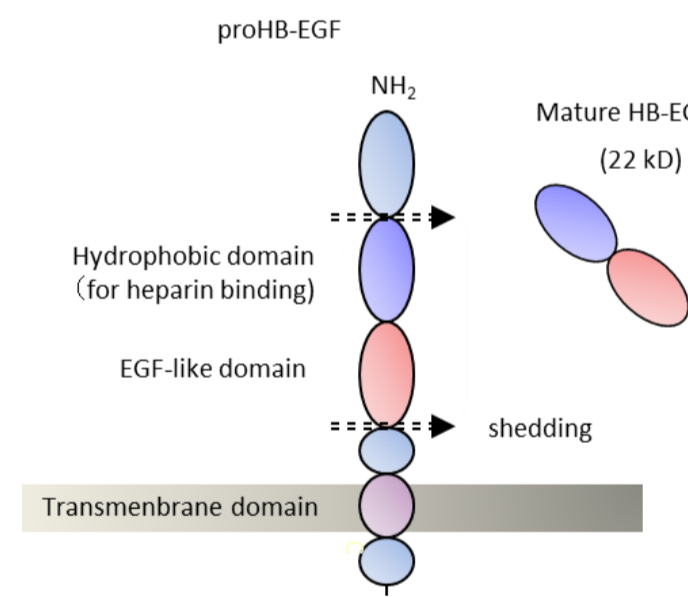


UPDATED ABSTRACT

Heparin-binding epidermal growth factor (HB-EGF), a member of the EGF family of growth factors, is more widely expressed in many human cancers than in normal tissues. It has been well-documented that HB-EGF acquires malignant phenotypes, such as tumorigenicity, invasion, metastasis, angiogenesis, and resistance to chemotherapy, which makes it an attractive target for a therapeutic antibody. Here, we demonstrate the potential for cancer therapy of a monoclonal antibody which neutralizes HB-EGF. Two monoclonal antibodies, HC15 and HE39, were generated and their blocking activity on HB-EGF was examined in vitro. Both antibodies efficiently inhibited HB-EGF from not only binding to EGFR-Fc, but also causing EGFR-expressing Ba/F3 transfectant lines to proliferate. Next, we investigated in vivo antitumor efficacy of HC15 and HE39 in several xenograft models and found that a similar inhibitory effect was observed in MCAS ovarian cancer xenograft. We also found that HC15 inhibited growth more potently than HE39 in the xenograft of HB-EGF-overexpressing SKOV3 transfectant, and could inhibit tumor growth in other human tumor models, such as pancreatic cancer (BxPC-3) and breast cancer (MDA-MB-231). These results indicate that mAbs would be useful to inhibit HB-EGF functions in cancer treatment.

INTRODUCTION

HB-EGF is synthesized as a transmembrane-anchored precursor protein (pro-HB-EGF). Mature soluble HB-EGF is later processed by an ectodomain-shedding mechanism, which exerts its biological activity through activation of EGFR and EGFR4.



Elevation of HB-EGF expression has been reported in many tumors, including hepatocellular carcinoma, colon cancer, pancreatic cancer, bladder malignancy, and ovarian cancer.

Mouse monoclonal antibodies against HB-EGF were obtained by mouse immunization with HB-EGF-mFc fusion proteins. By analyzing their ability to inhibit HB-EGF activity, two candidates from 5 different fusions (~7500 hybridomas) were chosen: HC15 and HE39.

HC15 and HE39 were then converted to human chimera IgG1, and generated in DG44 cells to move forward into pre-clinical study.

Table 1. Antibody character of anti-HB-EGF mAbs

| mAb | HB-EGF binding* (ED ₅₀ , nmol/L) | HB-EGF blocking [§] | |
|------|--|---|---|
| | | Receptor binding (IC ₅₀ , nmol/L) | Cell proliferation (EC ₅₀ , nmol/L) |
| HC15 | 0.65 | 0.52 | 2.06 |
| HE39 | 0.016 | 0.86 | 0.83 |

* Determined by direct binding ELISA. Numbers represent the mAb concentration that gives 50% of maximum binding (see also Fig.1A).

[§] Numbers represent mAb concentrations that give 50% inhibition of HB-EGF binding to immobilized EGFR or HB-EGF dependent cell growth (see also Fig.2 and 3).

Isolation and characterization of anti-HB-EGF mAbs

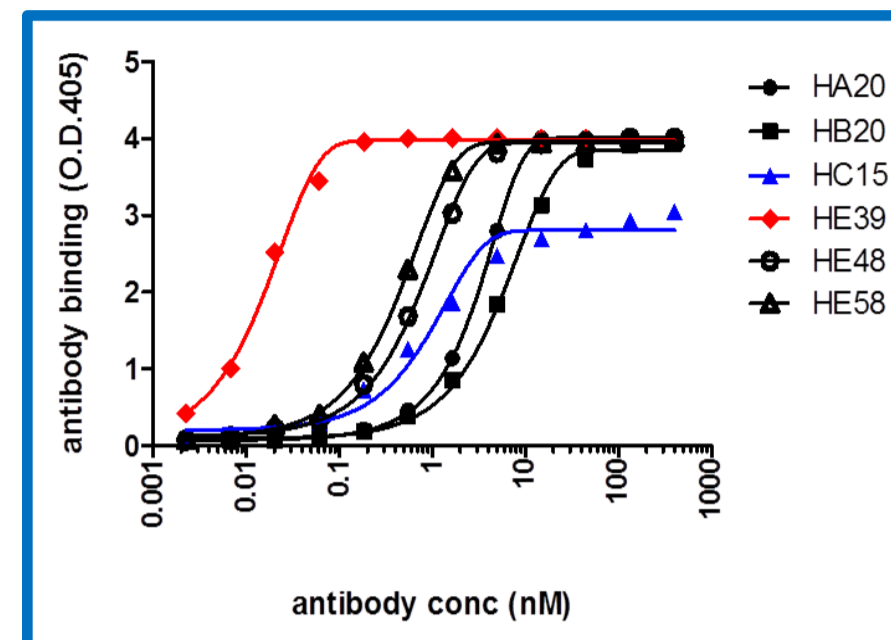


Fig.1. Comparison of mAbs binding activity to HB-EGF

Direct binding of mAbs to immobilized HB-EGF was compared by ELISA. Binding activity was detected by alkaline phosphatase conjugated goat anti-human IgG and substrate (see Table 1).

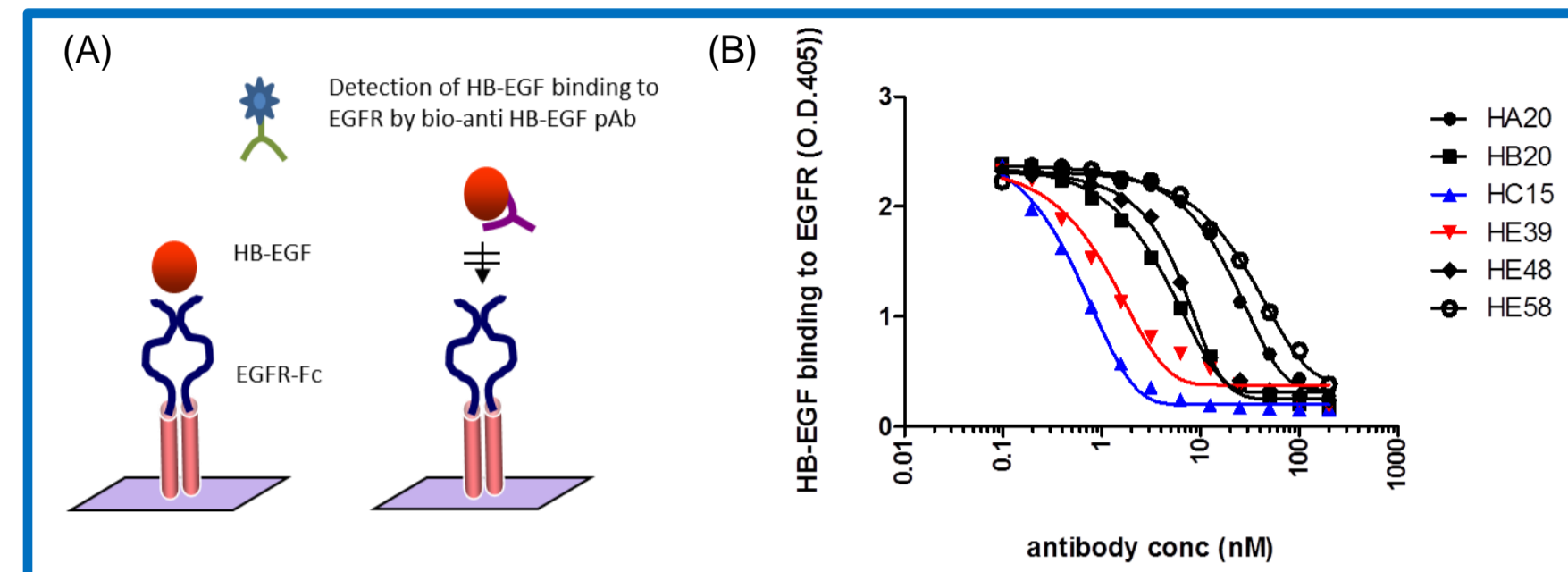


Fig.2. Inhibition of HB-EGF binding to EGFR

(A) Scheme of detecting HB-EGF binding to EGFR by ELISA. Extracellular domain of EGFR fused to human IgG Fc (EGFR-Fc) was prepared. The EGFR-Fc was captured on ELISA plates coated with goat anti-human IgG. The HB-EGF bound to EGFR-Fc was detected by incubating with biotin-conjugated anti-HB-EGF polyclonal antibody (R&D) and alkaline phosphatase conjugated streptavidin.

(B) HB-EGF (R&D) was preincubated with mAbs, and added to the wells. Then the bound HB-EGF was detected as indicated in (A). Binding of HB-EGF to the receptor was inhibited in the presence of increasing amounts of mAbs. (see Table 1)

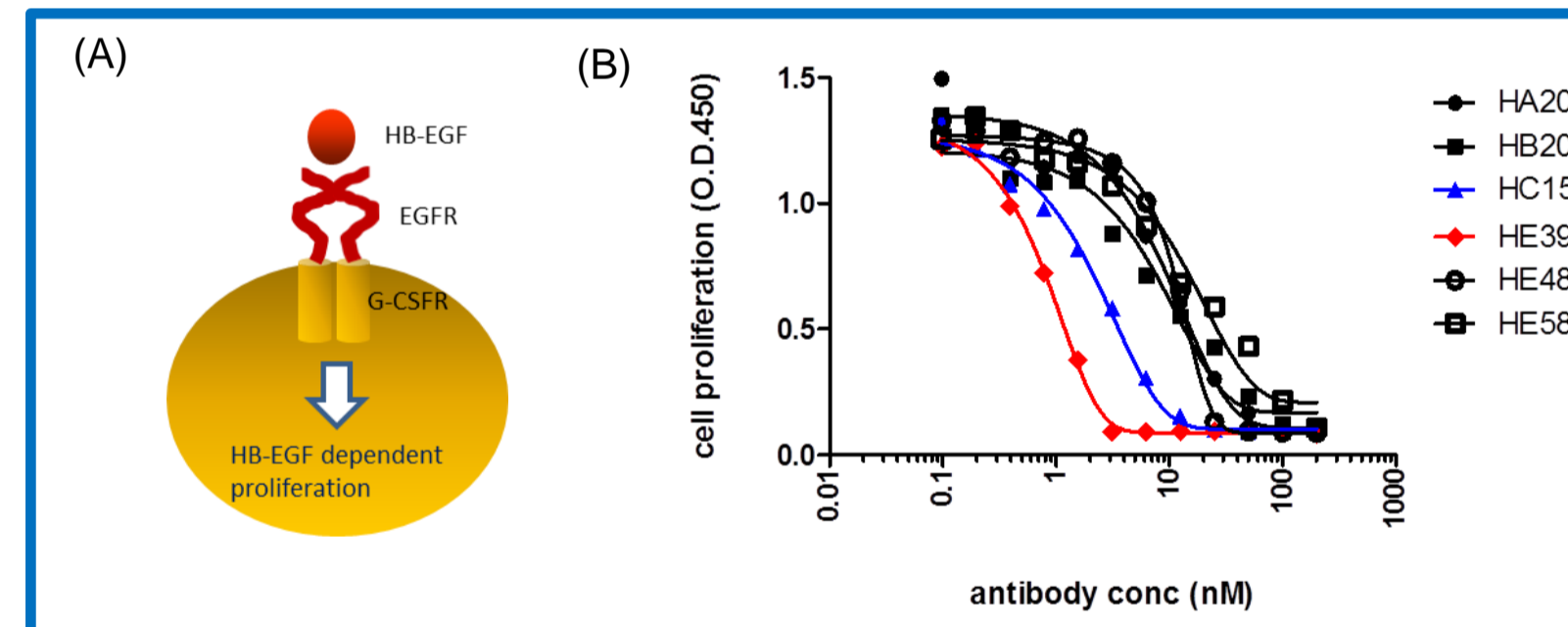


Fig.3. Inhibition of HB-EGF dependent proliferation in EGFR/BaF3

(A) Schematic illustration of an HB-EGF-dependent Ba/F3 cell line. The IL-3-dependent mouse proB cell line, in Ba/F3 cells, was transfected with chimeric receptor composed of the EGFR extracellular domain and G-CSFR cytoplasmic domain. The resultant cell line can proliferate specifically in response to HB-EGF, in the absence of IL-3.

(B) Cells were cultured with 20 ng/ml of HB-EGF in the presence of various concentrations of mAbs. After 3-day culture, cell proliferation was determined by adding WST-8. (see Table 1)

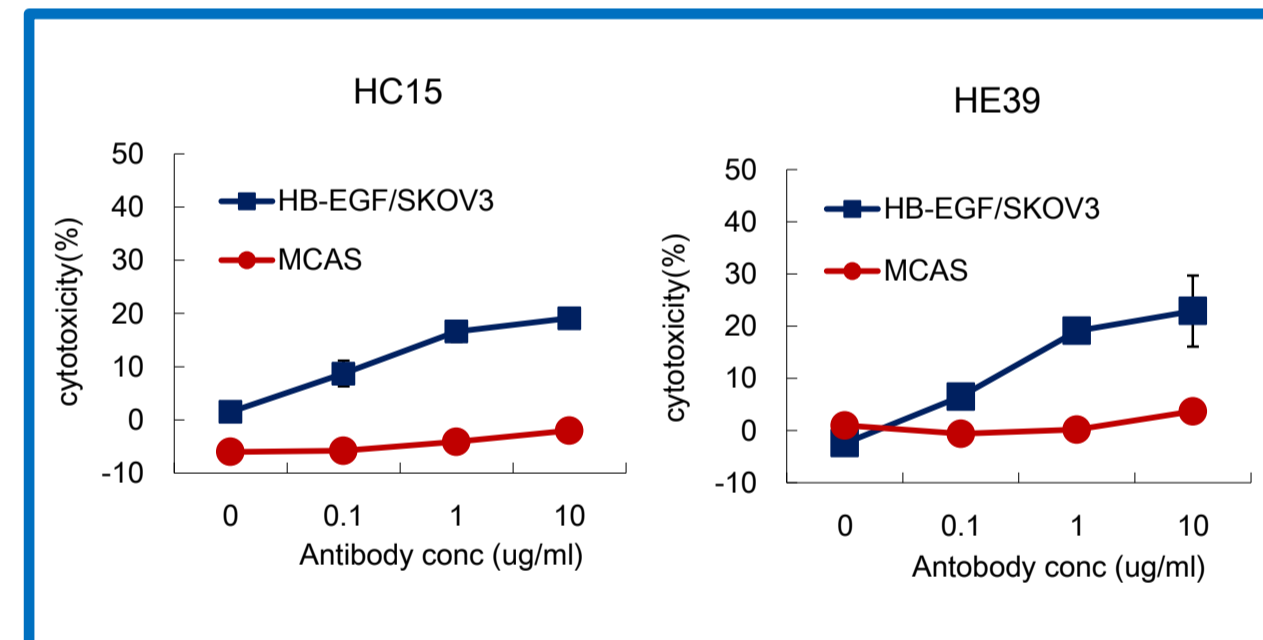


Fig.4. in vitro ADCC activity of anti-HB-EGF mAbs

Calcein AM-labeled HB-EGF-transfected SKOV3 cells or human ovarian cancer cell line MCAS were incubated with indicated antibodies and mFcγR3-transfected NK92 cells. Killing of target cell by ADCC is detected by release of fluorescence.

| Mature HB-EGF | Heparin-binding domain | EGF-like domain | polyclonal Ab (R&D) | HB20 | HC15 | HE39 | HE48 | HE58 | |
|-----------------|--|-----------------|---------------------|-------|-------|-------|-------|-------|-------|
| | | | | human | mouse | human | mouse | human | mouse |
| EGF-like domain | F115Y K122R V124L K125Q L127F A129T I133K H135L E141H S147T | human | F115Y | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | K122R | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | V124L | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | K125Q | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | L127F | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | A129T | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | I133K | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | H135L | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | E141H | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | H135L | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | E141H | ○ | ○ | ○ | ○ | ○ | ○ |
| | | human | S147T | ○ | ○ | ○ | ○ | ○ | ○ |

Summary of the binding results obtained, where "○" indicates that mAb binding was not affected despite the indicated mutation, "×" indicates that mAb binding was substantially eliminated by the indicated mutation.

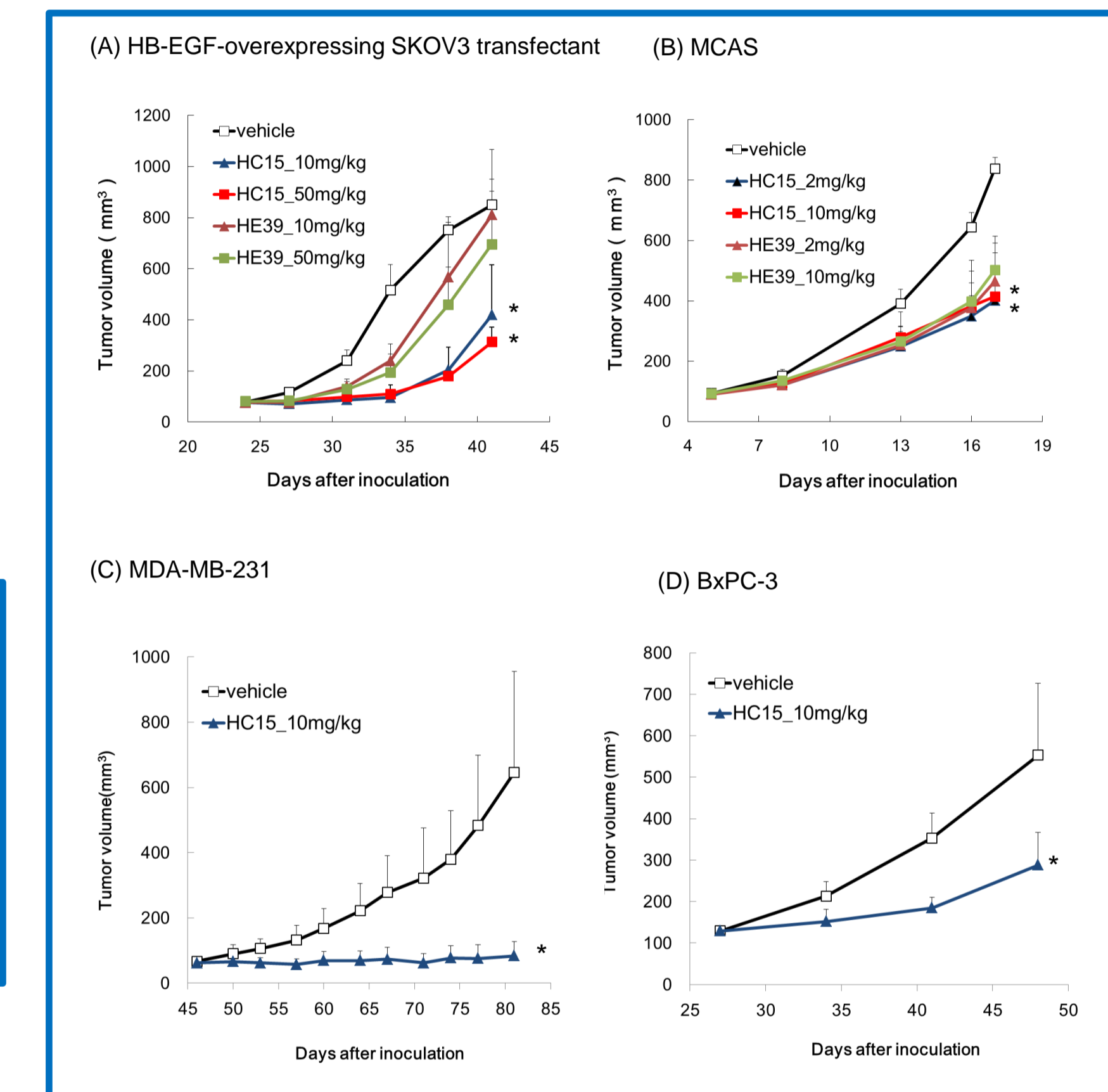
Table 2. Mapping the antibody binding site

First, to determine which domains of mature HB-EGF were recognized by present antibodies, Ba/F3 cells expressing human mature HB-EGF or human/mouse chimera HB-EGF were stained by the indicated mAbs and analyzed by flow cytometer. All mAbs recognized the EGF-like domain of HB-EGF, since replacement of EGF-like domain with the homologous mouse domain resulted in loss of binding to mAbs.

Next, to further identify the residues critical for antibody binding, 10 independent HB-EGF mutants were created by replacing one residue within EGF-domain with the corresponding amino acid in mouse HB-EGF. FACS analysis showed that amino acid H at position 135 was critical for HE39 binding. On the other hand, binding of HC15 to HB-EGF was not affected by any amino acid substitution in the EGF-like domain.

In vivo antitumor activity

Fig. 5. Growth inhibitory effect of HB-EGF-blocking mAbs in several human tumor xenografts



Growth inhibitory effect of human chimeric HC15 and HE39 was compared in xenograft models inoculated sc with human ovarian cancer cell line SKOV3 transfected with HB-EGF (A), and MCAS ovarian cancer cells (B). When the volume of tumors reached 80-100mm³, indicated antibodies were injected once-weekly (n=6). In the HB-EGF/SKOV3 transfectant xenograft model, HC15 had superior anticancer activity compared to HE39 but in the MCAS xenograft model their tumor growth inhibition was equal.

Tumor growth inhibition of human chimeric HC15 was further examined in MDA-MB-231 and BxPC3 xenografts. When the volume of tumors reached nearly 100mm³, HC15 was injected once-weekly at 10mg/kg (n=6). HC15 showed 96% tumor growth inhibition in the MDA-MB-231 breast cancer xenograft model (C), and 63% in the BxPC-3 pancreatic cancer xenograft model (D).

CONCLUSIONS

- ◆ Monoclonal anti-HB-EGF antibodies, HC15 and HE39, inhibit the binding of HB-EGF to the EGFR and also inhibit HB-EGF-dependent cell growth.
- ◆ HC15 and HE39 had different characteristics based on their epitope analysis.
- ◆ Although in vitro studies showed HC15 bound to HB-EGF and inhibited HB-EGF-dependent cell growth more weakly than HE39, in vivo the antitumor effect of HC15 is superior to that of HE39 in an HB-EGF/SKOV3 transfectant xenograft model.
- ◆ The ADCC-inducing activity of both antibodies was identical against HB-EGF/SKOV3 cells, but neither antibody elicited ADCC against MCAS cell lines.
- ◆ Antibodies blocking HB-EGF demonstrate potent antitumor activity in various xenograft models, suggesting that HB-EGF would be a potent target molecule for antibody-based cancer therapy.