Abstract. Monoclonal antibodies and tyrosine kinase inhibitors against ErbB receptors have been developed and have progressed to clinical applications following several decades of research in cancer cell biology. Inhibition of epidermal growth factor receptor (EGFR) signaling represents a particularly promising arena for the application of molecularly targeted cancer therapies. In EGFR signaling inhibition, EGFR itself has been recognized as a target in epithelial malignancies, though clinical studies using EGFR antagonists have not always resulted in favorable clinical outcomes. The aberrant enhancement of EGFR ligand expression is speculated to be one of several different molecular mechanisms accounting for the acquired resistance to EGFR antagonists. Recently, emerging evidence has indicated that EGFR ligands deserve considerable attention as potential targets for cancer therapy. In this review, we discuss the EGFR signaling inhibition strategies directed at EGFR ligands such as HB-EGF and amphiregulin.

ErbB receptors consist of four known proteins, including epidermal growth factor receptor (EGFR) (also known as HER1 or ErbB1), ErbB2 (p185\textsuperscript{neu} or HER2), ErbB3 (HER3) and ErbB4 (HER4) (1, 2) (Figure 1). Viral v-erbB1 was discovered as a potent oncogene and is the determinant for avian erythroblastosis virus-induced neoplasia (3). Another member of this family, p185\textsuperscript{neu}, was first isolated from chemically induced rat neuroblastomas (4, 5). Overexpression of EGFR or ErbB2 induces the transformation of NIH3T3 cells (6-8). In many types of human cancer, EGFR is overexpressed by more than 50% or 60% (9). ErbB2 expression is also enhanced in some types of human cancer, though sometimes at low frequencies (9). Although NIH3T3 cells are not transformed by transfection with ErbB3 or ErbB4 alone, ErbB3 or ErbB4 can promote the ability to transform NIH3T3 cells in the presence of EGFR or ErbB2 (8). No significant differences in the expression of ErbB3 or ErbB4 between cancer and normal tissues have been reported (10). On the basis of this evidence, EGFR and ErbB2 have been recognized as therapeutic targets for human cancer treatment (11). In particular, EGFR antagonists have been developed and prescribed for patients with epithelial malignancies (6-8).

In principle, EGFR transmits signals to the nucleus through the Raf-MEK-ERK mitogen-activated protein kinase cascade (12, 13). The activation of molecules involved in this pathway, such as by mutation or overexpression, induces cellular transformation (12, 13). Accordingly, the molecules associated with EGFR signaling have also been recognized as targets for cancer therapy (13). However, a loss of EGFR blocks the malignant transformation mediated by the activated form of Son-of-sevenless (SOS) (14). Additionally, EGFR only contributes to transformation of NIH3T3 cells in the presence of its cognate ligands (6-8). Therefore, malignant transformation requires both EGFR and its cognate ligands. Autocrine/paracrine production of EGFR ligands, as well as overexpression of EGFR, are two of the mechanisms most frequently implicated in cancer development and progression (15). According to these pieces of evidence, EGFR, as well as its cognate ligands, would be valid targets for cancer therapy.

ErbB receptors are activated by a number of ligands, termed epidermal growth factor (EGF)-related peptide growth factors (16-18). These ligands are produced as transmembrane...
precursors, which are processed and released by proteolysis (19). Within the numerous ErbB-specific ligands, each EGF-like domain is sufficient to confer binding specificity (1, 2). The ligands identified to date include EGF, amphiregulin (AR), epigen and transforming growth factor-α (TGF-α), which bind specifically to EGF-R, and betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF) and epiuregulin (EPR), which exhibit dual specificity for EGF-R and ErbB4 (1, 2). The neuregulins (NRGs) also comprise a family of ligands (2). NRG-1 and NRG-2 bind ErbB3 or ErbB4, whereas NRG-3 and NRG-4 bind to ErbB4 but not ErbB3 (2). Despite the large number of ligands identified for EGF-R, ErbB3 and ErbB4, no direct ligands for ErbB2 have yet been discovered. However, increasing evidence suggests that ErbB2 has a critical function as a co-receptor for all the other ErbB family members. In contrast to ErbB receptors, the ligands comprising the EGF family of growth factors have not yet been focused on as targets for cancer therapy. This is possibly due to the redundancy of EGF-R ligands for each receptor, which has led to a general perception that inhibiting receptor function is more effective than inhibiting multiple ligands for cancer therapy.

In this review, we highlight the validity of EGF-R ligands as therapeutic targets in human cancer, including the significance of specific EGF-R ligands in human cancer, the antitumor effects induced by the inhibition of EGF-R ligands, and preclinical studies using inhibitors of EGF-R ligands.

Involvement of EGF-R Ligands in the Progression of Cancer

Targeted disruption of the EGF-R gene is lethal during development in several strains of mice (20-22). It has been demonstrated that signals generated by EGF-R play pivotal roles in the development of epithelial organs (20-22). There has also been much research using targeted knockout mice and mice transgenic for EGF-R ligands. HB-EGF-null mice show a relatively severe phenotype, including an enlarged and dysfunctional heart, a heart valve malformation with enlarged semilunar and atrioventricular valves, thickened mesenchymal tissue and alveolar immaturity of the lungs. Most of these animals die at the neonatal stage (23-25). Transgenic expression of HB-EGF accelerates the proliferation of hepatocytes after partial heptectomy (26). AR knockout mice show impaired proliferative responses after partial liver resection (27, 28) and female mice exhibit impaired mammary gland development and/or function (29, 30). Neonatal mice lacking AR, EGF and TGF-α display spontaneous duodenal lesions, while the alveoli in the triple null mammary glands are poorly organized and differentiated (30). According to these lines of evidence, it is possible that AR is involved in the development of cancer originating from the breast, colon and liver. When AR or TGF-α are overexpressed in the exocrine pancreas in vivo, AR transgenic mice display small intralobular ducts and centroacinar cell proliferation, while TGF-α transgenic mice show tubular complex formation with a strong fibrogenic response, demonstrating enhanced expression of AR, suggesting that AR may be involved in the proliferation of pancreatic duct cells (31, 32). EGF or BTC knockout mice are healthy, fertile and display no adverse phenotypic effects (25, 29). Mice overexpressing BTC exhibit severe alterations in the lungs, accompanied by high early postnatal mortality, while EGF transgenic mice display no definite phenotypes (33, 34). EPR-null mice show no overt developmental defects, reproductive abnormalities or altered liver regeneration (35), while TGF-α knockout mice have a moderate phenotype, consisting of wavy hair and whiskers (36, 37). There have been no reports regarding the phenotypes of epigen knockout or transgenic mice, or epiuregulin transgenic mice.

On the basis of this evidence, the roles of individual EGF-R ligands which are predominantly expressed in cancer cells originating from individual organs are not always realized by the development of epithelia in the individual organs, or in the phenotypes of model transgenic mice, possibly due to compensation by other EGF-R ligands.

Many studies have analyzed the expression of EGF-R ligands in human cancer tissue specimens using immunostaining, northern blotting or reverse transcriptase-polymerase chain reaction (RT-PCR) (9, 11). Increased expression of EGF-R ligands, such as TGF-α, HB-EGF and AR, is associated with clinical prognosis in ovarian, endometrial, bladder, breast, gastric, colon, pancreatic and prostate cancer, as well as in renal cell carcinoma, glioblastoma and malignant melanoma (9, 11). Even in the same cancer types, some authors have reported that the expression of a certain EGF-R ligand is associated with the clinical outcome, while others have claimed that another EGF-R ligand is regarded as a prognostic factor (9, 11). Thus, the role of specific EGF-R ligands in different types of cancer remains to be elucidated. Recent studies have indicated that the expression levels of individual EGF-R ligands vary among different types of cancer (38-40). In ovarian cancer and bladder cancer, HB-EGF is specifically expressed at high levels (38-40). In addition, we analyzed the expression of seven EGF-R ligands in human tissue specimens of colon, breast and pancreatic cancer and found that in breast cancer tissues, HB-EGF was predominantly expressed, compared with the other EGF-R ligands (personal communication). In colon and pancreatic cancer, AR was recognized to be the EGF-R ligand with the most enhanced expression (personal communication). However, further experiments are needed to investigate the validity of EGF-R ligands as therapeutic targets for cancer treatment.

Validity of HB-EGF and AR as Targets for Cancer Therapy

To determine the increase in EGF-R ligand expression in various types of cancer, we examined the expression of EGF-R ligands using real-time PCR, ELISA and the Diphtheria toxin-
binding assay (41). Using real-time PCR in ovarian, endometrial, endocervical, bladder and gastric cancer cell lines, HB-EGF was found to be the predominant EGFR ligand. Analyzed by real-time PCR, HB-EGF and TGF-α were the main ligands expressed in melanoma and glioblastoma cell lines. Using ovarian, endometrial, endocervical, bladder, gastric cancer, melanoma and glioblastoma cells, the amount of HB-EGF in the culture medium was greater than that of AR, TGF-α and EGF. In seven breast cancer cell lines, HB-EGF was the most enriched EGFR ligand found in the culture medium, whilst real-time PCR demonstrated enhanced expression of HB-EGF, AR and EGF. In colon, pancreatic, prostate, liver and cholangiocarcinoma cell lines, amphiregulin was the most highly-expressed EGFR ligand, using real-time PCR or ELISA. In renal cell carcinoma cell lines, AR was abundantly secreted into the culture medium, and the amounts of AR and EPR RNA were significantly higher than those of other EGFR ligands, as shown by real-time PCR. Analysis of the expression of EGFR ligands suggests that HB-EGF and AR are potential therapeutic targets for many types of human cancer.

To reconfirm the validity of HB-EGF and amphiregulin as targets for cancer therapy, we investigated the alterations in tyrosine phosphorylation of EGFR and ERK using immunoblots and by examining the numbers of apoptotic cells by flow cytometry, after introduction of small interference RNA (siRNA) for EGFR ligands, in a variety of human cancer cells (41). In cancer cells with predominant expression of HB-EGF or AR, the transfection of siRNA for HB-EGF or AR induced a significant increase in apoptosis, with the attenuation of EGFR and ERK activation. The addition of CRM197, a specific inhibitor of HB-EGF, or an inhibitory antibody against AR to cancer cells, also promoted apoptosis and blocked the activation of EGFR signals. In lung cancer cells, no unusual expression of the EGFR ligands was found and no significant apoptosis was observed after transfection of siRNA for any EGFR ligand. According to this evidence, HB-EGF and AR are recognized as promising and valid targets for cancer therapy (Figure 2).
Synergistic Anticancer Effects of Conventional Agents and Inhibitors of HB-EGF or AR

Favorable interactions exist between molecularly targeted therapy and a variety of conventional chemotherapeutic agents, including cisplatin, doxorubicin, paclitaxel, irinotecan and topotecan (42). It has been speculated that molecularly targeted therapy, such as cetuximab and trastuzumab, potentiates the DNA-damaging, cytotoxic effects of cisplatin and other conventional agents (43, 44). The nature of the interaction between agents targeted to EGFR ligands and conventional chemotherapeutic agents remains unknown. Our previous results indicated that high expression of HB-EGF was significantly associated with poor clinical outcome in patients with ovarian cancer (39) and that cancer cells with elevated expression of HB-EGF were resistant to paclitaxel (45), suggesting that HB-EGF was involved in the resistance to conventional chemotherapy. To address the relationship between HB-EGF expression and drug-resistance, the antitumor effects of paclitaxel were evaluated in ovarian cancer cells with different degrees of HB-EGF expression. Paclitaxel significantly suppressed the in vitro cell proliferation rate and augmented apoptosis in ovarian cancer cells with low expression of HB-EGF (SKOV3 cells), compared with that in ovarian cancer cells with high expression of HB-EGF (SK-HB-1 cells), indicating that HB-EGF was involved in restoring the sensitivity to paclitaxel. In addition, paclitaxel induced transient ERK activation and sustained activation of JNK and p38 MAPK, through the ectodomain shedding of HB-EGF in the ovarian cancer cell line, SKOV3. The overexpression of HB-EGF in paclitaxel-treated SKOV3 cells (SK-HB-1 cells) resulted in modulation of paclitaxel-evoked MAPK signaling, including marked and persistent activation of ERK and Akt (antiapoptotic signals), and minimized activation of JNK and p38 MAPK (proapoptotic signals). According to this evidence, HB-EGF can modulate drug sensitivity through the balance of antiapoptotic (ERK and Akt) and proapoptotic (JNK and p38 MAPK) signals induced by paclitaxel (Figure 3). Other conventional chemotherapeutic agents, such as cisplatin and irinotecan, also promoted the ectodomain shedding of HB-EGF (personal communication).
To assess the synergistic anticancer effects, the in vitro as well as in vivo antitumor effects of paclitaxel were examined in combination with CRM197, a specific inhibitor of HB-EGF. This combination of paclitaxel with CRM197 inhibited cell proliferation and enhanced apoptosis in both SKOV3 and SK-HB-1 cells via the inhibition of ERK and Akt activation and the stimulation of p38 and JNK activation. In addition, tumor formation in xenografted mice was monitored after inoculation with SKOV3 and SK-HB-1 cells. The administration of paclitaxel with CRM197 resulted in synergistic antitumor effects in these xenografted mice. Accordingly, inhibitors of HB-EGF, such as CRM197, represent possible chemotherapeutic and chemosensitizing agents for ovarian cancer.

Figure 3. The balance between antiapoptotic signals (ERK and Akt) and proapoptotic signals (JNK and p38 MAPK). In the chemosensitive state (A), paclitaxel induces the activation of proapoptotic signals (JNK and p38 MAPK), whereas paclitaxel promotes the antiapoptotic signals (ERK and Akt) and inhibits the activation of proapoptotic signals (JNK and p38 MAPK) in the chemoresistant state (B), mediated by abundant expression of HB-EGF. Through the suppression of HB-EGF function in the presence of CRM197, cancer cells shift to the chemosensitive state (A) from the chemoresistant state (B).
AR is a promising target for therapy in pancreatic and colon cancer. We examined the ectodomain shedding of AR in pancreatic and colon cancer cells in the presence of conventional chemotherapeutic agents, including gemcitabine, irinotecan and oxaliplatin. These agents induced significant cleavage of pro-AR in both pancreatic and colon cancer cells. An inhibitory antibody against AR attenuated the activation of EGFR, ERK and Akt signalling and mediated significant apoptosis in pancreatic and colon cancer cells. In addition, strategies directed at the inhibition of AR provided additive or synergistic therapeutic value when combined with conventional chemotherapeutic agents (personal communication).

Preclinical studies of EGFR signaling inhibition through blockade of EGFR ligands combined with chemotherapy have shown considerable promise, indicating that the inhibition of HB-EGF or AR restores sensitivity to conventional chemotherapeutic agents. The development of inhibitors of HB-EGF or AR as EGFR antagonists may provide clinical possibilities for cancer therapy.

**Current Molecularly Targeted Therapies**

At present, molecularly targeted therapies are available and useful for patients with various malignancies. A monoclonal antibody against ErbB2 (trastuzumab) and a tyrosine kinase inhibitor against platelet-derived growth factor (PDGF) receptor (imatinib) have demonstrated major clinical benefits (46). Adjuvant trastuzumab therapy in a regimen with doxorubicin, cyclophosphamide, and paclitaxel in patients with resected ErbB2-positive breast cancer reduced the risk of recurrence, second primary cancer, or death before recurrence by 52% (p<0.0001), and reduced the risk of mortality by 33% (p=0.015) (47). In patients with Philadelphia chromosome-positive chronic myeloblastic leukemia unresponsive to interferon, imatinib had a significant survival advantage (hazard ratio=0.2; p<0.001) (48). Chimeric monoclonal antibody against CD20 (rituximab) has also shown a marked survival benefit in combination with chemotherapy (49). EGFR-targeted agents have already shown utility in different scenarios. Anti-EGFR monoclonal antibodies, including cetuximab and panitumumab, have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer (50). Cetuximab is also approved for head and neck cancer (51). Erlotinib, which is an EGFR tyrosine kinase inhibitor, is FDA-approved for advanced/metastatic lung cancer (52). Erlotinib, in combination with gemcitabine, is approved for advanced/metastatic pancreatic cancer treatment (53). However, EGFR-targeted therapies have not always held their promise. Cetuximab failed to influence overall survival in the European Prospective Investigation of Cancer (EPIC) study of second-line treatment of colorectal cancer, although the drug did increase progression-free survival by 55% (p<0.0001) (54). In refractory non-small cell lung cancer, erlotinib significantly prolonged survival, particularly in nonsmokers (55). The use of gefitinib has been particularly effective in patients of Asian ethnicity (56). Clinical studies using antagonists against RAS, ERK or Akt have not been successful in improving clinical outcomes (13). Accordingly, the further development of alternative agents based on EGFR signaling inhibition strategies is required in order to provide clinical benefit to patients with epithelial malignancies.

**Future Directions**

Despite major advances in the management of cancer, most types of solid tumors remain resistant to conventional treatment modalities. Although EGFR antagonists have been developed as therapeutic agents for epithelial malignancies, the current use of these agents does not greatly improve the clinical outcome in patients with cancer. In recent years, therefore, there has been substantial interest in developing novel therapeutic agents that specifically target growth factor pathways, which are dysregulated in tumor cells. Specific inhibitors of HB-EGF or AR could be considered as promising agents based on their EGFR signaling inhibition strategies. To date, a phase I study of the use of CRM197, a specific inhibitor of HB-EGF, has been performed in Fukuoka University (Japan) for patients with recurrent ovarian cancer, under the approval of the Ethical Committee. Improvements in the clinical efficacy of cancer treatment is anticipated in this clinical study.

Accumulation of the knowledge acquired from preclinical and clinical studies using agents based on EGFR inhibition strategies leads to definite understanding of the unique mechanisms of action, as well as the toxicity profiles, of molecularly targeted therapies that are generally different from those of standard therapies. Further research is therefore needed into specific aspects of existing molecular targeted therapies as follows: i) selection of appropriate patients who are most likely to benefit; ii) establishment of appropriate combination therapy using molecularly targeted agents and conventional chemotherapeutic agents. Further research is also required to identify possible new treatment targets. It is possible that integrated treatment of targeted agents with conventional agents can lead to dramatic improvements in clinical outcomes in patients with cancer.

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