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Clinical Cancer Research

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The EGFR Ligands Amphiregulin and Heparin-Binding EGF-like Growth Factor Promote Peritoneal Carcinomatosis in CXCR4-Expressing Gastric Cancer

Kazuo Yasumoto[1], Tadaaki Yamada[2], Atsuhiro Kawashima[3], Wei Wang[2], Qi Li[2], Ivan Shterev Donev[2], Shinji Tacheuchi[2], Hisatsugu Mouri[2], Kaname Yamashita[1], Koushiro Ohtsubo[2], and Seiji Yano[2]

Abstract

Purpose: Peritoneal carcinomatosis, often associated with malignant ascites, is the most frequent cause of death in patients with advanced gastric cancer. We previously showed that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis from gastric cancer. Here, we investigated whether epidermal growth factor receptor (EGFR) ligands are also involved in the development of peritoneal carcinomatosis from gastric cancer.

Experimental Design: The functional involvement of expression of the ErbB family of receptors and/or EGFR ligands was examined in CXCR4-expressing human gastric cancer cells and fibroblasts, clinical samples (primary tumors and ascites), and an animal model.

Results: High concentration of the EGFR ligands amphiregulin and heparin-binding EGF-like growth factor (HB-EGF), as well as of CXCL12, were present in malignant ascites. Human gastric cancer cell lines and primary gastric tumors, with high potential to generate peritoneal carcinomatosis, expressed high levels of EGFR and CXCR4 mRNA and protein. Both amphiregulin and HB-EGF enhanced the proliferation, migration, and functional CXCR4 expression in highly CXCR4-expressing gastric cancer NUGC4 cells. Amphiregulin strongly enhanced the proliferation of NUGC4 cells, whereas HB-EGF markedly induced the migration of fibroblasts. Moreover, HB-EGF and CXCL12 together enhanced TNFα-converting enzyme (TACE)-dependent amphiregulin shedding from NUGC4 cells. In an experimental peritoneal carcinomatosis model in mice, cetuximab effectively reduced tumor growth and ascites formation.

Conclusions: Our results strongly suggest that the EGFR ligands amphiregulin and HB-EGF play an important role, interacting with the CXCL12/CXCR4 axis, in the development of peritoneal carcinomatosis from gastric cancer, indicating that these two axes may be potential therapeutic targets for peritoneal carcinomatosis of gastric carcinoma. *Clin Cancer Res; 17(11); 3619–30. ©2011 AACR.*

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide, with peritoneal carcinomatosis, often associated with malignant ascites, being the most frequent cause of death in patients with advanced gastric cancer (1–3). The 5-year survival rate of patients with peritoneal carcinomatosis, including those with intraperitoneal free cancer cells and without macroscopic peritoneal carcinomatosis, is only 2% (4). Peritoneal carcinomatosis in gastric cancer patients may be due to the direct dissemination of cancer cells into the peritoneal cavity. We have previously shown that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis with malignant ascites from gastric cancer (5). CXCL12 produced by the tumor environment, in particular by peritoneal mesothelial cells, may enhance the proliferation and/or survival of CXCR4-expressing gastric cancer cells in the peritoneal cavity, leading to the survival and peritoneal dissemination of gastric cancer cells. Other tumor-associated factors, in addition to CXCL12, may be necessary to promote the malignant potential and survival of cancer cells (6). Peritoneal fluid acts as a rich source of growth factor activity for cancer cells (7). Thus, activation of disseminated gastric cancer cells by cancer-activating factors, including CXCL12, may lead to the peritoneal dissemination of gastric cancers. The development of effective therapies targeting peritoneal carcinomatosis from gastric cancer requires further understanding of the processes and molecules leading to its initiation and progression.

Activation of the ErbB family of receptors has been associated with the progression of various tumor types.

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Translational Relevance

Peritoneal carcinomatosis, often associated with malignant ascites, is the most frequent cause of death in patients with advanced gastric cancer. We have previously shown that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis form gastric cancer.

In this study, we showed that the EGFR ligands, amphiregulin and HB-EGF, are abundant in malignant ascites. Amphiregulin strongly enhanced the proliferation of CXCR4-expressing human gastric cancer NUGC4 cells, whereas HB-EGF markedly induced migration of fibroblasts. Moreover, HB-EGF and CXCL12 together enhanced TNFα-converting enzyme (TACE)-dependent amphiregulin shedding from functional CXCR4-expressing NUGC4 cells. Cetuximab, an anti-EGFR monoclonal antibody, effectively reduced tumor growth and ascites formation, and therefore dramatically prolonged survival in nude mice inoculated with NUGC4 cells. Our findings provide a novel insight into treatments that target tumor cells and their microenvironments during the development of peritoneal carcinomatosis from gastric cancer.

Epidermal growth factor receptor (EGFR) is a member of a family of closely related growth factor receptor tyrosine kinases, including EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Aberrant activation of EGFR signaling, such as that due to overexpression of EGFR or abnormal stimulation by autocrine growth factor loops, can contribute to constitutive EGFR signaling, resulting in the dysregulation of cell growth and ultimately leading to cancer (8). In particular, EGFR and ErbB2 have been recognized as targets in the treatment of various cancers (9). Although only about 20% of gastric cancers overexpress HER2, treatment with trastuzumab, a human monoclonal antibody specific for HER2, plus chemotherapy has shown survival benefits in patients with advanced, HER2-positive gastric cancer (10). To date, 7 ligands for EGFR have been identified: EGF, transforming growth factor α (TGFα); heparin-binding EGF-like growth factor (HB-EGF); amphiregulin; betacellulin; epiregulin; and epigen. Amphiregulin has been shown to play a crucial role in several types of tumors (11, 12), and HB-EGF has also been reported critical for tumor formation (13). However, the association between each EGFR ligand and tumor type, in particular the development of peritoneal carcinomatosis, is largely unknown. Moreover, there have been no comprehensive studies examining the role of EGFR ligands in gastric cancer progression driven by the tumor environment.

Here we report that the EGFR ligands amphiregulin and HB-EGF promote peritoneal carcinomatosis by stimulating the proliferation of tumor cells and their microenvironment and by enhancing functional CXCR4 expression in gastric cancers. Furthermore, these 2 EGFR ligands showed different biological activities in target cells. These findings indicate that EGFR and its ligands, especially amphiregulin and HB-EGF, are important in the development of peritoneal carcinomatosis in gastric cancer patients. Moreover, these findings provide a framework for future clinical trials of specific ErbB receptor inhibitors that target tumor cells and their environments.

Materials and Methods

Cell lines

The human gastric cancer cell lines, MKN28, MKN45, KKLS, NKPS (14), and NUGC4 (15), were purchased from the Health Science Research Resources Bank (Japan Health Science Research Resources Bank). All cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin G, and 100 units/mL streptomycin (Invitrogen Corp.). NKPS and NUGC4 are gastric cancer cell lines derived from malignant ascites or pleural effusions of patients with advanced gastric cancer and selectively express high levels of CXCR4. Following their inoculation into the abdominal cavity of nude mice, NUGC4 cells disseminate rapidly and form bloody ascitic fluid (16).

Tissue samples

Primary tumor tissue samples were obtained from the Division of Surgical Oncology, Cancer Research Institute, Kanazawa University, during gastrectomy of previously untreated gastric cancer patients. The histologic diagnosis of each patient was confirmed from the specimens. In addition, primary cultured fibroblasts were isolated from surgical specimens of human stomach and were identified by immunostaining with monoclonal antibodies against cytokeratin and vimentin. All patients provided written informed consent for molecular analysis of surgical samples.

Chemotaxis assay

Migration assays were carried out in 24-well Transwell plates (Costar Corp.) using inserts with 8 μm pore size membranes, as described (17). Cells were suspended in upper chambers at 1×10^6 cells/mL in 100 μL of RPMI 1640/0.1% bovine serum albumin. Where indicated, the cells were preincubated with 10 μg/mL monoclonal antibody against human CXCR4 (12G5, R&D) or EGFR (cetuximab, Merck Serono). The lower chambers contained 600 μL of RPMI 1640 supplemented with 100 ng/mL of CXCL12 (R&D Systems), 1 ng/mL of HB-EGF (R&D Systems), and 100 ng/mL of amphiregulin (R&D Systems). After incubation for 24 hours, the tumor cells remaining on the upper surface of the filters were removed by wiping with cotton swabs, and cells migrating to the lower surface of the membrane were stained with Giemsa solution (Wako) and at least 5 different fields were counted under a light microscope (original magnification, × 200). All assays were carried out in triplicate.

Proliferation assay

Gastric cancer cells (5×10^3) in RPMI 1640 supplemented with 10% charcoal treated FBS (Hyclone) were added to

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each well of a 96-well cell culture plate and cultured for 6 hours. Where indicated, the cells were preincubated with 10 μg/mL anti-EGFR monoclonal antibody (cetuximab, Merck Serono). The cells were stimulated with various concentrations of amphiregulin or HB-EGF and cultured for 72 hours. Two hours prior to the end of the assay, 10 μL of WST-8 (WST-8 Cell Counting Kit; Wako Pure Chemical) was added to each well, and the optical density at 450 nm was measured using an ELISA plate reader (BIO-RAD Laboratories, Inc.).

Cell proliferation was also measured using the MTT dye reduction method (18). Cells at 80% confluence were harvested, seeded at 2×10^3 per well in 96-well plates, and incubated in RPMI 1640 with 10% FBS for 24 hours. Various concentrations of amphiregulin and HB-EGF were added to each well, and incubation was continued for an additional 72 hours. A 50 μL aliquot of MTT solution (2 mg/mL; Sigma) was added to each well followed by Incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL dimethyl sulfoxide. Absorbance was measured using an ELISA plate reader (BIO-RAD). Percent growth was measured relative to untreated controls. Each condition was assessed in triplicate wells, and each experiment was carried out at least 3 times.

Antibodies and Western blot analysis

Cells were incubated in 10 mL RPMI 1640 supplemented with 10% FBS in the presence or absence of HB-EGF or amphiregulin. The cells were washed twice with PBS, harvested in cell lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL, leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride], and flash-frozen on dry ice. After allowing the cells to thaw, the lysates were collected with a rubber scraper, sonicated, and centrifuged at 14,000 × g (4°C for 20 minutes). Total protein concentrations were measured using a Pierce BCA Protein Assay kit (Pierce). For Western blotting analysis, 40 mg of total protein were resolved by SDS-polyacrylamide gel (Bio-Rad) electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad). After washing, the membranes were incubated for 1 hour at room temperature with Blocking One (Nacalai Tesque, Inc.) and then overnight at 4°C with the following primary antibodies: anti–phospho EGFR (Y1068), anti-ErbB2, anti-ErbB4, anti-Akt, phospho-Akt (Ser473), and anti-TACE (each 1:1,000 dilution, Cell Signaling Technology). The membranes were also incubated with anti-human EGFR (1 mg/mL), anti-CXCR4 12G5 (10 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase 1/2 (ERK1/2; 0.2 μg/mL), and anti–phospho-ERK1/2 (T202/ Y204; 0.1 μg/mL; R&D Systems); with anti-amphiregulin (1 μg/ml; Abcam), and with polyclonal anti–glyceraldehyde 3 phosphate dehydrogenase (anti-GAPDH; 1:1,000 dilution; Trevigen). After 3 washes, the membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with Super Signal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each assay was carried out independently in triplicate.

RNA interference

Duplexed Stealth RNA Interference (Invitrogen) against *EGFR* (5′-UUUAAAUUCACCAAUACCUAUUCCG-3′ and 5′-CGGAAUAGGUAUUGGUGAAUUUAAA-3′), *ErbB4* (5′-UAUAGAUGUUUCCUGCGCUGAUUUC-3′ and 5′-GAAAUCAGCGCAGGAAACAUCUAUA-3′), and TNFα-converting enzyme (*TACE*) (5′-AUGAGUUGUAACCAGGUACGCUUCC-3′, and 5′-GGAAGCUGACCUGGUUACAACUCAU-3′) and Stealth RNA Interference Negative Control Low GC Duplex 3 (Invitrogen) were used for RNA interference assay. Briefly, aliquots of 1×10^5 NUGC4 cells or human normal fibroblasts in 2 mL antibiotic-free medium were plated on 6-well plates and incubated at 37°C for 24 hours. The cells were transfected with small interfering RNA (siRNA; 250 pmol) or scramble RNA (siSCR) using Lipofectamine 2000 (5 μL) in accordance with the manufacturer's instructions. After 24 hours, the cells were washed twice with PBS and incubated with or without recombinant human amphiregulin (100 ng/mL) and/or HB-EGF (1 ng/mL) for an additional 72 hours in antibiotic-containing medium. These cells were then used for the proliferation (MTT) assay as described above. *EGFR*, *ErbB4*, and *TACE* knockdown were confirmed by Western blotting analysis. Each experiment was carried out at least 3 times using triplicate samples.

Animal studies

Pathogen-free, 6-week-old, female BALB/c (*nu/nu*) mice were obtained from Japan SLC (Hamamatsu) and quarantined for 1 week under pathogen-free conditions at the Advanced Science Research Center of Kanazawa University to confirm the absence of diseases. All animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. Mice weighting 18 to 22 g were injected intraperitoneally with 1×10^6 NUGC4 cells in 200 μL of PBS. NUGC4 cells can rapidly disseminate in the abdominal cavities of nude mice on i.p. inoculation and form bloody ascitic fluid (16). Beginning 3 days after NUGC4 cell injection, the mice were injected i.p. with 2 mg/kg cetuximab (19), a specific monoclonal anti-EGFR neutralizing antibody, or normal IgG (R&D Systems) twice weekly for 2 weeks. No *in vivo* toxicity of cetuximab was detected in these mice. To monitor the extent of development of peritoneal carcinomatosis, body weights were routinely measured. Mice were sacrificed 40 days after NUGC4 cell injection. Ascitic fluid was collected from each mouse, and its volume was recorded. Macroscopic peritoneal tumor dissemination and the size and number of tumors in the abdomen were also determined.

ELISA

The concentrations of CXCL12, EGF, TGFα, HB-EGF, and amphiregulin in ascitic fluid were measured using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol. Optical density at 450 nm was measured using an ELISA plate reader (BIO-RAD).

Immunohistochemistry

Surgical specimens of primary gastric tumors were routinely fixed in formalin and embedded in paraffin. Tissue sections (3 μm) were deparaffinized in xylene, rehydrated in an ethanol series, and treated for 30 minutes with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were subsequently washed with PBS, and sections being tested for EGFR expression were unmasked in citrate antigen unmasking solution (Target Retrieval Solution High pH; Dakocytomation) in an autoclave for 20 minutes at 120°C. Sections being tested for CXCR4 were not unmasked. The samples sections were immunohistochemically stained for EGFR with the EGFR pharmDx kit (Dakocytomation) or for CXCR4 with anti-human CXCR4 monoclonal antibody (44716; R&D Systems) using a standard indirect avidin-biotin HRP method. Sections were regarded as positively stained when the intensity of staining was 10% or more.

Statistical analysis

All parameters are reported as mean ± SD, with between-group differences compared using Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Presence of amphiregulin and HB-EGF in malignant ascitic fluid

Peritoneal carcinomatosis is often associated with malignant ascites, a rich source of growth factor activity for various cancer cells (7). To determine whether EGFR ligands are present in malignant ascites from patients with gastric cancer, we assayed the concentrations of the EGFR ligands EGF, TGFα, HB-EGF, and amphiregulin in these samples and in nonmalignant peritoneal exudates (Table 1). As we previously reported (5), the mean concentration of CXCL12 protein was markedly higher in ascitic fluid from gastric cancer patients (4,667 pg/mL) than in nonmalignant peritoneal exudates (<2,000 pg/mL). Although EGF and TGFα were barely detectable in any of these samples, the mean concentrations of amphiregulin (3,055 pg/mL vs. 784.2 pg/mL) and HB-EGF (346.5 pg/mL, vs. 69.7 pg/mL) were markedly higher in malignant ascites than in nonmalignant peritoneal exudates, suggesting that amphiregulin and HB-EGF may promote tumor growth during peritoneal dissemination.

EGFR ligands, amphiregulin and HB-EGF, enhance the proliferation and migration of CXCR4-expressing human gastric cancer cells

We previously reported that the CXCR4/CXCL12 axis is involved in the chemotactic response, proliferation and survival of gastric cancer in the peritoneal cavity (5). Because NUGC4 cells exhibit the highest levels of CXCR4 expression and rapidly disseminate in the abdominal cavity of nude mice on i.p. inoculation (5), we used this cell line as our model of CXCR4-expressing gastric cancer. To determine whether the EGFR ligands amphiregulin and HB-EGF promote tumor growth during peritoneal carcinomatosis, we assessed the proliferation of NUGC4 cells in response to amphiregulin and HB-EGF. We found that both of these ligands significantly and dose dependently enhanced the proliferation of NUGC4 cells when the cells were maintained in suboptimal serum-free growth conditions for 72 hours (Fig. 1A and B). Proliferation in response to amphiregulin was much greater than proliferation in response to HB-EGF and/or CXCL12. Furthermore, the anti-EGFR neutralizing antibody cetuximab significantly blocked the proliferation of NUGC4 cells induced by amphiregulin and HB-EGF (Fig. 1C). When we tested the effects of amphiregulin and HB-EGF in another CXCR4-expressing gastric cancer cell line, NKPS (5), we observed similar results. Both amphiregulin and HB-EGF enhanced the proliferation of

Table 1. Presence of amphiregulin and HB-EGF in malignant ascitic fluids

	Malignant ascites		Nonmalignant ascites	
Growth factors	Mean	Range, pg/mL	Mean	Range, pg/mL
CXCL12	4,667	2,530–7,320	1,958	1,408–2,443
EGF	<4		<4	
TGFα	<4		<4	
HB-EGF	346	30–2,500	52	45–164
Amphiregulin	3,055	288–13,850	776	320–1,600

NOTE: Concentration of the EGFR ligands (EGF, TGFα, HB-EGF, and amphiregulin) and of CXCL12 in malignant ascitic fluids from patients with gastric cancer ($n = 20$) and in nonmalignant exudates ($n = 10$), as determined by ELISA.

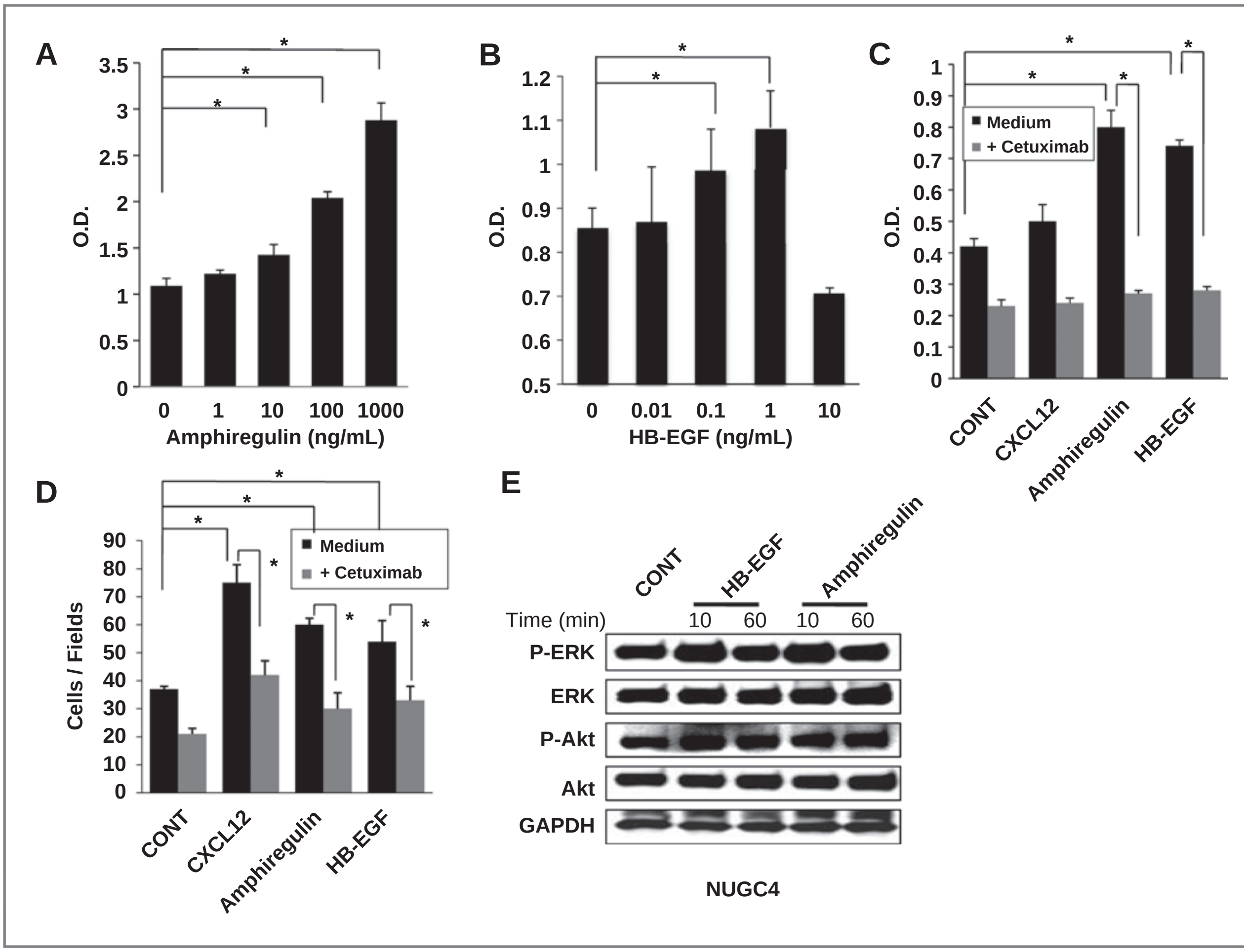

Figure 1. The EGFR ligands amphiregulin and HB-EGF enhance the proliferation, migration, and Akt and ERK phosphorylation of NUGC4 cells. NUGC4 cells were grown in medium containing 10% charcoal treated FBS, with or without amphiregulin (A) or HB-EGF (B). Cetuximab inhibits functional EGFR activities. NUGC4 cells were grown in serum-free medium with or without 10 μg/mL of the anti-EGFR antibody, cetuximab. Cetuximab significantly inhibited growth factor-induced cell proliferation (C). CXCL12, amphiregulin, and HB-EGF significantly enhance the migration of NUGC4 cells, a migration significantly inhibited by cetuximab (D). Immunoblotting analysis of Akt and ERK phosphorylation. NUGC4 cells were seeded in 10-cm dishes, and stimulated with HB-EGF (1 ng/mL) or amphiregulin (100 ng/mL) for 10 minutes and 1 hour. Whole cell lysates were electrophoresed, blotted onto filter membranes, and probed with primary antibodies against phospho-Akt, Akt, phospho-ERK, ERK, and GAPDH (E). Representative results from 3 separate experiments are shown. Bars, SD. *, $P < 0.05$.

NKPS cells, though these effects were weaker than in NUGC4 cells (data not shown).

Assays of the chemotactic responses of NUGC4 cells to EGFR ligands showed that these cells were highly responsive to amphiregulin (100 ng/mL), HB-EGF (1 ng/mL), and CXCL12 (100 ng/mL), with the strongest responses to CXCL12. Furthermore, the neutralizing anti-EGFR antibody cetuximab significantly inhibited the chemotactic responses of NUGC4 cells to these EGFR ligands (Fig. 1D).

We also tested the effects of EGFR ligands on the phosphorylation of Akt and ERK, molecules involved in cell survival signaling (20). We found that both amphiregulin and HB-EGF rapidly and strongly enhanced the phosphorylation of Akt and ERK in NUGC4 cells (Fig. 1E), occurring as soon as 10 minutes after exposure.

HB-EGF markedly enhances migration and proliferation of human normal fibroblasts, accompanied by increased phosphorylation of ERK and Akt

Tumor-stroma interactions play an important role in tumor development and progression, and stromal fibroblasts are frequently associated with cancer progression. When we tested the effects of amphiregulin, HB-EGF, and CXCL12, on the migration and proliferation of primary cultured human normal fibroblasts, we found that HB-EGF, but not amphiregulin, strongly enhanced the proliferation of human fibroblasts, up to 1.5-fold, and that this proliferation was slightly inhibited by cetuximab (Fig. 2A). Interestingly, human fibroblasts showed marked chemotactic responses to HB-EGF, but not to CXCL12 or amphiregulin, with this chemotaxis significantly inhibited by

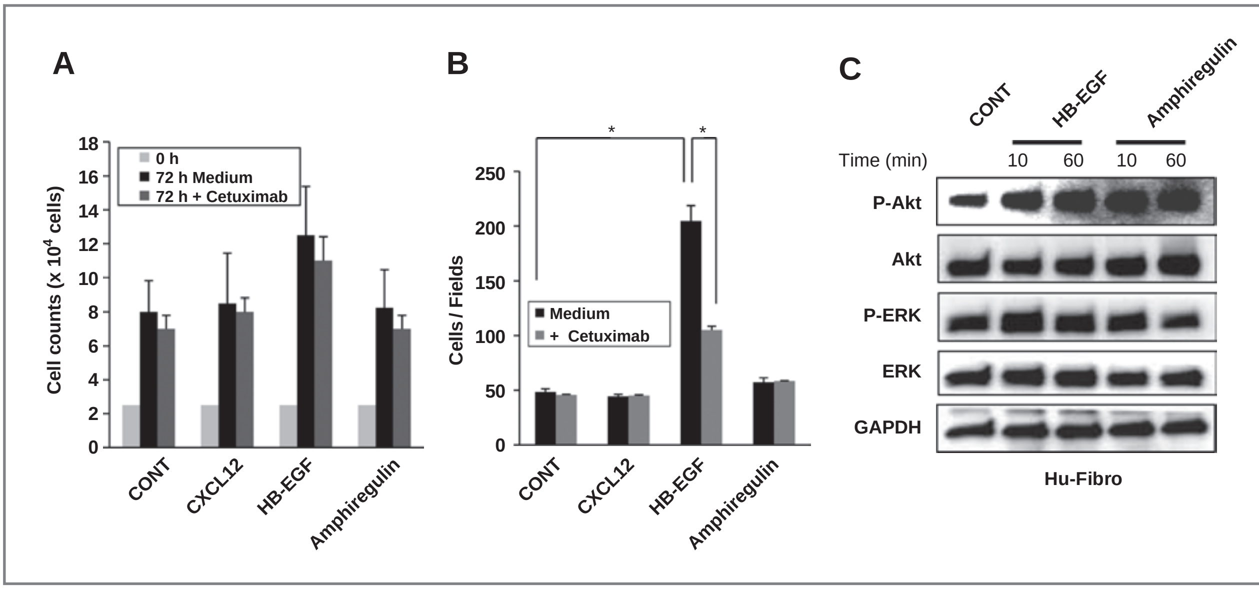

Figure 2. HB-EGF induces chemotactic response and cell proliferation, and rapid phosphorylation of Akt and ERK, in human normal fibroblasts. A, HB-EGF enhances proliferation of human normal fibroblasts. Normal human fibroblasts, seeded at 2.5×10^4 cells per well in 6-well plates, were stimulated with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 72 hours. HB-EGF stimulation resulted in an up to 1.5-fold increase in the number of human fibroblasts, compared with control or amphiregulin treated cells. Representative results from 1 of 3 independent experiments are shown. B, HB-EGF enhances migration of human normal fibroblasts. Induction of human fibroblast migration by CXCL12, HB-EGF, and amphiregulin. Neutralizing anti-EGFR antibody significantly blocked the enhancing effects of HB-EGF on migration of human normal fibroblasts. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$. C, HB-EGF enhances Akt and ERK phosphorylation of human normal fibroblasts. Immunoblotting analysis of the phosphorylation of Akt and ERK. Human normal fibroblasts were seeded in 10-cm dishes and stimulated with HB-EGF (1 ng/mL) or amphiregulin (100 ng/mL) for 10 minutes and 1 hour. Whole cell lysates were electrophoresed, blotted onto filter membranes, and probed with primary antibodies against phospho-Akt, Akt, phospho-ERK, ERK, and GAPDH. Representative results from 1 of 3 independent experiments are shown.

cetuximab (Fig. 2B). Moreover, stimulation of human fibroblasts with both HB-EGF and amphiregulin rapidly and strongly increased the phosphorylation of Akt and ERK (Fig. 2C).

Taken together, these findings showed that amphiregulin more effectively enhanced the proliferation of NUGC4 cells than HB-EGF, whereas HB-EGF more strongly enhanced human fibroblast migration than amphiregulin. These results strongly suggest that the EGFR ligands, amphiregulin and HB-EGF, both of which are abundant in malignant ascitic fluids, promote tumor progression driven by the tumor microenvironment during peritoneal dissemination.

Expression of EGFR, ErbB4, and CXCR4 in human gastric cancer cell lines and primary gastric tumors with peritoneal carcinomatosis

Amphiregulin and HB-EGF show different biological activities in target cells (21), with amphiregulin mainly stimulating cell proliferation (22) and HB-EGF stimulating chemotaxis (23). Amphiregulin binds to ErbB1 (EGFR), whereas HB-EGF binds to both ErbB1 (EGFR) and ErbB4 (24). To determine the molecular basis underlying these functional differences, we assayed the expression of EGFR, ErbB4, and CXCR4 in human gastric cancer cell lines and primary tumors. Western blotting using antibodies specific to EGFR showed that 4 of 5 human gastric cancer cell lines (MKN28, MKN45, NKPS, and NUGC4) expressed high levels of EGFR protein, whereas the fifth (KKLS) expressed little EGFR protein (Fig. 3A). We also found that normal human fibroblasts, which are present in the tumor microenvironment of the stomach and peritoneal cavity, express high levels of EGFR protein (Fig. 3A). RT-PCR showed similar results for EGFR mRNA (data not shown). When we assayed ErbB4 expression in these cell lines and primary tumors (Fig. 3A), we found that NKPS cells expressed a low amount, whereas MKN28, MKN45, KKLS, and NUGC4 cells essentially expressed none. In contrast, normal human fibroblasts expressed high levels of ErbB4 protein (Fig. 3A). NUGC4 and NKPS cells, which are highly efficient in generating malignant ascites in nude mice on i.p. inoculation, selectively expressed CXCR4 mRNA and protein (5). In particular, NUGC4 cells, which express high amounts of CXCR4, as well as EGFR mRNA and protein, disseminate soon after inoculation into the abdominal cavity of nude mice and form massive amounts of bloody ascitic fluid (5). Immunohistochemical staining showed that, of 29 primary stage IV gastric carcinomas with peritoneal carcinomatosis, 23 (79%) and 22 (76%) were positive for EGFR and CXCR4, respectively (Fig. 3B), whereas none was positive for ErbB4 expression (Supplementary Fig. S1).

We also used specific siRNA for EGFR and ErbB4 to knock down the expression of these proteins (Fig. 3C) and examined the effects of the amphiregulin

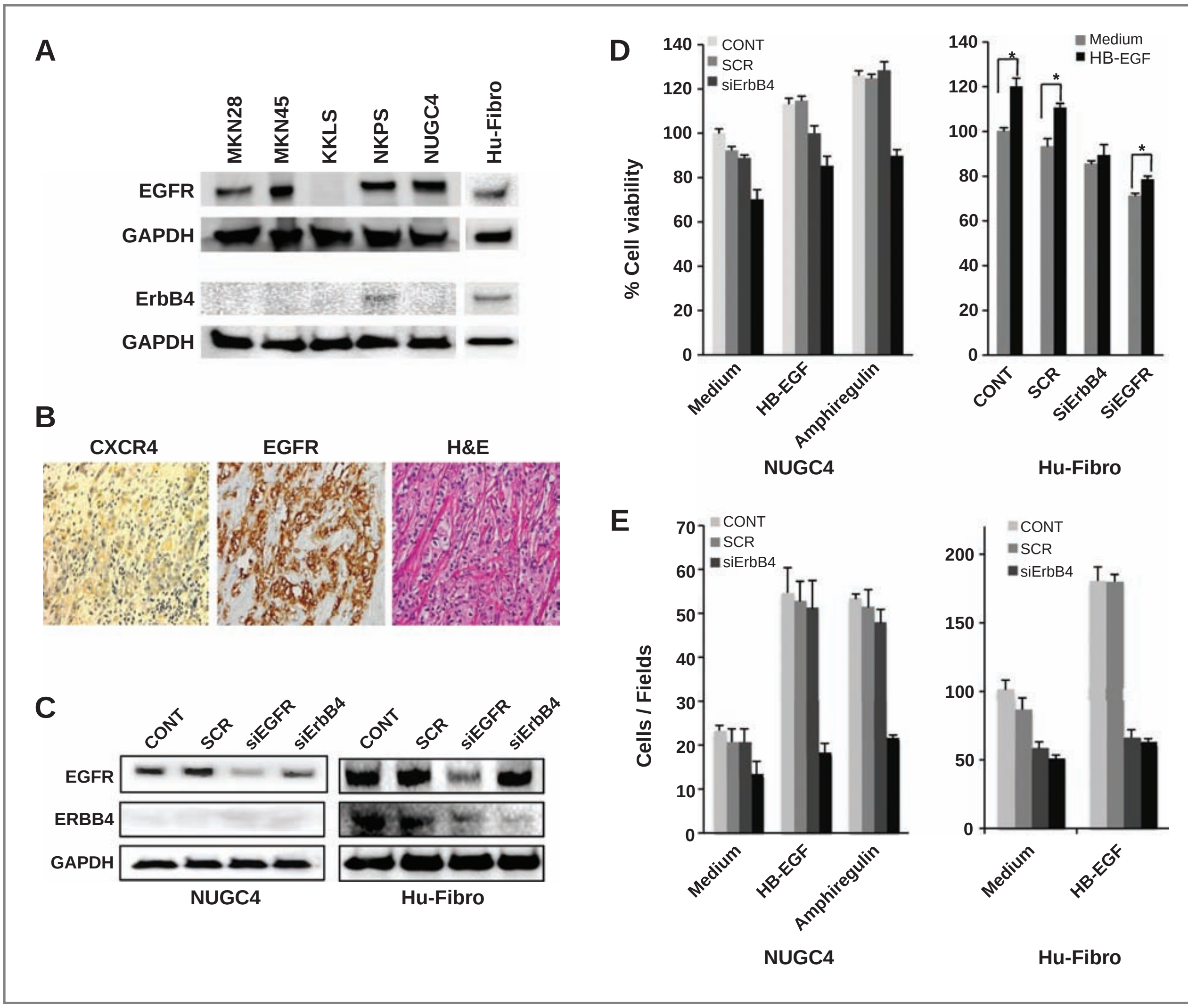

Figure 3. Expression of EGFR and ErbB4 in human gastric cancer cell lines and normal human fibroblasts. A, Western blotting analyses of EGFR and GAPDH expression in 5 human gastric cancer cell lines (MKN28, MKN45, KKLS, NKPS, and NUGC4) and normal human fibroblasts. The levels of EGFR protein in each sample were normalized relative to the levels of GAPDH protein. Representative results from 3 independent experiments are shown. B, immunohistochemical staining of CXCR4 and EGFR in human primary gastric tumors. Anti-CXCR4, anti-EGFR, and H&E staining of a representative CXCR4- and EGFR-positive gastric cancer. CXCR4 and EGFR were strongly localized in the membranes but weakly expressed in the cytoplasm of these cells. Original magnification, ×160. (C) Effects of EGFR and ErbB4 siRNA on NUGC4 cells and human normal fibroblasts. EGFR and ErbB4 knockdown was confirmed by Western blotting. Each experiment was done at least in triplicate and 3 times independently. D, knockdown of EGFR effectively reduced the amphiregulin-induced enhancement of NUGC4 cell proliferation, whereas knockdown of ErbB4 effectively reduced the HB-EGF enhancement of fibroblast proliferation. Cell proliferation was measured using the MTT dye reduction method. The percentage of growth is shown relative to untreated controls. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$. E, knockdown of EGFR effectively reduced the enhancing effects of amphiregulin and HB-EGF on NUGC4 cell migration, whereas knockdown of either EGFR or ErbB4 effectively reduced the enhancing effects of HB-EGF on fibroblast migration. Columns, mean number of migrated cells per field from a representative experiment in triplicate and repeated thrice; bars, SD. *, $P < 0.05$.

and HB-EGF on the proliferation and migration of NUGC4 cells and fibroblasts (Fig. 3D and E). In NUGC4 cells, knockdown of EGFR effectively reduced the enhancing effects of amphiregulin on proliferation compared with that of ErbB4. By sharp contrast, in fibroblasts, knockdown of ErbB4 effectively reduced the enhancing effects of HB-EGF on proliferation compared with that of EGFR (Fig. 3D). We also found that knockdown of EGFR, but not ErbB4, effectively reduced the enhancing effects of amphiregulin and HB-EGF on NUGC4 cell chemotaxis. Interestingly, in fibroblasts, knockdown of either EGFR or ErbB4 effectively reduced the chemotaxis enhancing effects of HB-EGF (Fig. 3E).

Collectively, these results indicate that, in NUGC4 cells, EGFR may be the predominant receptor mediating proliferation and migration induced by amphiregulin and HB-EGF. In fibroblasts, however, ErbB4 may be the predominant receptor mediating HB-EGF induced proliferation,

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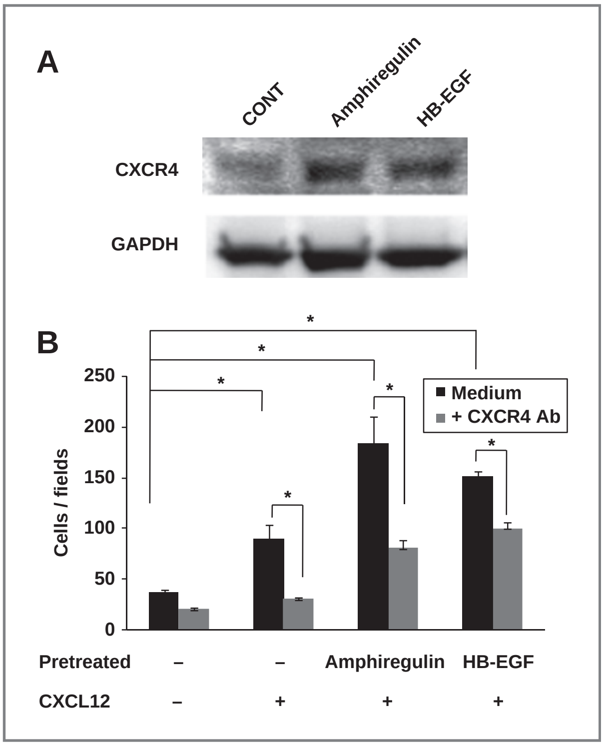

Figure 4. Amphiregulin and HB-EGF induce functional CXCR4 expression in NUGC4 cells. A, Western blot analysis of total proteins extracted from cells treated with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 1 hour and incubated with anti-CXCR4 antibody. B, pretreatment with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 24 hours significantly enhanced the migration of CXCR4-expressing NUGC4 cells. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$.

whereas both ErbB4 and EGFR are important for HB-EGF induced migration.

Amphiregulin and HB-EGF markedly enhance functional CXCR4 expression in NUGC4 cells

Because CXCR4 expression by primary gastric cancers promotes the development of peritoneal carcinomatosis (5), we examined whether amphiregulin and HB-EGF modulate CXCR4 expression in NUGC4 cells. We found that both ligands strongly increased CXCR4 protein concentrations in NUGC4 cells (Fig. 4A). We also found that NUGC4 cells pretreated with both ligands for 24 hours showed a significant and much higher chemotactic response to CXCL12 than non-pretreated NUGC4 cells (Fig. 4B). Tumor environmental stimulation, such as hypoxia and CXCL12 (SDF-1α), a growth factor abundantly produced in the peritoneal cavity, have been found to induce CXCR4 protein expression in cancer cells (25–27). These findings suggest that amphiregulin and HB-EGF may upregulate functional CXCR4 expression as well as the proliferation and/or survival of CXCR4-expressing gastric cancer cells in the peritoneal cavity, leading to the survival and peritoneal dissemination of gastric cancers.

TACE (ADAM17) cleaves amphiregulin produced by CXCR4-expressing gastric cancer NUGC4 cells

Using enzyme-linked immunosorbent assays (ELISA), we found that amphiregulin was present in the conditioned media of CXCR4-expressing NUGC4 cells and human fibroblasts (Fig. 5A). In contrast, none of these CXCR4-expressing gastric cancer cell lines and fibroblasts produced EGF, TGFα, or HB-EGF (data not shown). Interestingly, HB-EGF enhanced amphiregulin secretion from NUGC4 cells, particularly in the presence of CXCL12, but not from human fibroblasts. We also confirmed that costimulation with HB-EGF and CXCL12 resulted in a marked increase in amphiregulin production by all CXCR4-expressing gastric cancer cell lines, including NUGC4 (data not shown). Because HB-EGF enhanced amphiregulin secretion from NUGC4 cells, we also determined whether HB-EGF acts by increasing amphiregulin shedding alone or on ErbB signaling in NUGC4 cells. We confirmed that neutralizing anti-amphiregulin antibody could not block the ability of HB-EGF to enhance the proliferation of NUGC4 cells (Supplementary Fig. S2).

TACE has been shown to be a key regulator of amphiregulin cleavage (28). Because TACE was expressed in NUGC4 cells (Fig. 5B), we assessed whether TACE is the key regulator of these endogenously produced growth factors. We therefore used siRNA to knock down the expression of TACE and measured growth factor secretion by transfected cells (Fig. 5B and C). We found that amphiregulin shedding by NUGC4 cells was proportional to their level of TACE expression. Furthermore, Western blotting showed that the expression of TACE was highly increased in NUGC4 cells stimulated with amphiregulin and HB-EGF, as well with CXCL12 (Fig. 5D), suggesting that TACE is the primary regulator of amphiregulin secretion in NUGC4 cells. Collectively, these findings suggest that the potent proliferative EGFR ligands, amphiregulin and HB-EGF, both of which are present in ascitic fluids, synergize with CXCL12 in promoting the dissemination of CXCR4-expressing gastric cancer cells in an autocrine and/or paracrine manner.

Cetuximab, an EGFR monoclonal antibody, inhibits ascites formation by NUGC4 cells in nude mice

On i.p. inoculation into nude mice, CXCR4-expressing NUGC4 cells efficiently produce peritoneal carcinomatosis (5). We utilized this model to test whether EGFR blockade by cetuximab (19) affects the development of experimental peritoneal carcinomatosis in mice. Forty days after tumor inoculation, mice administered cetuximab showed consistently smaller disseminated tumors in the greater omentum and mesenterium than did mice administered control IgG (Fig. 6A). Furthermore, treatment with cetuximab significantly decreased the mean volume of ascitic fluid (0.24 ± 0.54 mL vs. 5.48 ± 1.86 mL, $P < 0.01$; Fig. 6B), resulting in significantly longer survival, than control IgG-treated mice (Fig. 6C).

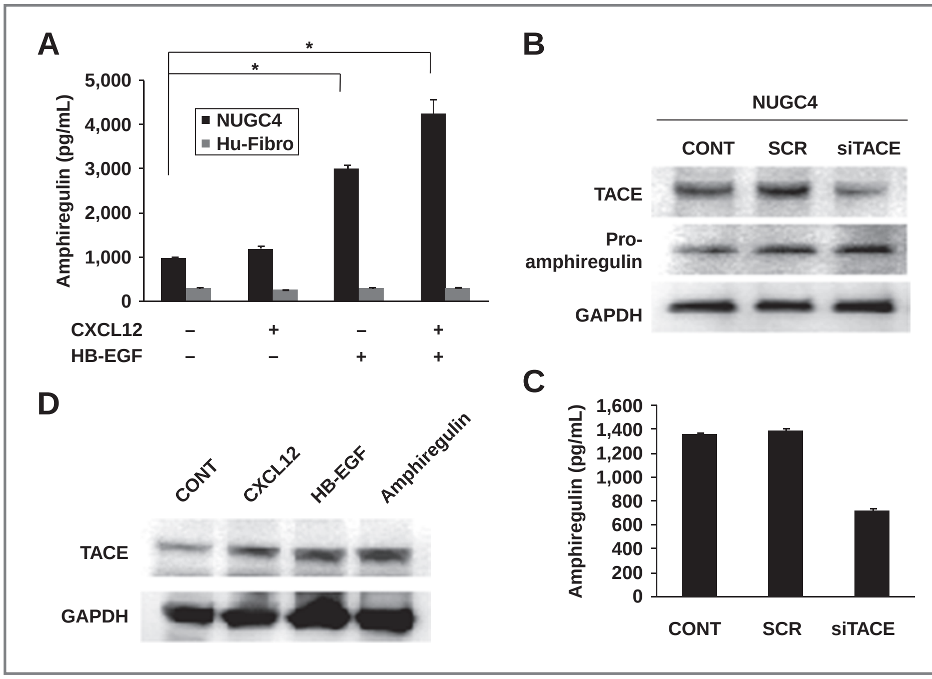

Figure 5. Presence of an autocrine amphiregulin loop in CXCR4-expressing NUGC4 cells and fibroblasts. A, synergistic production of amphiregulin by HB-EGF and CXCL12 proteins in NUGC4 cells, but not in fibroblasts. Bars, SD. *, $P < 0.05$. B and C, ELISA analysis of amphiregulin shedding in NUGC4 cells transfected with TACE siRNAs. Ligand shedding was proportional to the level of TACE expression. D, Western blot analysis showing strongly increased TACE protein levels in NUGC4 cells stimulated with amphiregulin (100 ng/mL), HB-EGF (1 ng/mL), and CXCL12 (100 ng/mL) for 1 hour. Representative results from 1 of 3 independent experiments are shown.

Discussion

We have shown here that the EGFR ligands amphiregulin and HB-EGF play important roles in the development of peritoneal carcinomatosis in patients with gastric cancer (Supplementary Fig. S3). In addition to CXCL12, amphiregulin and HB-EGF are abundantly present in malignant ascites from gastric cancer patients, whereas EGF and TGFα are not. Amphiregulin, HB-EGF, and CXCL12 stimulate CXCR4-expressing gastric cancer cells to migrate into the peritoneal cavity, as well as their proliferation. HB-EGF and CXCL12 cooperate and stimulate TACE expression, thereby upregulating amphiregulin secretion from gastric cancer cells. Secreted amphiregulin further stimulates the migration, proliferation, and amphiregulin production of gastric cancer cells in an autocrine and/or paracrine manner.

Gastric cancer and ovarian cancer are 2 major causes of malignant ascites (5, 29). In ovarian cancer, only HB-EGF is present at higher concentrations in malignant than in nonmalignant ascites (29), whereas, in gastric cancer, both HB-EGF and amphiregulin are present at higher concentrations in malignant than in nonmalignant ascites. Interestingly, though HB-EGF concentrations were about 10-fold higher in malignant ascites from ovarian than from gastric cancer patients, amphiregulin concentrations were 10-fold higher in malignant ascites from gastric than from ovarian cancer patients (3,055 pg/mL vs. 200 pg/mL; ref. 29). Moreover, amphiregulin enhanced the proliferation and/or survival signals (phosphorylated ERK and Akt) of CXCR4-expressing NUGC4 cells more strongly than did HB-EGF. These findings suggest that, though both amphiregulin and HB-EGF are involved, amphiregulin may be the predominant EGFR ligand in the development of malignant ascites in gastric cancer. Although HB-EGF did not enhance amphiregulin-stimulated proliferation or migration (data not shown) *in vitro*, it enhanced amphiregulin secretion from NUGC4 cells, particularly in the presence of CXCL12, suggesting that HB-EGF may cooperate with amphiregulin and facilitate malignant ascites formation *in vivo*.

Recent studies have revealed the process underlying the production of EGFR ligands. Amphiregulin and HB-EGF are synthesized as type I transmembrane protein precursors and expressed on the cell surface as pro-amphiregulin and pro–HB-EGF, respectively (30, 31). Shedding of pro-amphiregulin and pro–HB-EGF results in the plasma-membrane-anchored remnant carboxyl-terminal fragments amphiregulin-CTF and HB-EGF-CTF, as well as soluble EGFR ligands (32, 33). Interestingly, cleavage of membrane-anchored amphiregulin and HB-EGF induces translocation of their CTFs from the plasma membrane to the nucleus and regulates the cell cycle and/or global transcription (34, 35).

Amphiregulin is reported crucial in several types of tumors, including breast cancer, colon cancer, and hepatocellular carcinoma (11, 12). Although the expression of amphiregulin mRNA and protein has been assessed in gastric cancers (36), its role in gastric cancer pathogenesis, especially in the development of peritoneal carcinomatosis, remains poorly understood. We found that amphiregulin was present in malignant ascites from gastric cancer patients and that it could stimulate the proliferation and migration of gastric cancer cells. Amphiregulin is synthesized as a transmembrane precursor and is proteolytically processed to its mature secreted form (32) by TACE, also called disintegrin and metalloprotease-17

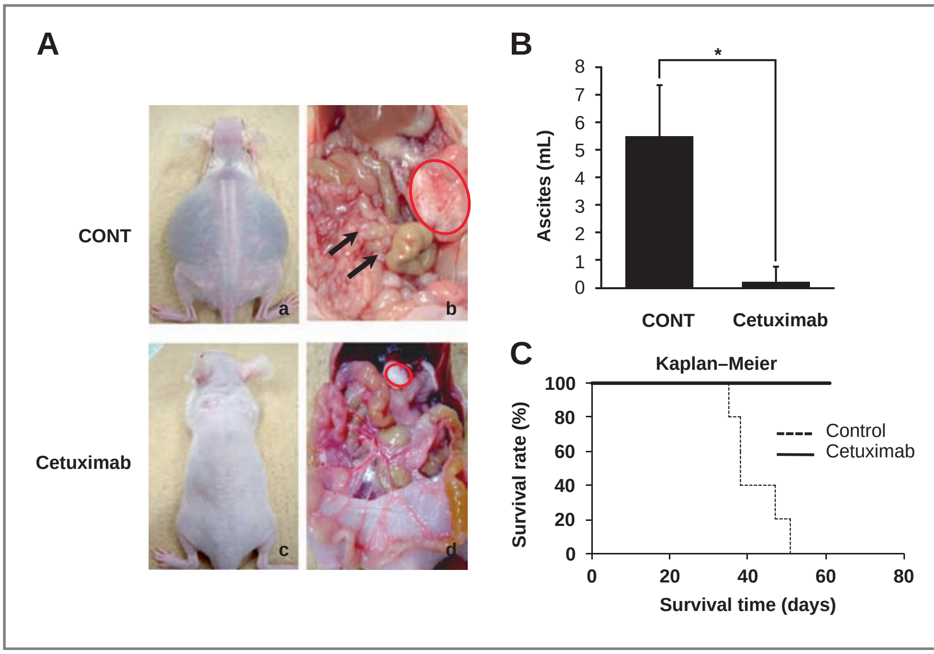

Figure 6. Cetuximab inhibits experimental peritoneal carcinomatosis. A, beginning 3 days after inoculation with CXCR4-expressing NUGC4 cells (1×10^6 per mouse), mice were injected intraperitoneally with normal IgG (a and b; 2 mg/kg) or cetuximab (c and d; 2 mg/kg) twice weekly for 2 weeks, and results were assessed 40 days later. b and d, omental tumors in the abdominal cavity. Arrows indicate disseminated tumors on the mesenterium in the peritoneal cavity. B, reduction of ascitic fluid formation by cetuximab. Representative results of 2 experiments (5 animals per group per experiment) are shown. Bars, SD. *, $P < 0.05$. C, real survival curves according to the Kaplan–Meier method. No cetuximab-treated mouse died for up to 60 days after tumor inoculation, whereas all mice treated with normal IgG were dead within 50 days.

(ADAM-17; refs. 37, 38). Interestingly, we found that HB-EGF and CXCL12 enhanced TACE expression and amphiregulin protein secretion from CXCR4-expressing NUGC4 cells and that amphiregulin itself enhanced TACE expression. These findings suggest that TACE-dependent amphiregulin shedding by NUGC4 cells is regulated in both an autocrine and a paracrine manner.

HB-EGF, which was initially reported to be produced predominantly by macrophages (39), was later found to be expressed in ovarian cancers, with ectodomain shedding of pro–HB-EGF shown to be critical for tumor formation (13). We found that malignant ascites from gastric cancer patients contained high concentrations of the soluble form of HB-EGF. Because HB-EGF was not detected in the conditioned media of any of the human gastric cancer cell lines tested, and pro–HB-EGF was not detected in NUGC4 cells (data not shown), HB-EGF may be produced predominantly by macrophages, especially regulatory macrophages, in the peritoneal cavity (40).

Interestingly, the 2 EGFR ligands, amphiregulin and HB-EGF, have shown different biological activities in target cells, with amphiregulin mainly stimulating cell proliferation (22) and HB-EGF mainly stimulating chemotaxis (23). Similarly, we found that amphiregulin more effectively cell enhanced NUGC4 cell proliferation than HB-EGF, whereas HB-EGF more strongly induced the migration of human normal fibroblasts than amphiregulin. These findings may be due to the preference of these ligands for certain receptors. Amphiregulin binds to ErbB1 (EGFR), whereas HB-EGF binds to both ErbB1 (EGFR) and ErbB4 (24). NUGC4 cells express EGFR, but little ErbB4, whereas normal human fibroblasts express both ErbB4 and EGFR. Furthermore, knockdown of EGFR in NUGC4 cells effectively reduced the amphiregulin-enhanced proliferation compared with ErbB4, whereas knockdown of ErbB4 in fibroblasts effectively reduced the HB-EGF-enhanced proliferation compared with EGFR. Furthermore, knockdown of EGFR, but not ErbB4, effectively reduced the chemotactic effects of amphiregulin and HB-EGF in NUGC4 cells, whereas knockdown of either EGFR or ErbB4 effectively in fibroblasts reduced the chemotactic effects of HB-EGF. These results suggest that the amphiregulin enhanced proliferation of NUGC4 cells is mediated predominantly by EGFR, whereas HB-EGF enhancement of fibroblast chemotaxis is mediated by both EGFR and ErbB4.

Tumor-stroma interactions play a significant role in tumor development and progression (41), and stromal fibroblasts are frequently associated with cancer progression at both primary and metastatic sites. In particular, scirrhous gastric cancers, diffusely infiltrating cancers, Borrmann type 4, also known as linitis plastica-type cancer, are characterized by rapid cancer cell infiltration and proliferation, accompanied by extensive stromal fibrosis (42). Peritoneal carcinomatosis occurs frequently in patients with scirrhous gastric cancer. To our knowledge, this study was the first to show that HB-EGF stimulated fibroblasts present in the stomach enhanced cell migration and proliferation, accompanied by increased phosphorylation of ERK and Akt. Therefore, HB-EGF produced in the peritoneal cavity may facilitate the accumulation and proliferation of fibroblasts and may induce fibrosis associated with peritoneal carcinomatosis in patients with scirrhous gastric cancer. It will therefore be important to clarify the mechanisms that regulate the expression of the soluble form of HB-EGF and the biological significance of stromal fibroblasts in gastric cancer.

We previously reported that the CXCR4/CXCL12 axis is involved in the chemotactic response, proliferation, and survival of gastric cancers in the peritoneal cavity (5). The results presented here extend our knowledge, showing that the EGFR/EGFR ligand axis is also important in these events and that these 2 axes interact with each other. CXCL12 stimulated the expression of TACE and enhanced the secretion of amphiregulin by CXCR4-expressing NUGC4 cells, especially in combination with HB-EGF. In NUGC4 cells, the EGF ligands amphiregulin and HB-EGF more potently stimulated proliferation than did CXCL12, whereas CXCL12 more potently enhanced motility. Because all 3 proteins, amphiregulin, HB-EGF, and CXCL12, are present in malignant ascites, further studies exploring the interaction between the CXCR4/CXCL12 and the EGFR/EGFR ligand axes may provide additional insights into the pathogenesis of peritoneal carcinomatosis of gastric cancer.

Cetuximab is a specific anti-EGFR monoclonal antibody, which is widely used to treat patients with several types of solid tumors, including colon, head and neck, and non–small cell lung cancers (43–45). We confirmed that cetuximab efficiently blocked the proliferation and migration of NUGC4 cells stimulated by amphiregulin and HB-EGF, indicating its anti-EGFR activity. Although cetuximab did not inhibit HB-EGF-induced fibroblast proliferation, this was not unexpected, because HB-EGF stimulated fibroblast proliferation predominantly through ErbB4. Importantly, cetuximab showed dramatic therapeutic efficacy in an experimental peritoneal carcinomatosis model in nude mice, as shown by the reduction in volume of ascitic fluids, the inhibition of disseminated tumor growth, and the prolongation of survival. These results suggest that cetuximab may be therapeutically useful for controlling peritoneal carcinomatosis of gastric cancer patients if amphiregulin and HB-EGF are highly expressed in their malignant ascites.

In conclusion, we have shown here that the EGFR ligands amphiregulin and HB-EGF collaborate with the CXCR4/CXCL12 axis in promoting peritoneal carcinomatosis by stimulating gastric cancer cell migration, proliferation, and survival. EGFR activation mediated by these EGFR ligands is a novel mechanism underlying the development of peritoneal carcinomatosis from gastric cancer. Therefore, inhibition of the EGFR/EGFR ligand axis may be a useful strategy for treating patients with gastric cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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