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Review

Efficacy of Ligand-based Targeting for the EGF System in Cancer

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Abstract. *Although drugs inhibiting ErbB receptors such as epidermal growth factor receptor (EGFR) and HER2 have been developed as anticancer agents targeting the EGF family, they are not effective for all types of cancer and instead target only certain types. We propose the following four main reasons for these observations: (i) although seven EGFR ligands exist, effective inhibition of specific EGFR ligands may occur because their expression levels differ in different malignancies; (ii) suppressing EGFR ligands inhibits aggregation of EGFR and other ErbB receptors and activation of ERK and Akt signals; (iii) EGFR ligands may have various combinations for signal transduction through the EGFR pathway and other receptor signals; and (iv) the intracellular C-terminals of EGFR ligands move into the nucleus and strongly regulate cell proliferation. In this review, we describe important implications for targeted cancer therapy against EGFR ligands and describe the current situation in the development of ligand-based therapies for cancer.*

Epidermal growth factor (EGF) ligands, comprising EGF-related peptides, activate EGF receptor (EGFR, also known as ErbB1/HER1) and other ErbB receptors (ErbB3/HER3 and ErbB4/HER4). They are broadly classified into EGFR family ligands that bind to EGFR (comprising EGF, transforming growth factor (TGF)-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin, betacellulin (BTC) and epigen) and neuregulin (NRG) family ligands that bind to ErbB3 or ErbB4 (comprising NRG1, NRG2, NRG3 and NRG4) (1). Binding of ligands to the extracellular domains of ErbB receptors initiates their homodimerization or heterodimerization with other ErbB receptors and phosphorylation of tyrosine residues within their cytoplasmic domains, which in turn activates downstream growth and survival signals such as the mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathways (2-4). No ligands that bind ErbB2 have been identified, and the kinase activity of ErbB3 is defective. These receptors are capable of generating intracellular signals by forming heterodimers with other ErbB receptors (5, 6). The EGF family members play important roles in normal tissue processes including ontogeny, morphogenesis, migration, differentiation and proliferation. Dysregulation of EGF family members and related signaling molecules can contribute to carcinogenesis and is associated with tumorigenesis, invasion and metastasis (2).

EGFR and ErbB receptors have been especially focused upon as target molecules for cancer treatments because overexpression and mutations of these receptors are frequently observed in human malignancies. A variety of small molecule kinase inhibitors targeting EGFR (*e.g.* erlotinib: Tarceva™) and monoclonal antibodies targeting EGFR (*e.g.* cetuximab: Erbitux) and HER2 (*e.g.* trastuzumab: Herceptin™) have been developed and some of them have already been used for treatment of lung cancer and breast cancer (7). However, these medicines have not exhibited the expected levels of clinical efficacy thus far, despite numerous cases of administration to patients with malignant tumors targeting EGFR and HER2 (8). One of the reasons for these observations is that EGFR and HER2 form complexes with HER3 and other signal receptors. The proliferation of cancer cells is subsequently accelerated by these complexes, whose formation cannot be inhibited by targeted therapies against EGFR and HER2. Another reason is that the anti-EGFR drugs can suppress the proliferation of

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extracellular signal-related kinase (ERK) signals located downstream of EGFR but cannot suppress protein kinase B (AKT) survival signals. This background has given rise to an increasing demand for further development of targeted medicines against the EGF family. The belief that cancer agents targeting EGFR ligands are less effective than those targeting receptors has delayed the development of such medicines until now, but our research has revealed that this notion is not necessarily true.

In this review, we will discuss the significance of targeting EGFR ligands for cancer therapy and describe the characteristics and present state of the development of anticancer agents targeting EGFR ligands.

Significance of Targeting EGFR Ligands for Cancer Therapy

Predominant expression of HB-EGF or AR in cancer (Figure 1a). Enhancement of EGFR ligand expression, an autocrine loop mediated by an EGFR ligand itself, is the main mechanism implicated in cancer development and progression (9-11). First, in order to identify the EGFR ligands forming autocrine loops in cancer, we examined the expression levels of EGFR ligands in a variety of cancer cell lines (12). HB-EGF expression was dominantly elevated in ovarian, gastric and breast cancer, melanoma and glioblastoma. In pancreatic, colon and prostate cancer, renal cell carcinoma and cholangiocarcinoma, the expression of AR was primarily enhanced. Next, in order to examine whether inhibition of EGFR ligands exerts antitumor effects, we transfected individual small interfering RNAs (siRNAs) for these EGFR ligands into the cancer cell lines. In the cell lines with dominant expression of HB-EGF, a siRNA for HB-EGF increased the number of apoptotic cells and suppressed the activation of EGFR and ERK, whereas transfection of siRNAs for other EGFR ligands had no effects. Similarly, in the cell lines with abundant expression of AR, apoptosis and attenuation of EGFR and ERK signals were significantly mediated by inhibition of AR, while inhibition of the other EGFR ligands had no effects. Taken together, these findings suggest that HB-EGF and AR play pivotal roles in cancer cell proliferation and should be considered as promising target molecules for cancer therapy.

Increases in the levels of HB-EGF or AR can also contribute to oncogenic transformation. In the absence of growth factors, *Ha-ras*-transformed human mammary epithelial cells do not form colonies in soft agar, and exogenous recombinant human HB-EGF is able to promote their anchorage-independent growth (13). AR is not expressed in the healthy liver, but is induced in the regenerating liver after partial hepatectomy and behaves as a pivotal mitogenic and antiapoptotic factor for normal hepatocytes (14, 15). Conversely, suppression of AR production dramatically reduces the aggressiveness of hepatocellular carcinoma cancer cells in anchorage-independent growth (16). Furthermore, EGFR ligands play important roles in inflammatory and neoplastic lesions in human tissues. Secretion of HB-EGF or AR, a heparin-binding EGFR ligand with undetectable expression in normal gastric tissues, is stimulated by the hormone gastrin, which induces gastric mucosa proliferation, and the inflammatory cytokine interleukin-1β. Inflammation of the gastric mucosa is itself associated with the proliferation of parietal cells in the gastric gland and the development of gastric malignancies (17). These pieces of evidence indicate that among the EGFR ligands, HB-EGF and AR are the main contributing growth factors for human carcinomas.

Activation of signals-mediated ErbB receptor heterodimerization resistance to anti-EGFR or anti-HER2 therapy (Figure 1b). Recently, there have been a large number of studies confirming the notion that dimerization of ErbB receptors is associated with resistance to anti-EGFR or anti-HER2 therapy (18-20). Dimerization is necessary for the signaling activity of ErbB receptors. The ligand-induced formation of a receptor complex stimulates the intrinsic tyrosine kinase activities of the receptors and induces autophosphorylation of specific tyrosine residues within their cytoplasmic domains. These phosphorylated residues serve as docking sites for various adaptor proteins and enzymes involved in potent signaling cascades, such as the raf proto-oncogene serine/threonine protein kinase/mitogen-activated protein kinase kinase/mitogen-activated protein kinase (Raf/MEK/MAPK) and phosphoinositide 3 kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathways (21, 22). When one receptor is functionally inactivated, its function as a receptor tyrosine kinase can be replaced by another receptor among the HER receptors. Gefinitib down-regulates the signaling pathway *via* EGFR, but does not block dimer formation between EGFR and other HER receptors. EGFR/HER2 (and EGFR/HER3) complex formation is increased in PC-9/ZD cells, a non-small cell lung cancer cell line with acquired gefinitib resistance (23). In addition, we tested the abovementioned hypothesis using MDA-MB-468 cells, a breast cancer cell line that secretes abundant soluble HB-EGF (12). In serum-free medium supplemented with HB-EGF, MDA-MB-468 cells formed EGFR/HER2 complexes, and this complex formation was enhanced by trastuzumab but reduced by CRM197, a specific HB-EGF inhibitor (unpublished data). Correspondingly, CRM197 attenuated the phosphorylation of ERK as well as AKT and led to significant apoptotic cell death compared with trastuzumab (unpublished data). Taken together, it is assumed that ligand-induced dimerization of ErbB receptors plays important roles in retrieving the intracellular signaling for cell survival against targeted

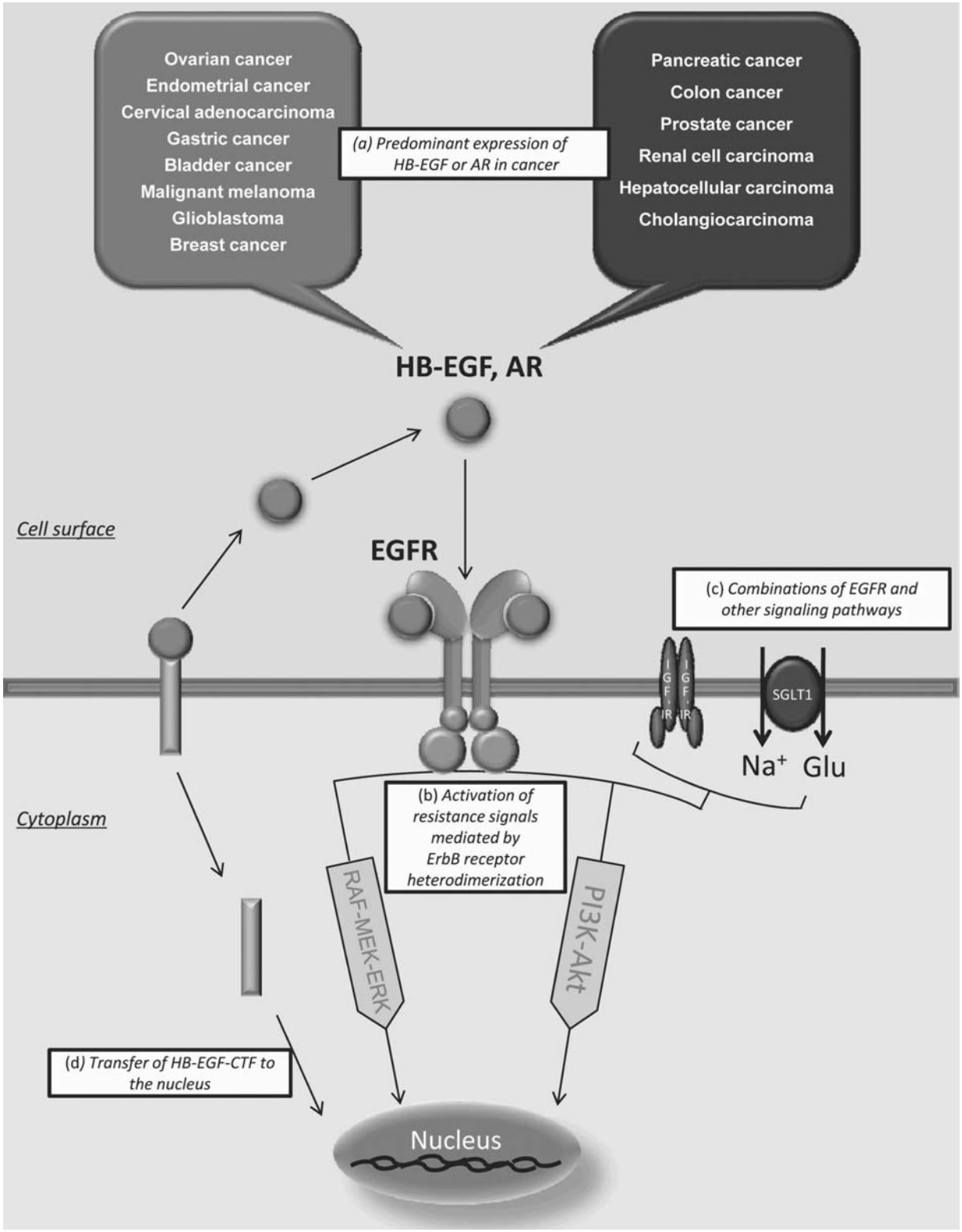

Figure 1. *Multidirectional functions of ligands in the EGF family. (a) Expression of a specific EGFR ligand (HB-EGF or AR) is enhanced in fourteen types of carcinomas and associated with cancer progression. (b) Binding of a ligand induces heterodimerization of ErbB receptors and subsequently activates resistance signals to targeted therapies for ErbB receptors. (c) Ligand-bound EGFR interacts with other growth factor receptors and SGLT1. (d) The shed extracellular domain and the CTF of HB-EGF transmit cell proliferation signals.*

therapy against EGFR or HER2 alone, and that targeting of a dominantly expressed EGFR ligand is an available strategy for the development of cancer therapies.

Combinations of EGFR and other signaling pathways (Figure 1c). EGFR signaling can be modulated by several mechanisms that include transactivation by or crosstalk with other growth factor receptors such as insulin-like growth factor-I receptor (IGF-IR) (24) or steroid hormone receptor (25). Enhancement of IGF-IR expression was reported to be associated with trastuzumab resistance in HER2-overexpressing breast cancer cells. Inhibition of IGF-IR signaling rescued trastuzumab sensitivity (26). In recent studies, IGF-I was identified as another ligand that uses EGFR for MAPK and AKT activation (27, 28). IGF-I binding to IGF-IR activates matrix metalloproteinase (MMP)-2 and MMP-9, which cleave HB-EGF and release it to bind to EGFR, thereby leading to stimulation of MAPK. In MCF-7 breast cancer cells, IGF-IR activation induced by 17β-estradiol/estrogen receptor α complexes on the cell membrane can also trigger a downstream signaling cascade through MMP-2, MMP-9, HB-EGF and EGFR and finally active MAPK (29). MDA-MB-468 cells are also a model for gefinitib resistance *via* constitutive activation of the intracellular signaling downstream of EGFR (*i.e.* the PI3K-AKT and MEK-MAPK pathways) (30-32). Under gefinitib treatment, MDA-MB-468 cells exhibit significant up-regulation (by up to 21-fold) of the EGFR ligands EGF, AR and BTC (33).

Although the significance of EGFR, an oncogenic protein, has been sufficiently proven, as described above, this review begins to clarify a new role of EGFR in malignancy. The Authors have demonstrated that the extracellular domain of EGFR associates with and stabilizes the sodium/glucose co-transporter SGLT1 to promote glucose uptake into cancer cells (34). In PC-3MM2 prostate cancer cells, a siRNA for EGFR induced autophagic cell death with a decrease in the intracellular glucose level, whereas inhibition of EGFR kinase did not exert these effects. Furthermore, EGFR increased its complex formation with SGLT1 and glucose uptake following stimulation of EGFR by exposure to EGF in serum-free medium. These novel insights into EGFR functions could widen its potential in the development of anticancer agents for EGF family members.

Transfer of HB-EGF-CTF to the nucleus (Figure 1d). Recent studies have reported that the intracellular HB-EGF carboxyl-terminal fragment (CTF) translocates from the plasma membrane to the nucleus and regulates the cell cycle when membrane-anchored HB-EGF is proteolytically cleaved by a disintegrin and metalloprotease (35, 36). BAG-1, promyelocytic leukemia zinc finger (PLZF) and B-cell leukemia 6 (Bcl6) have been identified as binding proteins for HB-EGF-CTF by yeast two-hybrid screening with the cytoplasmic region of HB-EGF (amino acids 185-208). Interactions between BAG-1, a prosurvival co-chaperone, and HB-EGF-CTF lead to attenuation of cell adhesion, resistance to apoptosis and enhancement of soluble HB-EGF expression (37). PLZF and Bcl6 are transcriptional repressors, and function as negative regulators within the cell cycle. Internalized HB-EGF-CTF co-localizes with PLZF or Bcl6 at the nuclear periphery, which releases suppression of the cell cycle. In addition, inhibition of HB-EGF-CTF nuclear translocation has been shown to reduce gastric cancer cell growth (38). Treatment using KB-R7785, an inhibitor of HB-EGF shedding, with or without EGFR activation by cetuximab interferes with the transfer of HB-EGF-CTF from the plasma membrane to the nucleus. As a result, KB-R7785 induces cell cycle arrest and increases the subG1 DNA content because PLZF remains in the nucleus and suppresses the cell cycle. Investigation of these functions of HB-EGF-CTF will lead to further understanding of the actions of EGFR ligands as growth factors and also provide new aspects for targeted therapies against EGFR ligands.

Present State of the Development of Anticancer Agents Targeting EGFR Ligands

Development of targeted therapeutic agents using CRM197. As discussed earlier, HB-EGF may be a promising target for ovarian cancer (39-41). Diphtheria toxin secreted by *Corynebacterium diphtheriae* binds to the EGF domain of HB-EGF and inhibits cell proliferation activity. Diphtheria toxin cannot be used as an HB-EGF inhibitor owing to its strong toxicity. However, cross-reacting material 197 (CRM197), a mutated diphtheria toxin, can be used because it is a non-toxic protein with a variation in the active site and binds to HB-EGF more strongly than, or at least as strongly as, diphtheria toxin. We investigated the anticancer effects of CRM197 on ovarian cancer by evaluating the proliferation of human ovarian cancer cell lines (namely SKOV3, RMG1 and OVMG1) subcutaneously implanted into nude mice. CRM197 significantly suppressed peritoneal dissemination in the nude mice peritoneally injected with RMG1 or high HB-EGF-expressing SKOV3 cells (42). Furthermore, concomitant administration of CRM197 and paclitaxel induced complete disappearance of tumors at concentrations that showed no satisfactory antitumor effects in single treatments with either agent (43). The above findings suggest that CRM197 should be considered as a promising antineoplastic agent against ovarian cancer because it shows synergistic antitumor effects with a conventional chemotherapeutic agent and exerts effects on peritoneal dissemination. We have already started phase 1 of a clinical trial of CRM197 administration for intractable advanced or recurrent ovarian cancer patients under the approval of an ethical committee.

Pan-ligand trapping as a targeted therapy for cancer. In 2008, a new strategy was reported that targets multiple ligands in the EGF family using a bispecific ligand trap, RB200 (44). RB200 was designed as a chimeric molecule composed of the full-length extracellular domains (ECDs) of EGFR and HER3 fused with the Fc domain of human immunoglobulin G1. cDNAs for EGFR and HER3 linked to the Fc domain (EGFR/Fc and HER3/Fc, respectively) were transiently co-transfected into HEK293T cells. RB200 was obtained by purification of conditioned medium harvested from the co-transfected cells. The purified RB200 bound EGFR ligands including EGF, TGFα and HB-EGF as well as HER3 ligands including NRG1-α, NRG1-β1 and NRG1-β3. Experiments were carried out to elucidate whether RB200 could inhibit ligand-stimulated phosphorylation of ErbB receptors and cell proliferation in a variety of cancer cells. Compared with C225 (the murine parent of cetuximab) and trastuzumab, RB200 successfully competed with the receptors for binding to ligands and suppressed both EGF- and NRG1-β1-induced tyrosine phosphorylation of EGFR, HER2 and HER3. Furthermore, RB200 exhibited inhibitory effects on cell growth in monolayer cultures of nine tumor cell lines and in vivo antitumor efficacy in two xenograft models (A431 human epidermoid carcinoma cells and H1437 non-small cell lung cancer cells). Furthermore, mutants with amino acid substitutions in the EGFR and HER3 ECDs showed enhanced ligand-binding affinities and were more powerful than RB200 for inhibition of cell proliferation (45). In conclusion, EGFR and HER3 ligand traps may be potent tools for cancer therapy.

Future Directions

Limitations of cancer treatments with conventional anticancer agents, such as nucleic acid analogs and cell division inhibitors, developed so far have already been proven, making it all the more crucial to develop molecular targeted medicines on the basis of cancer characteristics. The targeted therapies that have achieved certain treatment outcomes to date have involved either plasma membrane molecules (receptors: EGFR, HER2; ligands: vascular endothelial growth factor, HB-EGF) or nuclear receptors (estrogen receptor, androgen receptor, peroxisome proliferator-activated receptor γ). We have demonstrated through non-clinical basic science investigations that CRM197 targets HB-EGF, blocks the autocrine loop created by HB-EGF and shows efficacy exceeding that of targeted therapies for EGFR. As a consequence, CRM197 is currently undergoing a clinical trial for administration to cancer patients.

In future studies, we aim to elucidate the transcriptional mechanism involved in the acceleration of the autocrine loop mechanism by HB-EGF and identify novel target molecules that can inhibit nuclear signal transduction. The targeted medicines developed based on the results of these studies are expected to possess synergistic antitumor effects when used concomitantly with CRM197. Since these studies aim to identify transcription factors that induce the expression of HB-EGF, they appear to represent research into creating a novel concept targeting the molecules controlling the EGF system. According to our understanding, introduction of the *HER2* gene or a mutated *K-ras* gene accelerates HB-EGF expression in breast cancer. These observations suggest that the transcription factor controlling the expression of HB-EGF is likely to be a target molecule not only for breast cancer but also for stomach and pancreatic cancer with abnormalities in the *K-ras* gene. Consequently, we presume that these findings will help to improve the prognosis of cancer patients as well as clarify how EGF family members promote cancer progression.

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