Intratumoral Epiregulin Is a Marker of Advanced Disease in Non–Small Cell Lung Cancer Patients and Confers Invasive Properties on EGFR-Mutant Cells

Jie Zhang, Kentaro Iwanaga, Kuicheon C. Choi, Marie Wislez, Maria Gabriela Raso, Wei Wei, Ignacio I. Wistuba and Jonathan M. Kurie

Abstract: Non–small cell lung cancer (NSCLC) cells with activating epidermal growth factor receptor (EGFR) somatic mutations have unique biological properties, including high expression of the ErbB ligand epiregulin; however, the biological role of epiregulin in these cells has not been elucidated. To examine its role, we used an immunohistochemical approach to detect epiregulin expression in NSCLC biopsy samples and pharmacologic and genetic approaches to inhibit epiregulin in cultured NSCLC cells. In NSCLC biopsy samples, epiregulin was detected in 237 of 366 (64.7%) tumors, which correlated with nodal metastasis and a shorter duration of survival. In EGFR–mutant NSCLC cell lines, treatment with a small-molecule EGFR tyrosine kinase inhibitor diminished mRNA levels of the gene encoding epiregulin (EREG). The ability of EGFR–mutant NSCLC cells to invade through Matrigel in vitro was inhibited by treatment with an anti-epiregulin neutralizing antibody or by transfection with an EREG short hairpin RNA. Collectively, these findings show that epiregulin expression correlated with advanced disease, was EGFR dependent, and conferred invasive properties on NSCLC cells. Additional studies are warranted in NSCLC patients to evaluate whether epiregulin expression predicts the metastatic potential of primary tumors and whether anti-epiregulin treatment strategies are efficacious in the prevention of metastasis.

Treatments with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors lead to rapid and sustained tumor shrinkage in a subset of patients with non–small cell lung cancer (NSCLC; refs. 1–3). The tumor cells in these patients have somatic mutations in the EGFR kinase domain that constitutively activate EGFR (1–3). Mouse models constructed to investigate the oncogenicity of mutant EGFR develop invasive lung adenocarcinomas that regress after treatment of the mice with EGFR tyrosine kinase inhibitors (4, 5). Similarly, immortalized human bronchial epithelial cells acquire malignant properties after transfection with mutant EGFR (6). Treatment with EGFR tyrosine kinase inhibitors induces apoptosis of these EGFR-transfected cells and NSCLC cells with somatic mutations in EGFR (7, 8). Thus, evidence from human, murine, and cellular models indicates that mutant EGFR is oncogenic and confers EGFR dependence on NSCLC for cell survival.

Some of the downstream mediators of mutant EGFR that confer oncogenic properties on NSCLC cells have been identified. For example, EGFR forms a heterodimeric complex with ErbB3, which binds to and directly activates phosphatidylinositol 3-kinase and maintains cell survival through AKT-dependent mechanisms (9, 10). Other prosurvival signals in EGFR–mutant NSCLC cells are mediated through Src family kinases, which are constitutively activated (11, 12), and by the proapoptotic BH3-only BCL2 family member BIM, which is transcriptionally suppressed (13–16). Anchorage-independent growth of these cells is maintained by a different set of mediators, including cyclooxygenase-2, EphA2 receptor tyrosine kinase, and the EphA2 ligand ephrin A1 (17). Thus, mutant EGFR regulates the expression and activity of proteins involved in diverse cellular functions that together promote cellular transformation.

To date, there are few reports on downstream mediators of mutant EGFR that promote NSCLC metastasis. In breast cancer models, metastatic properties are conferred by a genetic signature encoding proteins involved in a broad range of cellular functions, including, among others, genes encoding the ErbB ligand epiregulin, cyclooxygenase-2, the chemokine CXCL1, and the metalloproteinase matrix metalloproteinase 1 (18). Epiregulin is a broad-specificity ErbB ligand and is one of several ErbB ligands that are highly expressed in EGFR–mutant NSCLC cells (19). In this study, we hypothesized that...
epiregulin confers metastatic potential on NSCLC cells and tested this hypothesis in vitro by carrying out studies on NSCLC biopsy samples and in vitro by using pharmacologic and genetic approaches to inhibit epiregulin in NSCLC cell lines.

Materials and Methods

Reagents

NSCLC cell lines were passaged in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO2. We purchased nonradioactive kinase assay kits for p38 (Cell Signaling Technologies, Inc.). We obtained small-molecule inhibitors of EGFR (gefitinib, AstraZeneca), mammalian target of rapamycin (CCI-779, Wyeth-Ayerst), mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (CI-1040, Pfizer Pharmaceuticals), c-jun NH2-terminal kinase (SP600125, Celgene Pharmaceuticals), and AKT (A838050.3, Abbott Pharmaceutical Products).

We purchased an antihuman epiregulin antibody for immunohistochemical and neutralization studies from R&D Systems; secondary antibody for fluorescent detection (murine IgG TRITC) from Sigma Chemicals; anti-total EGFR antibody for Western blotting from NeoMarkers; anti-phospho-EGFR (Y1068), anti-phospho-S6 (S236/235), anti-phospho-extracellular signal-regulated kinase (T202/Y204), anti-phospho-c-Jun (S63), anti-phospho-glycogen synthase kinase-3α/β (S21/9), anti-total S6, anti-total extracellular signal-regulated kinase-1/2, anti-total c-Jun, and anti-total glycogen synthase kinase-3β antibodies from Cell Signaling Technologies; and anti-Flag and anti-β-actin antibodies from Sigma-Aldrich.

Semiquantitative reverse transcription-PCR assays

Total RNA was prepared from cell lines using the TRIzol reagent protocol (Invitrogen). RNA (1 μg) was reverse transcribed and then subjected to PCR with the following epiregulin-specific primers: 5′-TGCCGGATGAACTTCTTGGT-3′ and 5′-TGTTTGCATGGACAGTGCATC-3′. The level of mRNA for each gene was measured with SYBR Green-based real-time PCR. The primers used for real-time PCR were designed by using Primer Express (Applied Biosystems). The EREG primer sequences used were 5′-TGTGGTATGACGATCAGTGATC-3′ and 5′-GCGATGATTTTGGATACATGGG-3′, and the L32 ribosomal protein primers were 5′-CCCTGTTGAGGCGGATCGT-3′ and 5′-TGCCGGATGACGATCAGTGATC-3′. Each cDNA sample (7 μL) was amplified by using SYBR Green PCR Master Mix according to the manufacturer’s instructions. The PCR products and their dissociation curves were detected with the 7500 Fast Real-time PCR System (Applied Biosystems). The level of the housekeeping gene ribosomal protein L32 in each sample was used as an internal control.

In vitro assays of cell proliferation and invasion

Cell proliferation was measured using the WST-1 [4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzenedisulfonyl] colorimetric dye reduction method (Roche). Cells (2.5 × 104) were subjected to PCR with the following epiregulin-specific primers: 5′-TGCCGGATGAACTTCTTGGT-3′ and 5′-TGTTTGCATGGACAGTGCATC-3′. The level of mRNA for each gene was measured with SYBR Green-based real-time PCR. The primers used for real-time PCR were designed by using Primer Express (Applied Biosystems). The level of the housekeeping gene ribosomal protein L32 in each sample was used as an internal control.

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Tissue microarrays of NSCLC biopsy specimens

Tissue microarrays were constructed from formalin-fixed, paraffin-embedded blocks using triplicate core samples from each tumor, as previously described (19).

Epiregulin immunochemistry

For immunohistochemical analysis, 4-μm tissue sections were deparaffinized, rehydrated, and washed with PBS. Antigens were retrieved by heating the slides in a steamer for 30 min. Samples were blocked for endogenous activity in 3% hydrogen peroxide/PBS, avidin/biotin solution (Zymed Laboratories), and DAKO serum-free protein block (DakoCytomation) and then incubated with monoclonal goat anti-human epiregulin antibody (R&D Systems) overnight at 4°C. After rinsing, the slides were incubated with a biotinylated link antibody and streptavidin peroxidase. For negative controls, the primary antibody was preincubated with recombinant epiregulin (R&D Systems). Staining intensity and extension were quantified by one investigator (M.G.R.) who was blinded to patient identity. Tissues were visualized at ×20 magnification for scoring.

EREGL short hairpin RNA transfection studies

pSM2 retroviral scrambled short hairpin RNA (shRNA) control and human Ereg shRNAmir clones were purchased from Open Biosystems. The four EREG-specific shRNAmir sequences used included EREG-1, TAAGAATCCGCTAAAGCCA; EREG-2, TATGCATTATGTTGTGT; EREG-3, TTACCGTCAAGATAATTCCGG; and EREG-4, TTGATACTACGTGTTACCCA. FTI-767 fibroblasts (Becton Dickson) were transfected with control or an EREG-specific shRNAmir construct (1, 2, 3, or 4) and selected for 21 d in 2 μg/mL puromycin to generate mass populations of retrovirus-producing cells. Supernatants from PT67 cells were concentrated using a 0.45-μm polyvinylidene difluoride filter. HCC827 cells were exposed to retrovirus in the presence of 5 μg/mL polybrene (Sigma) overnight. The media were then replaced with fresh media, and the transfectants were incubated overnight. This transfection process was repeated four times to increase the transfection efficiency. Mass populations of transiently transfected cells were used in the experiments.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

HCC827 cells were infected with virus supernatant of shRNA scrambled control, sh-Ereg-1, or sh-Ereg-3. After four cycles of virus infection, cells were plated in 100-mm dishes and grown for 3 d. The adherent and floating cells were then pooled and evaluated for induction of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Apo-BRDU kit, Phoenix Flow Systems). Briefly, the cells were fixed in 1% paraformaldehyde containing PBS and then in 70% ethanol, washed, and labeled with bromodeoxyuridine. After additional washing, labeled cells were treated with fluorescein-labeled anti-bromodeoxyuridine monoclonal
antibody and propidium iodide/RNase solution before flow cytometric analysis (BD CaliburTM) to determine the percentage of apoptotic cells.

**Statistical analysis**

Summary statistics were provided for continuous variables (mean, SD, and range) and categorical variables (frequency tables). Each tumor in the tissue microarray was represented by three core samples. The epiregulin staining score for each core sample was quantified on the basis of staining intensity and extension (intensity × extension), and the scores of the three core samples were averaged to determine the tumor score. Epiregulin scores were analyzed as continuous and dichotomized variables. Dichotomization was based on a cutoff score of 100 (positive if ≥100, negative otherwise). Comparisons of continuous variables across levels of clinical factors were made using ANOVA or the Kruskal-Wallis test, when appropriate. Associations between clinical factors were made using the $\chi^2$ test or Fisher’s exact test, when appropriate. Survival curves were estimated using the Kaplan-Meier method, and comparisons of survival curves between subgroups of patients were made using the log-rank test. The Cox proportional hazards model method was used to build multivariate survival models to assess the effects of biomarkers and clinical factors on patients’ overall survival. All tests were two sided, with $P \leq 0.05$ considered statistically significant. Statistical analysis was carried out using SAS version 9 software (SAS Institute).

**Results**

**Epiregulin expression correlates with advanced nodal disease**

To evaluate epiregulin expression in NSCLC, we performed immunohistochemical analysis on NSCLC biopsy samples. For these studies, we used a tissue microarray constructed

![Fig. 1. Intratumoral epiregulin expression correlates with poor prognosis in NSCLC. A, epiregulin detection by immunohistochemistry, a to c, anti-epiregulin–specific staining. Immunohistochemical analysis of a NSCLC biopsy sample in the presence (a) and absence (b) of primary antibody and in the presence of primary antibody preincubated with blocking peptide (c). d to g, intratumoral variability in epiregulin staining. Two adenocarcinomas (d and e) and two squamous cell carcinomas (f and g) in which epiregulin was detectable (d and f) or undetectable (e and g). B, intratumoral epiregulin expression correlated with a shorter duration of survival. Plots show Kaplan-Meier survival curves for tumors that were positive (+) or negative (−) for epiregulin staining (scores ≥100 were considered positive). E/N, deaths/total number of patients.**
from 366 randomly collected NSCLC biopsy samples from patients with localized disease (stages I-III). Most of the tumors had been annotated for relevant clinical variables (see Supplementary Table S1). Negative controls, including omission of the primary antibody and preincubation of the primary antibody with blocking peptide, confirmed the specificity of the immunohistochemical staining (Fig. 1A, a-c).

The staining varied in intensity and extent between tumor biopsy samples and was detected predominantly in the cytoplasmic compartment (Fig. 1A, d-g). Of the 366 tumors examined, 237 (64.7%) stained positively for epiregulin. Association of staining with clinical variables revealed a correlation between staining and the presence of advanced nodal (N2) disease ($P = 0.04; \text{Table 1}$) and a trend toward a shorter duration of survival ($P = 0.054; \text{Fig. 1B}$) that became more evident ($P = 0.014$) after we corrected for differences in covariates (age, pathologic nodal and tumor stage, and histologic subtype). We found no correlation of epiregulin expression with other variables (age, race, sex, smoking status, or histologic subtype; data not shown). Thus, epiregulin staining was detected in a subset of NSCLC biopsy samples, and this group of patients had a poorer clinical outcome than those patients with epiregulin-negative tumors had.

### Epiregulin expression is EGFR dependent

To evaluate the biological role of epiregulin in NSCLC, we used the NSCLC cell lines HCC827, H3255, and HCC2279, which express the gene encoding epiregulin (EREG; ref. 19). EREG expression was higher in these EGFR-mutant cell lines than in others that express wild-type EGFR (H1299, H460, A549, H322, and H596; Fig. 2A). To examine whether EREG expression was EGFR dependent, HCC827 cells were treated with gefitinib, an EGFR tyrosine kinase inhibitor. Gefitinib sharply reduced EREG mRNA levels (Fig. 2B). Treatment with selective inhibitors of certain serine/threonine kinases known to mediate EGFR actions, including mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (CI-1040) or p38 (SB202190), decreased EREG mRNA levels, whereas treatment with inhibitors of other EGFR-dependent kinases did not (Fig. 2B). Thus, EREG expression was EGFR dependent and was regulated by specific downstream mediators of EGFR.

### Table 1. Intratumoral epiregulin expression correlated with nodal status

<table>
<thead>
<tr>
<th>Epiregulin score</th>
<th>N stage (pathologic)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N0</td>
<td>N1</td>
</tr>
<tr>
<td>Negative</td>
<td>96</td>
<td>24</td>
</tr>
<tr>
<td>Positive</td>
<td>160</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>256</td>
<td>55</td>
</tr>
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$^a P = 0.04$ by $\chi^2$ test.

$^b$Only 222 of the 237 tumors that stained positively are included in this analysis because 15 of them had undetermined N stage.

$^c$Only 349 of the 366 tumors are included in this analysis because 17 of them had undetermined N stage.

### Epiregulin confers invasive properties and maintains cell survival

To inhibit the actions of epiregulin, we treated NSCLC cells with an anti-epiregulin neutralizing antibody. The cells were maintained in serum-free conditions to eliminate the

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**Fig. 2.** EREG expression is EGFR dependent. A, EREG mRNA was detected by semiquantitative PCR analysis of RNA prepared from a panel of NSCLC cell lines in which EGFR is mutant (HCC827, H3255, and HCC2279) or wild-type (H1299, H460, A549, H322, and H596). Actin was used as the control for RNA integrity. B, kinase-specific regulation of EREG expression. HCC827 cells were treated with inhibitors of EGFR (gefitinib), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (CI-1040), p38 (SB202190), mammalian target of rapamycin (CCI-779), c-jun NH₂-terminal kinase (SP600125), or AKT (A838450.3). Reverse transcription-PCR analysis of EREG and actin (top). EREG bands were quantified by densitometric analysis, normalized for loading differences based on actin, and expressed relative to that of untreated cells, which was set at 1.0. To evaluate dose-dependent target inhibition by the inhibitors, p38 kinase assays (KA) and Western blot analyses were done (bottom) of phosphorylated Y1068-EGFR (p-EGFR), T202/204-extracellular signal–regulated kinase (p-ERK), S235/236-S6 (p-S6), S63-c-Jun (p-cJun), and corresponding total proteins.
confounding effects of exogenous growth factors. Using a titer of anti-epiregulin antibody that decreased EGFR phosphorylation in HCC827 cells (Fig. 3A), showing its ErbB ligand-inhibitory effect, we examined whether treatment diminished cellular proliferation in monolayer cultures and invasion through Matrigel. We used the same titer of anti-epiregulin antibody on serum-starved HCC2279 cells, in which basal EGFR phosphorylation was at the lower limits of detection by Western blot analysis (data not shown). For these assays, we used HCC827 and HCC2279 cells but not H3255 cells because the latter exhibited no detectable invasive activity under these conditions. Relative to the effect of IgG control, the anti-epiregulin neutralizing antibody inhibited the ability of HCC827 and HCC2279 cells to invade (Fig. 3B) but not to proliferate (data not shown).

To evaluate the role of epiregulin by another approach, we depleted EREG from EGFR-mutant NSCLC cells by transiently transfecting them with EREG shRNA retroviral vectors that targeted different EREG coding sequences (constructs 1-4) to examine whether the different shRNA sequences induced consistent biological effects. We obtained efficient EREG depletion in HCC827 cells and H3255 cells (Fig. 4A) but not in HCC2279 cells (data not shown). EREG depletion diminished the number of HCC827 cells and H3255 cells in monolayer cultures (Fig. 4B) and induced apoptosis, as shown in HCC827 cells by cleavage of poly(ADP-ribose) polymerase (PARP), a caspase-3 substrate, and by TUNEL assays to quantify the percentages of cells with DNA fragmentation (Fig. 4C). EREG depletion caused no evidence of cell cycle arrest based on propidium iodide staining to assess DNA content in the transfectants (data not shown). Before the onset of apoptosis, EREG-depleted HCC827 cells exhibited reduced invasive ability, as shown by Matrigel assays done 24 hours after transfection (Fig. 4D). Thus, EREG was required for the invasive capacity and survival of these cells.

Discussion

Here, we showed that intratumoral epiregulin expression correlated with lymph node metastasis and a shorter duration of survival in NSCLC patients and that epiregulin enhanced the invasive and proliferative activities of NSCLC cell lines. Of note, cell proliferation was inhibited by EREG shRNA transfection but not by treatment with an anti-epiregulin neutralizing antibody. Although the reason for this discrepancy is not clear, one possibility is that EREG shRNA transfection achieved a more efficient inhibition of epiregulin than the neutralizing antibody did. The genetic focus of this study was on EGFR-mutant NSCLC cells, but the findings presented here might be relevant to NSCLC cells with other mutated proto-oncogenes. Supporting this possibility is the fact that epiregulin is highly expressed in K-ras-mutant cancer cells, including those of KrasAAV mice, which develop lung adenocarcinoma due to somatic K-ras mutations (19, 20). Moreover, these findings build on previous reports that ErbB ligands maintain the proliferation and survival of EGF-mutant NSCLC cells (7, 21) by showing that epiregulin is a crucial mediator of NSCLC invasion.
The biological effects of EREG depletion observed here were similar to those of the EGFR-neutralizing antibody cetuximab, which induces apoptosis of HCC827 cells (7). Epirapilin has no affinity for ErbB3 or ErbB4; low affinity for EGFR; and low to moderate affinity for the heterodimeric complexes EGFR/ErbB2, ErbB2/3, and ErbB2/4 (22, 23). The ErbB heterodimeric complexes identified thus far in EGFR-dependent NSCLC cells include ErbB2/3 and EGFR/ErbB3, both of which activate prosurvival signals through phosphatidylinositol 3-kinase (9, 10, 21). Collectively, these findings suggest that ErbB ligands promote the invasive capacity and survival of EGFR-mutant NSCLC cells through EGFR-dependent and EGFR-independent mechanisms.

We found that EREG expression was attenuated by specific inhibitors of EGFR and two of its downstream mediators, p38 and mitogen-activated protein kinase/extracellular signal-regulated kinase.
signal-regulated kinase 1. A similar pattern of regulation has been reported for the mRNA encoding the cytokine macrophage inflammatory protein-2 (MIP-2). The 3'-untranslated region of MIP-2 contains AU-rich elements that bind to heterogeneous ribonucleoprotein A0 in a p38- and mitogen-activated protein kinase/extracellular signal-regulated kinase 1-dependent manner and thereby stabilize MIP-2 mRNA (24–26). Through this mechanism, proinflammatory stimuli such as lipopolysaccharide regulate the expression of MIP-2 and other cytokines in macrophages (24–26). EREG also contains AU-rich elements in its 3'-untranslated region, raising the possibility that EREG is regulated through a posttranscriptional mechanism similar to that of MIP-2. If so, then proinflammatory and oncogenic stimuli converge on EREG and MIP-2 through a common posttranscriptional mechanism. Moreover, these findings indicate that EREG is part of a feed-forward loop by which EGFR maintains the activity of ErbB dimeric complexes in EGF-mutant NSCLC cells.

Findings reported here have potential clinical implications. In patients with EGF-mutant NSCLC who receive treatment with an EGFR tyrosine kinase inhibitor, their disease typically shrinks initially and then recurs due to the emergence of tyrosine kinase inhibitor–resistant clones that have undergone additional EGF mutations that abrogate tyrosine kinase inhibitor binding (27–29). Given the high likelihood of disease recurrence, treatment approaches are needed to prevent the invasion and subsequent metastasis of EGF-mutant NSCLC before the emergence of tyrosine kinase inhibitor–resistant disease. On the basis of the findings reported here, metastasis prevention might be achieved by the use of anti-epiregulin neutralizing antibodies alone or in combination with other agents that target the ErbB axis at multiple levels. For example, strategies have been developed to inhibit the tyrosine kinase activities of multiple ErbB family members (e.g., the EGFR/ ErbB2 inhibitor lapatinib), the formation of specific ErbB dimeric complexes (e.g., the ErbB2 dimerization inhibitor 2C4), receptor neutralization (e.g., cetuximab and the ErbB2 inhibitor Herceptin), or ErbB proapoptotic cleavage (e.g., inhibitors of specific proteases such as “a disintegrin and metalloproteases”; refs. 22, 30, 31). Clinical trials should be considered to investigate the efficacy of these approaches, alone and in combination, in patients with EGF-mutant NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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