A Possible Clinical Adaptation of CRM197 in Combination with Conventional Chemotherapeutic Agents for Ovarian Cancer

HIROSHI TSUJIOKA[1], TATSUYA FUKAMI[1], FUSANORI YOTSUMOTO[2], TAEKO UEDA[1], SHOKO HIKITA[1], YOKO TAKAHASHI[1], HARUHIKO KONDO[1], MASAHIDE KUROKI[2] and SHINGO MIYAMOTO[1]

[1]*Department of Obstetrics and Gynecology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan;*
[2]*Department of Biochemistry, Faculty of Medicine, Fukuoka University, Fukuoka, Japan*

Abstract. *Aim: Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a promising target for cancer therapy. We have already started a phase I study of CRM197, a specific HB-EGF inhibitor, for advanced ovarian cancer. In this study, we evaluated possible clinical adaptations of CRM197 in combination with conventional chemotherapeutic agents. Materials and Methods: CRM197, bevacizumab, and paclitaxel were intraperitoneally administered either alone or in combination with mice xenografted with ES2 human ovarian cancer cells. The tumor volumes and microvessel densities (MVD) were determined. Results: Enhanced antitumor effects were observed when paclitaxel was used in combination with bevacizumab or CRM197. The antitumor effect of paclitaxel/CRM197 was significantly higher than that of paclitaxel/bevacizumab. The tumor MVD of mice treated with paclitaxel/CRM197 was significantly lower than that of mice treated with paclitaxel/bevacizumab. Conclusion: CRM197 in combination with paclitaxel significantly blocked tumor formation and angiogenesis. These results suggest that paclitaxel is a suitable candidate for CRM197 combination therapy.*

Ovarian cancer is the leading cause of death among all gynecologic malignancies. The high mortality is due to occult tumor progression into the peritoneal cavity. Nearly 75% of ovarian cancer patients are diagnosed with advanced diseases on initial presentation (1). Current treatment of advanced ovarian cancer consists of surgical tumor resection, as complete as possible, followed by platinum/taxane-based chemotherapy. In this therapeutic procedure, conventional chemotherapy has not improved the prognosis of advanced ovarian cancer over the last 30 years; thus, novel effective treatment regimens for ovarian cancer are eagerly awaited.

The ErbB family of receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR; also known as ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4, mediates major cellular functions, including proliferation, differentiation, motility, and survival (2). These kinases are also implicated in the development and progression of most common human epithelial malignancies (3). Thus, ErbB receptors have been extensively studied as therapeutic targets. As described in the literature (3, 4), EGFR overexpression occurs in 35%-70% of all primary ovarian cancer cases. Statistical analyses have confirmed that EGFR overexpression is significantly associated with a high risk of progression in ovarian cancer patients (5). Various tyrosine kinase inhibitors targeting EGFR, *e.g.* erlotinib (Tarceva), and monoclonal antibodies targeting EGFR, *e.g.* cetuximab (Erbitux), and ErbB2/HER2, *e.g.* trastuzumab (Herceptin), have been developed and investigated for clinical application; however, clinical studies using EGFR antagonists have not always resulted in favorable clinical outcomes (6). The majority of patients who are initially responsive to ErbB receptor-targeted therapies experience tumor recurrence and their disease becomes refractory later (7). Despite favorable preclinical studies using EGFR antagonists, the overall clinical trial outcomes in ovarian cancer have been disappointing (8).

We previously proposed EGFR ligand-based targeted therapy for cancer (9, 10). The belief that ligand targeting is less effective than receptor targeting in the EGF signaling network has delayed the development of agents for EGFR ligand-based targeted therapy; however, our research has revealed this notion to be untrue (11). The speculation is that aberrant enhancement of EGFR ligand expression is one of the various molecular mechanisms accounting for acquired resistance to EGFR antagonists. Our reported evidence indicates that EGFR ligands deserve considerable attention as potential targets for cancer therapy, and we have discussed EGFR signaling inhibition strategies directed at EGFR ligands such as heparin-binding

Correspondence to: Dr. Hiroshi Tsujioka, Department of Obstetrics and Gynecology, Fukuoka University Faculty of Medicine, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011 ext. 3505, Fax: +81 928654114, e-mail: tsujioka@ fukuoka-u.ac.jp

Key Words: Ovarian cancer, HB-EGF, targeted therapy, CRM197.

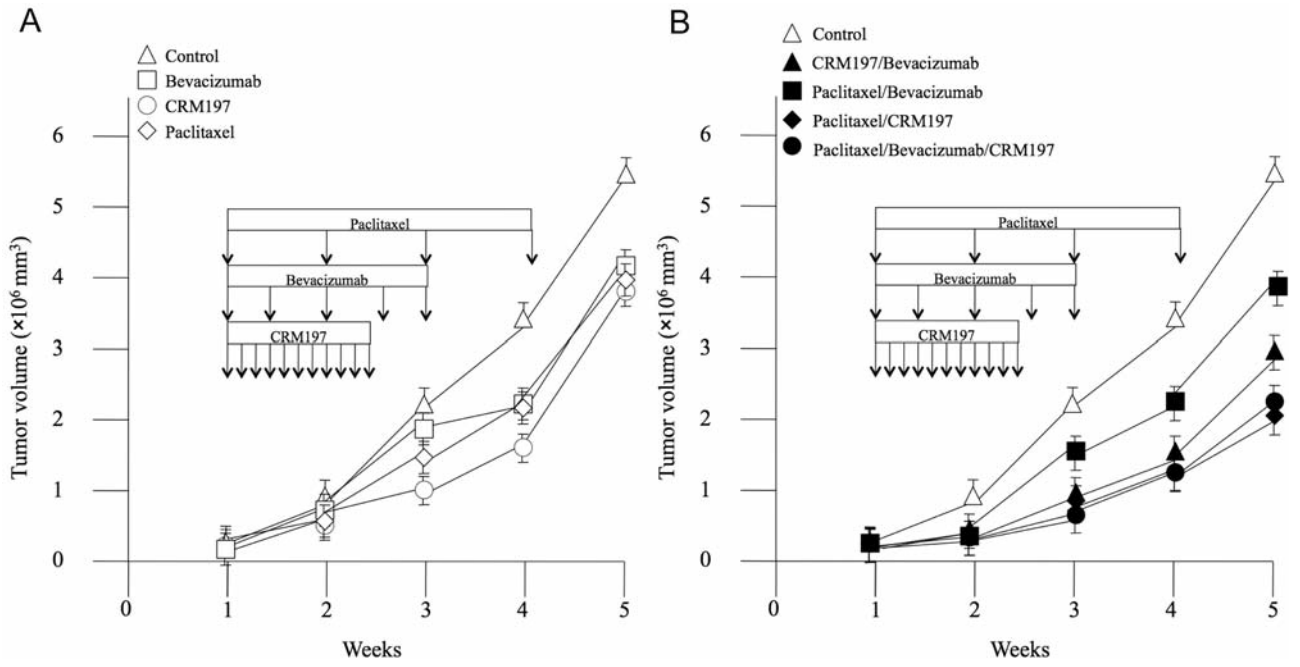

Figure 1. *Antitumor effects of CRM197 in combination with paclitaxel or bevacizumab. CRM197 (10 doses at 1 mg/kg, daily), bevacizumab (5 doses at 5 mg/kg, every fourth day), and paclitaxel (4 doses at 20 mg/kg, weekly) were intraperitoneally administered, either alone (A) or in combination (B), to mice xenografted with ES2 ovarian cancer cell. The tumor volumes were calculated. Each volume is expressed as the mean±SE (n=8) and compared with that of the control (no administration).*

EGF-like growth factor (HB-EGF) and amphiregulin (AR) (12). HB-EGF expression is remarkably elevated in ovarian, gastric, and breast cancer, as well as in melanoma and glioblastoma, whereas AR expression is primarily enhanced in pancreatic, colon, and prostate cancer, as well as in renal cell carcinoma and cholangiocarcinoma (13). In cell lines with dominant HB-EGF expression, transfection of small interfering RNAs (siRNAs) for HB-EGF increases the number of apoptotic cells and suppresses EGFR and extracellular signal-related kinase (ERK) activation, whereas transfection of siRNAs for other EGFR ligands has no effect. Similarly, apoptosis and attenuation of EGFR and ERK signals in cell lines with abundant AR expression are significantly induced by AR inhibition.

Receptor dimerization is essential for activation of the ErbB signaling network (14, 15). When a receptor becomes functionally inactivated, another ErbB receptor can substitute for its function. MDA-MB-468 breast cancer cells secrete abundant amounts of the soluble HB-EGF and form EGFR/HER2 complexes. This complex formation is accelerated by trastuzumab but inhibited by CRM197, a specific HB-EGF inhibitor. CRM197 attenuates the phosphorylation of ERK and v-Akt murine thymoma viral oncogene homolog (Akt), leading to significant apoptosis as compared with that induced by trastuzumab (16). We hypothesized that ligand-induced ErbB receptor dimerization plays pivotal roles in transmitting the intracellular signal for cell survival against therapy targeting EGFR or HER2 alone, and that targeting a dominantly expressed EGFR ligand is an acceptable strategy for cancer therapy.

We investigated the antitumor effects of CRM197 in ovarian cancer cells by evaluating the proliferation of the human ovarian cancer cell lines SKOV3, RMG1, and OVMG1, which were subcutaneously implanted into nude mice. CRM197 significantly suppresses peritoneal dissemination in nude mice peritoneally injected with ovarian cancer cells (17). A previous clinical research study reported CRM197 usage in cancer patients (18). A phase I study on CRM197 usage (the first targeting the ErbB ligand) for advanced ovarian cancer patients is currently being conducted, with the approval of the Ethics Committee, at Fukuoka University.

In this report, we discuss a possible clinical adaptation of CRM197 in combination with the conventional chemotherapeutics, paclitaxel and bevacizumab.

Materials and Methods

ES2 human ovarian cancer cells were maintained in RPMI-1640 supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, and 10% fetal bovine serum (ICN Biomedicals, Irvine, CA, USA). ES2 (5×10^6) human ovarian cancer cell, were subcutaneously administered to 5-week-old female BALB/c *nu/nu* mice. CRM197 (10 doses at 1 mg/kg, daily), bevacizumab (5 doses at 5 mg/kg, every 4th day), and paclitaxel (4 doses at 20 mg/kg,

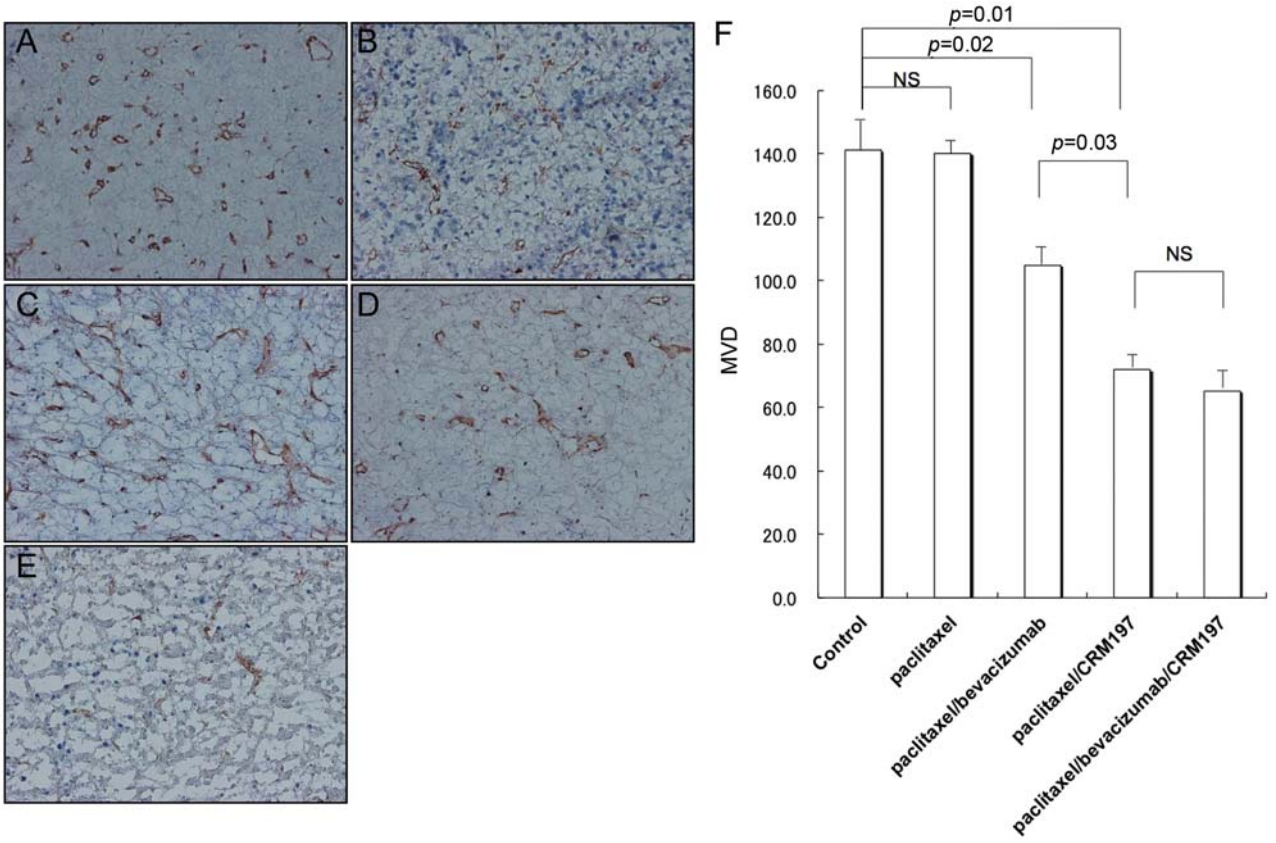

Figure 2. *Immunohistochemical staining for CD31 in tumors obtained from untreated xenografted mice (no drug administration; A) and mice treated with paclitaxel (B), paclitaxel/bevacizumab (C), paclitaxel/CRM197 (D), or paclitaxel/bevacizumab/CRM197 (E). Selected areas with the highest number of microvessels were scanned, and individual microvessels were counted in a ×200 field; the counts were expressed as microvessel density (MVD). Statistical analysis was performed to compare the MVD values among the treatment groups (F). The Mann–Whitney U–test was used to assess the association between categorical variables (n=3).*

weekly) were intraperitoneally administered, either alone or in combination, to tumor-bearing mice. These mice were examined weekly, and their tumor volumes were determined by two-dimensional tumor measurements using the following formula: tumor volume (mm^3)=length × $width^2/2$.

The microvessel detection and counting method has been described in a previous study (19, 20). Intratumor microvessels were highlighted with anti-CD31 monoclonal antibody (JC/70A; Dako, Carpinteria, CA, USA) and immunostaining was performed using biotin-streptavidin method. Sections were washed in Tris-buffered saline (TBS) at pH 7.6 and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, followed by incubation with streptavidin conjugated to alkaline phosphatase (Dako) for another 30 min. The reaction was revealed with naphthol AS-BI phosphate (Sigma Chemical Co., St, Louis, MO, USA) in 100 ml of 0.2M TBS (pH 8.2) containing 4% hydrochloric acid and 4% nitric acid and counterstained with methylgreen. The selected areas with the highest number of CD31 highlighted microvessels were scanned; individual microvessels were counted in a ×200 field and expressed as microvessel density (MVD). Statistical analysis was performed to compare the tumor volume and MVD among the different groups of xenografted mice.

Statistical analysis was performed using SPSS II 11.0.1 J (SPSS Japan, Tokyo, Japan). The Mann–Whitney *U*–test was used to assess the association between categorical variables. Statistical significance was set at $p<0.05$.

Results

The tumor volume (mean±SE, n=8), 5 weeks after cell injection, in each group was as follows: control (no drug administration): 5635±517 mm^3, paclitaxel, 3742±310 mm^3; paclitaxel/CRM197, 2154±132 mm^3; paclitaxel/ bevacizumab, 3885±350 mm^3; paclitaxel/bevacizumab/ CRM197, 2339±316 mm^3. Enhanced antitumor effects were observed when paclitaxel was used in combination with bevacizumab or CRM197. The antitumor effect of paclitaxel/ CRM197 was significantly higher ($p<0.05$) than that of paclitaxel/bevacizumab. These effects were not enhanced when paclitaxel was used in a combination with both bevacizumab and CRM197 (Figure 1).

MVD (mean±SE, n=8), 5 weeks after cell injection, in each group was as follows: control: 141.3±9.9; paclitaxel:

140.3±4.3; paclitaxel/CRM197: 72.0±4.3; paclitaxel/bevacizumab: 105.0±5.6; paclitaxel/bevacizumab/CRM197: 65.3±5.5. Enhanced inhibition of angiogenesis was observed when paclitaxel was used in combination with bevacizumab or CRM197. Angiogenesis was significantly inhibited ($p<0.05$) by paclitaxel/CRM197 compared to the inhibition induced by paclitaxel/bevacizumab. Inhibition was not enhanced when paclitaxel was used in combination with both bevacizumab and CRM197 (Figure 2).

Discussion

One of the causes of the development of resistance to EGFR antagonists is that EGFR and ErbB2/HER2 form complexes with ErbB3 and other signal receptors that cannot be inhibited by therapies targeted against EGFR and ErbB2/HER2. Cancer cell proliferation is subsequently accelerated by these complexes. Another is that anti-EGFR drugs reduce the proliferation of ERK signals located downstream of EGFR; however, they cannot suppress protein kinase B/Akt survival signals. Resistance may also arise in tumor cells through allelic and adaptive changes, leading to phosphoinositol 3-kinase (PI3K) activation through other RTKs. Down-regulation of insulin-like growth factor (IGF)-binding proteins 3 and 4, which are negative regulators of IGF-I receptor (IGF-IR) signaling, activates IGF-IR and the PI3K/Akt pathway, and contributes to the development of resistance to EGFR inhibitors (21).

CRM197, in combination with paclitaxel rather than bevacizumab, significantly blocked tumor formation and angiogenesis. These results suggest that HB-EGF controls the upstream signal of vascular endothelial growth factor (VEGF). Paclitaxel promotes the ectodomain shedding of proHB-EGF, induces the transient activation of ERK, and sustained activation of Jun-terminal kinases (JNK) and p38 mitogen-activated protein kinase (MAPK) through EGFR transactivation in tumor cells. HB-EGF overexpression in paclitaxel-treated cells modulated paclitaxel-evoked MAPK signaling, including marked activation of ERK and Akt, and minimized the activation of JNK and p38 MAPK. This indicates that HB-EGF influences drug sensitivity by balancing paclitaxel-induced anti-apoptotic and pro-apoptotic signals. The combination of paclitaxel and CRM197 had an inhibitory effect on cell proliferation and enhanced apoptosis by inhibiting ERK and Akt activation and activating p38 MAPK and JNK. More prominently, paclitaxel in combination with CRM197 exerted a synergistic antitumor effect in tumor cells (22). Although paclitaxel promotes the ectodomain shedding of proHB-EGF, resulting in synergism of the antitumor effect of CRM197, the same effect is not observed with all chemotherapeutic agents because the degree of ectodomain shedding of proHB-EGF varies with different chemotherapeutic agents. In contrast, reduced HB-EGF expression attenuated the expression of matrix metalloprotease-2 and VEGF (17). However, the most suitable adjunct in a combination therapy with CRM197 remains to be determined. This report suggests paclitaxel as a potential candidate in CRM197 combination therapy.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (no. 22591871) and FUKUOKA OBGYN Researcher's Charity Foundation Fund.

References

1 Penson RT, Shannon KE, Sharpless NE and Seiden MV: Ovarian cancer: an update on genetics and therapy. Compr Ther *24*: 477-487, 1998.

2 Blume-Jensen P and Hunter T: Oncogenic kinase signalling. Nature *411*: 355-365, 2001.

3 Salomon DS, Brandt R, Ciardiello F and Normando N: Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol *19*: 183-232, 1995.

4 Normanno N, Bianco C, De Luca A, Maiello MR and Salomon DS: Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer *10*: 1-21, 2003.

5 Scambia G, Benedetti Panici P, Battaglia F, Ferrandina G, Baiocchi G, Greggi S, De Vincenzo R and Mancuso S: Significance of epidermal growth factor receptor in advanced ovarian cancer. J Clin Oncol *10*: 529-535, 1992.

6 Imai K and Takaoka A: Comparing antibody and small-molecule therapies for cancer. Nat Rev Cancer *6*: 714-727, 2006.

7 Wang Q and Greene MI: Mechanisms of resistance to ErbB-targeted cancer therapeutics. J Clin Invest *118*: 2389-2392, 2008.

8 Zeineldin R, Muller CY, Stack MS and Hudson LG: Targeting the EGF receptor for ovarian cancer therapy. J Oncol *2010*: 414676, 2010.

9 Tsujioka H, Yotsumoto F, Shirota K, Horiuchi S, Yoshizato T, Kuroki M and Miyamoto S: Emerging strategies for ErbB ligand-based targeted therapy for cancer. Anticancer Res *30*: 3107-3112, 2010.

10 Tsujioka H, Yotsumoto F, Hikita S, Ueda T, Kuroki M and Miyamoto S: Targeting the heparin-binding epidermal growth factor-like growth factor in ovarian cancer therapy. Curr Opin Obstet Gynecol *23*: 24-30, 2011.

11 Miyamoto S, Hirata M, Yamazaki A, Kageyama T, Hasuwa H, Mizushima H, Tanaka Y, Yagi H, Sonoda K, Kai M, Kanoh H, Nakano H and Mekada E: Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. Cancer Res *64*: 5720-5727, 2004.

12 Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T and Mekada E: Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. Cancer Sci *97*: 341-347, 2006.

13 Yotsumoto F, Yagi H, Suzuki SO, Oki E, Tsujioka H, Hachisuga T, Sonoda K, Kawarabayashi T, Mekada E and Miyamoto S: Validation of HB-EGF and amphiregulin as targets for human cancer therapy. Biochem Biophys Res Commun *365*: 555-561, 2009.

14 Olayioye MA, Neve RM, Lane HA and Hynes NE: The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J *19*: 3159-3167, 2000.
15 Ferguson KM, Berger MB, Mendrola JM, Cho HS, Leahy DJ and Lemmon MA: EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell *11*: 507-517, 2003.
16 Yotsumoto F, Sanui A, Fukami T, Shirota K, Horiuchi S, Tsujioka H, Yoshizato T, Kuroki M and Miyamoto S: Efficacy of ligand-based targeting for the EGF system in cancer. Anticancer Res *29*: 4879-4885, 2009.
17 Yagi H, Yotsumoto F and Miyamoto S: Heparin-binding epidermal growth factor-like growth factor promotes transcoelomic metastasis in ovarian cancer through epithelial–mesenchymal transition. Mol Cancer Ther *7*: 3441-3451, 2008.
18 Buzzi S, Rubboli D, Buzzi G, Buzzi AM, Morisi C and Pironi F: CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients. Cancer Immunol Immunother *53*: 1041-1048, 2004.
19 Weidner N, Semple JP, Welch WR and Folkman J: Tumor angiogenesis and metastasis – correlation in invasive breast carcinoma. N Engl J Med *324*: 1-8, 1991.
20 Koga K, Nabeshima K, Nishimura N, Shishime M, Nakayama J and Iwasaki H: Microvessel density and HIF-1alpha expression correlate with malignant potential in fibrohistiocytic tumors. Eur J Dermatol *15*: 465-469, 2005.
21 Guix M, Faber AC, Wang SE, Olivares MG, Song Y, Qu S, Rinehart C, Seidel B, Yee D, Arteaga CL and Engelman JA: Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. Clin Invest *118*: 2609-2619, 2008.
22 Yagi H, Yotsumoto F, Sonoda K, Kuroki M, Mekada E and Miyamoto S: Synergistic antitumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. Int J Cancer *124*: 1429-1439, 2009.

Received April 7, 2011
Revised June 2, 2011
Accepted June 3, 2011