Effects of Cancer-Associated EPHA3 Mutations on Lung Cancer

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Background Cancer genome sequencing efforts recently identified EPHA3, which encodes the EPHA3 receptor tyrosine kinase, as one of the most frequently mutated genes in lung cancer. Although receptor tyrosine kinase mutations often drive oncogenic conversion and tumorigenesis, the oncogenic potential of the EPHA3 mutations in lung cancer remains unknown.

Methods We used immunoprecipitation, western blotting, and kinase assays to determine the activity and signaling of mutant EPHA3 receptors. A mutation-associated gene signature was generated from one large dataset, mapped to another training dataset with survival information, and tested in a third independent dataset. EPHA3 expression levels were determined by quantitative reverse transcription-polymerase chain reaction in paired normal-tumor clinical specimens and by immunohistochemistry in human lung cancer tissue microarrays. We assessed tumor growth in vivo using A549 and H1299 human lung carcinoma cell xenografts in mice (n = 7–8 mice per group). Tumor cell proliferation was measured by bromodeoxyuridine incorporation and apoptosis by multiple assays. All P values are from two-sided tests.

Results At least two cancer-associated EPHA3 somatic mutations functioned as dominant inhibitors of the normal (wild type) EPHA3 protein. An EPHA3 mutation–associated gene signature that was associated with poor patient survival was identified. Moreover, EPHA3 gene copy numbers and/or expression levels were decreased in tumors from large cohorts of patients with lung cancer (eg, the gene was deleted in 157 of 371 [42%] primary lung adenocarcinomas). Reexpression of wild-type EPHA3 in human lung cancer lines increased apoptosis by suppression of AKT activation in vitro and inhibited the growth of tumor xenografts (eg, for H1299 cells, mean tumor volume with wild-type EPHA3 = 437.4 mm³ vs control = 774.7 mm³, P < .001). Tumor-suppressive effects of wild-type EPHA3 could be overridden in trans by dominant negative EPHA3 somatic mutations discovered in patients with lung cancer.

Conclusion Cancer-associated EPHA3 mutations attenuate the tumor-suppressive effects of normal EPHA3 in lung cancer.

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Cancer initiation and malignant progression are multistep processes that involve loss of growth control, evasion of apoptosis, sustained angiogenesis, tissue invasion, and metastasis (1). Developing cancer cells stochastically acquire and selectively accumulate mutations in the genes that encode oncoproteins, tumor suppressors, and their regulators. Receptor tyrosine kinases (RTKs) are important regulators of signal transduction pathways that promote cell growth, survival, invasion, and motility during malignant progression of solid tumors (2). Dysregulation of RTKs, such as EGF receptor family members, by mutation, amplification, or overexpression, can result in increased kinase activity and ultimately oncogenic transformation. The generality of this paradigm of gain-of-function RTK signaling in cancer has been recently challenged by the discovery of the dual roles of the EPH receptors in both promoting and inhibiting oncogenesis and tumor progression in cell lines and mouse models. To date, there has been insufficient evidence from patient outcome-based studies to verify the findings from mechanistic analyses in tumor models.

EPH receptors and their membrane-bound ligands, the ephrins, were originally discovered in the 1990s as axonal guidance molecules, and since that time, the EPH proteins have been found to constitute the largest family of RTKs and to be key regulators of cell–cell communication both in development and disease (3,4). The role of EPH receptors in cancer models is complex; they can either promote or inhibit malignancy, depending on ligand stimulation, signaling cross-talk, and other contextual factors (5–8). For example, EPHA2 overexpression is associated with worsened survival in human breast, prostate, and lung cancers and in glioblastoma multiforme (9–17). Overexpression of EPHA2 can induce ligand-independent signaling, resulting in increased tumor cell malignancy in vitro and accelerated tumor growth and metastasis in vivo (18,19). In keeping with these findings, reduced
EPHA2 expression in the presence of short interfering RNA or targeted gene deletion inhibited tumor initiation and metastatic progression (19–21). However, ligand-dependent signaling by EPHA2 in both breast cancer and glioblastoma cell lines inhibited their malignant behavior in vitro and tumor growth in vivo (21,22).

The conundrum posed by these findings is not completely resolved, and mechanisms that account for these opposing activities are just beginning to be investigated.

Recently, “next-generation” DNA sequencing using large cohorts of human lung cancer samples identified various mutations in EPH receptor genes. Notably, somatic mutations in EPHA3, the gene for EPHA3, were present in 5% to 10% of lung adenocarcinomas (23–26). However, those mutations are associated with amino acid substitutions scattered throughout the receptor, and it is unclear whether these nonrecurrent mutations are “driver” or biologically neutral “passenger” genetic alterations. In addition, because EPH receptor signaling can cause either tumor promotion or tumor suppression, the biological impact of somatically mutated variants of EPHA3 in lung cancer remains unclear.

In this report, we sought to characterize the functional effects of EPHA3 mutations identified in primary tumors to distinguish between the oncogenic and tumor-suppressive roles of the protein that EPHA3 encodes. We used a combination of genomic and mutational analyses in cell lines and tumor specimens to investigate the role of EPHA3 and its somatic mutations in non–small cell lung cancer (NSCLC). We performed cell proliferation and apoptosis assays in NSCLC cell lines and used mouse xenograft models to assess the function of EPHA3 in vivo. Finally, we identified molecular mechanisms by which EPHA3 regulates tumorigenicity.

Materials and Methods

Cell Lines and Constructs

The human NSCLC lines H1299, A549, and H1975 were provided by the Specialized Programs of Research Excellence in lung cancer at Vanderbilt-Ingram Cancer Center. 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). H1299, A549, and H1975 cells were maintained in RPMI-1640 medium supplemented with l-glutamine (2 mM), penicillin (100 U/mL), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). 293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with l-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Invitrogen). Authenticity of the cells was verified by DNA profiling, flow cytometry, or immunohistochemistry.

EPHA3 cDNA was obtained from Open Biosystems (Lafayette, CO) and cloned into the pcDNA3.1/myc-His vector (Invitrogen). EPHA3 mutations were generated by the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by sequencing. Selection of mutants (T166N, G187R, S449F, T660K, D678E, R728L, K761N, and G766E) was based on somatic mutations discovered in human lung adenocarcinoma (23–26). Three additional mutations discovered in colorectal cancer (D806N, T37K, and N85S) were also included for comparison (30,31). The Myc, HA, or FLAG-tagged versions of EPHA3 were created by inserting oligonucleotides encoding epitope tags in the pcDNA3.1 vector (oligonucleotides for 2× HA: forward, 5′-TACCCATACG ATGTTCCTGTA CGTGCGGCC TATCTGGATG AAGTCGCCC GCGTA-3′; reverse, 5′-TGATAGTCC GGGAGTCTAGCGGTA CGCATATGCA GGAACCCTCGT ATGGGTA-3′; oligonucleotides for 3× FLAG: forward, 5′-GATTACAGG ATGACGACGA TAAAGTACG CTGACAGGA TTACAAAGAC GATGACAGF ATAAGGACTAT AAAGG-3′; reverse, 5′-CTTATCCTCA TGCTTTTGT GTATGCC GTCCCTCTCT ATCTGTCAGT TACCTGTGAA TCT-3′.) The expression of WT or mutant EPHA3 was verified by western blot and/or FACS analysis. Because NSCLC lines are difficult to transfet, WT EPHA3 or T166N, G187R, S449F, or G766E mutant EPHA3 were subcloned into the retroviral vector pBabe and introduced into H1299, A549, or H1795 lines via retroviral transduction.

Antibodies and Reagents

Antibodies against the following proteins were used: EPHA2 (D7, mouse monoclonal, 1:1000, Millipore, Billerica, MA); EPHA3 (rabbit polyclonal, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, for western blotting; mouse monoclonal, 1:50, Sigma-Aldrich, St. Louis, MO, for immunohistochemistry; mouse monoclonal, 1:500, Millipore Corporation, for immunoprecipitation); S6 ribosomal protein, pSer-235/236 S6, ERK, pThr-202/Ser-204 ERK, pSer-473 AKT, pThr-308 AKT, pThr-32 FOXO3A, pThr-1462 TSC2, TSC2, pSer-9 GSK3β, GSK3β, and cleaved caspase-3 (all rabbit monoclonal, 1:1000, Cell Signaling Technology, Danvers, MA); AKT (mouse monoclonal, 1:1000, Cell Signaling Technology); pThr-32 FOXO3A (rabbit polyclonal, 1:1000, Cell Signaling Technology); β-tubulin (mouse monoclonal, 1:500, Sigma-Aldrich); phosphorytosyne pY20 and pY99 (mouse monoclonal, 1:500, Santa Cruz Biotechnology); HA (mouse monoclonal, 1:5000, Sigma-Aldrich); Myc (mouse monoclonal, 1:1000, Cell Signaling Technology); and FLAG (mouse monoclonal, 1:5000, Sigma-Aldrich).

cDNA arrays containing paired normal lung tissues and NSCLC tumor samples or different stages of tumor specimens were purchased from Origene Inc (Rockville, MD). Tumor staging was based on American Joint Committee on Cancer Staging Manual (http://www.cancerstaging.org). NSCLC cell lines and paired lung tissue and tumor sections were provided by the Specialized Programs of Research Excellence in lung cancer at Vanderbilt University, Nashville, TN. For cell lines, authentication included testing for mycoplasma, confirmation of species identity by DNA profiling, and immunohistochemistry. Avidin peroxidase reagents were from Vector Laboratories (Burlingame, CA), and the liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit was from Zymed Laboratories/Life Technologies (Grand Island, NY). TO-PRO-3 iodide nuclear stain was purchased from Life Technologies/Invitrogen (Carlsbad, CA). The EPHA3 TaqMan gene expression assay was from Life Technologies/Applied Biosystems (Carlsbad, CA).
**Immunoprecipitations, Western Blots, and Kinase Assays**

HEK293 cells (500 000 per six-well plate) were transfected with 5 μg of plasmids encoding epitope-tagged wild-type (WT), mutant EPHA3, or a mixture of WT and mutant receptors using Lipofectamine 2000 transfection reagent (Invitrogen). Immunoprecipitation and immunoblotting were performed as described previously (27). Briefly, cells were lysed in Nonidet P-40 buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% Nonidet P-40) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Cell lysates were incubated on ice for 10 minutes and clarified by centrifugation at 15 000g for 10 minutes at 4°C. Protein concentrations in the supernatants were measured by the DC (detergent compatible) protein assay (Bio-Rad Laboratories, Hercules, CA). Epitope-tagged wild-type or mutant EPHA3 receptors were precipitated from 500 μL cell lysates (approximately 0.5 mg total protein) with 1 μg of anti-Myc, anti-HA, anti-FLAG, or anti-EPHA3 antibodies. The precipitated proteins were resolved by 8% sodium dodecylsulfate–polyacrylamide gel electrophoresis and subjected to western blot analyses. For kinase assays, EPHA3 receptors were immunoprecipitated as above and resuspended in 25 μL kinase buffer (20 mM HEPES, pH 7.6, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 mM MgCl2, 50 mM NaCl, 1 mM EDTA, 200 μM ATP, and 20 μCi γ-32P ATP), incubated at 30°C for 30 minutes, resolved on a 8% sodium dodecylsulfate–polyacrylamide gel, and transferred to a nylon membrane for autoradiography, followed by western blot analysis for total EPHA3. Quantification of phosphotyrosine levels and kinase activity were performed by the Odyssey Infrared Image System (LI-COR Biosciences, Lincoln, NE) and Image J, respectively.

**Analyses of an EPHA3 Mutation–Associated Gene Signature**

A linear model for microarray data (limma package in Bioconductor, Seattle, WA; http://www.bioconductor.org/) was used to select genes that were differentially expressed between 39 samples with wild-type EPHA3 vs three samples with EPHA3 mutations in the Ding dataset from the Gene Expression Omnibus (GEO ID: GSE12667) (26), based on the following criteria: 1) the signal was increased or decreased at least twofold in the samples containing EPHA3 mutations compared with those containing wild-type EPHA3, 2) the false discovery rate (FDR)-controlled P value of the moderated t test was less than .05, and 3) the log-posterior odds ratio of differential expression was greater than 1.

The selected genes were mapped to an independent training set of 104 lung adenocarcinoma samples with survival information (28). For each of the 32 genes, we estimated the coefficient using Cox proportional hazard models. The assumptions of proportionality for the Cox models were tested using Grambsch and Therneau’s method. Overall, the expression of genes with positive coefficients tended to be increased in samples with EPHA3 mutations in the Ding dataset, whereas the expression of genes with negative coefficients was more likely to be decreased. Based on this observation, the 32-gene signature was then refined by directional concordance examination comparing the coefficient positivity of each gene with its increased or decreased expression status in the Ding dataset, which resulted in a 27-gene EPHA3 mutation signature (Supplementary Table 1, available online).

A second independent dataset of 129 lung adenocarcinomas (29) was used to test whether the EPHA3 mutation–associated gene signature was associated with patient survival. A risk score for each patient was derived from the sum of multiplication of log, expression level of each gene by its corresponding coefficient generated in the training set. The patients were dichotomized into a high-risk and a low-risk group, using the median risk score as the threshold value. The log-rank test with Kaplan–Meier curves was used to test the differences in survival between the high-risk group and the low-risk group. Multivariable Cox proportional-hazards regression analysis was used to evaluate independent prognostic factors associated with survival, and the EPHA3 mutation–associated signature, sex, age, smoking status, and tumor stages were used as covariates. A P value of less than .05 was considered statistically significant, and all tests were two-tailed. Only the EPHA3 mutation–associated gene signature (P = .014) and tumor stages (P = .0496) were statistically significant determinants of survival in the multivariable analysis.

To rule out any potential overfitting of the identified mutation gene signature in predicting survival, the “bootstrapping” analysis was performed by randomly choosing 27 genes in the training set and conducting the compound risk score to fit the Cox model in the test set. This procedure was repeated 5000 times, generating 5000 resampling P values. Only 0.68% of resampling P values were smaller than the P value of EPHA3 mutation–associated 27-gene set, demonstrating that the EPHA3 mutation–associated gene signature performed statistically significantly better than randomly selected genes in predicting survival of patients with lung cancer (P = .002, Wald test).

**Immunohistochemistry**

For immunohistochemistry of human specimens, nine lung tumor samples that were paired with adjacent normal lung tissue and two tissue microarrays containing 104 NSCLC tumor samples and 26 normal lung tissues were provided by the Vanderbilt Specialized Program of Research Excellence in Lung Cancer. Immunohistochemistry was performed under antigen retrieval conditions in 2 mM citric acid, 10 mM sodium citrate buffer, pH 6.0, using the PickCell 2100 Retriever (PickCell Laboratories, Amsterdam, the Netherlands). Endogenous peroxidase was blocked by 3% H2O2 for 30 minutes, and the tumor sections were stained with mouse monoclonal antibodies against human EPHA3 (Sigma-Aldrich). Stained tumor and normal sections were scanned and analyzed using the Ariol SL-50 platform (Molecular Devices, Sunnyvale, CA).

**Tumor Studies**

To investigate the effects of wild-type and mutant EPHA3 on tumor growth in vivo, we used lung cancer xenograft models. Ten-week-old female nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). H1299 or A549 NSCLC tumor cells expressing wild type of EPHA3 were resuspended in
nude mouse (5 × 10⁶ cells per mouse), whereas tumor cells carry-

H1975, or H1299 lung cancer cells (5000) in 96-well plates were
grown. Briefly, A549, enzyme-linked immunosorbent assay (ELISA) kit (Roche)
to determine the extent of apoptosis, histone-associated DNA

Apoptosis Assays
To determine the extent of apoptosis, histone-associated DNA
fragments were quantified using the Cell Death Detection
enzyme-linked immunosorbent assay (ELISA) kit (Roche)
according to the manufacturer’s instructions. Briefly, A549,
H11975, or H1299 lung cancer cells (5000) in 96-well plates were
starved for 5 days or treated with GST-TRAIL (5 µg/mL) for 24 hours. Cells were lysed, and histone-complexed DNA fragments from the apoptotic cells were simultaneously bound by anti-histone (biotin-labeled) antibodies and anti-DNA (peroxidase-conjugated) antibodies. The histone–DNA–antibody complexes were transferred to streptavidin-coated microplates and detected by addition of peroxidase substrate ABTS (2,2’-azino-di-[3-ethylbenzthiazoline sul fonate]). The absorbance was measured on a microplate reader (Bio-Tek, Winooski, VT) at a wavelength of 405 nm.

Statistical Analysis
For analyses with multiple comparisons, analysis of variance (ANOVA) or Kruskal–Wallis tests were used to determine if there were differences among the groups. If a statistically significant effect was found, pairwise differences were tested with a Bonferroni multiple comparisons adjustment. The overall type I error constraint was set to 0.05. Bonferroni correction was applied to tests at the statistical significance level of .05/n, where n is the number of tests. Only P values less than .05/n were considered to be statistically significant. For comparisons between two groups, Student t tests or Mann–Whitney tests were used. χ² tests were used for categorical outcomes. In the analysis of high-dimensional microarray data, false discovery rate (FDR)-controlled P values were reported. All tests of statistical significance were two-sided, and the exact tests used for each experiment were listed in the text and the figure legends.

Results
Somatic Mutations in EPHA3, Receptor Activity, and
Prognosis of Patients
Although EPHA3 mutations are commonly present in NSCLC (23–26), their role in malignancy is not clear. Eleven cancer-associated mutations in the EPHA3 gene, including eight mutations in the extracellular domain and three mutations in the kinase domain that were identified in a screen of 188 lung cancer specimens (26) and additional EPHA3 mutations that were identified by other studies (23–26), are distributed throughout the EPHA3 receptor (Figure 1, A). To investigate whether these mutations affect ligand binding or kinase activity, we generated five cDNA constructs in which EPHA3 was mutated within the coding region for the kinase domain (T660K, D678E, R728L, K761N, and G766E) and seven constructs in which it was mutated in the coding region for the extracellular domain (T166N, G187R, W250R, N379K, T393K, A435S, and S449F). Three mutations (D806N, T37K, and N85S) that had been identified in colorectal cancer specimens (30,31) were also included for comparison.

Wild-type or mutant EPHA3 expression constructs were transfected into HEK293T cells, and receptor activity was measured by tyrosine phosphorylation levels and kinase assays. Activation of EPH receptors results in phosphorylation of cytoplasmic tyrosine residues [reviewed in (3,32)]. Like other RTKs, EPHA3 is constitutively tyrosine phosphorylated when the receptor is overexpressed in 293T cells by transient transfection. When mutant EPHA3 receptors were expressed in HEK293T cells, three mutations in the kinase domain, R728L, G766E, and D806N, markedly diminished tyrosine phosphorylation of the EPHA3 receptor (Figure 1, B). Other EPHA3 kinase domain mutants, T660K, D678E, and K761N, also appeared to be less phosphorylated than the wild-type receptor to a varying degree (Figure 1, B).

In principle, levels of EPHA3 tyrosine phosphorylation could be influenced by autophosphorylation of the activated receptor, phosphorylation by other cellular kinases, and dephosphorylation by phosphatases. To directly determine the effect of each EPHA3 mutation on receptor kinase activity, we performed in vitro kinase assays using immunoprecipitated EPHA3 protein (Figure 1, C). Consistent with their low levels of tyrosine phosphorylation, kinase activity appeared to be nearly absent in EPHA3 mutants R728L, G766E, and D806N. The other kinase mutants, T660K, D678E, and K761N, largely retained the ability to autophosphorylate. Most EPHA3 kinase domain mutants that we tested inhibited receptor tyrosine phosphorylation and/or kinase activity.

Among extracellular domain mutants, the G187R, W250R, and possibly S449F EPHA3 receptors exhibited less tyrosine phos-

Figure 1. Cancer-associated EPHA3 mutants, receptor kinase activity, and patient prognosis. A) Diagram showing locations of EPHA3 mutations relative to structural domains of the full length protein. Nine cancer-associated mutations mapped to the extracellular domain of EPHA3 and six mutations mapped to the intracellular kinase domain. B) Tyrosine phosphorylation of mutant EPHA3 receptors. Myc-tagged wild-type (WT) or mutant EPHA3 constructs were transfected into 293T cells. EPHA3 proteins were immunoprecipitated with anti-Myc antibodies, run on sodium...
(Figure 1, B and C). In principle, defects of extracellular domain mutants could be because of impaired cell-surface expression, loss of ligand binding, or more subtle structural changes within the receptor. In flow cytometry analyses, the ephrin-binding domain mutant, G187R, and the sushi domain mutant, W250R, showed severely impaired binding to ephrin-A5, whereas other mutants were expressed well on the cell surface and were recognized by ephrin-A5 (Supplementary Figure 1, available online). We concluded that overt loss of RTK function was a common attribute of EPHA3 receptors encoded with cancer-associated EPHA3 mutations. Although we can neither exclude nor prove the possibility that more subtle aspects of EPHA3 function might be altered in other EPHA3 receptors that are mutated in cancer, our finding suggests that lung cancer might select for attenuation of EPHA3 function.

To determine the impact of EPHA3 somatic mutations on human lung cancer biology, we analyzed genes from the dataset of Ding et al. (26) that were differentially expressed in human lung adenocarcinoma samples that contained wild-type vs mutant EPHA3 receptors and, as detailed in “Methods,” mapped them to an independent training set of 104 lung adenocarcinoma samples from patients for which survival information was available (28). This draft signature was then tested using a second independent dataset of 129 lung adenocarcinoma samples (29) to rule out potential problems with overfitting the identified gene expression signature in predicting survival. These analyses showed that the EPHA3 mutation–associated gene signature (Supplementary Table 1, available online) was highly statistically significant as an independent prognostic marker of survival (median survival time for low-risk [mutation-signature negative] vs high-risk [mutation-signature positive] group: 33 months vs not applicable [survival never reached 50%]; hazard ratio = 6.442, 95% CI = 2.196 to 18.9. P < .001; Wald test) (Figure 1, D) and demonstrated that EPHA3 mutations were clinically relevant to human lung cancer.

Expression of EPHA3 in Human NSCLC

The identification of loss-of-function mutations in EPHA3 in human NSCLC, together with the patient survival analysis, suggested that wild-type EPHA3 might inhibit lung cancer formation or progression. If wild-type EPHA3 were to act as a tumor suppressor, the tumorigenic process might generate or select for loss-of-function mutations, for decreased gene copy number, or for decreased levels of EPHA3 expression in tumors. If this model were correct, EPHA3 gene copy number or expression levels would often be altered in tumors. To investigate this possibility, first we analyzed gene copy number of EPHA3 in nine lung cancer cell lines from an NCI-60 human tumor cell line panel (33). The copy number analysis revealed that the region in chromosome 3p11 covering the EPHA3 gene was deleted in seven of the nine (78%) NSCLC cell lines (EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H222M, NCI-H522; Supplementary Figure 2, A, available online). Further analysis on a larger cohort of 84 NSCLC cell lines (34) supported the finding that EPHA3 is frequently deleted; it was deleted in 44 of 84 (52%) of these cell lines, with a median copy number below 1.87 (Supplementary Figure 2, B, available online). To assess the relevance of EPHA3 copy number loss in human lung cancer, we analyzed the EPHA3 gene on chromosome 3p11 in a large dataset of 371 lung adenocarcinoma samples (35). Consistent with the cell line data, the EPHA3 gene was deleted in 157 of 371 (42%) primary tumors (Supplementary Figure 2, C, available online).

To determine whether EPHA3 represents a target of 3p11 loss, we first asked whether EPHA3 expression was decreased in lung tumor samples relative to paired normal lung tissues. By quantitative PCR of a lung cancer tissue cDNA array with matched nonmalignant lung tissue in the patient sample, 19 of 24 (approximately 80%) samples showed twofold to 45-fold reduction of EPHA3 mRNA in tumors relative to nonmalignant tissues (Figure 2, A; note log scale of the Y-axis). Next, we compared EPHA3 expression in a panel of 85 clinical NSCLC specimens representing different stages of tumor progression relative to nonmalignant control tissues. EPHA3 expression was statistically significantly lower in tumors at all stages compared with nonmalignant control tissue (Figure 2, B; P < .001, Kruskal–Wallis test followed by individual Mann–Whitney tests). Using immunohistochemistry with a verified monoclonal antibody against EPHA3 (Supplementary Figure 3, B, available online), we also analyzed EPHA3 protein expression in nine NSCLC tumor samples vs paired adjacent nonmalignant lung tissue. Whereas eight nonmalignant tissues expressed EPHA3, only two of the tumor samples expressed detectable levels of EPHA3 (Figure 2, C; P = .015, Fisher exact test). Furthermore, EPHA3 expression was analyzed by immunohistochemistry in two lung tumor tissue microarrays of 104 cancer samples and 26 normal samples. These analyses revealed that EPHA3 expression was detectable in 16 of 26 (61.5%) nonmalignant lung tissue samples, compared with 33 of 104 (31.7%) tumor samples (Figure 2, D and E; P = .005, χ² test). Taken together, the copy
Figure 2. EPHA3 expression in human non–small cell lung cancer (NSCLC). A) EPHA3 expression in paired human lung tumors and normal tissues. A panel of cDNAs from 24 paired samples of human lung tumors and normal lung tissue was purchased from Origene Technologies Inc (TissueScan lung cancer tissue qPCR array IV-matched pairs). EPHA3 expression in these patient samples was measured by quantitative polymerase chain reaction (qPCR) and presented as relative expression level normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on a log2 scale. B) EPHA3 expression in different stages of lung cancer. Two panels of cDNAs from 15 normal lung tissue and 70 cancer samples were purchased from Origene Technologies Inc (TissueScan lung cancer tissue qPCR array III and V). EPHA3 expression was measured by quantitative PCR and is presented as the relative expression level normalized to GAPDH. Shown are the data from individual samples (each symbol representing one patient) and the median values stratified according to clinical stage and plotted relative to similar analyses of a tissue collection of normal lung samples.
number analysis and expression data indicated that EPHA3 expression was frequently reduced in human lung cancer, a finding that supported our hypothesis that wild-type EPHA3 acted to suppress the growth of NSCLC tumors.

Effect of EPHA3 Reexpression on Apoptosis and Growth of Tumor Xenografts

To gain further insight into the function of EPHA3 in tumor cells, wild-type and mutant receptors were expressed in three NSCLC tumor cell lines, A549, H1975, and H1299, which express relatively low amounts of endogenous EPHA3 (Figure 3, B and Supplementary Figure 3, A, available online). To test the effects of EPHA3 on cell growth, we initially measured clonal growth by a colony formation assay. Tumor cells expressing EPHA3 displayed a 30%–50% reduction in the number of colonies formed on a tissue culture dish. The size of colonies was not different between tumor cells expressing EPHA3 and vector control cells (Figure 3, A), indicating an equal growth rate but reduced plating efficiency. To determine whether the reduced number of cells was because of decreased proliferation or increased apoptosis, we used cellular incorporation of bromodeoxyuridine to measure proliferating cells and performed apoptosis assays. In all three lines (A549, H1975, and H1299), there was no change in tumor cell proliferation between control cells carrying the pBABE vector and cells expressing EPHA3 (Supplementary Figure 4, A, available online). By contrast, cells expressing EPHA3 displayed substantial enhancement of cellular apoptosis, as judged by both ELISA-based and TUNEL assays (Figure 3, B and Supplementary Figure 4, B and C, available online). These results indicated that decreased survival potential, rather than decreased cell proliferation, was responsible for the reduction in colony numbers.

To directly determine the function of EPHA3 in tumor cells in vivo, A549 and H1299 NSCLC cells expressing wild-type EPHA3 receptor were injected subcutaneously into a dorsal flank of nude mice, whereas control cells carrying the empty retroviral vector were injected contralaterally in the same mouse. Increased expression of wild-type EPHA3 in the A549 or H1299 cell lines was associated with statistically significant inhibition of tumor growth in the xenograft animal model (Figure 3, C; mean A549 tumor volumes, vector control = 373.3 mm³ vs EPHA3 = 249.3 mm³, difference = 124.0 mm³, 95% CI = 74.6 to 173.3 mm³, P < .001, paired t test; mean H1299 tumor volumes, vector control = 790 mm³ vs EPHA3 = 459.1 mm³, difference = 330.9 mm³, 95% CI = 193.8 to 468.0 mm³, P < .001, paired t test).

To determine whether EPHA3 mutations alter the growth of tumor cells in vivo, H1299 cells expressing wild-type or mutant EPHA3 (G187R, T166N, S449F, or G766E; 5 million per mouse) were each injected into an individual nude mouse (n = 10 per condition). Tumors were harvested 3 weeks after injection, tumor dimensions were measured, and tumor volumes were calculated. Consistent with our in vitro data that indicated that these were loss-of-function mutations, the mutant EPHA3 RTKs, when expressed at levels similar to the wild-type protein (Figure 3, D), were not associated with a statistically significant difference in tumor growth in vivo compared with control tumors carrying vector alone (Figure 3, E and F). By contrast, wild-type EPHA3 statistically significantly inhibited H1299 tumor growth (mean H1299 tumor volumes, vector control = 774.7 mm³ vs EPHA3 = 437.4 mm³, difference = 337.3 mm³, 95% CI = 216.8 to 457.8 mm³, P < .001, Kruskal–Wallis test followed by individual Mann–Whitney tests), confirming our previous studies (Figure 3, C).

To identify changes in the cancer cells within tumors, we analyzed cell proliferation and apoptosis in tissue sections by staining for Ki-67 and cleaved caspase 3, respectively. Quantitation of Ki67-positive nuclei revealed no apparent changes in cell proliferation between tumors expressing wild-type and mutant EPHA3 (data not shown). By contrast, apoptosis was increased approximately threefold in tumors expressing wild-type but not mutant EPHA3 receptors as compared with vector control cells (Figure 3, G; mean H1299 tumor apoptosis index, vector control = 5% vs EPHA3 = 15.25%, difference = 10.25%, 95% CI = 1.99 to 18.5%, P < .001, ANOVA followed by individual t tests). Taken together with the patient survival data, these results provided convergent evidence in support of a model in which wild-type EPHA3 suppresses tumor growth by enhancing apoptosis in vivo, leading to selection for tumors with decreased EPHA3 protein expression levels and/or loss-of-function mutations in the EPHA3 gene.

**EPHA3 Signaling, AKT Activity, and Apoptosis**

To gain mechanistic insight into the role of EPHA3 as a tumor suppressor, we used Western blot analysis to investigate potential links between EPHA3 and signaling molecules with known relevance to tumor growth and apoptosis (Figure 4). H1299 cells carrying wild-type EPHA3 expression constructs or the control vector were serum-starved overnight and stimulated with ephrin-A5 following a time course, and activities of AKT, ERK, and S6 were inferred from phosphorylated protein levels. In the serum-starved and unstimulated state, EPHA3 expression in H1299 cells had little or no effect on the activation of many key molecules. Stimulation with ephrin-A5, a ligand for EPHA3-expressing tumor cells, was followed by a rapid decrease in phosphorylation of AKT at Thr308 and Ser473, indicating inactivation of its kinase activity (Figure 4, A). There was a particularly sustained reduction in phosphorylation of the critical activating residue T308, and expression of higher levels of wild-type EPHA3 accentuated this inhibitory effect.
Figure 3. Effects of wild-type EPHA3 on apoptosis and tumor growth. A) Effects of wild-type EPHA3 on colony formation. Three NSCLC lines (A549, H1975, and H1299) were transduced with a retrovirus encoding wild-type EPHA3 or the pBABE vector as a control and were analyzed by colony formation assays. Representative photomicrographs of colony formation are shown from two independent experiments. B) Effects of wild-type EPHA3 on cellular apoptosis. NSCLC lines (A549, H1975, and H1299) were transduced with wild-type EPHA3–encoding or pBABE control vectors and analyzed for cellular apoptosis. Top panel: EPHA3 expression in the transduced cells was assessed by western blotting for levels of EPHA3 and tubulin, as indicated. Bottom panel: cells were treated with GST-TRAIL (5 µg/mL) for 24 hours to induce apoptosis, which was then analyzed using
Thus, although ligand stimulation could, in principle, affect both the transduced EPHA3 receptors and endogenous EPHA2 receptors, EPHA3 has a higher affinity than EPHA2 for ephrin-A5 (36), so the timing and magnitude of ligand-induced decrease in AKT phosphorylation was EPHA3-dependent. EPHA2 levels were not altered in the presence of transduced EPHA3 or in response to ephrin-A5 stimulation during these experiments.

To test this model further, we examined AKT activation in cells that were stimulated with ephrin-A1 compared with ephrin-A5, a ligand that has higher affinity for EPHA3 (Figure 4, B). Relative to ephrin-A1 treatment, ephrin-A5 stimulation inhibited AKT activation in cells expressing EPHA3, but it had notably less effect on vector control cells, consistent with ephrin-A5 being a preferred ligand for EPHA3. As further evidence of ligand-induced reduction of AKT activity, ephrin inhibited phosphorylation of the transcription factor FOXO3A, a direct target of AKT. However, phosphorylation of two other targets of AKT, TSC2 and GSK-3β, was not changed substantially. These findings suggested that other signaling pathways, such as WNT and ERK or RSK, can regulate these targets [reviewed in (37)], consistent with previous evidence in lymphocytes and/or murine embryonic fibroblasts that ablation of rictor or mLST8 resulted in reduced AKT activity, independent of any changes in TSC2 and GSK-3β activity (38,39).

To investigate the effects of mutant EPHA3 on AKT signaling, H1299 cells expressing either wild-type or mutant EPHA3 (G187R or G766E) were serum-starved overnight and stimulated with ephrin-A5 for 15 minutes, and pS473 AKT levels were measured by western blot analysis. Ephrin-induced inhibition of AKT activity was greater in H1299 cells transduced with wild-type EPHA3 but not the G187R or G766E mutant receptors, compared with control cells transduced with empty vector (Figure 4, B and C). To directly investigate the effects of mutant EPHA3 on apoptosis, apoptosis in H1299 cells expressing either wild-type or mutant EPHA3 (G187R or G766E) was measured by an ELISA-based assay. Consistent with known roles of AKT and its phosphorylation of FOXO3A in survival signaling, addition of ephrin-A5 was associated with increased apoptosis of H1299 tumor cells that expressed low levels of EPHA3. Apoptosis levels in these cells were further increased when transduced wild-type EPHA3, but not cancer-associated mutant EPHA3 proteins (G187R, G766E), was expressed in these cells (Figure 4, D). These studies provide evidence that links EPHA3 levels and activity (WT vs mutant) to tumor cell survival, and they suggest that EPHA3-dependent apoptosis is mediated at least in part by ligand-induced inhibition of AKT activity.

**Effect of EPHA3 Somatic Mutations on Tumor Suppression**

Activation of EPH receptors requires oligomerization of receptor monomers, so we reasoned that loss-of-function EPHA3 mutants may function in a dominant negative manner if they can form heteromeric complexes with the wild-type EPHA3 receptor. If this scenario were to occur in human tumor cells, mutation of one EPHA3 allele could attenuate the ability of the product of the wild-type EPHA3 allele to restrain tumor growth, and thus transdominant inhibition would increase as expression of the wild-type allele decreased. To explore this possibility, we simultaneously expressed epitope-tagged wild-type and mutant EPHA3 in 293T cells, then coimmunoprecipitated both receptors and performed western blots to reveal complexes of mutant EPHA3 with the wild-type EPHA3 receptor (Figure 5, A). To investigate whether the activity of wild-type EPHA3 was affected by the formation of mutant:wild-type complexes, Myc-tagged wild-type EPHA3 receptor was cotransfected with increasing amounts of HA-tagged mutant EPHA3. Wild type:wild type or mutant:wild type complexes were immunoprecipitated with anti-Myc antibody, and kinase activity of wild-type EPHA3 was measured by phosphotyrosine blot analysis. Tyrosine phosphorylation of wild-type EPHA3 was decreased in the presence of increasing amounts of mutant EPHA3 (Figure 5, B), suggesting that the mutant receptors inhibit wild-type receptor signaling in a dominant negative fashion. To complement this study, Myc-tagged mutant EPHA3 was also cotransfected with increasing amounts of HA-tagged wild-type EPHA3, and mutant:mutant or mutant:wild type complexes were immunoprecipitated with anti-Myc antibody. Despite the presence of increasing amounts of wild-type EPHA3, phosphorylation was not detected in the Myc-tagged
Figure 4. EPHA3 signaling, AKT activity, and apoptosis. A) Time course of EPHA3 receptor signaling in response to ephrin-A5. H1299 cells expressing EPHA3 (+) or carrying control vector (−) were stimulated with ephrin-A5 (100 ng/mL) for 0, 5, 15, and 30 minutes. Cell lysates were subjected to western blot analyses using anti-phosphopeptide-specific and total protein-directed antibodies as indicated. Immunoblots representative of three independent experiments are shown. B) EPHA3 receptor signaling in response to ephrin-A1 vs ephrin-A5. H1299 cells expressing EPHA3 or carrying control vector were stimulated for 15 minutes with 100 ng/mL of either ephrin-A1, ephrin-A5, or control IgG, after which cell lysates were analyzed by immunoblotting with the indicated anti-phosphopeptide-specific and total protein-directed antibodies. Shown are representative immunoblots of two independent experiments. C) Effect of mutant EPHA3 on AKT activity. H1299 cells expressing either wild-type (WT) or mutant EPHA3, as indicated, were stimulated with either 100 ng/mL of ephrin-A5 or with 100 ng/mL control IgG for 15 minutes, after which cell lysates were analyzed by immunoblotting with the anti-phospho AKT, anti-total AKT, and anti-EPHA3 antibodies. Shown are representative immunoblots of two independent experiments. D) Effect of wild-type or mutant EPHA3 on apoptosis. H1299 cells expressing WT or mutant EPHA3 were treated with 100 ng/mL ephrin-A5 in the presence of 5 µg/mL GST-TRAIL for 24 hours and then apoptosis was analyzed by a Cell Death Detection ELISA kit. Data from one experiment representative of two independent experiments are shown.

mutant immunoprecipitates, indicating that mutant: wild-type complexes were not functional (Figure 5, C).

To determine whether cancer-associated EPHA3 mutants can attenuate biological functions downstream of EPHA3, we stably coexpressed wild-type and G187R or G766E mutant EPHA3 receptors in H1299 lung cancer cells via retroviral transduction, followed by an apoptosis assay in vitro and tumor xenograft experiments in vivo. Consistent with in vivo data, expression of wild-type EPHA3 alone amplified apoptosis in these tumor cells. However, coexpression of mutant EPHA3 (G187R or G766E) eliminated the effect of wild-type EPHA3 on apoptosis in vitro (Figure 5, D). To investigate whether mutant EPHA3 could inhibit the wild-type EPHA3 tumor-suppressive function in vivo, H1299 cells that expressed either wild-type or mutant EPHA3 alone or that coexpressed wild-type and mutant EPHA3 (G187R or G766E) were subcutaneously injected into nude mice. Strikingly, the capacity of wild-type EPHA3 to suppress tumor growth in vivo was blocked (Figure 5, E; at day 10, pBabe control vs WT EPHA3 = 292.8 mm³ vs 194.1 mm³, difference = 98.76 mm³, 95% CI = 59.18 to 138.3 mm³, P = .001; at day 13, 375.8 mm³ vs
Figure 5. Effect of EPHA3 somatic mutations on tumor suppression. A) Interaction between wild-type and mutant EPHA3 receptors. HA-tagged wild-type EPHA3 and Myc-tagged mutants were cotransfected into 293T cells, after which lysates were used for immunoprecipitation with either anti-Myc or anti-HA followed by immunoblotting with each of these antibodies. Shown are blots representative of two independent experiments. B and C) Effect of EPHA3 mutants (G187R or G766E) on wild-type (WT) receptor phosphorylation. B) Myc-tagged WT EPHA3 was cotransfected with HA-tagged mutants at a ratio of 1:0.5, 1:1, 1:2, and 1:4. WT receptors were immunoprecipitated with anti-Myc antibody and subjected to anti-phosphotyrosine blot analysis. Shown are blots representative of two independent experiments. C) Myc-tagged mutant EPHA3 were cotransfected with HA-tagged WT receptors at a ratio of 1:0.5, 1:1, and 1:2. Myc-tagged WT EPHA3 receptors were also cotransfected with HA-tagged WT proteins as a control. Mutant or WT receptors were immunoprecipitated with anti-Myc antibody and subjected to anti-phosphotyrosine blotting.
Discussion

Recent sequencing of the cancer genome has identified the EPHA3 RTK as one of the most frequently mutated genes in lung cancer. However, the oncogenic potential of the EPHA3 mutations remains unknown. In this study, we found that EPHA3 somatic mutations impair receptor activity and that an EPHA3 mutation–associated gene signature predicts poor patient survival. In addition, both gene copy number and expression levels of EPHA3 were reduced in large cohorts of human tumors. Reexpression of wild-type EPHA3 in lung cancer cells increased apoptosis and inhibited tumor growth in vivo. EPHA3-induced tumor suppression is mediated through enhanced apoptosis via inhibition of AKT activity. The products of the somatically mutated EPHA3 genes discovered in the cancer genome, however, can form complexes with wild-type EPHA3 and inactivate its tumor-suppressive function.

This body of convergent studies leads to a cohesive model that elucidates the complex roles of EPHA3 receptors and of the somatic mutations that have been identified in patients with NSCLC. Collectively, these analyses provide genetic, functional, and mechanistic evidence that wild-type EPHA3, expressed at normal levels, suppresses the emergence of lung cancer. EPHA3 mutations or decreased receptor expression appear to be selected for in clinically evident NSCLC, and they are associated with a more aggressive course of the disease. The results further indicate that one mechanism leading to the biological behavior is that wild-type EPHA3 promotes tumor cell apoptosis by a mechanism that can be overridden in trans by a dominant negative action of EPHA3 somatic mutations identified in samples from patients with lung cancer. Intriguingly, one rational target identified by these analyses is the suppression of AKT activation by ephrin action on wild-type but not mutant EPHA3. This latter finding suggests a rationale for testing the therapeutic benefit of AKT inhibition in NSCLC, especially when the tumor expresses a somatically mutated EPHA3 receptor.

Because both tumor-promoting and tumor-suppressive functions have been assigned to EPH receptors, the discovery of EPHA3 mutations at a high frequency in human lung cancer was an indication of the clinical relevance, but not the function, of EPHA3 receptors. Indeed, one EPHA3 mutation, K761N, is located in the kinase domain at a highly conserved position analogous to FGFR2 (K641) (40), which was predicted to function as an activating mutation that would disrupt part of a “molecular brake” (26). However, in a direct analysis of EPHA3 (K761N), we found no apparent increase in RTK activity. In addition, some reports showed elevated expression of EPHA3 in lymphoid cell lines and tumor tissues, suggesting a possible role for EPHA3 as a “proto-oncogene” (41–43). Here, we found that many of the EPHA3 mutations in NSCLC displayed decreased kinase activity or reduced tyrosine phosphorylation, and that none of the mutations exhibited increased activities. These data indicate that, at least in this clinical setting, somatic EPHA3 mutations tend to encode loss-of-function mutants but not gain-of-function mutants. Our finding that expression of wild-type EPHA3 receptor, but not four EPHA3 mutants, in two NSCLC cell lines inhibited their ability to grow as tumors in vivo suggests that the EPHA3 RTK functions as a tumor inhibitor in lung cancer.

Recent structural analyses of EPH receptors have identified residues involved in ligand binding, receptor–receptor interaction, and kinase activation (44–46). Of the somatic mutations in the EPHA3 receptor that were discovered in human lung cancer, only W250R is located in the receptor–receptor interface formed by a sushi domain that connects neighboring EPHA molecules for receptor clustering. None of the other extracellular mutations mapped to recently discovered receptor–ligand or receptor–receptor interfaces, suggesting that these mutations could affect receptor activities through other mechanisms. For example, the G187 residue is located two amino acids away from the ligand–receptor interface modeled from an EPHA2:ephrin-A5 cocrystal structure, so that this mutation could subtly affect the fine structure of the ligand–EPHA3 interface, thereby inhibiting binding to ligand.

In the EPHA receptor kinase domain, R728, K761, G766, and D806 are highly conserved. Mutation of K761, as noted, might be expected to increase kinase activity by lifting a molecular brake inferred from homology with FGFR2, but our direct experimental analysis did not detect this effect. G766 is located in an absolutely conserved DFG segment of the activation loop and is predicted to affect kinase activity. D806, situated in the middle of kinase domain, forms two hydrogen bonds with the neighboring chain. A D806N mutation, discovered in colon cancer, might be expected to disrupt the folding of the kinase domain. Indeed, G766E and
D806N mutations destroyed kinase activities, as judged by in vitro kinase assays and phosphotyrosine blots (Figure 1). R728 is a highly conserved residue that is located in the αE helix, and our work shows that the R728L cancer-associated mutation also markedly inhibited kinase activity, but the mechanism for that is currently unclear because it resides on the surface of the kinase domain.

Not all mutated EPHA3 receptors affect tyrosine phosphorylation levels or kinase activity. It remains possible that these other mutations retain kinase activity yet inactivate EPHA3 function through other mechanisms, such as weakened heterotypic association with adaptors, other cytoplasmic signaling molecules, or receptor substrates. Further investigation will be needed to detect their function. For example, T166N only slightly decreased kinase activity (Figure 1), but it behaved similarly to a kinase-deficient mutant in vivo and was unable to inhibit tumor growth in xenografts (Figure 3, E and F). The possibility that some EPHA3 mutations cause little alteration in receptor function cannot be excluded. Nonetheless, the frequency of EPHA3 mutations in lung cancer is substantially higher than would be expected by chance (26), consistent with our observation that EPHA3 mutations serve as “drivers” in lung cancer. Overall, our analyses indicate that the EPHA3 receptors affect receptor activities through multiple molecular mechanisms for which a common thread is diminished function, including impairments of ligand binding, receptor–receptor clustering, receptor surface expression, kinase activity, and structural integrity.

Although EPHA3 somatic mutations by themselves are loss-of-function mutations, based on our data we propose that EPHA3 mutations contribute to tumorigenesis in human lung cancer. Specifically, mutant EPHA3 receptors were able to form heteromeric complexes with their wild-type counterparts and inhibit receptor tyrosine phosphorylation, with biological effects of mitigating the apoptosis in vitro and suppression of tumor growth in vivo that were promoted by wild-type EPHA3. In this sense, the mutant EPHA3 acts in a dominant negative fashion to suppress wild-type EPHA3 function. This scenario, applied to human NSCLC, would mean that mutation of one EPHA3 allele could inactivate the tumor-suppressive function of wild-type EPHA3 documented in our data. Consistent with our hypothesis, analyses of lung cancer mutation datasets revealed that EPHA3 mutations were not accompanied by loss of heterozygosity (26). Together, the findings identify a novel mechanism by which loss-of-function RTK mutations could play an active role in tumor promotion. In addition, the fact that an EPHA3 mutation–associated gene signature predicts poor patient survival highlights the clinical relevance of EPHA3 mutations in human lung cancer.

Because the frequency of EPHA3 mutation in lung cancer is approximately 5–10%, we hypothesized that tumor cells may develop other means to inactivate the EPHA3 tumor suppressor function. Using quantitative PCR analyses and immunostaining of EPHA3 in human lung cancer tissue microarrays, we found a statistically significant reduction of EPHA3 expression in approximately 68% of tumor samples, suggesting that reducing EPHA3 expression is another mechanism for tumor cells to inhibit EPHA3 activity. Modulation of EPH receptor levels in tumor cells could be achieved through either genetic alterations or epigenetic regulation (6,7). Our copy number analyses indicate that the EPHA3 gene is often deleted in lung cancer cell lines (52%) and human tumor specimens (42%). Although EPHA3 was also reported to be amplified in two lung cancer specimens (Ding et al., 2008), it is unclear whether the amplified allele carried a mutation and whether EPHA3 protein levels were elevated in those two samples. Furthermore, EPHA3 expression is reportedly silenced by promoter hypermethylation in leukemia and hematopoietic tumor cells (47), suggesting that lung cancer cells may use similar strategies. Thus, EPHA3 mutation or deletion or decreased EPHA3 expression could all contribute to tumor development.

Although our studies suggest that wild-type EPHA3 plays tumor-suppressive role in lung cancer, it cannot be excluded that EPHA3 may have tumor-promoting activities in some cancers or during particular stages of tumorigenesis. For example, although the mutational status of the receptor is unclear in these settings, elevated EPHA3 expression has been reported in melanomas and some hematopoietic malignancies (42,43,48,49). It is possible that EPHA3, like EPHA2, has a dual role in tumor suppression and tumor promotion, depending on ligand-dependent vs ligand-independent signaling [reviewed in (5,6,8)]. Therefore, further studies are needed to determine how EPHA3 expression and signaling might regulate tumor initiation and progression of different types of cancer.

The identification of a suppressive role for EPHA3 in NSCLC tumor biology opens up exciting questions that have not been answered by the present study. First, because EPH receptors regulate communication between different cell types, the full impact of EPHA3 mutations in vivo will require careful studies in inducible and tissue-specific transgenic or “knock-in” tumor models. Second, the mechanism by which EPHA3 expression is diminished in lung cancer is unknown. Although we found that chromosome 3p11 covering the EPHA3 gene is often deleted, our results do not exclude the possibility of regulation of EPHA3 expression via epigenetic changes such as promoter hypermethylation or inhibition of transcription. Because reexpression of the EPHA3 gene in patients with lung cancer may be beneficial, defining the mechanisms of EPHA3 gene silencing may lead to novel therapeutic strategies to restore expression in tumor cells. Third, because another EPH family member, EPHA2, appears to function as a tumor promoter in NSCLC (13,14,50), the discovery of the EPHA3 tumor-suppressive function suggests that future therapeutic strategies targeting EPH receptors in cancer need to be directed at individual EPH molecules, rather than multiple or pan-EPH receptor inhibition strategies. EPHA3 mutations have also been identified in other tumor types (24,25,30), so it will be critical to determine whether EPHA3 plays a tumor-suppressive role in those cancers. Finally, our data that show that loss-of-function mutations of EPHA3 block the capacity of wild-type EPHA3 to engage ephrin ligands, thus resulting in the continued activity of AKT, suggest a potentially important means of optimizing patient selection for studies of the efficacy of AKT inhibitors in NSCLC.

References


G. Zhuang and J. Chen designed the experiments. G. Zhuang, W. Song, K. Amato, Y. Hwang, L. Lin, K. Lee, and D. M. Brantley-Sieders performed experiments and analyzed and interpreted data. F. Ye, Y. Guo, and Y. Shyr performed bioinformatic analysis on mutation-associated gene signature and interpreted data. D. Carbone provided critical reagents and helpful discussion. G. Zhuang, M. Boothby, and J. Chen wrote the manuscript.

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