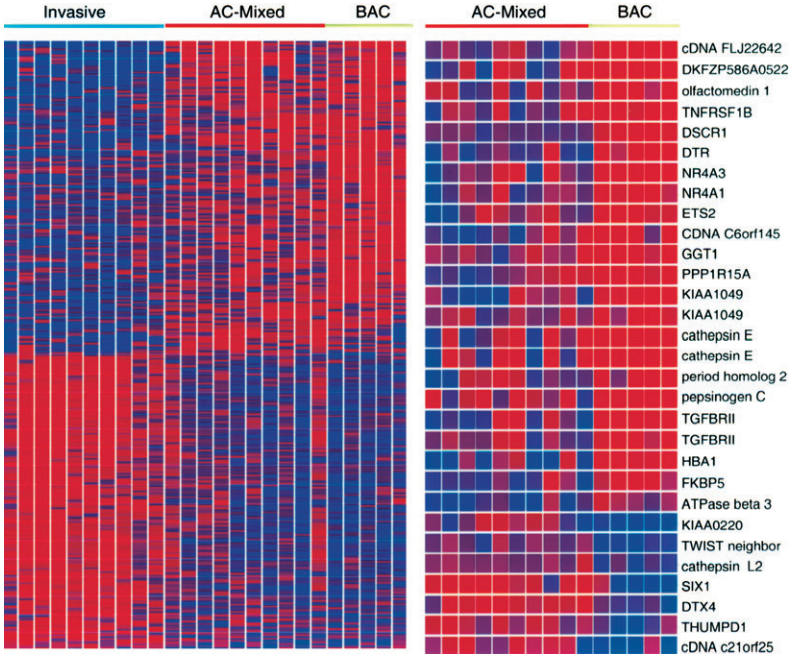


Figure 3. Supervised clustering of differentially expressed genes for the three subclasses of adenocarcinoma. Genes differentially expressed in three histologic subtypes (*left panel*) and in BAC versus AC-mixed (*right panel*) were identified using BRB-array tools, with $p < 0.01$. To determine that the class assignments were robust and that multiple testing was implicitly taken into account, class labels were randomly permuted 1,000 times and a permutation p value less than 0.01 was associated with each gene in the lists. The probability of obtaining at least 319 genes (*left*) and 30 genes (*right*) significant by chance (at the 0.01 level), if there were no real differences between the classes, was 0 and 0.002, respectively. Genes are on the *y axis* and tumors on the *x axis*. *Red* indicates high expression and *blue* indicates low expression, with the color scale at 0 to 5 relative to the experiment median.

TABLE 2. GENES DIFFERENTIALLY EXPRESSED IN BRONCHIOLOALVEOLAR CARCINOMA VERSUS ADENOCARCINOMA-MIXED SUBTYPE

Probe	Gene Description	GenBank	Gene Category	p Value
	Genes Highly Expressed in AC-Mixed Type Compared with BAC Tumors			
4095_at	PI-3-kinase–related SMG-1-like (KIAA0220)	AL049250	Unknown function	0.0003181
40004_at	Sine oculis homeobox homolog 1 (SIX1)	X91868	Transcription factor	0.0007989
35369_at	Deltex 4 homolog (DTX4)	AB023154	Unknown function	0.0018299
40617_at	THUMP domain containing 1	AC004381	Unknown function	0.0022976
35007_at	TWIST neighbor	AC004940	Unknown function	0.0025262
32107_at	cDNA c21orf25	AL050173	Unknown function	0.0029529
40717_at	Cathepsin L2	AB001928	Cysteine proteinase	0.0073467
	Genes Highly Expressed in BAC Compared with AC-Mixed Type Tumors			
34840_at	cDNA FLJ22642	AI700633	Unknown function	9.51E-05
32168_s_at	Down syndrome critical region gene 1	U85267	Calcineurin-mediated signaling regulation	0.0005758
41267_at	mRNA for KIAA1049	AB028972	Unknown function	0.0015624
280_g_at	NR4A1	L13740	Steroid-thyroid hormone-retinoid receptor superfamily	0.0018289
31525_s_at	Hemoglobin, α pseudogene 1	J00153	Unknown function	0.0020264
206_at	Cathepsin E (CTSE)	M84424	Aspartic proteinase	0.0024424
40659_at	NR4A3	U12767	Steroid-thyroid hormone-retinoid receptor superfamily	0.0032117
38037_at	Diphtheria toxin receptor (DTR)	M60278	EGF-like growth factor	0.0039134
34246_at	cDNA C6orf145	AA418437	Unknown function	0.0039684
1814_at	TGFBRII	D50683	Serine/threonine kinase receptor	0.0043177
37028_at	Protein phosphatase 1, regulatory subunit 15A	U83981	Serine threonine kinase/protein phosphatase	0.0044977
271_s_at	CTSE	J05036	Aspartic proteinase	0.0047981
35008_at	Period homolog 2 (PER2)	AB002345	Ciracadian regulator	0.004922
33699_at	Pepsinogen C (PGC)	M18667	Aspartic proteinase	0.0049418
1815_g_at	TGFBRII	D50683	Serine/threonine kinase receptor	0.0050199
34721_at	FKBP5	U42031	Peptidylprolyl isomerase	0.0052014
38717_at	DKFZP586A0522	AL050159	Methyltransferase	0.0061461
36134_at	Olfactomedin 1 (OLFM1)	U79299	Neuronal glycoprotein	0.0062414
1583_at	TNFRSF1B	M32315	TNF-receptor superfamily	0.0071696
32563_at	ATPase, β3	U51478	Na+/K+ and H+/K+ ATPase	0.0076784
32893_s_at	γ-glutamyltransferase 1 (GGT1)	M30474	Transferase protein	0.007909
1519_at	ETS2	J04102	Transcription factor	0.0095507
41268_g_at	mRNA for KIAA1049 protein	AB028972	Unknown function	0.0099509

Definition of abbreviations: AC = adenocarcinoma; BAC = bronchioloalveolar carcinoma; EGF = epidermal growth factor; TNF = tumor necrosis factor.

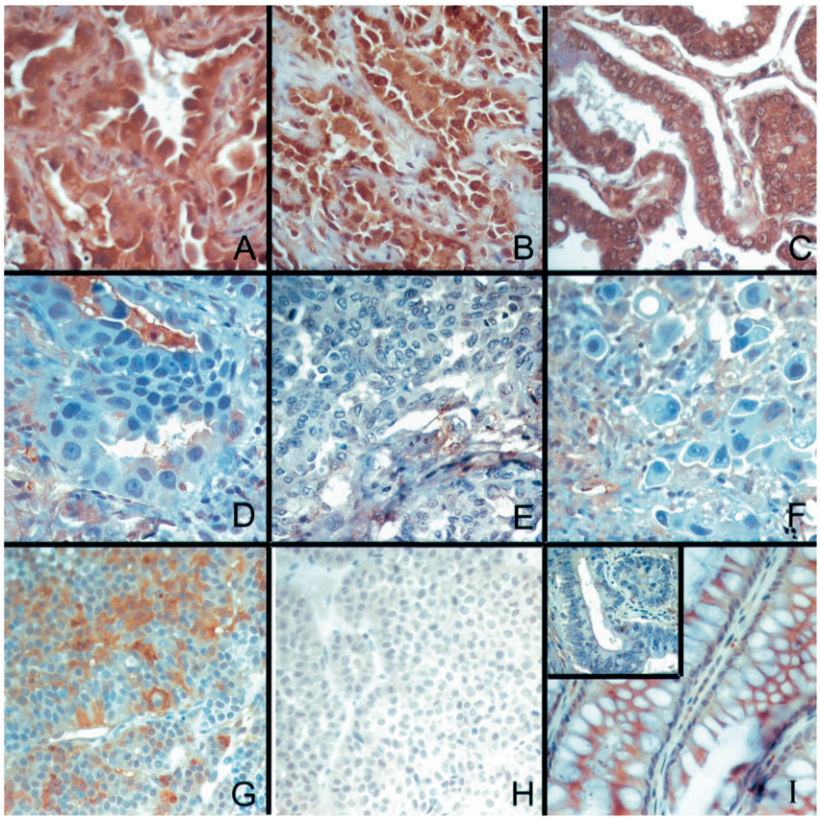
The response of lung tumor cells to TGF-β is complex, with signaling mediated through Smad proteins, Akt, and mitogen-activated protein kinases (MAPK). We examined the consequences of decreased *TGFβRII* expression on TGF-β signaling pathways (Figure 6). We observed that Smad2 activation was diminished after TGF-β stimulation in cells treated with *TGFβRII* siRNA, whereas total Smad2 was unchanged. Smad2 signaling pathways are involved in growth regulation, but no alteration in cell proliferation was observed in our cell lines. We next examined activation of other TGF-β signaling pathways associated with tumor cell mobility and invasion. We observed that Akt activation, similar to Smad2, was diminished in siTGFβRII cells. However, consistent with reports in breast carcinoma (19), activation of p38 MAPK was slightly increased relative to total p38 in siTGFβRII cells after stimulation with TGF-β, suggesting TGF-β–mediated p38 MAPK activation may play an important role mediating invasiveness in cells with repressed TβRII expression.

To identify potential mediators of an invasiveness transcriptional program downstream of type II TGF-β receptor, we examined gene expression profiles of knocked-down TβRII expression in H23 cells. Genes with greater than twofold differences in expression were identified using Significance Analysis of Microarrays version 1.2.1 (http://www-stat.stanford.edu/~tibs/SAM) (20), with a median false-discovery rate of 1.5% (Table E2). To identify those genes most likely to be important in mediating invasiveness *in vivo*, we restricted the gene list to those that were also differentially expressed in lung adenocarcinoma tissues, as identified by an F test in BRB array tools, $p < .01$ (Table 4). Two members of the Nur77 nuclear receptor subfamily 4, group A family of transcription factors (NR4A2, NR4A3) were repressed in knocked-down cells. This orphan receptor subfamily of steroid thyroid hormone receptors all bind to a Nur77-binding response element to activate target gene expression (21). Although it is unknown if these regulatory elements and signaling pathways are functional in this context, the classifiers represent transcriptional regulation steps that are potentially important in TGF-β–mediated invasiveness. Among the genes negatively correlated with *TGFβRII* expression in knocked-down cells were the chemokine CCL5 (RANTES [regulated upon activation, T-cell secreted and expressed]) and the lysyl oxidase enzyme family members LOX and LOXL2, all of which encode secreted proteins that are associated with tumor invasiveness (22, 23).

DISCUSSION

The spectrum of intrahistologic heterogeneity in adenocarcinoma tumors suggests invasiveness may represent a continuum of disease from noninvasive BAC, to adenocarcinoma-mixed subtype with BAC component, to pure invasive adenocarcinoma. This study examined lung adenocarcinoma specimens encompassing a spectrum of invasion and identified distinct molecular signatures of histologic subtype and of invasiveness. Because we are interested in studying the biology of tumor progression and metastasis in early clinical stage tumors, we focused our investigations on identifying gene signatures correlated with the acquisition of invasiveness using the theoretic transition of *in situ* BAC tumors to adenocarcinoma-mixed subtype as a model.

In contrast to genes in the pure invasive tumor subtype cluster, which predominantly represented functional categories of proliferation and DNA repair, genes differentially expressed in



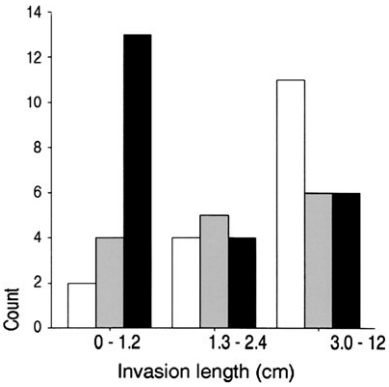


Figure 4. Top: TβRII immunostaining is decreased in invasive lung adenocarcinoma tumors. TβRII is expressed in BAC tumor cells (2+ immunoreactivity), localized to the cytoplasmic membrane and cytoplasm, with less intense staining detected in macrophages and endothelial cells (*A*–*C*); immunoreactivity is decreased or absent in well (*D*), moderately (*E*), and poorly (*F*) differentiated lung adenocarcinoma tumors. (*G*) Pituitary adenoma with 2+ immunoreactivity for TβRII (positive control). (*H*) Pituitary adenoma with isotype-matched, concentration-matched antibody showing no immunoreactivity (nonimmune antibody control). (*I*) Colonic mucosa showing 2+ immunoreactivity for TβRII, and *inset* showing invasive colonic adenocarcinoma from same section with no immunoreactivity for TβRII. Hematoxylin–eosin stain; original magnification, ×150. *Bottom*: TβRII immunostaining was highest in tumors with the least invasion. TβRII intensity: *white bars* = 0; *gray bars* = 1; *black bars* = 2. Slides of 55 independently acquired primary lung adenocarcinoma specimens were reviewed. The greatest linear dimension of histologic invasiveness was measured, which was subset into tertiles. The Spearman correlation coefficient of the relation between staining intensity and length of invasion was −0.36, p = 0.007.

the BAC and adenocarcinoma-mixed classes represented functional categories of signal transduction, regulation of cell adhesion, and proteolysis. Within this latter gene signature, repression of *TGFβRII* was identified as an important, reproducible molecular alteration in lung adenocarcinoma that was associated with invasiveness. TGF-β receptor, type II, is a transmembrane receptor serine threonine kinase that mediates TGF-β signaling (24). On TGF-β binding, TβRII phosphorylates the type I receptor, which then activates direct phosphorylation of downstream effector signaling molecules, such as Smad2 and Smad3. *TGFβRII* genetic alterations have been well characterized in gastrointestinal tumors in which 25% colorectal carcinomas have missense mutations associated with microsatellite instability. Animals with targeted deletion of *Tgfbr2* in the colonic epithelium demonstrate increased tumor progression from adenomas to invasive carcinomas (25), similar to human colorectal tumors with loss of TβRII (18). In breast carcinoma, mammary tumors in animals with targeted deletion of *Tgfbr2* demonstrated increased progression and metastases (26). A recent case-control study indicated that, within breast hyperplasia specimens, the proportion of cells with decreased TβRII immunostaining was associated with increased risk for the development of invasive breast cancer (27). In lung cancer, TβRII repression is detectable in approximately 40% of lung adenocarcinomas overall and in up to 100% of poorly differentiated adenocarcinomas (28), and is frequently caused by epigenetic silencing (29). To our knowledge, our report is the

TABLE 3. TβRII IMMUNOSTAINING IN LUNG ADENOCARCINOMA TUMORS

Staining Intensity	BAC	AC-Mixed	Invasive
High 2+	7 (78)	9 (39)	7 (30)
Low 1+/0	2 (22)	14 (61)	16 (70)

Definition of abbreviations: AC = adenocarcinoma; BAC = bronchioloalveolar carcinoma.

Percentages are shown in parentheses.

TABLE 4. TGFβRII-DEPENDENT GENES DIFFERENTIALLY EXPRESSED IN LUNG ADENOCARCINOMA SUBTYPES

Affymetrix Probe Set ID	GO Function/Gene Name	Gene Symbol	Fold Change*
	Adhesion		
201130_s_at	Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	0.23
205328_at	Claudin 10	CLDN10	2.94
204750_s_at	Desmocollin 2	DSC2	0.42
204751_x_at	Desmocollin 2	DSC2	0.42
202267_at	Laminin, γ2	LAMC2	0.38
203726_s_at	Laminin, α3	LAMA3	0.29
208083_s_at	Integrin, β6	ITGB6	0.13
208084_at	Integrin, β6	ITGB6	0.24
226535_at	Integrin, β6	ITGB6	0.42
215446_s_at	Lysyl oxidase	LOX	4.00
202998_s_at	Lysyl oxidase-like 2	LOXL2	2.70
214154_s_at	Plakophilin 2	PKP2	0.17
207717_s_at	Plakophilin 2	PKP2	0.42
	Transcription Factor		
204622_x_at	Nuclear receptor subfamily 4, group A, member 2	NR4A2	0.31
204621_s_at	Nuclear receptor subfamily 4, group A, member 2	NR4A2	0.37
216248_s_at	Nuclear receptor subfamily 4, group A, member 2	NR4A2	0.40
209959_at	Nuclear receptor subfamily 4, group A, member 3	NR4A3	0.42
209291_at	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	ID4	0.41
209292_at	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	ID4	0.28
202599_s_at	Nuclear receptor interacting protein 1	NRIP1	0.38
213139_at	Snail homolog 2 (*Drosophila*)	SNAI2	0.20
206261_at	Zinc finger protein 239	ZNF239	0.24
	Signal Transduction		
202609_at	Epidermal growth factor receptor pathway substrate 8	EPS8	0.36
204396_s_at	G protein–coupled receptor kinase 5	GRK5	0.43
202948_at	Interleukin-1 receptor, type I	IL1R1	0.46
202388_at	Regulator of G-protein signaling 2, 24 kD	RGS2	0.48
207334_s_at	Transforming growth factor, β receptor II (70/80 kD)	TGFBR2	0.34
208944_at	Transforming growth factor, β receptor II (70/80 kD)	TGFBR2	0.45
	Cell–Cell Signaling		
205290_s_at	Bone morphogenetic protein 2	BMP2	0.18
205289_at	Bone morphogenetic protein 2	BMP2	0.22
1405_i_at	Chemokine (C-C motif) ligand 5	CCL5	3.70
1555759_a_at	Chemokine (C-C motif) ligand 5	CCL5	2.94
	Cell Communication		
205490_x_at	Gap junction protein, β3, 31 kD (connexin 31)	GJB3	0.28
215243_s_at	Gap junction protein, β3, 31 kD (connexin 31)	GJB3	0.26
206156_at	Gap junction protein, β5 (connexin 31.1)	GJB5	0.33
	Immune Response		
202948_at	Interleukin-1 receptor, type I	IL1R1	0.46
212013_at	Melanoma-associated gene (IL1R antagonist)	D2S448	2.63
	Metabolism		
209608_s_at	Acetyl-coenzyme A acetyltransferase 2	ACAT2	0.38
202739_s_at	Phosphorylase kinase, β	PHKB	0.48
203217_s_at	Sialyltransferase 9	SIAT9	0.37
	RNA Processing		
207836_s_at	RNA binding protein with multiple splicing	RBPMS	0.48
	Transport		
217785_s_at	SNARE protein Ykt6	YKT6	0.45
	Unknown		
212830_at	Epidermal growth factor–like domain, multiple 5	EGFL5	0.48
212471_at	KIAA0241 protein	KIAA0241	0.33
213929_at	mRNA; cDNA DKFZp586F1223 (from clone DKFZp586F1223)		0.48

*Fold change = siTGFβ/control.

first to associate TβRII repression with invasiveness in lung carcinoma.

Depending on context, TGF-β signaling may alternatively function to suppress tumor growth or to promote tumor cell invasiveness and metastasis (30–33). The signaling pathways essential to the prometastatic phenotype of TGF-β are not well characterized, but recent evidence suggests Smad activation mediated by TGF-β signaling is not required and alternative, parallel pathways involving MAPK and phosphatidylinositol 3-kinase are operative (34). Bhowmick and colleagues (19) demonstrated p38 MAPK was required for TGF-β–mediated epithelial mesenchymal transition and progression in mammary tumor cells

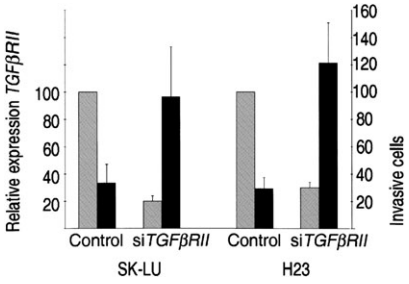


Figure 5. Repression of *TGFβRII* increases lung tumor invasiveness. *TGFβRII* expression, as measured by quantitative real-time polymerase chain reaction (*gray bars*), was knocked down in SK-LU and H23 cells. The transwell matrigel migration assay was used to measure invasive cells after 48 hours of transfection. The number of invasive cells (average of five [5×] fields ± SEM) at 22 hours is indicated (*black bars*). There was no difference in the number of migrating cells through the control insert between the knocked-down and control cells. The results are representative of experiments performed in triplicate. *TGFβRII* = *gray bars*.

expressing a dominant negative TGF-β type II receptor. These observations are in line with our results suggesting that p38 MAPK activation is required for TGF-β–mediated invasiveness in lung cancer cells with decreased TGF-β responsiveness. The importance of context, in terms of cell type and extent of TβRII repression, is demonstrated by other reports showing that, in advanced breast carcinoma cells, total blockade of TGF-β signaling reduces invasion and metastasis (35).

To more completely characterize the context of our observations in lung cancer cells, we investigated potential downstream transcriptional events associated with TβRII repression by examination of the transcriptional profile of knocked-down cells. These profiles indicate that the transcriptional activators NR4A2 and NR4A3, and E-cadherin expression were lower in knocked-down cells and in invasive lung adenocarcinoma tissues. The latter is consistent with prior reports demonstrating that TGF-β can physically interact with members of the Wnt/β-catenin pathway and lead to downregulation of E-cadherin (36). Among genes negatively correlated with TGFβRII expression, we noted increased expression of chemokine CC subfamily member CCL5, which encodes RANTES. RANTES may promote tumor cell migration via autocrine and/or paracrine effects. RANTES secretion by breast carcinoma cells is associated with disease progression (37, 38) and with enhanced tumor migration mediated via paracrine actions on monocytes and via autocrine effects

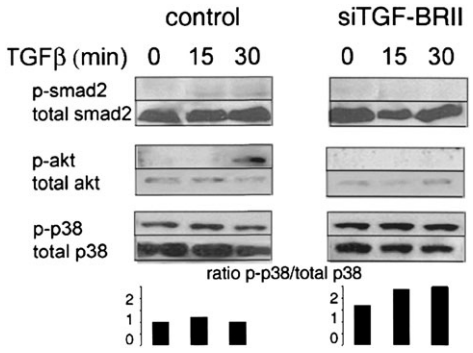


Figure 6. Western analysis of TGF-β signaling pathway activation. H23 cell cultures transfected with control (*left*) or siRNA (*right*) constructs were incubated with TGF-β for the indicated times. Protein extracts were subjected to Western blot analysis with the indicated antibodies. The ratio of the intensity (densitometric units) of activated p38 relative to total p38 is indicated in the *bottom panel*.

on CCR5 receptor–bearing cells (22). Recent reports indicate that CCL5 expression in the lung is regulated in part by p38 MAPK (39); because H23 lung cells express CCR5 (data not shown), it is possible that an autocrine pathway of RANTES-associated tumor cell invasion mediated by TGF-β signaling is operative in our *in vitro* system. Although our results suggest RANTES may play a role in the promotion of invasiveness and metastasis, a recent report noted that RANTES expression was associated with longer survival in lung adenocarcinomas with active lymphocytic response (40). Clarification of the role of RANTES in mediating *in vivo* lung tumor invasion and disease progression will require further study.

The identification of molecular pathways associated with the biological process of invasiveness acquisition in lung adenocarcinoma has potentially important clinical implications. The prevalence of noninvasive lung adenocarcinoma, usually represented by ground glass opacities on chest imaging studies is increasing, presumably as a result of increased detection by screening (41). Clinical studies indicate that the prognosis of noninvasive BAC is favorable and suggest that treatment approaches in terms of surgical procedure and adjuvant chemotherapy may be tailored to biological properties of these tumors (42). More recent evidence suggests ascertainment and application of biologically and clinically important molecular programs, such as the invasiveness signature, into clinical decision making may further enhance the effective allocation of treatment (43) for patients with lung carcinoma.

In summary, we have identified a transcriptional profile that distinguishes invasive from noninvasive lung adenocarcinoma, includes reduced expression of a previously identified tumor suppressor, *TGFβRII*, and suggests that downregulation is an early step in adenocarcinoma metastasis. Potential mediators of invasion resulting from suppression of *TGFβRII* in tumors were identified and include several transcriptional factors and E-cadherin, which were lower in *TGFβRII* knock-down cells and in primary invasive tumor specimens. The clinical implementation of these invasiveness signatures, once refined and tested, has the potential to improve lung carcinoma diagnostics and therapeutics.

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