

Review

## New Approach to Cancer Therapy: Heparin Binding-Epidermal Growth Factor-like Growth Factor as a Novel Targeting Molecule

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**Abstract.** Heparin binding-epidermal growth factor-like growth factor (HB-EGF) is one of the EGF receptor ligands and possesses several functional domains. It is involved in diverse biological processes, including wound healing, blast implantation, atherosclerosis and tumor formation, through its interactions with various molecules. We have reported that HB-EGF gene expression is significantly elevated in human ovarian cancer, and further demonstrated that HB-EGF plays key roles in the acquisition of malignant phenotypes, such as cell survival in peritoneal fluid, cell adhesion on extracellular matrices, invasion, angiogenesis, tumorigenicity, and chemoresistance in ovarian cancer. Thus, HB-EGF was considered as a promising target for cancer therapy. *In vitro* as well as *in vivo* experiments have revealed that cross-reacting material 197 (CRM197), a specific inhibitor of HB-EGF, or a small interfering RNA for HB-EGF can block each step involved in peritoneal dissemination. According to these pieces of evidence, the development of targeting tools against HB-EGF, such as CRM197, could allow us to improve the prognosis of cancer patients.

The ErbB receptors belong to the tyrosine kinase family and consist of four members, designated epidermal growth

factor receptor (EGFR)/ErbB1, ErbB2, ErbB3 and ErbB4 (1-3) (Figure 1). The activation of these receptors is controlled by the spatiotemporally regulated expression and liberation of their ligands. Ligand binding induces the formation of homo- or heterodimeric complexes of the receptors and activation of their intrinsic kinase domains. This activation results in the phosphorylation of specific tyrosine residues that serve as docking sites for adaptor molecules, thereby leading to the activation of intracellular signaling pathways. Thus, the ErbB receptor, family members transmit signals to the cells for processes, such as cell proliferation, survival, apoptosis and tumor formation. In particular, EGFR and ErbB2 can induce malignant transformation of NIH-3T3 cells (4), and have also been found to be altered in a variety of human carcinomas (5, 6). On the basis of these lines of evidence, EGFR and ErbB2 have been proposed as targets for cancer therapy.

Several anti-EGFR or anti-ErbB2 agents are available in the clinic. The two classes of anti-EGFR and anti-ErbB2 agents that show clinical activity and have achieved regulatory approval for cancer are monoclonal antibodies (mAbs) directed against the extracellular domains of the receptors and low molecular weight ATP-competitive inhibitors of the tyrosine kinases of specific ErbB receptors. Anti-EGFR or anti-ErbB2 mAbs, such as cetuximab or trastuzumab, have now been approved for the treatment of advanced colorectal and head and neck tumors, and breast cancer (7). The tyrosine kinase inhibitors include: erlotinib (OSI-774) and gefitinib (ZD1839) which inhibit EGFR; lapatinib (GW5720167), PKI-166 and PD168393 which inhibit EGFR and ErbB2; and PD12878 and CI-1033 (PD183805) which

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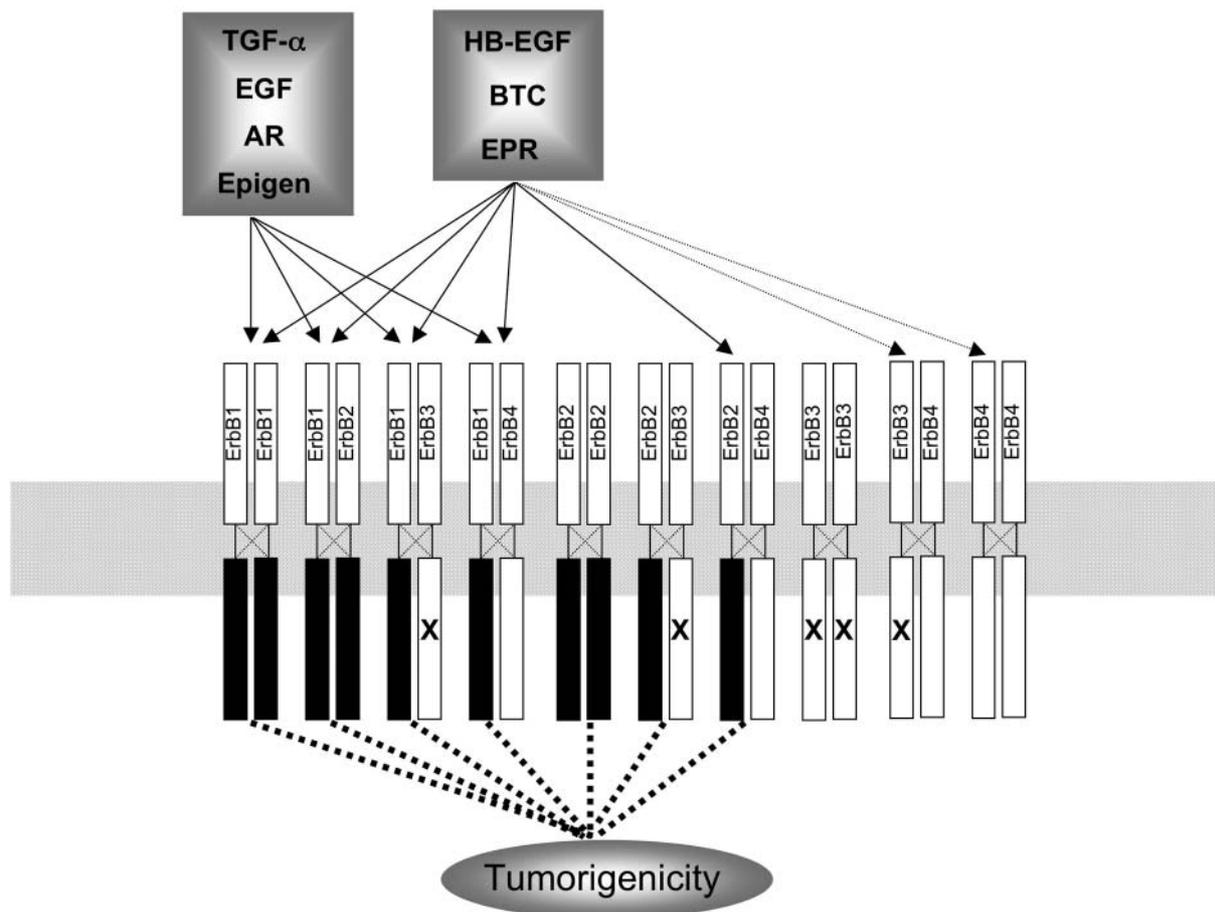


Figure 1. Binding specificities of members of the ErbB receptor family for their cognate ligands. ErbB receptor homo- and heterodimer complexes are activated by EGFR ligands. EGF: epidermal growth factor; TGF- $\alpha$ : transforming growth factor- $\alpha$ ; HB-EGF: heparin-binding EGF-like growth factor; AR: amphiregulin; BTC: betacellulin; EPR: epiregulin. ErbB3 is deficient in kinase activity (X). Black bars indicate the transforming activity for NIH3T3 cells in ErbB receptors (EGFR and ErbB2). Black solid or dotted arrows indicate binding of the EGFR ligands to ErbB receptors with or without the transforming activity for NIH3T3 cells.

inhibit all members of the ErbB receptor family (8). EGFR tyrosine kinase inhibitors, such as erlotinib, have been approved for the treatment of advanced and non-small cell lung carcinomas and pancreatic carcinoma (7). However, subsets of cancer patients seem to benefit the most from the above-mentioned therapies, and they are not always useful for most cancer patients. Therefore, further clinical studies are required to identify the most effective antibody- or small-molecule-based treatments for particular tumor types as well as for particular patients.

The EGFR ligands and EGFR play fundamental roles in development, proliferation and differentiation. To date, there are seven known EGFR ligands, namely EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), amphiregulin, epigen, heparin binding-EGF-like growth factor (HB-EGF), epiregulin and betacellulin (Figure 1). HB-EGF, epiregulin and betacellulin can also bind to ErbB4. EGFR gene

overexpression and EGFR activation by its cognate ligands in an autocrine loop are the two main mechanisms frequently implicated in cancer development and progression (9-11). Autocrine loops, in which both the receptor and its ligands are mutually activated in the same tumor cells, are recognized as important contributors to the growth autonomy of cancer cells (12). In contrast to their receptors, however, the ligands comprising the ErbB receptor family of growth factors have not yet been focused on as targets for cancer therapy. This is possibly due to the abundance of ErbB ligands for each receptor, since it is generally recognized that inhibiting receptor function is more effective than inhibiting multiple ligands for cancer therapy. However, recent studies have indicated that the expression levels of the individual EGFR ligands varies in carcinomas, and that a particular ligand is specifically expressed in some human carcinomas (13, 14). This of evidence may allow us to

develop therapeutic strategies that target EGFR ligands in some human carcinomas. In this review, we would like to highlight the features of HB-EGF among the EGFR ligands as a candidate target for cancer therapy.

### Structural and Functional Features of HB-EGF

HB-EGF is initially synthesized as a membrane-bound precursor (pro-HB-EGF), similar to other EGFR ligands (15). A soluble form of HB-EGF (sHB-EGF) is released from the cell membrane by ectodomain shedding of pro-HB-EGF, in a similar manner to that for the other EGFR ligands (16). Several physiological and pharmacological stimuli, including G protein-coupled receptor ligands, such as lysophosphatidic acid, induce ectodomain shedding of pro-HB-EGF (17). Ectodomain shedding of pro-HB-EGF is critical for growth factor activity, and unregulated release of sHB-EGF results in lethal severe hyperplasia in mice (18). Interestingly, the transmembrane form of HB-EGF (pro-HB-EGF) also acts in a juxtacrine manner to transmit signals to neighboring cells (19). The transmembrane type of HB-EGF forms complexes with several molecules. In epithelial cells, pro-HB-EGF interacts with CD9 (motility-related protein 1), integrin- $\alpha$ 3 $\beta$ 1 and heparan-sulfate proteoglycan (HSPG) (20). CD9 modulates the juxtacrine activity of pro-HB-EGF, and integrin- $\alpha$ 3 $\beta$ 1 and HSPG may also be involved in biological functions mediated by HB-EGF, such as adhesion and signaling (21). A yeast two-hybrid screening analysis has demonstrated BAG1 and promyelocytic leukemia zinc finger (PLZF) to be proteins that bind to the cytoplasmic domain of pro-HB-EGF (22, 23). BAG-1, which binds to Bcl-1 and several other signaling molecules, is capable of suppressing apoptosis (24). PLZF is recognized as a transcriptional repressor and a negative regulator of the cell cycle (25). Recently, two different strains of HB-EGF knockout mice have been established (26, 27). HB-EGF-null mice show the following phenotypes: i) an enlarged and dysfunctional heart; ii) heart valve malformation including enlarged semilunar and atrioventricular valves; and iii) thickened mesenchymal tissue and alveolar immaturity in the lungs. No abnormal cardiovascular phenotypes are present in triple null mice lacking three other EGFR ligands, namely TGF- $\alpha$ , EGF and amphiregulin (28). HB-EGF, which is recognized as unique among the EGFR ligands, participates in a variety of physiological and pathological processes, including wound healing, blast implantation, atherosclerosis and tumor formation through its multiple functions (29, 30).

### HB-EGF as a Target for Ovarian Cancer Therapy

*Clinical significance of HB-EGF expression in ovarian cancer.* Ovarian cancer, which is one of the most highly malignant carcinomas in women, is characterized by an extremely poor

prognosis. The reasons for this poor prognosis are that ovarian cancer cells are widely spread throughout the peritoneal cavity by the peritoneal fluid, thereby leading to peritoneal dissemination and ascites, and that most patients show late-stage ovarian cancer at the time of diagnosis. Accumulating evidence from many studies has revealed that peritoneal fluid from patients with ovarian cancer is an abundant source of growth factors activating ovarian cancer cell survival and proliferation, termed ovarian cancer activating factors (OCAFs) (31). The dissemination of cancer cells activated by OCAFs results in an exaggerated increase in the peritoneal fluid, which in turn leads to tumor extension of the ovarian cancer. To gain further insight into the role of HB-EGF as an OCAF, we examined the expression levels of EGFR ligands in cancer tissues using real-time polymerase chain reaction (PCR), immunohistochemistry and *in situ* hybridization (32). Real-time PCR analysis revealed that HB-EGF expression was significantly increased in advanced ovarian cancer, compared with that in normal ovaries, and significantly associated with the clinical outcome. In addition, there were large differences in expression between HB-EGF and the other EGFR ligands. By immunohistochemistry, abundant positive staining of HB-EGF protein was seen in interstitial tissues, but not in cancer cells, while diffuse staining for HB-EGF mRNA was detected in cancer cells, but not in interstitial tissues by *in situ* hybridization. These results suggest that HB-EGF protein is only produced by cancer cells, and not by interstitial tissues, and that the cleaved form of HB-EGF accumulates in the extracellular matrix with heparin sulfate in the interstitial tissues surrounding cancer cells.

The proliferation-promoting activity in peritoneal fluid obtained from patients with ovarian cancer was much higher than that in peritoneal fluid from patients with benign ovarian cysts or normal ovaries, and the activity was only suppressed by antibodies against EGFR and HB-EGF (33). In addition, cell survival activity mediated by peritoneal fluid obtained from patients with ovarian cancer was significantly elevated, compared to that mediated by peritoneal fluid from patients with benign ovarian cysts or normal ovaries. This cell survival activity was also inhibited by an antibody against HB-EGF. Large differences in the EGFR ligand level were observed between HB-EGF and TGF- $\alpha$  or amphiregulin in patients with ovarian cancer. Based on these results, we propose that HB-EGF in the peritoneal fluid of ovarian cancer patients is sufficiently enriched for cancer cells to survive and proliferate, suggesting that HB-EGF in the peritoneal fluid plays a key role in the extension of ovarian cancer. Taken together, these clinical studies suggested that HB-EGF may contribute to the progression of ovarian cancer and that HB-EGF is a putative target molecule for ovarian cancer therapy.

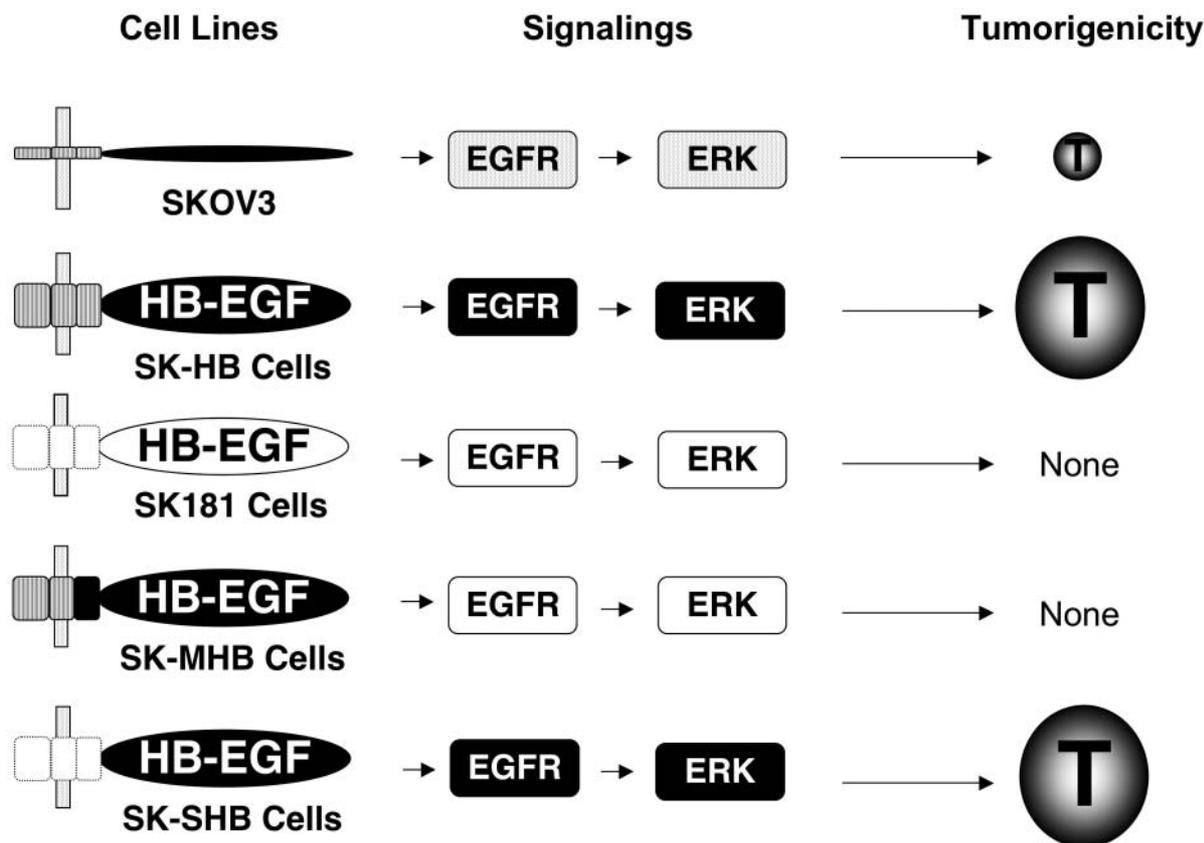


Figure 2. Schemes of the alterations in EGFR and ERK activation and tumorigenic properties of SKOV3, SK-HB, SK181, SK-MHB and SK-SHB cells. SKOV3 cells show EGFR and ERK activation and significant tumor formation in nude mice. In SK181 and SK-MHB cells, the activation of EGFR and ERK is completely suppressed and no tumor formation is detected, while SK-HB and SK-SHB cells show huge tumor growth in nude mice, accompanied by remarkable activation of EGFR and ERK. EGFR: epidermal growth factor receptor; ERK: extracellular-related kinase; T: tumor growth in nude mice.

*HB-EGF expression and tumorigenicity in ovarian cancer.* To confirm HB-EGF as a novel target for ovarian cancer therapy, we investigated the relationship between HB-EGF expression and signal transduction or tumorigenicity. We transfected various plasmids into SKOV3 (low HB-EGF expression cells) cells and established transfected cells harboring plasmids encoding human pro-HB-EGF (SK-HB cells), an uncleavable pro-HB-EGF mutant (SK-MHB cells), or an HB-EGF mutant with its transmembrane domain deleted (SK-SHB cells), as well as cells harboring a plasmid coexpressing a small interfering RNA (siRNA) against pro-HB-EGF (SK181 cells) (14) (Figure 2). The SK-MHB cells are resistant to the ectodomain shedding induced by various stimuli, while the SK-SHB cells secrete sHB-EGF in the absence of shedding stimuli (18). The EGFR and extracellular-related kinase (ERK) activation in parental SKOV3 cells was completely diminished in the SK-MHB and SK181 cells, while EGFR and ERK were remarkably activated in the SK-HB and SK-SHB cells, compared to the levels in the parental SKOV3 cells. In

addition, the SK-MHB and SK181 cells did not form tumors until 10 weeks after subcutaneous injection, whereas the SKOV3 cells formed significant tumors in nude mice at a much earlier time-point. The SK-HB or SK-SHB cells induced markedly more rapid tumor growth in nude mice, compared to that induced by the parental SKOV3 cells. These results indicated that the release of sHB-EGF is essential for tumor formation, suggesting that HB-EGF is a promising target for ovarian cancer therapy.

*HB-EGF expression and acquisition of chemoresistant properties in ovarian cancer.* Paclitaxel (taxol) has been widely used as a therapeutic agent for various carcinomas including those of the ovary, breast and lung (34, 35). In our clinical study, the enhanced expression of HB-EGF was significantly associated with the clinical outcome as well as with chemoresistance in ovarian cancer. Paclitaxel inhibited tumor formation in SKOV3 cells growing subcutaneously in nude mice, but only weakly inhibited tumor formation by the transfected SKOV3 cells overexpressing HB-EGF (SK-

HB cells) (14, 32). Paclitaxel partially, but dose-dependently, suppressed the *in vitro* proliferation of SKOV3 cells, while no inhibitory effect was observed for SK-HB cells, even at the highest concentration of paclitaxel. In a comparison of SKOV3 cells with SK-HB cells, paclitaxel increased the number of apoptotic cells and levels of c-Jun N-terminal kinase (JNK) and p38 activation in SKOV3 cells, compared to the corresponding values in SK-HB cells, whereas Akt activation was clearly detected in the SK-HB cells, but not in the SKOV3 cells. Accordingly, enhanced expression and/or presence of HB-EGF modulates the paclitaxel-induced anti-apoptotic signaling molecules, such as ERK and Akt, or pro-apoptotic signaling molecules, such as JNK and p38, thereby leading to the acquisition of chemoresistant properties in the cells.

*Involvement of HB-EGF in peritoneal dissemination of ovarian cancer.* To further confirm that HB-EGF is a promising target for patients with advanced ovarian cancer, we needed to prove that HB-EGF is intensely involved in peritoneal dissemination as well as chemoresistance of ovarian cancer through *in vitro* experimental procedures. The acquisition of a malignant phenotype for peritoneal dissemination of ovarian cancer mainly involves four key steps as shown in Figure 3.

Previous studies indicated that HB-EGF is sufficiently enriched for cancer cells to survive in the peritoneal fluid of ovarian cancer patients (33). For the cell adhesion step, spreading assays were performed on fibronectin, collagen type I and collagen type III, which are all components of extracellular matrices in the abdominal peritoneum, using RMG1 (high HB-EGF expression cells) and SKOV3 ovarian cancer cells. Transfection of a siRNA for HB-EGF or EGFR, but not for TGF- $\alpha$  or amphiregulin into the RMG1 ovarian cancer cells significantly decreased the cell adhesive properties on extracellular matrices. The suppression of HB-EGF expression in the RMG1 cells also inhibited EGFR and focal adhesion kinase (FAK) activation as well as integrin  $\beta$ 1 expression (personal communication). In addition, the presence of sHB-EGF enhanced the cell adhesive properties of SKOV3 and RMG1 cells on extracellular matrices. These results suggested that HB-EGF is responsible for the cell adhesive properties on extracellular matrices in the abdominal peritoneum. For invasion assays, the RMG1 and SKOV3 ovarian cancer cells were added to the Matrigel coated wells. The numbers of migrated cells were significantly lower following transfection of a siRNA for EGFR or HB-EGF, compared to the numbers of migrated cells among untransfected cells and those after transfection of a siRNA for TGF- $\alpha$  or amphiregulin (personal communication). The addition of sHB-EGF to the SKOV3 cells also significantly increased the cell invasive properties. The transfection of a siRNA for HB-EGF or EGFR, but not

for TGF- $\alpha$  or amphiregulin into ovarian cancer cells significantly decreased the expression levels of VEGF and interleukin (IL)-8. Constitutive suppression of HB-EGF after transfection of the HB-EGF siRNA vector markedly inhibited tumor growth on xenografted mice. Taken together, these results indicated that HB-EGF can contribute to the aggressive behavior of a tumor, such as invasiveness, angiogenesis and tumorigenicity. According to these accumulating results, HB-EGF is involved in each key step of peritoneal dissemination.

To further confirm the promotion of peritoneal dissemination of ovarian cancer mediated by HB-EGF, tumor volumes in the peritoneal cavity were analyzed using RMG1 cells alone and RMG1 cells transfected with a siRNA for HB-EGF, TGF- $\alpha$ , or amphiregulin. The RMG1 cells, which show a high level of HB-EGF expression, exhibited definite peritoneal dissemination in mice following intraperitoneal inoculation. The RMG1 cells transfected with a siRNA for HB-EGF failed to form disseminated tumors in the peritoneal cavity, while the RMG1 cells transfected with a siRNA for TGF- $\alpha$  or amphiregulin formed similar tumor volumes by peritoneal dissemination to those formed by the parental cells. The SKOV3 cells harboring relatively low expression of HB-EGF displayed no peritoneal dissemination in mice following intraperitoneal inoculation. After transfection of the plasmid containing a human pro-HB-EGF cDNA into the SKOV3 cells, the transfected cells exhibited a high level of HB-EGF expression and formed a significant tumor volume in the peritoneal cavity in mice following intraperitoneal inoculation. According to our observations, HB-EGF plays a pivotal role in peritoneal dissemination, including cell survival, cell adhesion, angiogenesis and tumorigenicity.

*CRM197 as an anticancer agent for ovarian cancer.* Cross-reacting material 197 (CRM197) is a nontoxic mutant of diphtheria toxin that shares the immunological properties of the native molecule. It binds to human HB-EGF and blocks its mitogenic activity by prohibiting its binding to EGFR (36). CRM197 has been used as a specific inhibitor of HB-EGF, since it does not inhibit the mitogenic activities of other EGFR ligands. We found that CRM197 effectively blocked all the steps of disseminated metastasis including cell survival, adhesion, invasion, angiogenesis and tumorigenicity (Figure 3). Thus, CRM197 attenuated the cell survival properties of the peritoneal fluid of ovarian cancer patients. In addition, CRM197 blocked the cell adhesion mediated by integrins on extracellular matrices, accompanied by inhibition of FAK and EGFR activation in the RMG1 cells. The number of migrated ovarian cancer cells in was also significantly reduced in the presence of CRM197. Moreover, the expression levels of VEGF and IL-8 were suppressed in the presence of CRM197.

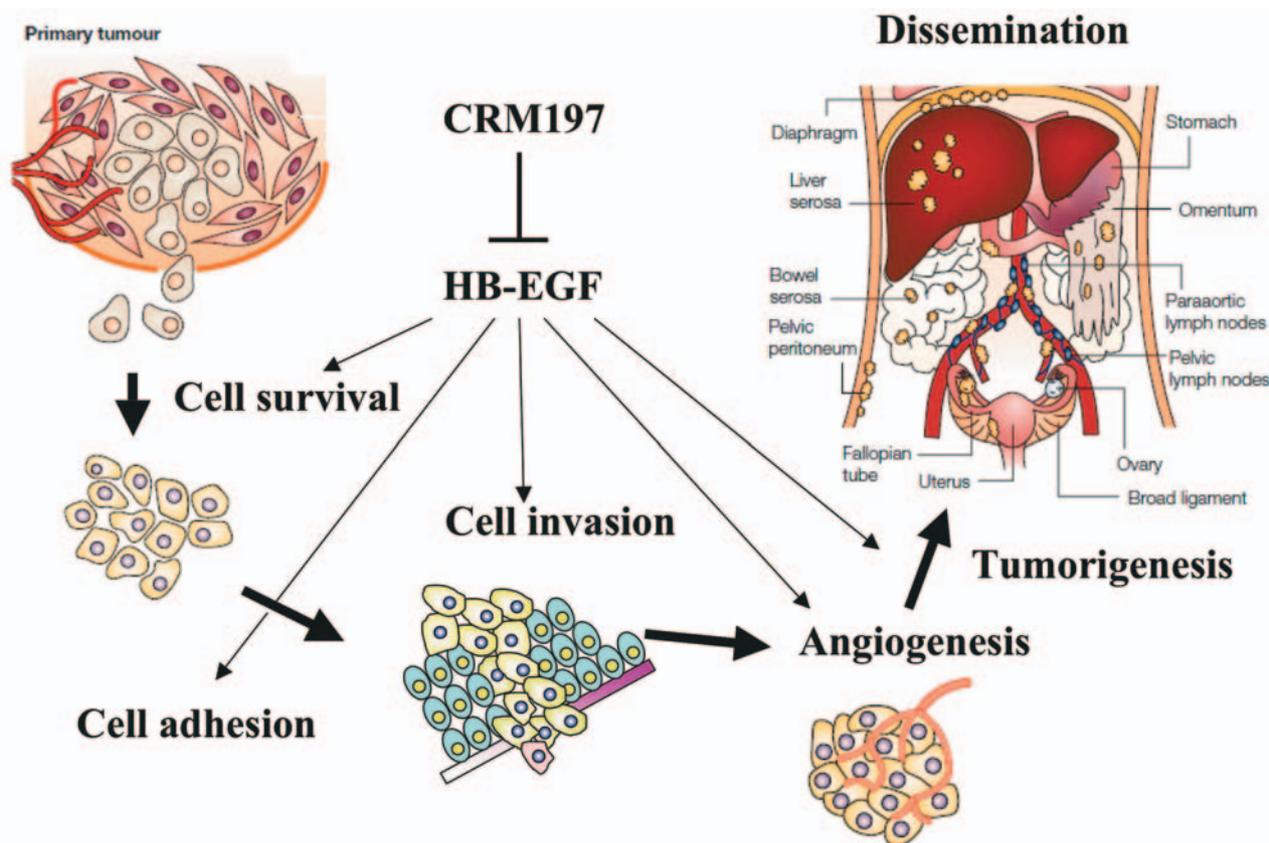


Figure 3. Schemes for each step of peritoneal dissemination of ovarian cancer. Peritoneal dissemination mainly consists of four steps: i) survival of cancer cells in peritoneal fluid after detachment from the primary cancer lesion; ii) adhesion of cancer cells to extracellular matrices of the peritoneum covering the peritoneal cavity; iii) motility and invasion of cancer cells into the extracellular matrices of the peritoneum and iv) angiogenesis and tumorigenicity mediated by cancer cells at the dissemination sites. Our studies have revealed that HB-EGF is intricately involved in each step of peritoneal dissemination and that the behavior of the cancer cells at each step is suppressed by CRM197.

To investigate the antitumor effects of CRM197 on xenografted mice, RMG1, SKOV3 and OVMG1 cells were injected subcutaneously into nude mice, and the tumor sizes were examined at the injection sites each week (14). The tumor formation by SKOV3, RMG1 or OVMG1 cells was completely suppressed by CRM197 treatment. Furthermore six weeks after intraperitoneal injection of RMG1 cells, hardly any tumor burden occurred in the abdominal cavity when CRM197 treatment was administered, while a significant tumor burden was detected in control mice. To further examine the combined antitumor effects of CRM197 and paclitaxel on xenografted mice, SKOV3 and OVMG1 cells were injected subcutaneously into nude mice, and the tumor sizes were measured at the injection sites each week. When CRM197 was administered alone, tumor growth was suppressed in a dose-dependent manner for both cell types, whereas paclitaxel alone did not significantly inhibit tumor formation by the SKOV3 or the OVMG1 cells. However, co-administration of paclitaxel and CRM197 completely

blocked tumor formation by both the SKOV3 and the OVMG1 cells, indicating a synergistic *in vivo* antitumor effect for the combined treatment. Moreover, CRM197 displayed antitumor effects on mice xenografted with human cancer cells, including gastric, bladder, prostate, breast and endometrial cancer cells, as well as melanoma and glioblastoma.

A clinical trial of CRM197 treatment has been carried out on patients with advanced cancer (37). Out of a total of 25 outpatients with advanced cancer, who were refractory to standard therapies or had refused conventional therapies, 2, 1 and 6 patients showed a complete response, partial response and stable disease, respectively following subcutaneous CRM197 injection. The toxicities were minimal, since only 1 patient had skin irritation at the injection sites and a flu-like syndrome with fever. Taken together, these results suggest that CRM197-mediated inhibition of HB-EGF contributes to the loss of *in vitro* and *in vivo* tumor formation in ovarian

cancer as well as other carcinomas and that HB-EGF is a potential target for cancer therapy. Since CRM197 is available for patients with cancer, its use could allow improvement of the clinical outcomes of patients with many types of carcinomas.

### HB-EGF and Other Human Carcinomas

To date, the emerging evidence has revealed that HB-EGF expression is increased in tumors, including pancreatic, liver, esophageal, colon, gastric, ovarian and bladder carcinomas, as well as melanoma and glioblastoma, compared with normal tissues (30). The relevance of HB-EGF expression in human cancer has been minutely investigated in human ovarian cancer. It has been demonstrated that only HB-EGF is abundantly expressed among the EGFR ligands, and that HB-EGF, but not other EGFR ligands, possibly contributes to tumor growth signaling *via* EGFR activation in both ovarian and bladder carcinomas. Several laboratories have reported high levels of HB-EGF mRNA expression in pancreatic cancer tissues, compared with those in normal tissues (38). The level of HB-EGF mRNA expression was also significantly correlated with clinical prognosis in patients with gastric cancer (39). HB-EGF gene expression, as measured by *in situ* hybridization and immunostaining, was found to be elevated in 100% (17/17) of human hepatocellular carcinoma biopsies, compared with the surrounding liver tissues that only showed faint positivity in normal hepatocytes (40). The dominant expression of HB-EGF was only detected at the early stage in colon and pancreatic carcinomas (41). According to these reports, it is plausible that HB-EGF expression, which is elevated in a variety of human carcinomas, is associated with the aggressive behavior of a tumor.

In human gastric cancer, HB-EGF was identified as one of the candidate DDP(cisplatin)-resistance-related genes (42). Chemotherapy treatment induced the elevated expression of HB-EGF, which was largely dependent on the activation of chemotherapy-resistant genes, including activator protein-1 and nuclear factor- $\kappa$ B (NF- $\kappa$ B), suggesting that chemotherapy-induced HB-EGF activation represents a critical mechanism for inducible chemotherapy resistance (43). Thus, HB-EGF has emerged as a key molecule in the resistance to cancer agents.

### Future Directions

Many clinical cancer therapy trials using tools against EGFR have been performed. However, in most of these clinical trials, the therapies have not always been successful. In principle, EGFR antagonists interfere with the activation of several intracellular pathways that control cell proliferation, survival, apoptosis, invasion and metastasis.

Acquisition of resistance to EGFR antagonists can occur as a result of several different molecular mechanisms including autocrine/paracrine production of ligands, receptor mutations, constitutive activation of downstream pathways and activation of alternative pathways (44). Two types of EGFR mutations exist in human carcinomas. The first type is EGFRvIII (variant III), which is generated by deletion of exons 2 to 7 of the EGFR gene and overexpressed in glioblastoma multiforms (45). This mutant EGFR does not bind to EGFR ligands and is more tumorigenic than wild-type EGFR. However, EGFRvIII induces the expression of HB-EGF as well as other genes and the inhibition of HB-EGF activity with neutralizing antibodies reduces the cell proliferation induced by expression of EGFRvIII, suggesting that the EGFRvIII-HB-EGF-wild-type EGFR autocrine loop plays an important role in signal transduction by EGFRvIII in glioma cells (46). The second type occurs in lung carcinomas, in which three different kinds of mutations that are all located in exons 18-21, have been identified, missense mutations, deletions and in-frame insertions (47, 48). These mutant EGFRs are hyperreactive to EGFR ligands, compared to wild-type EGFR, and selectively activate the Akt and STAT pathways. Mutations of K-ras, a signaling molecule in the EGFR downstream pathway, are detected in most patients with pancreatic cancer, approximately 50% of patients with colon cancer and 20-30% of patients with other carcinomas (49). Ras point mutations have been implicated in accelerated signaling for tumor growth, not only through the downstream signaling pathways of Ras/Raf/ERK, but also through the upstream signaling pathways of EGFR mediated by increased expression levels of EGFR ligands (50, 51). Furthermore, increased expression of EGFR ligands has also been reported to occur in ErbB2-transformed human mammary epithelial cells (52). Therefore, in some epithelial carcinomas harboring ras point mutations or enhanced expression of ErbB2, the autocrine loops of EGFR/EGFR ligands may play pivotal roles in the cancer progression. In breast and prostate cancer cells, acquired resistance to gefitinib or trastuzumab is associated with increased signaling *via* the insulin-like growth factor I receptor (IGF-IR) pathway (53, 54). Crosstalk between IGF-IR and EGFR occurs *via* an autocrine mechanism involving matrix metalloprotease-dependent release of HB-EGF and accounts for the majority of IGF-I-stimulated Shc phosphorylation and activation of the ERK cascade in COS-7 cells (55).

On the basis of these lines of evidence, an abundant increase in a particular EGFR ligand should contribute to resistance to EGFR targeting therapies. In the near future, the development of targeting agents against EGFR ligands, such as EGFR antagonists, should be a requisite for improving the clinical outcomes of cancer patients.

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