Epiregulin as a therapeutic target in non-small-cell lung cancer

Noriaki Sunaga1,2
Kyoichi Kaira2,3
1Oncology Center, Gunma University Hospital, 2Department of Medicine and Molecular Science, 3Department of Oncology Clinical Development, Gunma University Graduate School of Medicine, Gunma, Japan

Correspondence: Noriaki Sunaga
Oncology Center, Gunma University Hospital, 3-39-15, Showa-machi, Maebashi, Gunma 371-8511, Japan
Tel +81 27 220 7928
Fax +81 27 220 8609
Email nsunaga@gunma-u.ac.jp

Abstract: Epiregulin (EREG) belongs to the ErbB family of ligands. EREG binds to EGFR and ErbB4 receptor and stimulates homodimers of EGFR and ErbB4 in addition to all possible heterodimeric ErbB complexes, resulting in the activation of downstream signaling pathways. EREG is overexpressed in various human cancers and has been implicated in tumor progression and metastasis. Oncogenic activation of the MEK/ERK pathway plays a central role in the regulation of EREG expression. Non-small-cell lung cancers (NSCLCs) harboring KRAS, BRAF, or EGFR mutations overexpress EREG, and abrogation of such mutations or inhibition of MEK or ERK downregulates the expression of EREG. Elevated EREG expression in NSCLC is associated with aggressive tumor phenotypes and unfavorable prognosis, especially in oncogenic KRAS-driven lung adenocarcinomas. The finding that attenuation of EREG inhibits cell growth and induces apoptosis in KRAS-mutant and EREG-overexpressing NSCLC cell lines suggests that targeting EREG might be a treatment option for KRAS-mutant NSCLC, although further studies are necessary to elucidate its therapeutic value. These observations suggest that oncogenic mutations in the EGFR, KRAS, or BRAF genes induce EREG upregulation through the activation of MEK/ERK pathway in NSCLC cells, whereas overproduced EREG stimulates the EGFR/ErbB receptors and activates multiple downstream signaling pathways, leading to tumor progression and metastasis of these oncogene-driven NSCLCs. This paper reviews the current understanding of the oncogenic role of EREG and highlights its potential as a therapeutic target for NSCLC.

Keywords: epiregulin, NSCLC, KRAS mutation, therapeutic target

Introduction
Lung cancer is the leading cause of cancer mortality worldwide.1 Lung cancer is categorized into two main subtypes: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), the latter accounts for 80%–85% of all lung cancers.2 Lung adenocarcinoma is a major histological subtype of NSCLC, and its incidence is increasing in both men and women.3 The majority of patients with NSCLC have locally advanced or metastatic disease at initial diagnosis, and systemic cytotoxic chemotherapy such as platinum doublets has limited efficacy, with a median overall survival (OS) of 8–11 months.4 Therefore, there is an urgent need for the development of effective treatment modalities to improve the survival of patients with NSCLC.

The development of NSCLC involves a number of genetic and epigenetic alterations that accumulate over time.5 One of the functions of these molecular alterations is the activation of driver oncogenes that are essential for maintaining...
the malignant phenotype. Inactivation of a single oncogene is sufficient to kill cancer cells due to the phenomenon of “oncogene addiction”. Recent studies have identified several driver oncogenes that are potential therapeutic targets for NSCLC. \(^6^,\ldots^,^10\) KRAS and EGFR mutations are the common driver mutations in lung adenocarcinomas, and several fusion genes, including ones formed by rearrangements of ALK, RET, and ROS1, have also been identified as key driver oncogenes in terms of their therapeutic implications.\(^11^,\ldots^,^16\)

To overcome such oncogene-driven tumors, molecularly targeted drugs, including tyrosine kinase inhibitors of EGFR and ALK, have been approved and are currently being used in the clinic.\(^17\) Mutations in the tyrosine kinase domain of EGFR have been widely studied;\(^18\) sensitive EGFR mutations such as in-flame deletions in exon 19 and L858R substitutions in exon 21 are well-known predictive biomarkers of the efficacy of EGFR-tyrosine kinase inhibitors (EGFR-TKIs).\(^19^,\ldots^,^23\) Soda et al identified ALK rearrangements\(^11\) that have been found as predictive biomarkers of the therapeutic efficacy of ALK-tyrosine kinase inhibitors in NSCLC.\(^24^,^25\) Currently, molecular testing for sensitizing EGFR mutations and EML4–ALK fusion oncogenes is performed in tumor samples.\(^26\)

Although “personalized medicine” such as the use of EGFR-TKIs against EGFR-mutated NSCLC and ALK-tyrosine kinase inhibitors against ALK fusion-positive NSCLC is being applied into clinical practice, therapeutic modalities for KRAS-mutant NSCLC have not yet been established. KRAS encodes a small GTP-binding protein that is involved in many cellular processes, including cell growth, differentiation, and apoptosis.\(^27^,^28\) Wild-type KRAS has intrinsic GTP hydrolysis activity that catalyzes the conversion of KRAS into its GDP-bound (inactive) form, and KRAS mutations lock KRAS into its GTP-bound (active) form, resulting in oncogenic activation of downstream signaling pathways. KRAS mutations are attractive therapeutic targets because they are present in many human cancers, including cancers of the pancreas, colon, and lung.\(^27^,^28\) To establish therapeutic strategies for KRAS-mutant NSCLC, we performed a microarray analysis to compare the gene expression profiles of mutant KRAS-disrupted NSCLC clones with those of the mutant KRAS-expressing clones.\(^29\) Consequently, we identified epiregulin (EREG) as one of several putative transcriptional targets of oncogenic KRAS signaling.\(^30\) In this review, we describe the current understanding of the oncogenic role of EREG and its relationship with oncogenic KRAS, and we highlight the potential of EREG as a therapeutic target for NSCLC.

### Epiregulin

EREG belongs to the ErbB family of ligands and was originally purified from conditioned medium of NIH 3T3 mouse tumorigenic fibroblasts.\(^31\) The human EREG gene is located on chromosome 4q13.3, and the AREG and BTC genes are also clustered at that location.\(^32\) EREG has 46 amino acid residues, and 24%–50% of its sequence is shared with those of other EGF family members.\(^33\) EREG is capable of binding to EGFR and ErbB4 receptor and stimulates homodimers of EGFR and ErbB4 in addition to heterodimers of ErbB2 and ErbB3, leading to the activation of their intrinsic kinase domain and the phosphorylation of specific tyrosine residues in the cytoplasmic tail of their receptors (Figure 1).\(^33^,^34\) Those phosphorylated residues serve as docking sites for intracellular signaling molecules, and therefore activate downstream signaling pathways, including the MEK/ERK pathway.\(^33\)

Previous studies have reported the physiological role of EREG in the control of cell proliferation and differentiation of human airway epithelial cells. Coculturing human airway epithelial cells with lung fibroblasts, which express EREG, induces human airway epithelial differentiation accompanied by ErbB2 phosphorylation.\(^35\) Exposure of compressive stress increases EREG expression, and this phenomenon was shown to be suppressed by an EGFR inhibitor in human bronchial epithelial cells.\(^36\) These findings suggest that EREG activates ErbB receptors and their downstream signaling pathways in bronchial epithelial cells.

### Role of EREG in cancer

EREG/EGFR pathways regulate diverse cellular processes, including cell proliferation, invasion, metastasis, angiogenesis, and resistance to apoptosis, conferring aggressive tumor behavior.\(^37\) EREG is overexpressed in many human cancers, such as pancreatic cancer, colon cancer, NSCLC, breast cancer, bladder cancer, prostate cancer, kidney cancer, liver cancer, ovarian cancer, oral cancer, thymic cancer, salivary adenoid cystic carcinoma, and malignant glioma, whereas EREG expression levels in normal adult tissues are extremely low.\(^38^,^39^,^43^–^49\) For instance, thymic carcinomas had a high percentage (91.7%) of immunohistochemical expression of EREG.\(^40\) EREG has been identified as one of the highly expressed genes in the hTERT-immortalized fibroblasts, and blockage of EREG inhibits the in vitro growth of the hTERT-immortalized cells, suggesting the critical role of EREG in hTERT-mediated immortalization and transformation.\(^50\) Zhu et al reported that pancreatic ductal adenocarcinomas (PDAs) exhibit higher levels of EREG mRNA than normal pancreatic and chronic pancreatitis tissues.\(^39\) They also found...
that in vitro cell growth of pancreatic cancer is significantly increased by human recombinant EREG in a dose-dependent manner. Given that whole-exome sequencing analysis of PDAs detected KRAS mutations in >90% of PDAs,\textsuperscript{96} it is possible that EREG is involved in the development of oncogenic KRAS-driven PDAs.

An oncogenic role of EREG has also been suggested in other human cancer types. In a COX2-overexpression mouse model of bladder carcinoma, EREG is the most significantly upregulated gene, and the expression of a recombinant EREG increases cell proliferation in bladder cancer cell lines.\textsuperscript{57} In a mouse model of hepatocellular carcinomas, EREG-knockout mice have fewer tumors that are smaller in size than EREG-wild-type mice.\textsuperscript{58} In addition, siRNA-mediated EREG knockdown suppresses in vitro hepatoma growth.\textsuperscript{47} These findings suggest that EREG is involved in bladder and hepatocellular carcinogenesis.

Several lines of evidence have implicated the role of EREG in tumor metastasis. A previous study of breast cancer cells with potential for lung metastasis identified the lung metastasis signature (LMS) genes, which include EREG, COX2, MMP1, and MMP2.\textsuperscript{59} shRNA-mediated simultaneous knockdown of these four genes in a xenograft model suppressed in vivo tumor growth, angiogenesis, and metastasis,\textsuperscript{60} indicating that EREG confers metastatic potential in breast cancer. Similarly, in a bladder cancer mouse model with lung metastasis, microarray analysis identified EREG as one of the upregulated genes in lung metastatic tumors.\textsuperscript{42} Gene expression profiling comparing colon cancers with and without liver metastasis also identified EREG as a metastasis-associated gene.\textsuperscript{61} Moreover, salivary adenoid cystic carcinoma cells with lung metastatic potential overexpress EREG, resulting in the promotion of migration and invasion through the activation of ERK and Akt.\textsuperscript{46} Recently, EREG was found to be upregulated by KAP1, a transcriptional regulator that promotes proliferation and metastasis in breast cancer.\textsuperscript{62} Collectively, these findings imply that EREG plays a key role in tumor progression and metastasis and confers high malignant potential in human cancers.

Interestingly, a possible link between EREG and cancer cell stemness has been suggested. A previous study that included microarray and immunohistochemistry analyses showed that EREG is expressed in LGR5-positive colon cancer cells, which possess cancer stem cell properties.\textsuperscript{63} Furthermore, in a metastatic xenograft model, an anti-EREG antibody exhibited antitumor activity against tumors derived from human colorectal cancer cells.
from LGR5-positive colon cancer cells. Notably, LGR5 expression was reported to be related to larger tumor size, more advanced stage, and poor prognosis in lung adenocarcinoma. Therefore, EREG may be a therapeutic target for lung adenocarcinoma stem cells.

In contrast to the oncogenic roles of EREG, the negative aspect was also reported. EREG expression levels are undetectable in most SCLC cell lines, and EREG does not seem to be necessary for SCLC carcinogenesis. EREG expression was shown to be epigenetically silenced in gastric cancer cell lines by aberrant DNA methylation and histone demethylation. In addition, EREG promoter was hypermethylated in 30% of primary gastric tumor tissues. Thus, it is possible that EREG is inactivated in some human cancers, including gastric cancer and SCLC, and aberrant promoter methylation might be one of the mechanisms for EREG inactivation.

**EREG as oncogenic KRAS-regulated gene**

The Ras gene family includes three genes, *KRAS, HRAS,* and *NRAS,* all of which share very similar molecular structures and a common GTPase domain that binds to and hydrolyze guanine nucleotides. Oncogenic mutations of the Ras genes mainly occur at codons 12, 13, and 61, and these mutations lock Ras proteins into their GTP-bound (active) form, resulting in the constitutive activation of Ras downstream pathways and the promotion of oncogenesis.

*KRAS* is the most commonly mutated isoform of the Ras genes, and *KRAS* mutations have been found in a variety of human cancers, including cancers of the pancreas and colon, and NSCLC. Several studies have found a relationship between EREG and KRAS. Baba et al. conducted a polymerase chain reaction-based cyclic DNA subtraction library to compare genes that were differentially expressed between HCT116 colon cancer cells and KRAS-disrupted clones derived from HCT116 cells, and EREG was found to be upregulated by activation of Ras signaling pathways. In the KRAS-disrupted HCT116 clones, forced expression of exogenous EREG partially recovered in vivo tumorigenicity, indicating that EREG is involved in the tumorigenesis of KRAS-mutant colon cancer. Similarly, a previous microarray analysis demonstrated that *EREG* expression is upregulated in KRAS-transformed human prostate cancer cell; furthermore, MEK inhibition downregulated *EREG* expression, accompanied by downregulation of the ETS1 transcription factor, which binds to the EREG promoter. EREG expression is also upregulated in lung tumors from mice carrying mutant KRAS alleles.

In addition to these findings, we identified *EREG* as one of the transcriptional targets of oncogenic KRAS signaling in *KRAS*-mutant NSCLC cells and immortalized bronchial epithelial cells, expressing ectopic mutant *KRAS.* In *KRAS*-mutant and *EREG*-overexpressing NSCLC cells, *EREG* expression is reduced by siRNAs targeting mutant *KRAS* (but not by siRNAs targeting wild-type *KRAS*) and by inhibitors of MEK and ERK. EREG is preferentially expressed in *KRAS*-mutant NSCLC cell lines and tumor specimens. Importantly, *EREG* expression is significantly correlated with *KRAS* copy number in a subgroup of *KRAS*-mutated NSCLC cell lines. Given that *KRAS* copy number gains are associated with increased mutant allele transcription and gene activities in NSCLC cells, *KRAS* copy number gains appear to enhance oncogenic KRAS-induced activation of the MEK/ERK pathway, resulting in the upregulation of EREG in NSCLC cells. Collectively, these findings suggest that EREG overexpression is likely induced by oncogenic KRAS via activation of the MEK/ERK pathway and plays an essential role in oncogenic KRAS-mediated tumorigenesis.

**Clinicopathological and prognostic significance of EREG expression in cancer**

Clinicopathological studies have suggested that EREG is associated with aggressive tumor phenotypes and unfavorable prognoses in several human cancers. The previous studies that had showed the prognostic significance of EREG expression were summarized in Table 1. *EREG* mRNA is highly expressed in bladder tumors with advanced T stages, and elevated *EREG* expression is significantly correlated with shorter survival. Elevated *EREG* expression is also associated with shorter survival in patients with oral squamous cell carcinoma. In colon cancer, *EREG* expression is significantly correlated with the depth of tumor invasion and distant metastasis. Furthermore, *EREG* expression was found to be associated with higher tumor grade and worse survival in glioblastoma.

With regards to the prognostic significance of EREG in NSCLC, an immunohistochemical analysis of NSCLC biopsy samples revealed that 64.7% of the tumors stained positively for EREG, and that patients with EREG-positive tumors had poorer clinical outcomes than those with EREG-negative tumors. We also conducted a gene expression analysis of surgical tumor specimens to determine the clinicopathological and prognostic features of EREG expression in NSCLC. *EREG* mRNA expression levels are significantly
higher in lung adenocarcinomas than in lung squamous cell carcinomas. EREG is predominantly expressed in NSCLCs with pleural involvement, lymphatic permeation, and vascular invasion, all of which confer aggressive tumor phenotypes. When we extended this clinicopathological study to include 136 surgical specimens from patients with lung adenocarcinoma, EREG expression was found to be significantly higher in tumors from elderly patients (≥70), male patients, and smokers.49 In this series, elevated EREG expression was also associated with pleural involvement positivity, lymphatic permeation positivity, and vascular invasion positivity. Patients with lung adenocarcinoma with EREG-high tumors have significantly shorter disease-free survival (DFS) and OS compared to those with EREG-low tumors. When the patients were divided into four groups according to the EREG expression and the KRAS mutation status, the patients with EREG-high/KRAS-mutant tumors had significantly shorter DFS and OS compared to those with EREG-low/KRAS-wild-type tumors. Multivariate analyses showed that EREG expression is a significant prognostic factor for DFS and OS. Taken together, EREG appears to contribute to the acquisition of aggressive tumor phenotypes and serves as a prognostic marker in NSCLC, especially KRAS-mutant lung adenocarcinoma.

Therapeutic potential for targeting EREG in NSCLC

Several lines of evidence suggest that targeting EREG in NSCLC has therapeutic potential. KRAS-mutant NSCLC cells preferentially express EREG, and siRNA-mediated EREG knockdown inhibits anchorage-dependent and anchorage-independent growth and induces apoptosis in KRAS-mutant NSCLC cells,49 suggesting that EREG is a therapeutic target for oncogenic KRAS-driven NSCLC. EREG overexpression was also found in the EGFR-mutant NSCLC cells,29,45,69 in the BRAF-mutant NSCLC cells, and in a subset of NSCLC cells with wild-type EGFR/KRAS/BRAF.29 In EREG-overexpressing NSCLC cells, inhibition of MEK or ERK reduces EREG expression, irrespective of mutation status.30 Thus, activation of the MEK/ERK pathway seems to be a common mechanism of EREG upregulation in NSCLC.

Previous studies have suggested that EREG has therapeutic potential for EGFR-mutant NSCLC. EREG is downregulated by siRNA-mediated EGFR knockdown and EGFR inhibitors in EGFR-mutant NSCLC cells.29,41 Lung tumors of mutant EGFR transgenic mice exhibit high levels of EREG.72 In EGFR-mutant NSCLC cells, both shRNA-mediated EREG knockdown and an anti-EREG antibody inhibit cell proliferation and invasion and induce apoptosis.43 These findings indicate that targeting EREG is a good therapeutic option for EGFR-mutant NSCLC cells with resistance to EGFR-TKIs.

BRAF mutations occur in 2%–3% of NSCLCs (predominantly in lung adenocarcinomas)73,74 and are potential therapeutic targets. BRAF-mutant NSCLC cells were found to have high EREG expression at similar levels to those in KRAS-mutant NSCLC cells.30,49 In BRAF-mutant NSCLC cells, siRNAs targeting BRAF and the inhibitor of MEK or ERK reduce EREG expression, showing that oncogenic BRAF upregulates EREG expression via activation of the MEK/ERK pathway. Thus, targeting EREG might also be effective for BRAF-mutant NSCLC cells.

Conclusion

Accumulated evidences suggest that oncogenic mutations in the EGFR, KRAS, or BRAF genes induce EREG overexpression via activation of the MEK/ERK signaling pathway. Overproduced EREG can stimulate the EGFR/ErbB receptors

Table 1 The studies evaluating the association between EREG expression and survival in human cancers

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Method</th>
<th>Cutoff value</th>
<th>Number of points</th>
<th>Survival for EREG positive</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC</td>
<td>IHC</td>
<td>≥ score 100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>356</td>
<td>Poor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>qRT-PCR</td>
<td>≥ median</td>
<td>119</td>
<td>Poor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>qRT-PCR</td>
<td>First cutoff: 2.4 and second cutoff: 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73</td>
<td>Poor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma</td>
<td>qRT-PCR</td>
<td>≥ 0.1</td>
<td>30</td>
<td>Poor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>IHC</td>
<td>≥ score 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>73</td>
<td>Poor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>a</sup> Notes: In all studies, overall survival was analyzed by the Kaplan-Meier method, and comparison between subgroups was examined by the log-rank test. The staining score was quantified on the basis of staining intensity and extension (intensity × extension); Cox regression analysis was also performed, and the hazard ratio, adjusted by tumor stage, was 8.71 (95% CI: 1.90–39.80); the first cutoff point was median, and the second cutoff point was arbitrarily chosen; IHC positivity was determined according to the total score (intensity score + extension score); P=0.054 for the difference of overall survival, whereas the difference was more evident (P=0.014) after correction for differences in covariates (age, pathological nodal, tumor stage, and histological subtype).

Abbreviations: EREG, epiregulin; NSCLC, non-small-cell lung cancer; IHC, immunohistochemistry; CI, confidence interval; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.
to activate multiple downstream signaling pathways, including the MEK/ERK and PI3K/Akt pathways through an autocrine loop mechanism (Figure 2). Thus, EREG is likely to play diverse oncogenic roles, including the regulation of cell proliferation, invasion, and metastasis as a potent pan-ErbB ligand; therefore, it may contribute to the acquisition of highly malignant phenotypes and the development of human cancers, including NSCLC. Considering that half of the lung adenocarcinomas have mutations in EGFR, BRAF, or KRAS in a mutually exclusive manner \(^7,9,10\) and that tumors with such driver mutations overexpress EREG, it is plausible that the majority of NSCLCs could benefit from EREG-targeted therapy. Although the exact mechanism behind the regulation of EREG is still unclear, EREG may be an excellent target for anticancer therapies, especially for NSCLCs. Furthermore, in vivo studies and clinical trials are warranted to clarify the effectiveness of EREG-targeted therapy for NSCLCs.

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