EphA2 Mutation in Lung Squamous Cell Carcinoma Promotes Increased Cell Survival, Cell Invasion, Focal Adhesions, and Mammalian Target of Rapamycin Activation*

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Non-small cell lung cancer (NSCLC) has a poor prognosis and improved therapies are needed. Expression of EphA2 is increased in NSCLC metastases. In this study, we investigated EphA2 mutations in NSCLC and examined molecular pathways involved in NSCLC. Tumor and cell line DNA was sequenced. One EphA2 mutation was modeled by expression in BEAS2B cells, and functional and biochemical studies were conducted. A G391R mutation was detected in H2170 and 2/28 squamous cell carcinoma patient samples. EphA2 G391R caused constitutive activation of EphA2 with increased phosphorylation of Src, cortactin, and p130Cas. Wild-type (WT) and G391R cells had 20 and 40% increased invasiveness; this was attenuated with knockdown of Src, cortactin, or p130Cas. WT and G391R cells demonstrated a 70% increase in focal adhesion area. Mammalian target of rapamycin (mTOR) phosphorylation was increased in G391R cells with increased survival (55%) compared with WT (30%) and had increased sensitivity to rapamycin. A recurrent EphA2 mutation is present in lung squamous cell carcinoma and increases tumor invasion and survival through activation of focal adhesions and actin cytoskeletal regulatory proteins as well as mTOR. Further study of EphA2 as a therapeutic target is warranted.

Lung cancer is the leading cause of cancer death in the United States (1) and is a growing health epidemic worldwide (2). Metastatic lung cancer is virtually incurable. Even when lung cancer is detected early and aggressive intervention is undertaken, its outcome remains disappointing. Patients with completely resected Stage I non-small cell lung cancer (NSCLC)2 have a 5-year overall survival of 55–70% (3–5). Locoregional and, more commonly, distant recurrence limits the curative potential of surgery.

The Eph receptor family represents the largest group of receptor tyrosine kinases (6). The Eph receptors are divided into EphA and EphB based on structural homology and ligand-binding affinity. In all, there are 16 Eph receptors (Eph A1–A10 and B1–B6), but humans lack EphA9 and EphB5 (7). The ligands for the Eph receptors are ephrins (7). The ephrins fall into two subclasses, ephrin-A and -B, based on their mode of membrane attachment and receptor affinity. The physiologic role of Eph receptors is crucial in embryonic developmental processes, such as cell migration, vascular development, tissue-border formation, axonal and synaptic network development, and adult processes such as regulation of neuronal plasticity (7). A peculiarity of the Eph-ephrin system is that signals are transduced by both the receptor (forward signaling) and ligand (reverse signaling) (7).

EphA2 is overexpressed in glioblastoma (8), breast (9, 10), colon (9, 11), ovarian (12), pancreatic (13), and prostate (14) cancer. EphA2 is overexpressed (staining intensity 2+/3+) in 70% of NSCLC (15). This receptor promotes cell proliferation (16), motility (16, 17), invasion (8), metastasis (18, 19), and angiogenesis (20) in malignant tumors. Furthermore, somatic mutations in the Eph (EphA3 and -A5) family have been previously described in lung cancer; however, mutations in EphA2 have not been described (21). The presence and role of such mutations remain undefined.

In an initial study, Harpole and coworkers (15) showed that EphA2 is expressed in lung cancer tumor tissues. In particular, the overexpression of EphA2 correlated with metastasis to the brain. Overexpression of EphA2 did not correlate with histologic type or tumor differentiation but strongly predicted survival and disease recurrence. Furthermore, EphA2 expression in matched sets of primary lung tumor and brain metastases from the same patients was significantly higher in the metastatic lesions (15). EphA2 expression has also been found to be positively correlated with smoking history and with poorer prognosis in lung cancer patients (22). These studies suggest that EphA2 regulates cellular behavior that promotes a metastatic phenotype in lung cancer. In this study, we have systematically studied the expression, mutation, and biological functions of EphA2 in lung cancer.

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‡The abbreviations used are: NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; WT, wild type; PBS, phosphate-buffered saline; mTOR, mammalian target of rapamycin.
EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—NSCLC and BEAS-2B cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were cultured with RPMI media supplemented with 10% fetal bovine serum at 37 °C with 5% CO2. All histologic subtypes of NSCLC were evaluated: squamous cell carcinoma (H226 and H2170), adenosquamous carcinoma (A549 and H1703), adenocarcinoma (H1838, H1975, H522, H1993, SKLU-1, and H1437), bronchoalveolar carcinoma (H358 and SW1573), large cell carcinoma (H661), and the non-cancerous human lung epithelial cell line BEAS-2B.

Mutational Analysis—Lung cancer tumor samples were collected with informed consent and in conformation with institutional guidelines. Genomic DNA from cell lines and tumor tissue samples were prepared by using the QIagen DNA Mini-kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA from 13 lung cancer cell lines (H226, H2170, A549, H1703, H1838, H1975, H522, H1993, SKLU-1, H1437, H358, SW1573, and H661) and 52 NSCLC tumors were PCR-amplified and sequenced.

Mutational screening for the coding regions of the EphA2 gene from Exons 2–16 was performed with standard PCR and direct DNA sequencing. Sequencing was done with an Applied Biosystems 3730XL capillary sequencer at the University of Chicago DNA Core Facility. The proofreading Pfu polymerase was used (Stratagene, La Jolla, CA). All PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH) was mixed with an equal volume of 2 M okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 250 mM N-acetyl-L-cysteine, and 10 mM β-mercaptoethanol (Cell BioLabs Inc., San Diego, CA) was mixed with an equal volume of 2 M okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, Complete™ protease inhibitor tablet). The samples were then subjected to SDS-PAGE in 4–15% polyacrylamide gels, transferred onto Immobilon™ membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences). In some cases, a standardized average gray value was generated for immunoreactive protein bands of interest for graphical quantitation.

For EphA2 stimulation experiments, subconfluent BEAS-2B cells were grown for 24 h in serum-free RPMI media. Cells were stimulated with serum-free RPMI media containing 1 µg/ml Ephrin-A1-Fc for 15 min. At the designated time point, cells were harvested according to the protocol described above and separated by SDS-PAGE under reducing conditions.

The following antibodies were used for immunoblotting: anti-EphA2, anti-phospho-cortactin (Tyr421), and anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-EphA2 antibody (Tyr994, Cell Applications, Inc.); anti-p130Cas and anti-phospho-p130Cas (either tyrosines 165, 249, or 410), anti-phospho-Src (Tyr416), anti-mTOR, anti-phospho-mTOR (serines 2448 and 2481), anti-p70 S6 kinase, anti-phospho-p70 S6 kinase (Thr389), and anti-vinculin antibody (Cell Signaling Technology, Danvers, MA).

Transfection of siRNA against EphA2, Src, Cortactin, and p130Cas—Individual siRNAs were purchased from either Santa Cruz Biotechnology (siRNA#1) or Thermo Scientific (ON-TARGETplus, siRNA#2). BEAS-2B cells (with or without pCDNA6.2 empty vector, EphA2 WT, or EphA2 G391R mutant expression) were transfected with siRNA using siPORTamine™ as the transfection reagent (Ambion, Austin, TX) according to the protocol provided by Ambion. Cells (~40% confluent) were serum-starved for 1 h followed by incubation with 250 nM of target siRNA (or scrambled siRNA or no siRNA) for 6 h in serum-free media. Serum-containing media was then added (10% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

Overexpression of EphA2 WT and EphA2 Fibronectin III Domain (G391R) Mutant—For the purpose of overexpressing EphA2 in the BEAS-2B cell line, which is a human immortalized bronchial epithelial cell line, we produced a plasmid containing the EphA2 open reading frame by TOPO-cloning methodology (Invitrogen). After obtaining human EphA2 cDNA (Origene), it was PCR-amplified and cloned into the pCDNA6.2 vector (Invitrogen), which contains a C-terminal V5 tag as well as antibiotic resistance genes for selection of stable transfectants in mammalian cells. All vectors were sequenced to confirm proper cloning and the absence of artificially introduced mutations. The resulting plasmids were transfected into BEAS-2B cells using Fugene HD (Roche Life Sciences), and cultured in the presence of Blasticidin for more than 2 weeks to select only plasmid-containing cells. In parallel experiments, we created an EphA2 mutant construct containing the G391R identified in the H2170 squamous cell carcinoma cell line.

Soft Agar Colony Formation Assay—Soft agar assays to monitor in vitro anchorage-independent tumor cell growth were performed as previously described (15). Briefly, a 2× agar solution (Cell BioLabs Inc., San Diego, CA) was mixed with an equal volume of 2× Dulbecco’s modified Eagle’s medium and 20% fetal bovine serum at 37 °C. A cell solution was prepared containing (80,000 cells/ml) for each cell line (BEAS-2B control, empty vector, EphA2 WT, and EphA2 G391R). The agar/Dulbecco’s modified Eagle’s medium solution was then mixed with the cell solution in a ratio of 2:1, and 75 µl was added to each well of a 96-well plate pre-coated with 25 µl of the agar/Dulbecco’s modified Eagle’s medium solution (2,000 cells/well final concentration). Once the agar solidified, 100 µl of culture medium was added to each well. Culture media was changed every 4 days. Cells were allowed to grow for 2 weeks until colonies were visible. Colony pictures were taken using QImaging IEEE 1394 FireWire™ Digital CCD Camera, and cell colonies were counted manually. Each assay is set up in triplicate.
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Expression of EphA2 in Lung Cancer Tumor Tissue—We examined EphA2 expression in tumor samples. Formalin-fixed, paraffin-embedded specimens were obtained from a retrospective cohort of 105 NSCLC and 109 corresponding normal specimens. Adenocarcinomas accounted for the most common histology \((n = 46)\), followed by large cell carcinoma \((n = 35)\) and squamous cell carcinoma \((n = 24)\). Staining for EphA2 was specific for the carcinoma component of the tumor, with minimal staining of surrounding connective tissues (Fig. 1A). The staining pattern of the specimens was uniform, with prominent membranous and cytoplasmic staining. Immunohistochemical analysis of EphA2 on the tumor microarray showed that expression in tumors was significantly higher than in normal adjacent tissues (Fig. 1, A and B). All subtypes of NSCLC demonstrated relatively similar rates of EphA2 overexpression (Fig. 1B).

Overall survival was estimated for 94 patients stratified by EphA2 expression above or below average (Fig. 1C). Survival for patients with high EphA2 tumor expression was shorter than that of patients whose tumors had lower expression (16.4 months versus 53.2 months, \(p = 0.053\), log-rank test). When patients were stratified by histologic subtype, those with squamous cell carcinoma were found to have the poorest overall survival (squamous cell carcinoma = 18.0 months; adenosquamous cell carcinoma = 55.2 months; large cell carcinoma = 54.7 months; \(p = 0.027\), log-rank test; Fig. 1C).

The expression of EphA2 in lung cancer cell lines was determined using standard immunoblotting with an anti-human polyclonal EphA2 antibody (Fig. 1D). Robust expression of EphA2 was identified in 9 of 13 NSCLC cell lines. Expression of EphA2 was also seen in the BEAS-2B cell line, an immortalized bronchial epithelial cell line (data not shown).

Mutational Analysis of EphA2 in Cell Lines and Lung Cancer Tumor Tissues—As mutations have been identified for several receptor tyrosine kinases in lung cancer, the EphA2 gene was analyzed for mutations in NSCLC initially in 13 lung cancer cell lines. Mutations were identified in 4 of 13 cell lines (30%) and in 5 of 45 primary lung cancers (11%). All mutations were missense, and none were recurrent. All of the mutations were identified in NSCLC cell lines. A single mutation was identified in each of the 4 cell lines. Two mutations were identified in each of 3 tumor samples. A cell line and a tumor sample both contained a mutation at amino acid 577, which corresponds to residue 552 in the published EphA2 sequence (GenBank accession number X90185).

The EphA2 gene was amplified by PCR and sequenced using standard methods. The wild-type EphA2 sequence and the mutant sequences were compared with published sequences to determine the amino acid changes caused by the mutations. The mutations were all missense, and none were recurrent. Two of the mutations were found in primary lung cancer samples and 2 were found in NSCLC cell lines (Table I).
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A.

EphA2 IHC

Normal Lung | Adeno | Squamous | Large Cell

B.

EphA2 Immunohistochemical Staining in NSCLC

C.

All Histologies

D.

1. Anti-EphA2

2. Anti-Actin

TABLE 1

Tumor histology and frequency of G391R mutation

| Tumor histology            | No. samples | No. samples with G391R |
|----------------------------|-------------|------------------------|
| Squamous cell carcinoma    | 28          | 2                      |
| Adenocarcinoma             | 16          | 0                      |
| Large cell carcinoma       | 8           | 0                      |
| Small cell carcinoma       | 8           | 0                      |
| Lung cancer cell lines     | 13          | 1                      |
| Total samples              | 71          | 3                      |

FIGURE 1. EphA2 expression and correlation with survival. A and B, immunohistochemical analysis of EphA2 staining in patient tumor tissue sections. C, Kaplan-Maier analysis of EphA2 expression versus survival. Left panel: patients stratified by EphA2 expression levels, where low and high indicate expression relative to mean expression. Right panel: patients stratified by lung cancer histology subtypes. D, immunoblot analysis of EphA2 expression in various cell lines. Lysates from various cell lines were obtained, run on SDS-PAGE, and immunooblotted with either anti-EphA2 (1) or anti-actin (2) antibody. The cell lines are: A549, SK-LU-1, H1703, H358, H1993, H661, SW1573, H522, H226, H1437, H1838, H1975, and H2170.

We identified one mutation, G391R, within the first fibronectin III domain of EphA2 in the H2170 squamous cell lung carcinoma cell line. Further, we evaluated the EphA2 mutations in lung tumor tissues. We examined EphA2 mutations in 28 squamous cell carcinomas, 16 adenocarcinomas, and 8 large cell carcinomas. We found the G391R mutation in 2 of 28 screened squamous cell carcinoma samples and none in any other lung cancer subtypes (Table 1). It is to be noted, based on the complete sequencing of all EphA2 exons in cell lines, that mutations only occurred in exon 5. Each mutation was confirmed with a second PCR and sequenced bidirectionally.

The Role of WT and G391R EphA2 on Anchorage-independent Growth—We next transfected non-cancerous BEAS-2B cells with EphA2 WT and EphA2 G391R overexpression vectors. Fig. 2A indicates that we can successfully express EphA2 proteins in similar quantities in BEAS-2B cells. Because in vitro anchorage-independent growth is often correlated with in vivo oncogenic potential, we performed soft agar growth assays and observed increased colony formation with EphA2 WT and
G391R overexpression compared with BEAS-2B control or empty vector (Fig. 2B). In addition, the EphA2 G391R had a statistically significant increase in colony formation compared with WT (Fig. 2C).

The Role of WT and G391R EphA2 on Focal Adhesion Dynamics—EphA2 has been implicated in regulating actin cytoskeleton and focal adhesion dynamics (23). We therefore examined the role of EphA2 WT and G391R on focal adhesions in BEAS-2B cells. Fig. 3 (A and B) indicates that overexpression of either WT or G391R mutant EphA2 caused an increased number of focal adhesions (as measured by vinculin immunocytochemistry) that were larger in size compared with untransfected BEAS-2B cells. Phenotypically, the EphA2 WT-overexpressing cells tended to be more elongated and contain invadopodia, suggesting a more malignant cellular behavior (Fig. 3A). In addition, cell size tended to be increased in these EphA2-overexpressing cells.

EphA2 G391R Leads to Increased Cellular Invasion and Phosphorylation of p130Cas—Based on phenotypic changes we observed with EphA2 WT- and EphA2 G391R mutant-overexpressing cells suggesting a more malignant cellular behavior (see Fig. 3A), we examined the role of EphA2 in cell invasion through basement membrane components. Fig. 4A indicates that overexpression of EphA2 WT increases cellular invasion by ~20% while overexpression of EphA2 G391R increases invasion by ~50%. There is a statistically significant increase in EphA2 G391R versus EphA2 WT invasive properties. In addition, silencing (siRNA) EphA2 expression in BEAS-2B cells inhibited basal invasion by ~50%.

We further evaluated the biochemical aspects of EphA2 overexpression and its relation to proteins known to regulate tumor cell invasion (24, 25). For that purpose, we used ephrin-A1 as an EphA2 ligand to stimulate kinase activity (Fig.
FIGURE 4. EphA2 and EphA2 G391R mutation promote invasion through activation of Src, cortactin, and p130Cas. A, graphical representation of the percent invasion (y axis) of either control, EphA2 WT, or EphA2 G391R mutant BEAS-2B cells as determined by using the QCM ECMatrix Cell Invasion Assay (Millipore), which measures cell invasion through a 8-μm pore filter covered with basement membrane components. Cells were added to the upper chamber in 0.2% medium, and 10% medium was added to the lower chamber. The cells were analyzed for invasion through basement membrane into the bottom chamber after 24 h. Statistical significance is indicated by bars above each experimental condition; n = 5 per condition. B and C, Immunoblotting of BEAS-2B cell lines (control, empty vector (EV), EphA2 wild-type (WT), EphA2 G391R mutation, with and without Ephrin-A1 (1 μg/ml)) and its effect on downstream signaling. B, cell lysates from Ephrin-A1-treatment (0, 5, and 15 min) were obtained, run on SDS-PAGE, and immunoblotted with anti-EphA2, anti-phospho-EphA2 (pTyr594) or anti-actin antibody. C, cell lysates from Ephrin-A1-treatment (0 and 15 min) were obtained, run on SDS-PAGE, and immunoblotted with anti-phospho-Src (pY418), anti-phospho-cortactin (pY421), anti-phospho-p130Cas, pY249, pY410, anti-EphA2, or anti-actin antibody. D, graphical representation of the ratio of phospho-protein immunoreactivity versus total EphA2 immunoreactivity from experiments described in C. Standardized average gray values for immunoreactive bands of total EphA2, pY418Src, pY421Cortactin, pY165p130Cas, pY249p130Cas, and pY410p130Cas, were obtained from BEAS-2B control, empty vector (EV), EphA2 wild-type (WT), and EphA2 G391R mutant cells treated with Ephrin-A1 for 0 or 15 min, and ratios were calculated. The asterisks indicate a statistically significant difference (p < 0.05) between untreated and Ephrin-A1-treated cells; n = 3 per condition.
and p130Cas revealed that EphA2 overexpression leads these molecules to become activated (phosphorylated) in a ligand-independent fashion (Fig. 4). While knockdown of Src and cortactin significantly reduced cellular invasiveness shown in the functional studies above. This mechanism could be responsible for the increased cellular invasiveness shown in the functional studies above.

We disrupted downstream signaling pathways with two different siRNAs against Src, cortactin, and p130Cas and examined downstream signaling with immunoblot analysis of BEAS-2B cell lysates indicating complete knockdown of Src, cortactin, and p130Cas protein expression with siRNA treatment. BEAS-2B cells were transfected with siRNA (Santa Cruz Biotechnology) using siPORTamine™ as the transfection reagent (Ambion). Cells (~40% confluent) were serum-starved for 1 h followed by incubation with 250 nM of target siRNA (or scramble siRNA or no siRNA) for 6 h in serum-free media. The serum-containing media was then added (10% serum final concentration) for 42 h. Cell lysates were obtained, run on SDS-PAGE, and immunoblotted with anti-Src (1), anti-cortactin (2), anti-p130Cas (3), or anti-actin antibody. B, graphical representation of invasion assay results with WT-EphA2 and G391R-EphA2 cells lines showing significant difference in inhibition of invasion between the groups in cells transfected with siRNAs against p130Cas. Knockdown of Src and cortactin significantly reduced cellular invasion by ~80% with similar effects in WT and G391R cells. However, with p130Cas silencing, the G391R had significantly less (p < 0.05) invasive function compared with the WT-EphA2-overexpressing cells, siRNA#1 refers to siRNA purchased from Santa Cruz Biotechnology, siRNA#2 refers to siRNA purchased from Thermo Scientific; n = 5 per condition.

4B) and examined downstream signaling with immunoblotting (Fig. 4C). Evaluation of phosphorylated Src, cortactin, and p130Cas revealed that EphA2 overexpression leads these molecules to become activated (phosphorylated) in a ligand-independent fashion (Fig. 4C). Furthermore, EphA2 G391R was shown to cause significantly stronger activation of the mTOR pathway than its wild-type counterpart even with normalization for total EphA2 expression (Fig. 6, A and B).

Activation of mTOR and p70 S6 kinase can regulate cell survival (27, 28). Therefore, we examined the role of EphA2 overexpression on cell survival and discovered that EphA2-overexpressing cells displayed significantly increased survival rates. Furthermore, EphA2 G391R-expressing cells had even higher survival rates when compared with EphA2 WT-expressing cells. In the presence of Fas ligand, an apoptosis-inducing molecule, although untransfected, empty vector, and EphA2 WT-transfected BEAS-2B cells showed decreased cell survival due to increased apoptotic events, EphA2 G391R-transfected cells showed minimal cell death despite the pro-apoptotic signal (Fig. 6C). In addition, we observed the EphA2 G391R transfectants also had increased sensitivity to rapamycin (Fig. 7), demonstrating the central role of the mTOR pathway for EphA2 G391 mutant oncogenic activities.

**DISCUSSION**

In this study, we sought to describe the expression and functional significance of EphA2 and EphA2 mutations in cancer cell lines and NSCLC tumor tissue arrays. Our data indicate that EphA2 is specifically overexpressed in human NSCLC cell lines and tumor samples. Patients whose tumors overexpress EphA2 tended to have a worse prognosis. We identified one EphA2 mutation, G391R in the H2170 squamous cell cancer cell line, and two silent mutations in the SW900 cell line (A216A and T784T), and further confirmed the presence of this mutation in samples from patients exhibiting NSCLC with squamous histology. We have demonstrated that overexpression of EphA2 increases cellular invasion and that the
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A.

| Ephrin-A1 (15') | BEAS-2B | EphA2 WT | EphA2 G391R |
|----------------|---------|----------|-------------|
| 1. Anti-pSer2448mTOR | - | + | - |
| 2. Anti-pSer2481mTOR | - | + | - |
| 3. Anti-mTOR | - | + | - |
| 4. Anti-pThr389 p70 S6K | - | + | - |
| 5. Anti-p70 S6 Kinase | - | + | - |
| 6. Anti-Vinculin | - | + | - |
| 7. Anti-EphA2 | - | + | - |
| 8. Anti-Actin | - | + | - |

BEAS-2B Cell Lysates

B.

| Ephrin-A1 | BEAS-2B | EphA2 WT | EphA2 G391R |
|-----------|---------|----------|-------------|
| C | - | + | - |
| EV | - | + | - |
| WT | - | + | - |
| G391R | - | + | - |

Ratio Phospho-Protein / Total EphA2

C.

% Fas Ligand-mediated Apoptosis

- BEAS-2B Control
- Empty Vector
- EphA2 WT Overexp.
- EphA2 G391R Overexp.
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G391R mutation further amplifies this phenomenon. Silencing (siRNA) Src, cortactin, or p130Cas inhibits EphA2-mediated invasion with the EphA2 G391R mutant being more sensitive to p130Cas expression. Both EphA2 WT and G391R also have increased area of focal adhesions, cell survival, and growth. Biochemically, the G391R mutation was shown to specifically increase the tyrosine phosphorylation of p130Cas (pY165 and pY249) and mTOR (pSer2448 and pSer2481). Cells harboring the G391R mutation are more susceptible to treatment with rapamycin, an mTOR inhibitor.

An important consideration of EphA2 as a potential molecular target is that EphA2 is detected at very low levels in normal lung tissues while expressed abundantly in tumors. It is clear that EphA2 is highly expressed in more than half of NSCLC and only at low levels in normal lung tissue. Our data, in contrast to Kinch et al. (15), did not show that EphA2 expression was predictive of survival. There could be multiple reasons for this difference. Of note, our series had a larger number of tumor tissues from patients with Stage IV NSCLC (24 patients versus 4 patients), and the various NSCLC stages in our series are more equitably represented, allowing for better inter-stage comparisons.

Consistent with the known function of EphA2, we showed that the invasive tumor edge and metastatic sites have higher EphA2 expression compared with the primary tumor. In our tumor-metastasis array, brain and lymph node metastatic sites demonstrated significantly higher rates of EphA2 overexpression compared with the primary tumor (91.4% versus 79.2%), but matched-pair analysis of primary tumors and lymph node metastases do not consistently show higher EphA2 expression in metastases compared primary tumors. Our results contrast those of Kinch et al. in that metastatic tumors to the brain showed higher EphA2 expression compared with the primary lung tumor. Our finding is further supported by the observations of Mudali et al. showing that pancreatic cancer metastases have a lower expression of EphA2 (29). The mechanism of EphA2 overexpression is undefined, but gene amplification is known to account for only a small subset in pancreatic cancer (29). Gene amplification of EphA2 in NSCLC is unknown and should be elucidated.

Mutations in EphA3 and EphA5 have been described in NSCLC (21). To our knowledge, we are the first group to demonstrate the presence of a mutation in EphA2 in NSCLC. The G391R mutation occurs in the fibronectin III domain of EphA2. The fibronectin III domain is a domain of ~100 amino acids, different tandem repeats of which contain binding sites for DNA, heparin, and the cell surface (30). This mutation is hypothesized to alter the adhesion of lung cancer cells to its microenvironment. The exact nature of this missense mutation and other Eph family mutations will need to be explored further, but our structural data indicate for a number of reasons that this mutation produces a structurally different protein product, a notion supported by our functional studies and clinical observations. First, a glycine to arginine amino acid change replaces a neutral, compact residue with a basic, bulky one. Second, the proximity of Arg391 and the adjacent His390 introduces the possibility of hydrogen bonding, leading to stabilization of the mutant protein structure; however, this hypothesis remains to be tested. Third, the position of the G391R mutation in the global fibronectin domain structure suggests that the Arg391 residue may play some role in aberrant interactions with other growth factors or aberrant homo- or heterodimerization with other Eph receptors; these manifestations could serve as the basis for the abnormal functions of EphA2 G391R suggested by our functional assays. Finally, the incidence of EphA2 mutation in NSCLC needs to be further defined to elucidate its clinical significance.

p130Cas is a docking protein containing multiple protein-protein interaction domains. The N-terminal SH3 domain may function as a molecular switch regulating its tyrosine phosphorylation, because it interacts with focal adhesion kinase (FAK) (31) and the FAK-related kinase PYK2 (32). Tyrosine phosphorylation of p130Cas has been implicated as a key signaling step in integrin control of normal cellular behaviors, including motility, proliferation, and survival (33). FAK and p130Cas

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**FIGURE 6.** The EphA2 G391R mutation increases cell survival through activation of the mTOR pathway. A, immunoblotting of BEAS-2B cell lines (controls, WT-EphA2, SNP (G391R)-EphA2), with and without ephrin-A1 (1 µg/ml) for 15 min and its effect on mTOR signaling. Cell lysates were obtained, run on SDS-PAGE and immunoblotted with anti-phospho-mTOR (pSer2448) or (pSer2481), anti-mTOR (5 M rapamycin concentrations), or anti-actin antibody. B, graphical representation of the ratio of phospho-protein immunoreactivity versus total total EphA2 immunoreactivity from experiments described in A. Standardized average gray values for immunoreactive bands of total EphA2, pSer2448mTOR, and pThr389p70S6K were obtained from BEAS-2B control, empty vector (EV), EphA2 wild-type (WT), and EphA2 G391R mutant cells treated with Ephrin-A1 for 0 or 15 min, and ratios were calculated. The asterisks indicate a statistically significant difference (p < 0.05) between untreated and Ephrin-A1-treated cells; n = 3 per condition. C, graphical representation of the percent survival (y axis) of control, empty vector (EV), EphA2 WT, or EphA2 G391R mutant BEAS-2B cells in serum-free media with or without 500 ng/ml Fas ligand treatment for 24 h. EphA2-overexpressing cells displayed significantly increased survival rates (p < 0.05). Furthermore, the mutant (G391R) EphA2 had even higher survival rate when compared with the WT-EphA2 cells (p < 0.05). Untransfected, EV, and EphA2 WT-transfected BEAS-2B cells showed decreased cell survival due to increased Fas ligand-mediated apoptotic events. However, EphA2 G391R-transfected cells showed minimal cell death despite the pro-apoptotic signal.

**FIGURE 7.** G391R enhances sensitivity to mTOR inhibition. Cell survival of BEAS-2B control, empty vector (EV), EphA2 wild-type (WT), or EphA2 G391R mutant-expressing cells in culture was assayed in the presence of varying rapamycin concentrations. Survival drops sharply with a 10⁻³ M (10 µM) rapamycin dose; this effect is more pronounced in cells harboring the G391R mutation. Statistical significance between WT and G391R cells is indicated for 10⁻³ M (1 µM) and 10⁻⁵ (10 µM) rapamycin concentrations.
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are known downstream effectors of activated EphA2. Ephrin-A1 stimulation of EphA2 induces cell adhesion and actin cytoskeletal changes in fibroblasts in a FAK- and p130Cas-dependent manner (23). In H358 NSCLC cells, ephrin-A1 stimulation results in increased phosphorylation of p130Cas, but paradoxical to fibroblasts, NSCLC cell adhesion to vitronectin and motility are reduced. A similar observation was made in PC3 prostate cancer cells (34). This paradox may represent a characteristic aberrant function of EphA2 in malignant cells.

With EphA2 stimulation, NSCLC cells tend to be more motile and less adherent. This corresponds to the metastatic and invasive nature of malignant tumors and is consistent with our data indicating that silencing of the non-receptor tyrosine kinase Src and the actin cytoskeletal regulator cortactin inhibited EphA2-mediated cellular invasion. To elucidate this relationship clinically, we analyzed matched primary tumors and metastatic lymph nodes for EphA2 overexpression. However, the level of EphA2 phosphorylation did not correspond to EphA2 overexpression, and efforts to elucidate this relationship are hampered by the fact that there is no phospho-EphA2 antibody available to date. Future efforts will be focused on evaluation of the difference between activated EphA2 in metastatic versus primary tumors. More importantly, knockdown of EphA2 was feasible in BEAS-2B cells. Similar findings have been reported in pancreatic (13) and ovarian cancers (35). The absence of EphA2 resulted in a reduction of cell invasion, which has been reported in pancreatic (13) and ovarian cancers (35). The EphA2 overexpression, and efforts to elucidate this relationship were hampered by the fact that there is no phospho-EphA2 antibody available to date. Future efforts will be focused on evaluation of the difference between activated EphA2 in metastatic tumors.

The G391R mutation in EphA2 was shown by us to increase the phosphorylation of two serine residues (pSer2448 and pSer2481) within mTOR. mTOR has been identified as an important therapeutic target in cancer, with several compounds being actively investigated in clinical settings, such as rapamycin, everolimus, and temsirolimus (39). mTOR has been extensively studied as a molecular therapeutic target, with several compounds being actively investigated in clinical settings, such as rapamycin, everolimus, and temsirolimus (39). mTOR inhibition may be clinically effective in NSCLC, and several clinical trials are currently ongoing. In our study, the cells containing the EphA2 G391R mutation were shown to have increased sensitivity to rapamycin, indicating that mTOR inhibition might be more efficacious against tumors with this specific mutation.

In summary, we showed that EphA2 is overexpressed in lung cancer cells. EphA2 and the EphA2 G391R mutation seem to be functionally important in regulating cellular proliferation and invasion due, in part, to downstream effects on p130Cas and mTOR. EphA2 represents a novel molecule potentially involved in progression of lung cancer and may be a useful molecular therapeutic target.

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