Anti-tumor Effect of Intravenous Administration of CRM197 for Triple-negative Breast Cancer Therapy

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Abstract. *Background/Aim: Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which belongs to the epidermal growth factor family, is a rational therapeutic target for triple-negative breast cancer (TNBC). This study aimed to assess the anti-tumor efficacy of intravenous (i.v.) HB-EGF-specific inhibitor (CRM197) for TNBC. Materials and Methods: NOD/SCID mice were subcutaneously injected withTNBC cells, MDA-MB-231, and, then, treated with i.v. CRM197 in either dose- or frequency-dependent manners, using an advanced cancer model and an adjuvant therapy model. Tumor volume and mouse body weight were calculated weekly. Statistical significance was assessed by the Mann-Whitney U-test. Results: Mice that received i.v. CRM197 showed a significant anti-tumor effect in dose- and frequency-dependent manners in both models. However, their body weight did not differ significantly among groups. Conclusion: These results suggest that i.v. CRM197 is an effective treatment for TNBC.*

Breast cancer makes up approximately one-quarter of all cancers in women having the highest cancer-related mortality rate in the world (1, 2). Breast cancer mortality is also increasing steadily worldwide. Breast cancer is a heterogeneous disease that encompasses different morphological features and clinical behaviors, reflecting distinct patterns of genetic, epigenetic and transcriptomic aberrations. The identification of gene expression through microarray-based characteristics has led to characterization of five breast cancer subtypes: luminal A, luminal B, normal breast-like, human epidermal growth factor receptor 2 (HER2) and basal-like. Triple-negative (TNBC), which is recognized as the same subtype as the basal-like, is a complex disease, diagnosed immunohistochemically by the lack of estrogen and progesterone receptors, along with the absence of HER2 overexpression or gene amplification. TNBC is biologically aggressive neoplasia associated with distant recurrence, visceral metastases and high death rate compared with other breast cancer subtypes (3). The median survival of advanced TNBC is at best 12 months, which is much shorter than the median survival duration of survival of other advanced breast cancer subtypes (4). Therefore, development of molecularly targeted therapies is needed to improve the prognosis of TNBC.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a ligand of epidermal growth factor (EGF) receptor and a member of the EGF superfamily. Pro-HB-EGF is cleaved by a member of the disintegrin and metalloproteinases (ADAMs) family (5) and membrane type 1-matrix metalloproteinase (MT1-MMP) (6) at the cell surface (a process called "ectodomain shedding"), resulting in the induction of mature soluble HB-EGF (sHB-EGF) (7, 8). sHB-EGF binds to both EGFR and human epidermal growth factor receptor 4 (HER4) and, then, activates their tyrosine kinase receptors, resulting in a variety of biological processes, including inflammation (9), wound healing (10) and carcinogenesis (8, 11-13). In particular, HB-EGF is highly expressed in patients with ovarian, gastric and breast cancers; the blockade of HB-EGF suppressed the tumorigenicity of ovarian, gastric, breast cancers *etc*. indicating that HB-EGF is a rational therapeutic target for these cancers (14-18). CRM197, recognized as a specific inhibitor of HB-EGF, has recently been made using a Good Manufacturing Practice and designated as BK-UM (19, 20). Accordingly, BK-UM (CRM197) is a candidate agent for TNBC treatment; however, it warrants further investigation. We previously

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Key Words: CRM197, HB-EGF, triple-negative breast cancer.

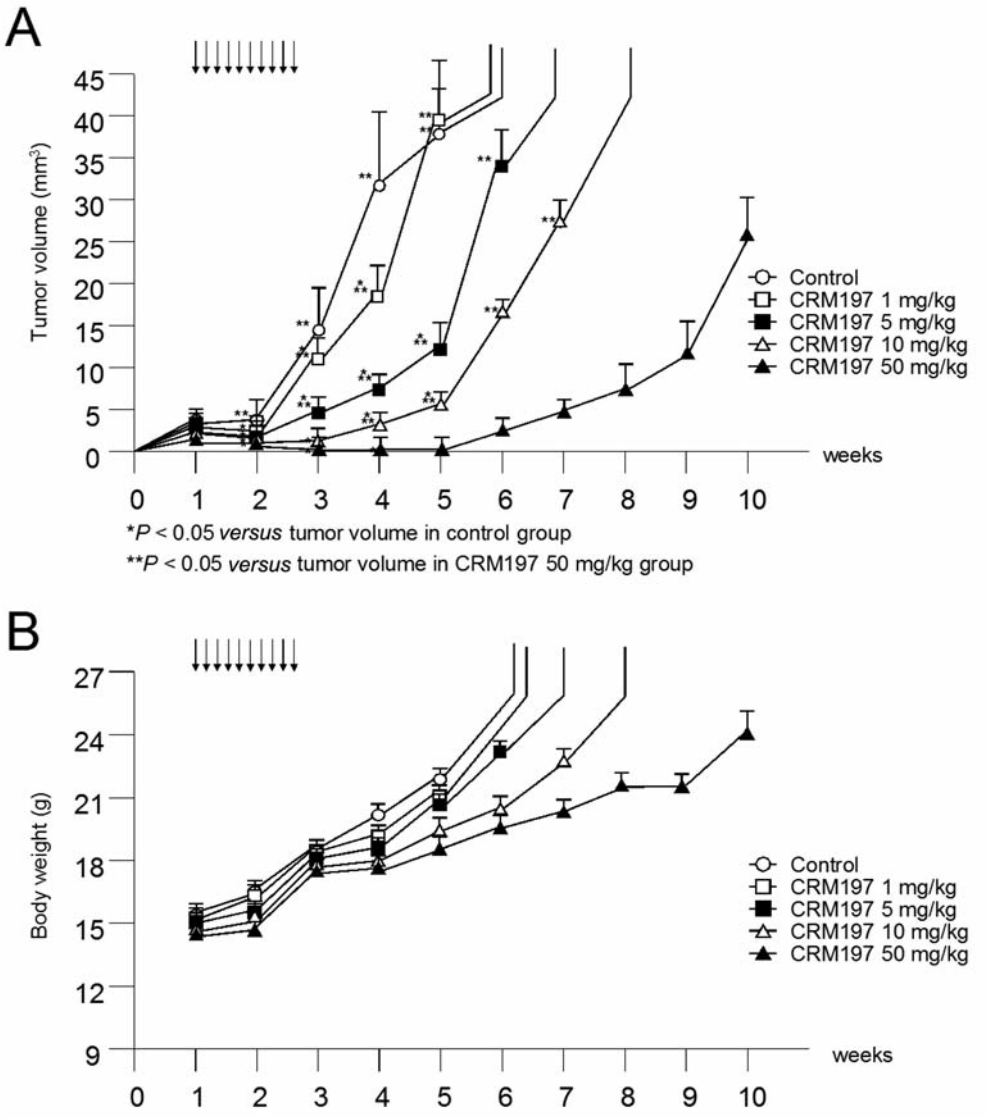

Figure 1. *Week-by-week effects of four different intravenous doses (1, 5, 10 and 50 mg/kg) of CRM197 on volumes of xenograft tumors of MDA-MB-231 cells in NOD/SCID mice as an adjuvant therapy model. Black arrows: days when CRM197 was administered. A: Tumor volume; B: mouse body weight. Data represent mean±standard error (SEM); n=9. *p<0.05 versus tumor volume in control group; **p<0.05 versus tumor volume in CRM197 50 mg/kg group.*

reported that intraperitoneal administration of CRM197 blocked tumor formation completely in MDA-MB-231 cells in mice (21). Nevertheless, the anti-tumor effect of intravenous (*i.v.*) CRM197 administration remains unclear.

A pre-clinical study suggested that daily intraperitoneal administration of BK-UM (CRM197) might be the optimal schedule for clinical application to treat peritoneally disseminated tumors, such as ovarian and gastric cancer. In breast cancer, cancer cells generate from epithelial cells in mammary glands that metastasize to lymph nodes through lymphatic ducts and various organs, including lung, brain and so on *via* vessels. Intravenous chemotherapy is clinically important as a systemic therapy for breast cancer. Therefore, this study aimed to evaluate the optimal *i.v.* BK-UM dosing as a preliminary investigation for a phase I clinical trial of patients with advanced or recurrent TNBC.

Materials and Methods

Reagent. CRM197, an anti-cancer agent, was obtained from the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

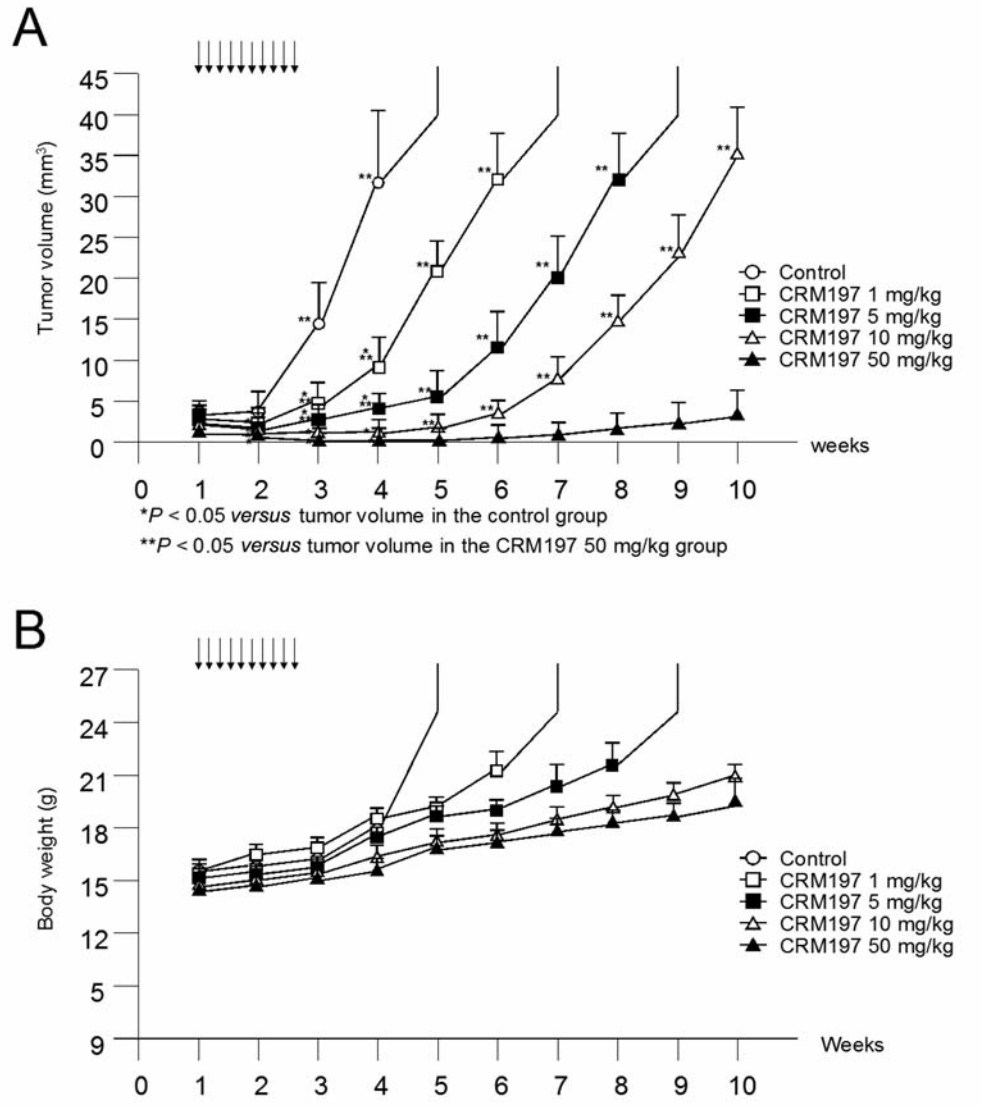

Figure 2. *Week-by-week effects of four different intravenous doses (1, 5, 10 and 50 mg/kg) of CRM197 on volume of xenograft tumors of MDA-MB-231 cells in NOD/SCID mice as an advanced cancer model. Black arrows: days when CRM197 was administered. A: Tumor volume; B: mouse body weight. Data represent mean±SEM; n=9. *p<0.05 versus tumor volume in the control group; **P<0.05 versus tumor volume in the CRM197 50 mg/kg group.*

Cells and cell culture. A human TNBC cell line, MDA-MB-231, was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (ICN Biomedicals, Irvine, CA, USA), 100 U/ml of penicillin G and 100 μg/ml of streptomycin (Invitrogen Corp., Carlsbad, CA, USA), in a humidified atmosphere of 5% CO_2 at 37˚C.

Anti-tumor effects of CRM197 in a mouse xenograft model. Subconfluent cell cultures were detached from plates with trypsin-EDTA. Five-week-old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River Laboratories Japan Inc., Yokohama, Japan) were each subcutaneously injected with 250 μl serum-free RPMI 1640 in which 1×10^7 cells were suspended. To assess the inhibitory effects of CRM197 on tumor growth, different doses (1, 5, 10 or 50 mg/kg) of CRM197 were injected intravenously into tumor-bearing mice by two different schedules. In one schedule, called the adjuvant therapy model, CRM197 was injected at the same time as MDA-MB-231 was injected subcutaneously. In the other schedule, the advanced cancer model, CRM197 was injected after the tumor reached an estimated volume > 100 mm^3. CRM197 was injected daily for 5 or 10 days during the treatment. Tumor size and mouse body weight were measured weekly. Tumor volume was estimated from two-dimensional tumor measurements as: tumor volume (mm^3)=(length × $width^2$)/2.

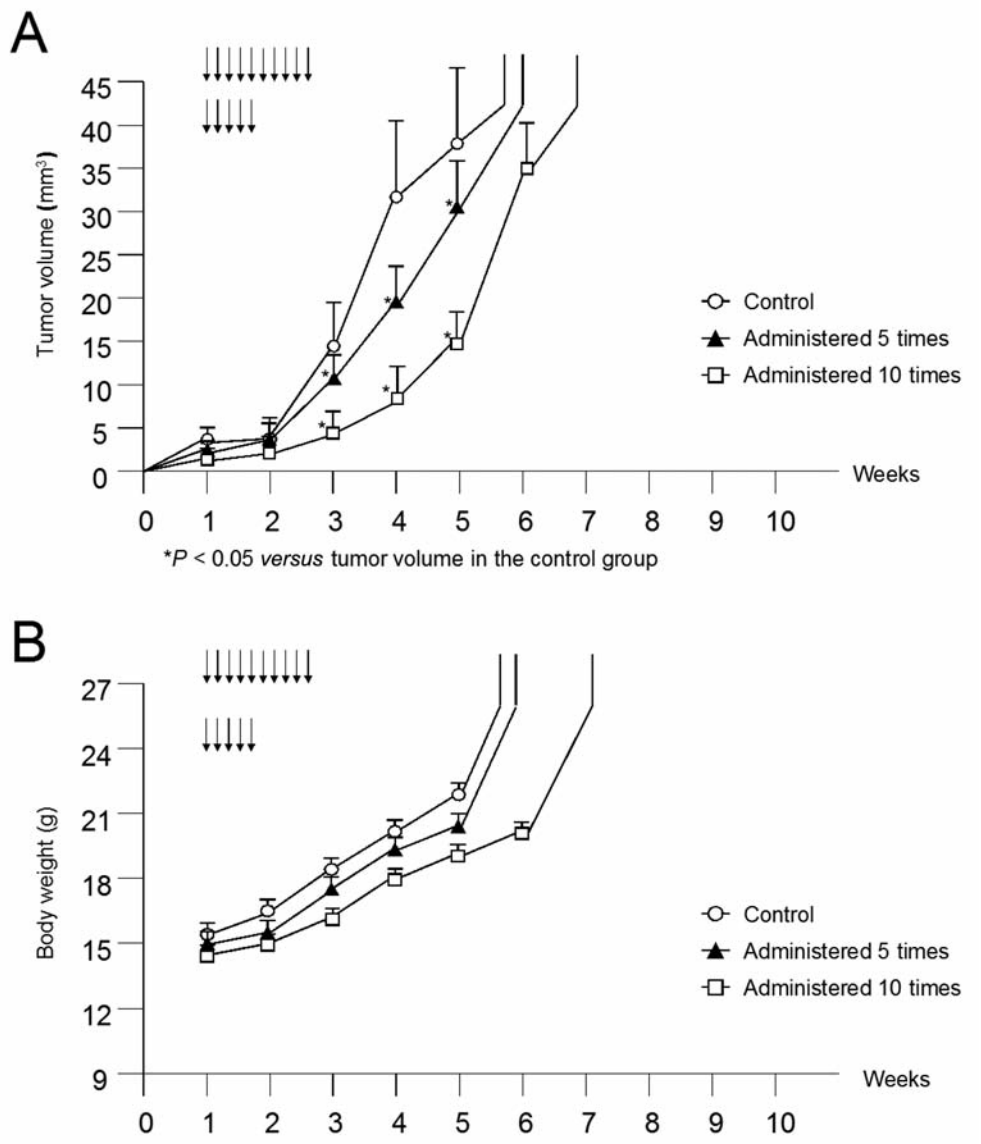

Figure 3. *Week-by-week effects of administering 5 mg/kg CRM197 on volume of xenograft tumors of MDA-MB-231 cells in NOD/SCID mice as an adjuvant therapy model (n=9 per group). Black arrows: days when CRM197 was administered (5× or 10×). A: Tumor volume; B: mouse body weight. Data represent mean±SEM of tumor volumes. *p<0.05 versus tumor volume in the control group.*

Statistical analysis. Statistical significance of differences among the groups was assessed using the Mann–Whitney *U*-test. $p<0.05$ was considered significant.

Results

To validate the anti-tumor effect of *i.v.* CRM197 and confirm that CRM197 was used safely without side-effects, such as weight loss caused by administration of anti-cancer drugs for TNBC, we examined tumor size and body weight in NOD/SCID mice with xenograft tumors created with MDA-MB-231 cells, which is a TNBC cell line. The tumors were treated with 1, 5, 10 or 50 mg/kg of CRM197. Two therapy models to validate the anti-tumor effect of CRM197 for TNBC were used: the adjuvant therapy model (in which CRM197 was injected intravenously at the same time as the subcutaneously injected MDA-MB-231 cells) and the advanced cancer model (in which CRM 197 was injected after the tumor reached an estimated volume >100 mm^3). The adjuvant therapy model showed significantly smaller tumors for all CRM197 doses from week 2 compared to control mice (Figure 1A).

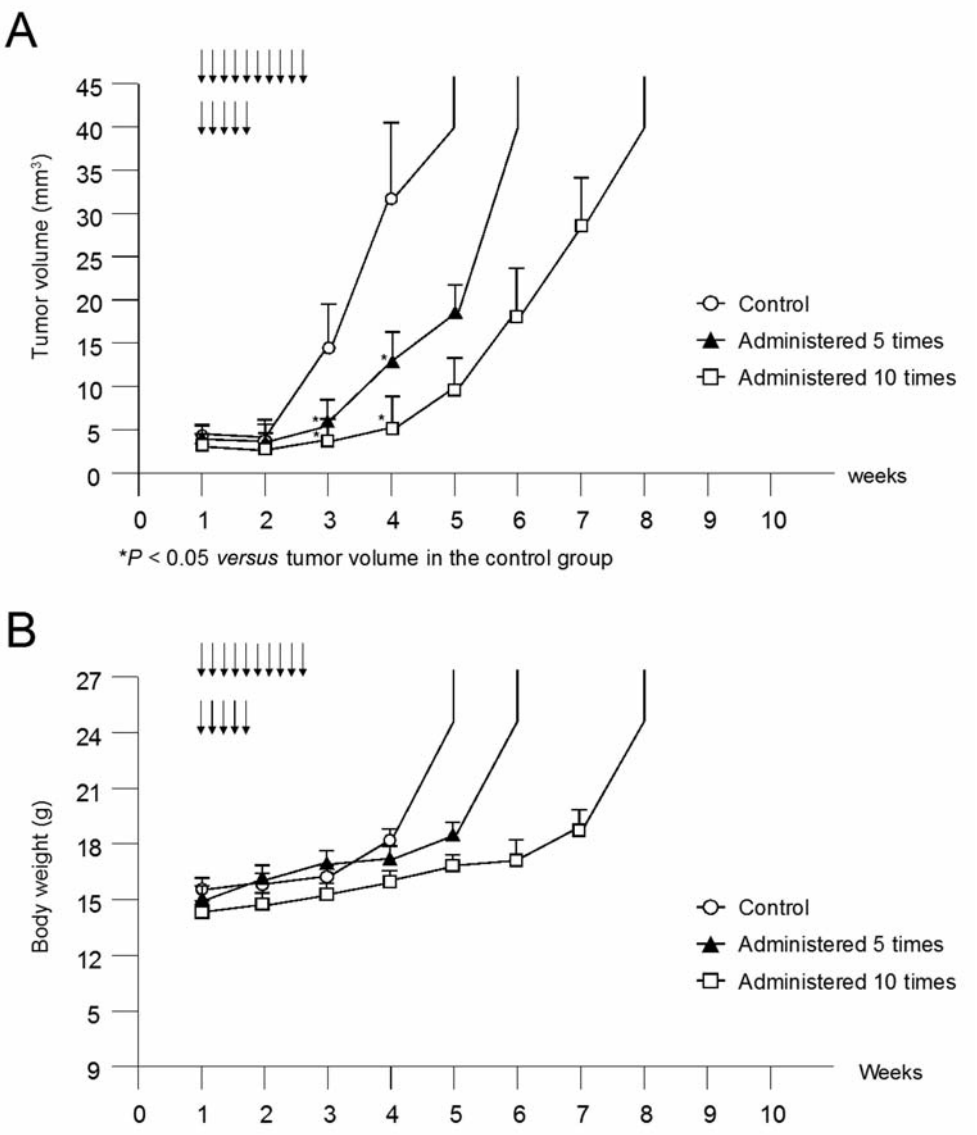

Figure 4. *Week-by-week effects of administering 5 mg/kg CRM197 on volume of xenograft tumors of MDA-MB-231 cells in NOD/SCID mice as an advanced cancer model (n=9 per group). Black arrows: days when CRM197 was administered (5× or 10×). A: Tumor volume; B: mouse body weight. Data represent mean±SEM of tumor volumes. *p<0.05 versus tumor volume in the control group.*

To further confirm the safety of *i.v.* CRM197 and the lack of apparent side-effects, the body weight of mice was monitored every week. The body weight of control mice did not differ significantly from that of mice treated with any of the CRM197 doses in the adjuvant therapy model (Figure 1B). Similarly, tumors in the advanced cancer model group were significantly smaller for all CRM197 doses from week 2 compared with control mice (Figure 2A). Furthermore, as with the adjuvant therapy model, body weights of mice in the advanced cancer model groups did not differ significantly between control and treated mice for all CRM197 doses (Figure 2B).

In our results, the anti-tumor effect of CRM197 was more significant in the advanced cancer therapy model than in the adjuvant therapy model. These results also indicate that *i.v.* CRM197 has a dose-dependent anti-tumor effect on TNBC in the adjuvant and advanced cancer therapy models. Moreover, CRM197 seemed to be safe and without side-effects, such as weight loss caused by administration of anti-cancer drugs for TNBC. Although useful for adjuvant therapy, our results suggest that *i.v.* CRM197 is more effective for advanced cancers.

Next, to assess whether the effect of *i.v.* CRM197 was frequency-dependent, 5 mg/kg of CRM197 was administered

daily for 5 or 10 days during treatment. The adjuvant therapy model group showed significantly smaller tumors from week 3 compared to control mice, with the10-day administration schedule being more effective than the 5-day administration (Figure 3A). In addition, body weight of control mice did not differ significantly from that of mice treated with CRM197 in the adjuvant therapy model, at all doses (Figure 3B). Tumors in the advanced cancer model group were also significantly smaller from week 3 than those in the control mice (Figure 4A) and body weight of the advanced cancer model group did not differ significantly among control and treated mice (Figure 4B). These results show *i.v.* CRM197 to have a frequency-dependent anti-tumor effect.

Discussion

In the present study, *i.v.* CRM197 induced significant anti-tumor effects in mice with MDA-MB-231 xenograft tumors, in both dose- and frequency-dependent manners. Additionally, *i.v.* CRM197 was found to be safe and without side-effects, such as weight loss.

In this study, tumor growth in the advanced cancer model was significantly more suppressed than in the adjuvant model. Expression of HB-EGF is much higher in three-dimensional culture or *in vivo* tumor growth on mice indicating that HB-EGF plays a pivotal role in *in vivo* tumor growth (22). According to this evidence, CRM197 may be more effective in the advanced model than in the adjuvant model. The combination of CRM197 and paclitaxel also induces synergistic anti-tumor effects (23). With regard to the body weight of the mice, *i.v.* CRM197 had similar safety to intraperitoneal administration of CRM197 (24). Together, these results suggest that CRM197 is a promising anti-cancer agent for patients with advanced or recurrent TNBC.

Previously, we reported that HB-EGF is predominantly expressed in all breast cancer subtypes (21). Generally, breast cancer patients with positive expression of estrogen and progesterone receptors are treated with surgery, chemotherapy, radiotherapy and molecularly targeted therapy, including selective estrogen receptor modulators; patients with HER2 over-expression are also treated with these conventional therapies, in addition to HER2 inhibitors, such as trastuzumab. HB-EGF expression affects HER2 expression in MCF7 breast cancer cells, (21). Based on these lines of evidence, BK-UM may be available for all breast cancer subtypes

In conclusion, we showed that *i.v.* administration of CRM197 was effective on mice in both the advanced cancer model and the adjuvant model. Recently, we performed a phase I study for intraperitoneal administration of CRM197 in patients with recurrent ovarian cancer in the Fukuoka University Hospital (Japan) under the approval of the Institutional Ethical Committee and completed the Good Laboratory Practice for *i.v.* administration of BK-UM. A clinical phase I study of BK-UM will start at the Fukuoka University Hospital for patients with advanced and recurrent TNBC.

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