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Preclinical Development

Molecular Cancer Therapeutics

Global Evaluation of Eph Receptors and Ephrins in Lung Adenocarcinomas Identifies EphA4 as an Inhibitor of Cell Migration and Invasion

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Abstract

The Eph family of receptors is the largest family of receptor tyrosine kinases, but it remains poorly studied in lung cancer. We aimed to systematically explore the human Eph receptors and their ligands, the ephrins, in lung adenocarcinoma. The prognostic impact of Eph receptor and ephrin gene expression was analyzed using 2 independent cohorts of lung adenocarcinoma. Gene expression profiles in lung adenocarcinoma compared with normal adjacent lung were studied in 3 independent cohorts and in cell lines. Gene expression profiles were validated with quantitative polymerase chain reaction (qPCR) and Western blotting in cell lines. Functional studies to assess the role of Eph receptor A4 (EphA4) were carried out *in vitro*. The biological effects of EphA4 in lung cancer cell lines were assayed following overexpression and knockdown. Of the 11 Eph receptors and 8 ephrins analyzed, only EphA4 and ephrin A1 gene expression were consistently associated with an improved outcome in patients with lung adenocarcinoma. Expression levels of EphA4 by microarray correlated well with expression levels measured by qPCR and Western blotting. EphA4 overexpression reduced cell migration and invasion but did not affect cell cycle, apoptosis, or drug sensitivity. Surprisingly, EphA4 was expressed at higher levels in cancer compared with non-cancer tissues and cell lines. EphA4 gene expression is associated with an improved outcome in patients with resected lung adenocarcinoma, possibly by affecting cancer cell migration and invasion. *Mol Cancer Ther; 11(9); 2021–32. ©2012 AACR.*

Introduction

The most successful examples of targeting tyrosine kinases in non–small cell lung cancer (NSCLC) are epidermal growth factor receptor (EGFR) and ALK tyrosine kinase inhibitors, both of which are associated with dramatic responses in patients with mutant *EGFR* or translocations involving *ALK*, respectively (1). Understanding the role of other tyrosine kinase families may lead to the identification of new therapeutic targets (2).

The Eph family of receptors is the largest group of receptor tyrosine kinases (3, 4). Eph receptors are classified into 2 subfamilies, A and B, on the basis of sequence similarity and ligand affinity. Their ligands, called ephrins, are membrane bound and divided into 2 subfamilies on the basis of how they are attached to the membrane via a glycosylphosphatidylinositol anchor (A-type) or a transmembrane domain (B-type). Receptor–ligand interactions result in bidirectional signals: a forward signal that depends on Eph kinase activity and propagates in the receptor-expressing cell, and a reverse signal that depends on Src family kinases and propagates in the ephrin-expressing cell. In general, EphA receptors bind ephrin A ligands; however, there are a few exceptions, including EphA4, that can also bind Ephrin B2 and B3 (4).

Eph receptors are expressed in numerous tissue types and have distinct, albeit frequently overlapping, patterns of expression, which indicates a level of redundancy (3). They have been found to be overexpressed or downregulated in cancer cells and tumor stroma and, often, Eph receptor and ephrin levels are discordantly regulated. In addition, Eph receptor mutations are likely to have a role in cancer pathogenesis (4). In many cellular contexts, Eph bidirectional signaling promotes an epithelial phenotype and suppresses cancer cell–substrate adhesion, migration, invasion, and growth. In addition, Eph receptors and ephrins can promote

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cancer progression through cross-talk with oncogenic signaling pathways. In addition, Eph bidirectional signals have been shown to promote tumor angiogenesis (4).

We recently reported that EphA2 expression was observed in lung adenocarcinoma premalignant lesions and was associated with smoking history, *KRAS* mutation, and poor survival in lung adenocarcinoma (3, 5). EphA2 knockdown decreased proliferation and cell migration through ERK1/2 activation in lung adenocarcinoma cells (3). *EphA2* mutations have been recently reported in 7% of squamous cell carcinomas and are associated with increased cancer cell invasion and cancer cell survival (6). Ephrin B3–EphA2 interaction has been reported to drive survival signaling *in vitro* (7). EphB3 expression has been shown to be increased in NSCLC and to be associated with metastasis (8). For these reasons, as well as for their role in angiogenesis, Eph family receptors are considered valuable potential targets (9). In contrast, EphB6 has been shown to be frequently silenced by promoter DNA methylation in NSCLC and to act as a metastasis suppressor (10). Finally, some Eph receptors have been identified as potential targets of dasatinib (11, 12). The role of EphA4 in cancer has not been studied extensively. Most of the studies focused on gastrointestinal cancer and report an overexpression of EphA4-promoting cancer cell growth (13–16).

To increase our understanding of the complexity of this family, we carried out a global evaluation of Eph/ephrins gene expression in clinical samples to identify the family members most important for NSCLC progression. Using multiple and independent datasets, we found that EphA4 was overexpressed in cancer versus normal cell lines and clinical samples. In contrast, EphA4 gene expression was associated with improved outcome in lung adenocarcinomas. Consistent with this latter observation, loss- and gain-of-function experiments *in vitro* showed that EphA4 inhibits tumor cell migration and invasion.

Materials and Methods

Gene expression analysis

Gene expression profiles of a panel of 61 lung cell lines (17, 18), 432 resected stage I–III lung adenocarcinomas from the National Cancer Institute (NCI) Director's Challenge Consortium for the Molecular Classification of Adenocarcinoma (DCC; ref. 19), 147 resected stage I–III lung adenocarcinomas, and 17 normal lungs from the Dana–Farber Cancer Institute (20), 26 paired adjacent normal tumor samples (21), and 45 lung adenocarcinomas with 62 normal adjacent lung samples (22) were used as described in the Supplementary Material.

Distribution of Eph receptor gene mutations in NSCLC

Data from the Catalog of Somatic Mutations in Cancer (COSMIC) database (23) were extracted on July 15, 2011 (24–26).

Statistics

Data analysis was carried out using R packages in Bioconductor (27) and GraphPad Prism version 5.00. In 2 independent cohorts of patients (19, 20), multicovariate Cox models were fitted to estimate the effects of prognostic factors, including age, gender, disease stage, institution, and individual Eph receptor or ephrin status (continuous variable and trichotomized on tertiles) on time-to-event endpoint for both overall survival (OS) and relapse-free survival (RFS). The Kaplan–Meier method was used to construct overall and progression-free survival curves, and the log-rank test was used to test the difference in survival by EphA4 gene expression stratified using tertiles. Summary statistics, including median and range values, were used to describe the distribution of Eph receptors and ephrins in different datasets. Cancer and normal samples were compared using a $\log_2$ fold-change and a paired or non-paired 2-sided Student *t*-test. Pearson correlations were computed among gene expressions by using Affymetrix platforms and quantitative polymerase chain reaction (qPCR). All statistical tests were 2-sided, and *P* values of 0.05 or less were considered to be statistically significant.

Cell culture and cell transfection

Human NSCLC cell lines (A549, H1792, H1299, H460, H661, H2009, and H1711) were obtained from Drs. John Heymach and John Minna and maintained using standard cell culture techniques (3, 5). All cell lines were authenticated by cross-comparing their allelic short-tandem repeat patterns generated using the PowerPlex 1.2 system (Promega) with the American Type Culture Collection repository database.

EphA4 cDNA cloned in Lenti6/Ubc/V5/DEST and LacZ control plasmids were kindly provided by Drs. Li Jiannong and Eric B. Haura. EphA4 siRNAs were predesigned sets of 4 independent sequences (siGENOME SMARTpool; Dharmacon Inc.). Non-targeting scrambled siRNA was transfected as a control. Cells were transfected according to the manufacturer's instructions using Amaxa Nucleofector System (Lonza Walkersville, Inc.) or Attractene (Qiagen).

Real-time PCR analysis of Eph receptor mRNA expression

The RNeasy Mini Kit (Qiagen) was used to extract total mRNA from cultured cells, and the Access RT-PCR System (Promega) was used to synthesize cDNA according to the manufacturers' instructions. The PCR primers were designed by using Primer-Blast (28). The sequences of the primers are provided in Supplementary Table S1. SYBR Green chemistry was used for RT-PCR (Applied Biosystems) according to the manufacturer-recommended protocol. PCR products and their dissociation curves were detected with the ABI Prism 7500 fast real-time PCR system. Housekeeping gene L32 ribosomal gene (*RPL32*) was used as an internal control. Individual datasets were

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normalized with control vehicle–treated cells; the absolute quantity was normalized with L32 as an internal control.

Cell migration and invasion assays

Modified Boyden chamber migration and Matrigel invasion assays were conducted using Biocoat Matrigel invasion chamber inserts and control uncoated inserts containing 8-μm pores (Becton Dickinson Transduction Laboratories), as previously described (3, 5). The average number of migrating or invading cells per chamber in triplicate samples is presented.

Western blot analysis

Western blot analysis of protein expression in NSCLC cell lysates was carried out as previously described (3, 5). Antibodies included EphA4 (Becton Dickinson Bioscience); β-actin, calnexin, phospho-ERK (T202/Y204), phospho-AKT (S473), phospho-SRC (Y416), phospho-FAK (Y861), phospho-paxillin (Y118), and α-smooth muscle actin (Sigma Chemical); E-cadherin, vimentin, α5-integrin, and occludin (Cell Signaling Technology). Protein expression was quantified using the ImageJ software program (National Institutes of Health, Bethesda, MD) and expressed as a ratio of the target protein divided by the loading control after Western blotting.

Cell-cycle and apoptosis assays

For the cell-cycle analysis, cells were harvested, fixed, and stained with propidium iodide, and DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACS; Becton Dickinson) using ModFit software (Verity Software House). To measure apoptosis, fixed cells were subjected to terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining according to the manufacturer's instructions (APO-BRDU kit; Phoenix Flow Systems, Inc.) and quantitated with FACS.

Cytotoxicity assay

Cytotoxicity in NSCLC cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (29). Under each experimental condition, at least 4 independent wells were treated. The median effects of drugs on viability were calculated using the Chou–Talalay equation with the CalcuSyn software program (Biosoft).

Results

Expression of EphA4, Ephrin A1, EphB2, and Ephrin B2 correlates with survival in patients with resected early-stage lung adenocarcinoma

We analyzed the gene expression of all Eph and ephrin family members in 2 independent cohorts of adenocarcinoma patients. Supplementary Table S2 shows the hazard ratio and Ward *P*-value for each Eph receptor and ephrin family member in the DCC cohort (19), for both OS and RFS, adjusted for age, gender, stage, and institution. When several probesets were available for one unique gene, they were usually consistent in terms of effect on prognosis. As expected, hazard ratios (HR) for OS and RFS were strongly correlated overall ($R^2 = 0.68$, data not shown). EphA5, EphB2, and EFNB2 (ephrin B2) expression were associated with shorter OS, whereas EphA4 and EFNA1 (Ephrin A1) expression were associated with longer OS. EphA5, EphB2, EphB6, and EFNB3 (ephrin B3) were significantly associated with a higher risk of relapse. To validate these observations, a similar analysis was carried out in an independent cohort of patients with resected lung adenocarcinomas (Boston cohort; ref. 20; Supplementary Table S3). Consistently, EphA4 ($P = 0.063$) and EFNA1 ($P = 0.007$) were associated with improved OS, and EphB2 ($P = 0.059$) and EFNB2 (ephrin B2; $P = 0.139$) were associated with decreased OS.

For a more clinically relevant interpretation of EphA4's impact on survival, we stratified its gene expression into tertiles that allowed the comparison of 3 groups of patients with high, intermediate, or low expression levels using Kaplan–Meier curves. In DCC, higher levels of EphA4 expression were associated with longer OS (Fig. 1A) and RFS (Fig. 1B). In patients with low versus high EphA4 expression, median OS was 50 and 81 months, respectively, and median RFS was 44 and 64 months, respectively. After adjusting for age, gender, disease stage, and institution, low EphA4 was associated with an HR of 1.57 (95% confidence intervals [CI] 1.13, 2.18; $P = 0.007$] for OS and 1.46 (95% CI 1.01, 2.11; $P = 0.045$) for RFS (Table 1). A similar analysis was done in the Boston cohort. A Kaplan–Meier curve for OS, with EphA4 stratified by tertiles, showed a similar trend (Supplementary Fig. S1), and patients with low EphA4 expression had a median OS of 30 months compared with 67 months in patients with high EphA4 expression. Multicovariate analysis for OS confirmed that low EphA4 gene expression was associated with an increased risk of death, with an HR of 2.20 (95% CI 1.27, 3.80; $P = 0.005$; Supplementary Table S4).

EphA4 gene expression was higher in well-differentiated than in moderately differentiated lung adenocarcinoma, and in moderately, compared with poorly, differentiated lung adenocarcinoma (Fig. 1C). No significant difference in *EphA4* gene expression was observed according to smoking status (Fig. 1D), although EphA4 expression was slightly higher in NSCLCs with mutant *EGFR* compared with wild-type *EGFR* (Fig. 1E).

Eph receptors and ephrins are expressed in NSCLC cell lines and differentially expressed in lung cancer versus noncancerous epithelial samples

We next studied the expression profile of Eph receptors and ephrins in a panel of 52 NSCLC cell lines and 9 bronchial epithelial cell lines (18). With few exceptions, NSCLC and bronchial epithelial cell lines had a wide range of Eph receptor and ephrin expression (Supplementary Fig. S2). The vast majority of Eph receptors and ephrins were found to be differentially expressed in

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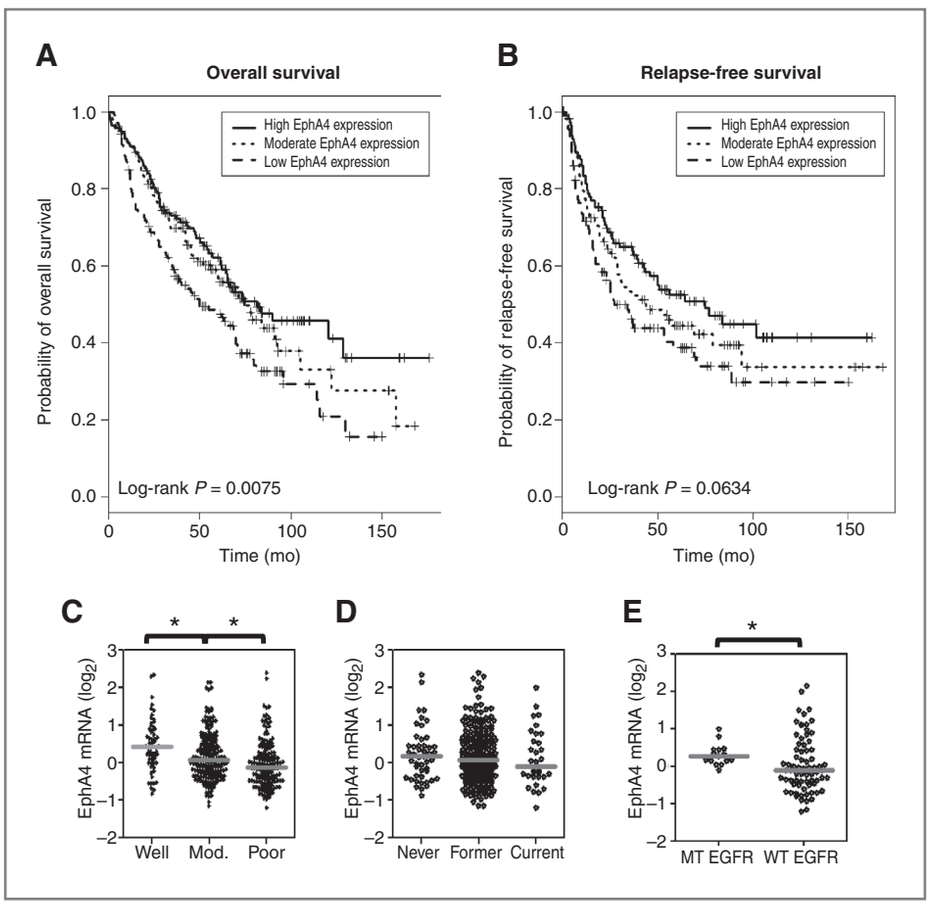

Figure 1. The expression of EphA4 is associated with an improved outcome. A and B, Kaplan–Meier curves for overall survival ($n = 422$; A) and relapse-free survival ($n = 327$; B) as a function of EphA4 expression in patients who underwent surgical resection of lung adenocarcinoma (19). EphA4 expression was stratified using tertiles: high, moderate, or low expression. C, *EphA4* gene expression and tumor cell differentiation in lung adenocarcinoma (well vs. moderately differentiated, $P = 0.0053$; moderately vs. poorly differentiated, $P = 0.0022$). D, *EphA4* gene expression and smoking status ($P > 0.05$). E, *EphA4* gene expression and *EGFR* mutation status ($P = 0.0277$). Mod., moderately; MT, mutant; WT, wild-type.

NSCLC versus bronchial epithelial cell lines and, in general, were downregulated in NSCLC cell lines (Fig. 2A). Unexpectedly, EphA4 was found to be significantly upregulated in NSCLC cell lines.

We then looked at the distribution of the expression of Eph receptors in lung adenocarcinoma versus histologically normal lung tissue samples using publicly available data from 3 independent studies and 2 different platforms (refs. 19, 21, 22; Fig. 2B–H; Supplementary Fig. S3). One study was particularly informative, as we were able to pair the cancer samples with the corresponding normal-appearing lung samples (21). Similar to what we observed *in vitro*, Eph receptor and ephrin expression varied widely *in vivo*. EFNB1, EFNB2, and EphB6 were downregulated in cancer versus noncancer samples, both in cell lines and in tissue, whereas EphA4 was consistently overexpressed in cancer versus normal samples, both in cell lines and in tissue. However, in general, Eph receptors and ephrins were overexpressed in lung adenocarcinoma compared with normal lung tissue, as opposed to what was seen in the cell lines, and this was consistent across the 3 independent studies. EFNA3, EFNA4, EFNA5, EFNB3, EPHA1, EPHB1, EPHB2, EPHB3, and EPHB4 were found to be upregulated in lung adenocarcinoma compared with normal lung *in vivo* in all 3 datasets (Fig. 2B–D); in the vast majority of the tissue sample pairs analyzed, EphA4 was upregulated in lung adenocarcinoma compared with adjacent normal lung (paired *t*-test $P = 0.0007$; Fig. 2H).

Eph receptor mutations in NSCLC

Supplementary Tables S5 and S6 describe the frequency and details of the mutations identified in Eph receptor genes in NSCLC. No mutation has been reported in the ephrin family. The majority of the mutations described in the Eph receptor family affect *EphA5* (mutation frequency 5%) and *EphA3* (mutation frequency 4%), with no definitive association with any histological subtype. The mutations are almost all missense mutations, distributed along the length of the gene, and their functional significance is unknown.

EphA4 RNA and protein expression were strongly correlated in NSCLC cell lines

The association between EphA4 gene expression and improved outcome in 2 independent cohorts of patients with lung adenocarcinoma led us to study further its expression and biological role in 7 NSCLC cell lines: A549, H460, H661, H1299, H1792, H2009, and H1711, which were selected to construct a panel with a wide range of EphA4 expression. EphA4 expression was not

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Table 1. Multicovariate Cox model fitted to estimate the effects of age, gender, disease stage, institution, and EphA4 expression on time to death or relapse

Covariates	HR	95% CI	*P*
Overall survival ($N = 422$)			
Low vs. high EphA4	1.57	1.13–2.18	0.007
Moderate vs. high EphA4	0.98	0.70–1.39	0.922
Age	1.04	1.02–1.05	<0.001
Male vs. female	1.27	0.96–1.67	0.090
Stage III vs. I-II	4.27	3.05–5.98	<0.001
Moffitt Cancer Center	1.56	0.99–2.47	0.057
MI	0.87	0.57–1.33	0.520
MSKCC	0.61	0.37–1.01	0.053
Relapse-free survival ($N = 327$)			
Low vs. high EphA4	1.46	1.01–2.11	0.045
Moderate vs. high EphA4	1.18	0.81–1.72	0.388
Age	1.01	1.00–1.03	0.092
Male vs. female	1.08	0.80–1.47	0.608
Stage III vs. I-II	2.47	1.63–3.74	<0.001
Moffitt Cancer Center	0.99	0.62–1.56	0.949
MI	0.67	0.43–1.04	0.074
MSKCC	0.57	0.36–0.90	0.016

NOTE: EphA4 expression was stratified using tertiles, and 3 groups with high, moderate, or low levels of EphA4 expression were compared (19). 95 CI, 95% confidence interval; *P*-value was from the Wald test.

Abbreviations: MI, University of Michigan Cancer Center; MSKCC, Memorial Sloan Kettering Cancer Center.

detected in H661 cells, was low in A549, was intermediate in H1299, H2009, and H1711, and was high in H460 and H1792 (Fig. 3A). EphA4 mRNA levels by qPCR were very well correlated with mRNA levels measured on the array (Fig. 3B). This was the case as well for EFNA1 and EFNB2, but not for EphB2 (Supplementary Fig. 4A–C). Further, EphA4 mRNA levels were well correlated with protein expression (Fig. 3C).

EphA4 did not affect proliferation or apoptosis in NSCLC cells

The correlation between shorter patient survival and low tumor expression of EphA4 indicates that it may function as a tumor suppressor. We manipulated EphA4 expression levels using transfection of the full-length gene under the control of a constitutive promoter into NSCLC cell lines with low endogenous EphA4 (A549 and H661) and using EphA4-specific siRNA in NSCLC cells with high endogenous EphA4 (H460 and H1792). Following transfection, we measured cell number, cell-cycle progression, and apoptosis and found no significant effect on apoptosis or cell cycle in any of the cell lines studied (Supplementary Fig. S5A–B).

EphA4 inhibits the migration and invasion of NSCLC cells

We measured the effects of EphA4 manipulation on NSCLC cell migration and invasion. Knockdown of EphA4 in NSCLC cells with high endogenous levels of EphA4 led to increased cell invasion and migration, whereas overexpression of EphA4 in NSCLC cells with low levels of EphA4 led to decreased cell migration and invasion (Fig. 4). Slight changes were observed in colony formation in 3 of 4 cell lines following EphA4 manipulation (Supplementary Figure S5C).

Because epithelial-to-mesenchymal transition (EMT) plays a major role in cancer cell metastasis, we examined various EMT markers following the manipulation of EphA4 expression. No consistent change in expression was observed for markers of epithelial (E-cadherin) or mesenchymal (vimentin) phenotypes (Fig. 5A). However, expression of the tight-junction protein occludin (epithelial marker) did increase with EphA4 overexpression. EphA4 transient overexpression or depletion did not affect cell morphology (Supplementary Fig. S6). Together, these data do not support a role for EphA4 in EMT.

To understand the mechanism by which EphA4 decreases cell migration and invasion, we looked at the effects of EphA4 on signaling pathways known to affect adhesion and migration (Fig. 5). Knockdown of EphA4 led to an increase of ERK1/2 activation, with inconsistent effects on FAK and paxillin activation. Similarly, EphA4 overexpression decreased activation of ERK1/2. Together, these data indicate that the changes in migration and invasion are mediated by EphA4-dependent ERK1/2 inactivation. EphA4 overexpression resulted in increased phosphorylation of FAK at Y861 (Fig. 5A) but

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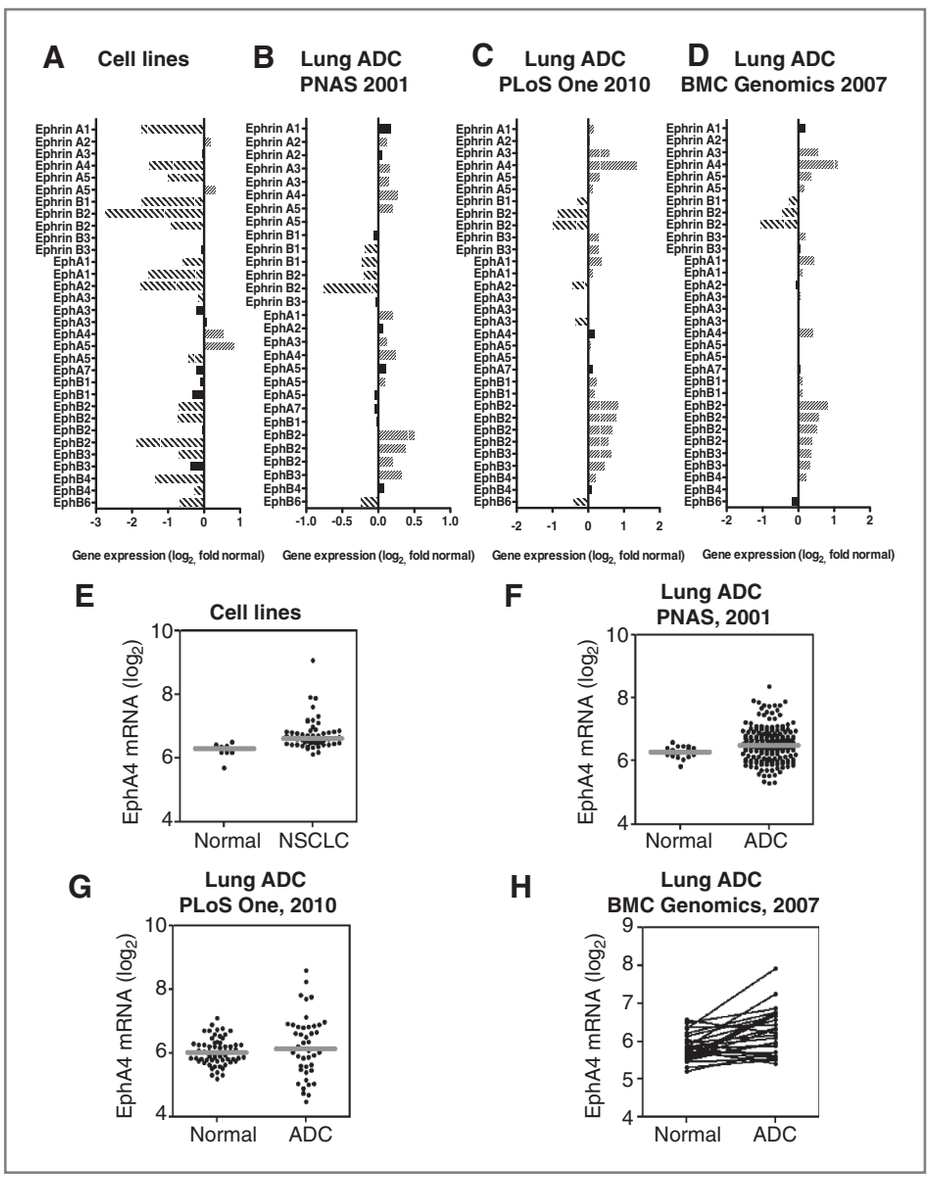

Figure 2. Distribution of Eph receptor and ephrin gene expression in NSCLC versus bronchial epithelial cell lines or normal-appearing lung. Gene expression profiles were processed from 4 publicly available studies including a large panel of NSCLC cell lines (A; refs. 17, 18) and 3 independent studies comparing lung adenocarcinomas and normal lung (B–D; refs. 19, 21, 22). E–H, *EphA4* gene expression in NSCLC versus normal bronchial epithelial cells (E; refs. 17, 18) and in lung adenocarcinoma versus normal lung in 3 independent studies (F–H; refs. 19, 21, 22). Log_2 fold-change of Eph receptors and ephrins in cancer versus normal cells/tissues are displayed in the same order to allow comparison. Striped bars represent genes downregulated in cancer versus normal. Bars striped in the other direction represent genes upregulated in cancer versus normal. Black bars represent no significant change. Statistical significance was determined by a paired (D, H) or non-paired (A–C and E–G) two-sided Student *t*-test.

no effect on FAK phosphorylation at Y407, Y576, Y397, or Y925 (Fig. 5B). Although we were initially surprised that the effect of EphA4 manipulation on pFAK (Y861) did not correlate with the effect on migration, one must consider that FAK can affect migration without a change in its phosphorylation status and can act as a scaffold (30). In addition, FAK Y861 is essential for RAS transformation and does promote the association of FAK with integrin alphaVBeta5 – although neither of those functions explains the marked decreased in Y861 phosphorylation in H1792 cells following EphA4 knock down.

Basal phosphorylated EphA4 was not detected using phospho-specific antibodies in any of the cell lines and only detected in 1 cell line (H1792) by immunoprecipitation. The level of phosphorylated EphA4 did not change following knock down in H1792. Phosphorylated EphA4 was detectable after EphA4 overexpression, suggesting that in H460, A549, and H1792, the portion of phosphorylated protein is low (Fig. 5B).

Effect of EphA4 manipulation on the gene expression of other Eph receptors and ephrins

One possible explanation for EphA4's lack of an effect on cell-cycle progression and survival is via the compensatory expression of other Eph receptors and ephrins. We examined the levels of several Eph receptors and ephrins in NSCLC cell lines in which we altered EphA4 expression as described above. Although some changes were observed, there was no dramatic or consistent change in the expression of any member of the family or in their ligands (Supplementary Fig. S7).

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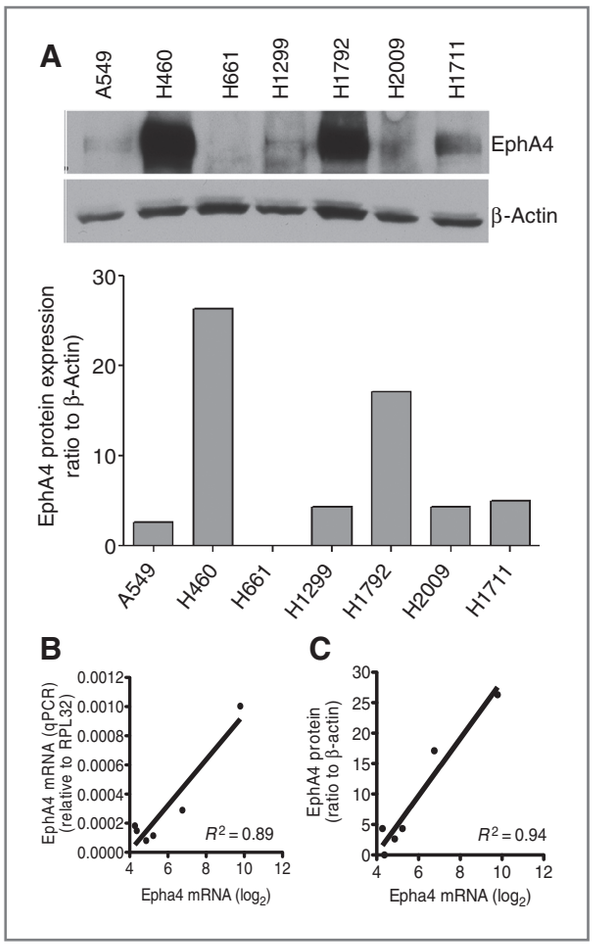

Figure 3. *EphA4* gene expression correlates well with protein expression. A, EphA4 protein expression in 7 NSCLC cell lines by Western blotting. The bottom panel is a quantitation of the blot. B, *EphA4* gene expression on the Affymetrix platform, by qPCR and by Western blotting, was compared with the noted correlation coefficients. C, comparison of EphA4 protein and gene expression.

EphA4 does not affect erlotinib and cisplatin sensitivity

Another possible mechanism for the effect of EphA4 tumor expression on patient outcome is an EphA4-mediated increase in sensitivity to anticancer therapy. Information about systemic therapy was not available in the cohorts we studied. We tested whether EphA4 was associated with response to 2 standard drugs in the treatment of lung adenocarcinoma: erlotinib, an EGFR tyrosine kinase inhibitor, and cisplatin. However, knockdown or overexpression of EphA4 did not affect cancer cell sensitivity to either of these drugs (Supplementary Fig. S8).

Discussion

We report the results of a global evaluation of Eph receptors and ephrins in lung adenocarcinoma using publicly available gene expression profiles. We showed, in 2 independent cohorts of patients, that EphA4 and EFNA1 gene expression were correlated with improved survival, and that EphB2 was correlated with worse survival. Surprisingly, in cell lines and tissues, EphA4 expression was higher in cancer versus noncancer samples. Overexpression of EphA4 decreased cell migration and invasion in our functional studies, and EphA4 knockdown increased migration and invasion; this was consistent with the correlation between EphA4 and survival. However, EphA4 did not affect cancer cell survival, cell-cycle progression, or drug sensitivity. We did not detect a compensatory effect of EphA4 on other family members or ligands that may have abrogated the biological effects of EphA4 manipulation. Finally, we report that EphA4 may decrease migration and invasion through downregulation of activated ERK1/2.

Eph receptors and ephrins remain poorly studied in lung cancer and other cancers (4). Our study represents a first step toward an understanding of the role of this family using a global approach. This study would have been difficult to complete at the protein level. Because false discovery is inherent to any high-throughput method, we used multiple independent datasets to validate our initial observations (31). *EphA4* gene expression was associated with a decreased risk of death or relapse after surgery for lung adenocarcinoma in 2 independent cohorts. Moreover, we found a strong correlation between *EphA4* gene expression by qPCR and by array, as well as a strong correlation between EphA4 mRNA and protein levels. This led us to carry out more in-depth *in vitro* studies. Consistent with our results in tumor samples, we showed that EphA4 reduced cell migration and invasion in 4 different cell lines. Further studies will be necessary to investigate the mechanism by which EphA4 may be downregulated in cancer (10, 32).

The role of EphA4 in cancer development and progression is context dependent. EphA4 signaling is an important component of the molecular mechanisms driving somite morphogenesis, establishing cell polarity during mesenchymal-to-epithelial transition of the paraxial mesoderm (33), and it promotes adhesion in the zebrafish (34). In a different context, EphA4 has been shown as an effector of Twist1, one of the major transcription factors associated with EMT (35), and it downregulates cell–cell adhesion in the *Xenopus* embryo (36). EphA4 has been shown to both promote cancer progression in various cancers (13–16, 37–39), but to behave as a tumor suppressor in vestibular schwannomas (40). To the best of our knowledge, its role in NSCLC has not been previously reported, and this is of particular importance because Eph receptor inhibitors are currently under development (41, 42). It has been proposed that cancer cells can inhibit the antimigratory functions of the Eph–ephrin system in various ways: decreasing the expression of the genes encoding either the ligands or the receptors, co-opting Eph receptors into oncogenic partners, or promoting cross-talk with other pathways (4). In this regard, EphA4

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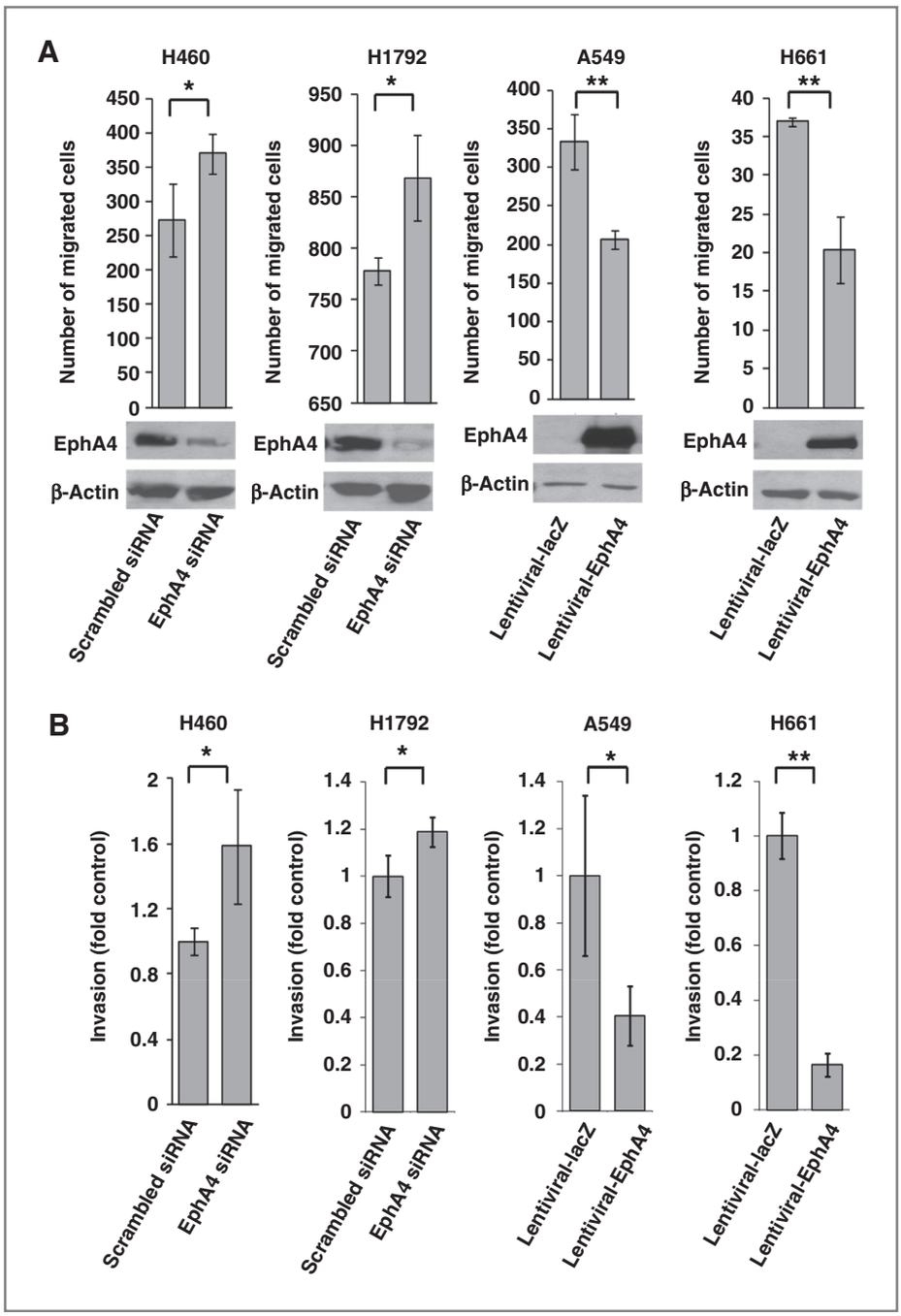

Figure 4. EphA4 decreases NSCLC cell migration and invasion. A, effect of EphA4 knockdown (H460, H1792) and overexpression (A549, H661) on cell migration. B, effect of EphA4 knockdown (H460, H1792) and overexpression (A549, H661) on cell invasion. Data are obtained from 3 independent assays. *, $P < 0.05$; **, $P < 0.01$.

and FGFR1 cross-talk identified in studies showing that EphA4 promotes tumor progression might be an important mechanism for further study (13, 15, 43).

It is interesting to note that, in our survival analysis, both EphA4 and EFNA1 were consistently and significantly associated with improved OS. EFNA1 was not differentially expressed in cancer versus normal tissues and cell lines, but its level of expression was one of the highest among the Eph receptors and ephrins. Eph receptors typically interact with the cell-surface ephrins at sites of cell–cell contact, and forward signaling is often detrimental to tumor progression and may be silenced in cancer cells (44). It is known that EphA4 can bind both A- and B-class ephrin ligands owing to its structural plasticity (45). Evidence from a prostate cancer model (PC-3) established that EphA4–ephrin A interactions mediate contact inhibition of locomotion; in addition, PC-3 cells have endogenous ephrin As that can activate EphA4, leading to contact inhibition of locomotion through activation of RhoA (46). Thus, we hypothesize

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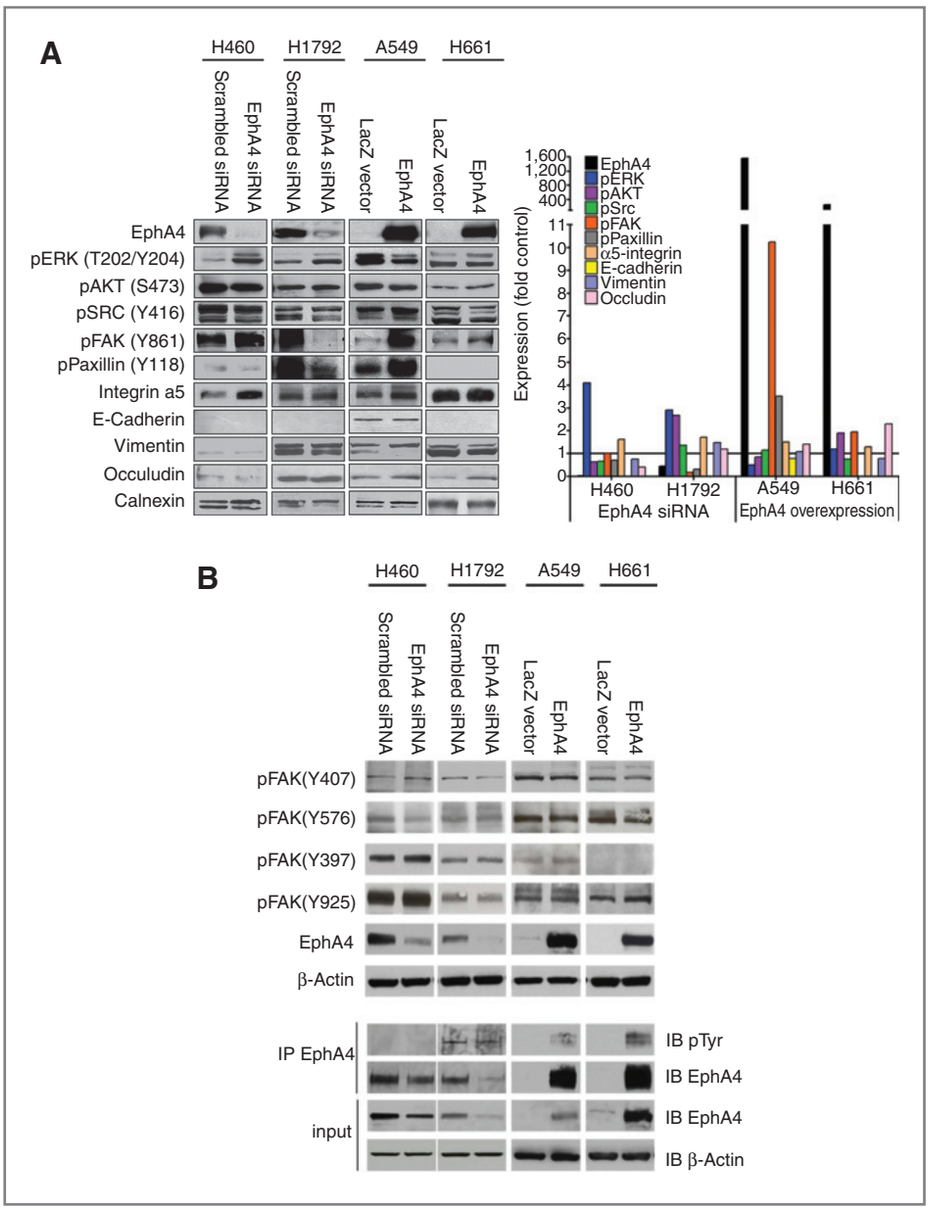

Figure 5. EphA4 increases occludin expression and decreases ERK activation. Following EphA4 knockdown (H460, H1792) or overexpression (A549, H661), the expressions of the noted proteins and phospho-proteins were measured using Western blotting. Quantitation of noted proteins is shown in A. IP, immunoprecipitation; IB, immunoblot.

that coexpression of EphA4 and EFNA1 in NSCLC cells inhibits cell migration and invasion.

Surprisingly, although increased expression of EphA4 was associated with a better outcome, EphA4 was consistently upregulated in cancer versus noncancer samples. We are confident that both of these results are accurate, as EphA4 mRNA levels were strongly correlated with protein levels. It is possible that EphA4 plays a dual role in tumorigenesis, switching from acting as a tumor promoter early in the process to functioning as a migration-inhibitory tumor suppressor in late-stage disease. Such a dual role has been demonstrated for transforming growth factor-β (47) and the process of autophagy. The interaction of EphA4 with the stroma *in vivo* might also explain this apparent conflicting result. In addition, the "normality" of HBECs and BEAS-2B cell lines is questionable as they have been immortalized with various retroviral expression vectors (48–50). This might affect the results observed for EphA4, as well as the general observation that Eph receptors and ephrins were upregulated in lung adenocarcinoma versus normal lung as opposed to the result *in vitro*. Many of the *in vitro* findings involving Eph receptors and ephrins remain to be shown *in vivo* (44). An Eph forward signaling inhibitory effect was initially hypothesized on the basis of the observation that Eph receptors have been reported to be highly expressed with a low level of tyrosine phosphorylation due to poor activation by ephrins (51, 52). In our study, Eph receptors did not seem to be expressed at higher

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levels compared with ephrins in general. However, our study was limited in that we studied mRNA as opposed to protein levels.

In our previous studies, we reported that EphA2 was associated with poor survival in lung adenocarcinoma (3, 5). In the present study, we did not observe any significant association between EphA2 mRNA levels and OS in 2 cohorts of patients. There are several possible explanations for these discordant results. First, the correlation between EphA2 mRNA levels by qPCR and array was lower than that with EphA4. Second, the strong correlation of EphA4 mRNA levels with protein levels might be different in the case of EphA2. Third, we only included lung adenocarcinoma in our study, whereas both adenocarcinoma and squamous cell carcinoma were included in our previous studies of EphA2. Finally, the HRs for EphA2 in our previous publication were 1.61 for progression-free survival and 1.66 after adjusting for patient age and disease stage, which are in the same range as that reported in the present study for EphA4. In any case, EphA2 is not a very potent prognostic factor, and that may underlie the lack of reproducibility from 1 study to another.

EphA2 mutations have been reported in 7% of squamous cell carcinomas and are associated with increased tumor invasion and survival (6). *EphA3* mutations have been reported in 10% of lung adenocarcinomas. In 188 lung adenocarcinomas, 37 mutations in 10 of the 13 Eph receptors sequenced were reported (25). Many of those mutations were in functional domains (ephrin-binding domain, kinase domain), strongly indicating a causal role in the oncogenic transformation that remains to be shown.

Our study has several limitations. First, the high level of correlation we observed between EphA4 protein and mRNA expression might not be the case for other Eph receptors and ephrins. Second, Eph receptors and ephrins are known for their redundancy, which cannot be appreciated in our study. It would be interesting to study whether some combinations of Eph receptors and ephrins affect survival more than individual genes. Large-scale RNA interference screens might be useful to improve our understanding of the complex interactions among all these different genes and their impact on the tumor phenotype. Third, we only included lung adenocarcinoma in our study; the role of Eph receptors and ephrins in squamous cell carcinoma remains to be studied.

Given the complexity of the interactions and the redundancy among different Eph receptors and ephrins, our study represents an initial step toward improving our understanding of the role of these families in lung cancer. Future studies will need to integrate genetic and epigenetic analyses, as well as high-throughput functional studies, to fully appreciate the complex role of this family in lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Saintigny, B. Sen, F. M. Johnson
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Analysis and interpretation of data: P. Saintigny, S. Peng, L. Zhang, B. Sen, L. Girard, F. M. Johnson
Writing, review, and/or revision of the manuscript: P. Saintigny, B. Sen, S. M. Lippman, L. Girard, J. D. Minna, F. M. Johnson
Administrative, technical, or material support: P. Saintigny, S. Peng, B. Sen
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Molecular Cancer Therapeutics

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