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HER2 Is Frequently Over-expressed in Ovarian Clear Cell Adenocarcinoma: Possible Novel Treatment Modality Using Recombinant Monoclonal Antibody against HER2, Trastuzumab

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Ovarian clear cell adenocarcinoma (CCA) is generally chemo-resistant. Recently the poor prognosis and resistance to chemotherapeutic agents of HER2/*neu* over-expressing tumors have become clear. Thus, we investigated the expression level of HER2 in surgically resected CCA and ovarian serous adenocarcinoma, endometrioid adenocarcinoma, and mucinous adenocarcinoma specimens, as well as CCA cell lines, by an immunohistochemical method. HER2 was over-expressed in 42.9% of CCA (*P*=0.026, vs. ovarian serous adenocarcinoma), 20.8% of ovarian serous adenocarcinoma, 23.1% of ovarian endometrioid adenocarcinoma, and 30.0% of mucinous adenocarcinoma specimens. Three CCA cell lines, RMG-1, HAC-II and KK were also positively stained for HER2. A flow-cytometric study of HER2 revealed 7.2-, 6.4- and 4.5-fold greater expression of HER2 than that of normal mammary gland, respectively. Trastuzumab, a humanized recombinant monoclonal antibody against HER2 significantly and dose-dependently reduced the growth of CCA cell lines *in vitro*. The extent of the inhibitory effect of trastuzumab was dependent on the expression level of HER2. Trastuzumab also dose-dependently inhibited the growth of xenografted RMG-1 tumor. The survival period of trastuzumab-treated mice was longer than that of the control group. From these findings, trastuzumab appears to be a candidate as a treatment modality for HER2 over-expressing ovarian CCA.

Key words: Ovarian clear cell adenocarcinoma — HER2 — Trastuzumab — HERCEPTIN — Growth inhibition

Clear cell adenocarcinoma (CCA) of the ovary is generally chemo-resistant,[1)] and is one of the most difficult tumors to treat in the field of gynecologic oncology.[2)] The preferred treatment of CCA is complete resection of the tumor, but this is difficult to accomplish in patients with advanced disease. Effective treatment for advanced cases is urgently needed.[3)] The natural mechanisms of growth and metastasis of this tumor should also be established. Komiyama *et al.* reported a better prognosis of stage I ovarian CCA patients with endometriosis compared with those without endometriosis.[4)] They reported growth inhibition of tumor cells by transforming growth factor-β (TGFβ) produced by associated endometrial tissue. We demonstrated that estrogen receptor-α (ERα) was absent in clinically resected ovarian CCA, in contrast with a high incidence in serous (SAC), endometrioid (EAC) and mucinous (MAC) adenocarcinoma specimens in our previous study.[5)] The activation of several growth factor genes after stimulation of ER was documented as a mechanism of ovarian carcinogenesis.[6)] This may explain why the growth of CCA is regulated by some growth factors in an estrogen-independent manner. AIB1, which is synonymous with SRC-3, i.e., steroid receptor coactivator-3, was reportedly up-regulated after HER2 activation in breast cancer.[7)] This may indicate the presence of a signaling pathway other than the ER-dependent pathway, and HER2 may regulate the cellular growth independently of ER.

Trastuzumab is a newly developed recombinant monoclonal antibody against HER2. Investigation revealed that patients with malignant tumors which over-express HER2 showed poor sensitivity to known anti-cancer agents and had a worse prognosis.[8–13)] When this compound was used combined with paclitaxel, it gave a clinically identifiable good response in patients with metastatic breast cancer.[14, 15)]

Here, we investigated the over-expression of HER2 in clinical ovarian CCA and CCA cell lines, and examined a possible novel treatment for this tumor using trastuzumab, a newly developed recombinant monoclonal antibody against HER2.

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MATERIALS AND METHODS

Culture conditions of human ovarian CCA cell lines, RMG-1, HAC-II and KK Cell lines KK,[16] which was kindly provided by Dr. H. Kikuchi, Department of Obstetrics and Gynecology, National Defense Medical College, RMG-1,[17] which was kindly provided by Dr. S. Nozawa, Department of Obstetrics and Gynecology, Keio University, and HAC-II,[18] which was also kindly provided by Dr. M. Nishida, Department of Obstetrics and Gynecology, University of Tsukuba, were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) supplemented with 10% fetal calf serum (Mitsubishi Chemicals Co., Tokyo). All of these cell lines were proved to be resistant to known anti-cancer drugs in our previous study.[19] The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Background of CCA patients Thirty-five ovarian CCA patients, who had been treated initially in the Department of Obstetrics and Gynecology, Toyama Medical and Pharmaceutical University Hospital and affiliated hospitals from 1982 to 1999, were registered in this study. The age of these patients ranged from 32 to 65 years. The mean age of patients was 51.9 and the median age was 51.0 years. They were basically treated with abdominal total hysterectomy and bilateral salpingooophorectomy. Pelvic and para-aortic lymphadenectomy were performed in some cases when intra-abdominal staging was more than T2.

Clinical specimens Thirty-five cases of CCA, 53 cases of SAC, 13 cases of EAC and 10 cases of MAC were investigated. After pathological review by one of the authors (M. F.), an appropriate block was selected for each specimen, then continuous paraffin sections of 5 μm thick were obtained from each block.

Immunohistochemical staining Immunohistochemical staining was performed by the usual strepto-avidin-biotin (SAB) method using an immunohistochemical staining kit (Nichirei Co., Tokyo) after a high temperature antigen unmasking procedure (121°C, 15 min) and blocking of endogenous peroxidase. The primary antibody was rabbit anti-human c-erbB-2 oncoprotein (DAKO, Glostrup, Denmark) at a dilution of 1:100, at 4°C overnight. Diaminobenzidine (DAB) was used as the staining substrate. The result was interpreted as negative (−), weakly positive (+), moderately positive (++) or strongly positive (+++) depending on the immunostaining pattern of the cellular membrane under a high-power microscope field, following the diagnostic criteria of "HercepTest."[20] Then, moderately and strongly positive cases were determined as HER2-over-expressing. Immunohistochemical study for HER2 was also performed on the three ovarian CCA cell lines cultured on glass slides, using the same procedure as described above.

Measurement of HER2 expression by flow cytometry CCA cell lines, RMG-1, HAC-II and KK, and HER2 over-expressing breast cancer cell lines SKBR3, which has 926 650 receptor sites/cell[21] and 33-fold greater expression of normal mammalian glandular cells, BT474, which has 702 000 receptor sites/cell, and MCF7, which has only 5525 receptor sites/cell,[21] were studied. A normal mammalian glandular cell, NME, was calculated to express 28 080 receptor sites/cell. Cells were suspended in phosphate-buffered saline (PBS) containing 1% fetal calf serum and 0.02% sodium azide. A cell suspension was incubated for 20 min at room temperature with phosphatidylethanolamine (PE)-conjugated anti-HER2/*neu* antibody (Becton Dickinson, San Jose, CA). Cells were analyzed on a FACS Calibur cyto-fluorimeter using Cell Quest software (Becton Dickinson, La Jolla, CA). The degree of HER2 protein expression was calculated as the mean fluorescence index (ΔMFI) of CCA cell lines, converted to the receptor number, and expressed relative (fold) to the number in normal mammalian glandular cells.

Treatment of cultured CCA cells with trastuzumab CCA cell lines were suspended in DMEM medium supplemented with 2% fetal calf serum to a concentration of 5×10^3/ml. Then 100 μl of the cell suspension (500 cells/well) was poured into each well of a 96-well flat culture plate (Corning, Inc., NY). Trastuzumab (Roche Co., Basel, Switzerland) was added to the cells at a concentration of 0.1, 1.0, 10, or 100 n*M*/ml. The cells were incubated for 72 h at 37°C in 5% CO_2. Cellular growth was measured by using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Twenty microliters of the solution was added to each well. After 3 h of incubation at 37°C in 5% CO_2, A_{490} was measured by using a micro plate reader (Tosoh Corp., Tokyo). Then the cell numbers in each well were calculated.

The study was done in triplicate by using three consecutive wells and the average was used as representative. The cell numbers after incubation with each concentration of trastuzumab were plotted as a histogram and compared with the control.

Identification of apoptotic cells after trastuzumab treatment Apoptotic cells were identified with the TUNEL method using ApopTag *In Situ* Apoptosis Detection Kits (Intergen Co., Purchase, NY) on glass slides, following the manufacturer's instructions. The number of apoptotic cells per 1000 cells was counted under the microscope and expressed as a percentage. This procedure was performed at 6, 12 and 24 h after trastuzumab addition.

Treatment of an RMG-1 human ovarian CCA xenograft model with trastuzumab SCID mice inoculated with RMG-1 were used for the current study. After the tumor volume had reached 100 mm^3, trastuzumab (0.1, 1.0, or 10 mg/kg) was administered into the peritoneal

cavity of the mice twice a week for 4 weeks. The same culture medium was injected into the mice of the control group in the same way as above. The tumor size was monitored twice a week by measuring the shorter diameter (SD) and longer diameter (LD). The tumor volume was calculated according to formula (i).

$$\text{Tumor volume} = LD \times SD^2/2 \quad \text{(i)}$$

The tumor volume of the trastuzumab-treated group was compared with that of the control group by using a modification of Houchens' methods.[22)]

Statistical analysis To evaluate the statistical significance of the difference of tumor volume between the trastuzumab-treated and control groups, a χ^2 test was performed, and $P<0.05$ was taken as the criterion of significance. To evaluate cell growth after administration of trastuzumab, a 2-sided Student's *t* test was performed. For evaluation of the tendency of dose-dependency, ANOVA was performed. All statistical analysis procedures were performed using StatView v4.5 for Macintosh (Abacus Concepts, CA).

Table I. HER2 Over-expression in Clinical Specimens (Immunohistochemistry)

Histological type	Over-expression (%)
Clear cell adenocarcinoma	15/35 (42.9)*
Serous adenocarcinoma	11/53 (20.8)
Endometrioid adenocarcinoma	3/13 (23.1)
Mucinous adenocarcinoma	3/10 (30.0)

∗ $P=0.026$ vs. serous adenocarcinoma.

RESULTS

HER2 status in clinically resected specimens and cell lines: Immunohistochemical study HER2 over-expression on the cell membrane was detected in 15 of 35 CCA (42.9%), 11 of 53 ovarian SAC (20.8%), 3 of 13 ovarian EAC (23.1%) and 3 of 10 ovarian MAC (30.0%) (Table I and Fig. 1, a–d). Over-expression in CCA was more frequent than in serous adenocarcinoma ($P=0.026$). No correlation was observed between HER2 over-expression or HER2 negativity and association of endometriosis in the cases examined (data not shown). HER2 was also detected in three ovarian CCA cell lines with an immunohistochemical method (Fig. 1, f–h).

HER2 expression measurement by flow cytometry The level of HER2 expression was examined in all cell lines (Fig. 2). Measured ΔMFI for MCF7, BT474 and SKBR3 was 2.3, 593.5 and 915.8, respectively. Also ΔMFI for three CCA cell lines, RMG-1, HAC-II and KK, was 119.7, 102.3 and 63.4, respectively. The expression levels of HER2 of three CCA cell lines expressed relative (fold) to that of normal mammalian cells were 7.2-, 6.4- and 4.5-fold, respectively (Fig. 2f).

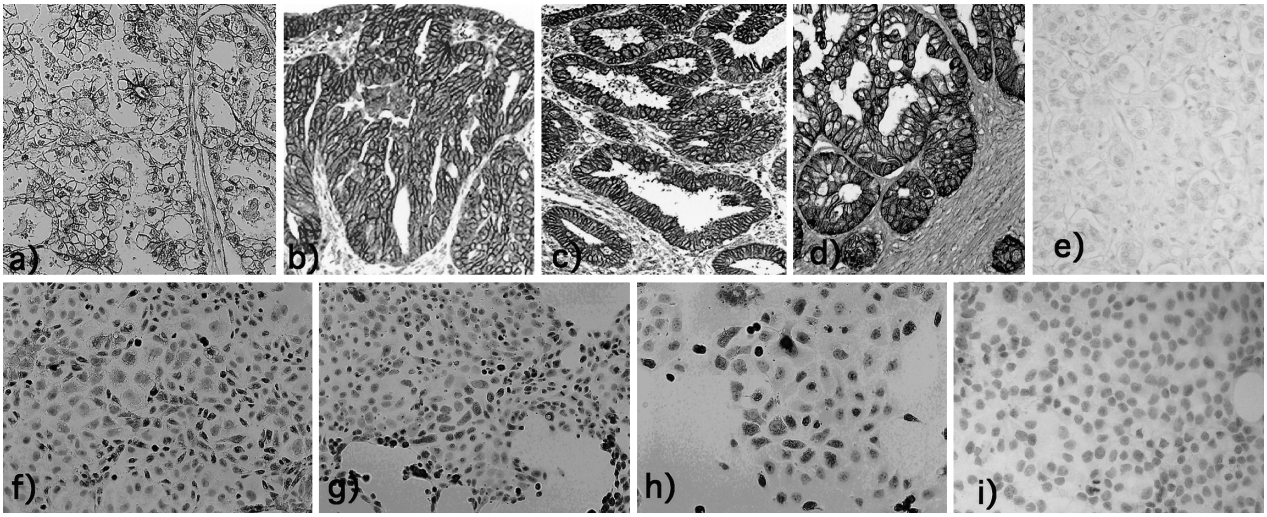

Fig. 1. Immunohistochemical staining for HER2 in clinical specimens of clear cell adenocarcinoma (a), serous adenocarcinoma (b), endometrioid adenocarcinoma (c) and mucinous adenocarcinoma (d), and negative control (clear cell adenocarcinoma) (e). The result was interpreted as HER2 over-expression when the immunostaining pattern of the cell membrane in a high-power microscope field was moderately and strongly positive, following the diagnostic criteria of "HercepTest." Immunohistochemical staining for HER2 in clear cell adenocarcinoma cell lines, KK (f), RMG-1 (g), HAC-II (h) and negative control (RMG-1) (i) is also presented. A positive staining pattern was observed in all the cell membranes of the cell lines examined.

Inhibition of CCA cell growth by trastuzumab addition We examined the anti-proliferative activity of trastuzumab in these CCA cell lines. Trastuzumab inhibited proliferation of these cell lines (Fig. 3). The most potent inhibition was seen with RMG-1, which expressed HER2 at about 7.2 times higher level than normal mammary gland. The inhibitory effect of trastuzumab was also correlated with HER2 expression determined by flow cytometry (RMG-1>HAC-II>KK) (Fig. 3).

Induction of apoptosis *in vitro* by trastuzumab To identify the mechanism of the inhibitory effect of trastuzumab on these CCA cell lines, the possibility of induction of apoptosis was examined by means of the TUNEL method. The CCA cells were exposed to 10 μg/ml trastuzumab.

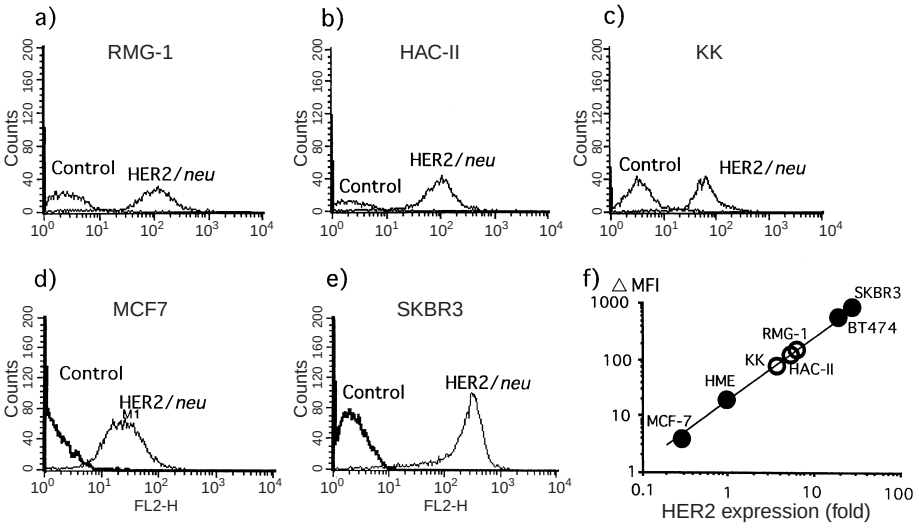

Fig. 2. HER2 expression in CCA (a, b, c) and breast cancer cell lines MCF7 (d) and SKBR3 (e). SKBR3 showed the strongest expression of HER2, whereas MCF7 expressed very low levels of HER2. HER2 expression of RMG-1, HAC-II and KK was calculated from the ΔMFI-HER2 expression curve (f). RMG-1 showed 7.2 times, HAC-II showed 6.4 times, and KK showed 4.5 times greater HER2 expression than normal mammalian cells.

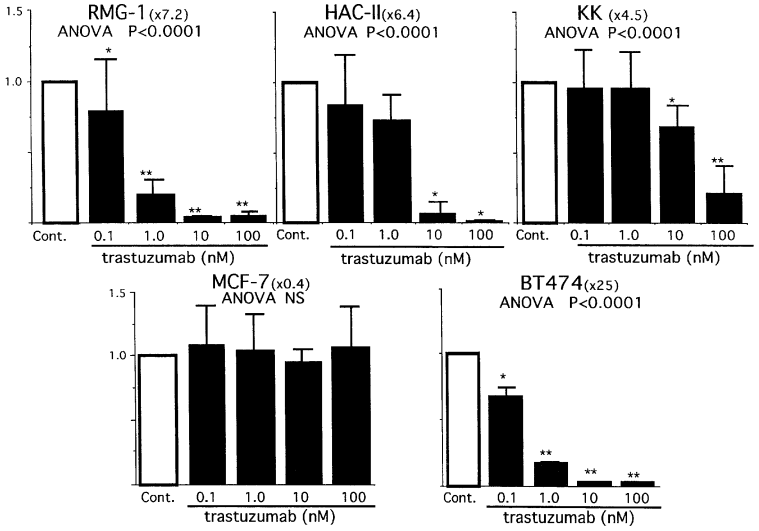

Fig. 3. Cell growth inhibition after administration of trastuzumab. Cell numbers of the CCA cell lines and HER2 over-expressing breast cancer cell line BT474 decreased dose-dependently after addition of trastuzumab (* $P<0.05$, ** $P<0.0001$ vs. control, ANOVA). No decreasing effect was observed in MCF-7.

Apoptosis was induced after 12 to 24 h (P=0.0021 at 12 h, P=0.0002 at 24 h vs. control) (Fig. 4).

Inhibition of CCA tumor growth *in vivo* by trastuzumab The tumor volume of the control group increased after administration of DMEM solution as a control. However, trastuzumab obviously inhibited tumor growth compared with the control (vs. trastuzumab 1.0 mg/kg, P=0.034; vs. trastuzumab 10 mg/kg, P=0.026) (Fig. 5). In the 0.1 mg/kg trastuzumab group, tumor volume was decreased, but without statistical significanse. In the 0.01 mg/kg trastuzumab group, no tumor-inhibitory effect was observed. Dose-dependent tumor volume reduction was observed in the trastuzumab groups. Treatment with trastuzumab was well tolerated in terms of maintenance of body weight (data not shown). Survival of mice in each group was also compared. SCID mice with 1.0 and 10 mg/kg of trastuzumab administration survived longer than the mice of the control group (Fig. 6).

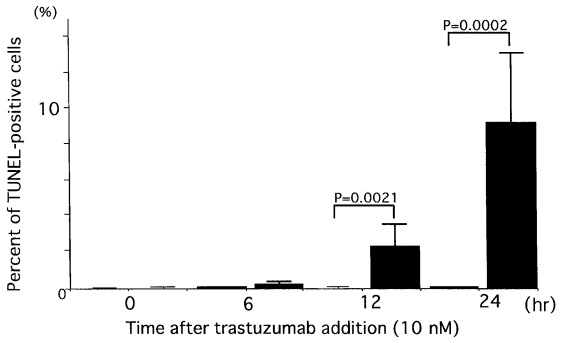

Fig. 4. Apoptosis induction after addition of trastuzumab (RMG-1). Apoptosis was induced in the CCA cell line after 12 h of exposure to trastuzumab. The TUNEL method was used to detect apoptotic cells (□ control, ■ trastuzumab).

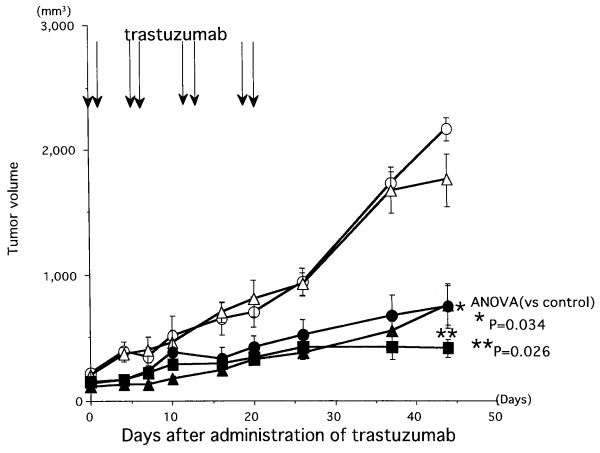

Fig. 5. Growth of xenografted CCA (RMG-1) after administration of trastuzumab to SCID mice. The tumor volume after administration of DMEM (control) or trastuzumab into the peritoneal cavity of SCID mice bearing a xenograft twice a week for 4 weeks was plotted. Significant dose-dependent reduction of tumor volume after administration of trastuzumab was observed (* P=0.034, ** P=0.026 vs. control, ANOVA) (○ control, △ trastuzumab 0.01 mg/kg, ● trastuzumab 0.1 mg/kg, ▲ trastuzumab 1.0 mg/kg, ■ trastuzumab 10 mg/kg).

DISCUSSION

HER2, which is one of a family of trans-membrane-type epidermal growth factor receptors, is over-expressed in many human malignant neoplasms, such as breast,[23] ovary,[24] endometrium,[25] lung,[26] stomach,[27] colon,[28] esophagus,[29] bladder[30] and pancreas.[31] Also HER2 protein over-expression was reported as a prognostic factor in patients with breast,[8] endometrium,[9] urinary bladder,[11] pancreas[13] and non-small cell lung cancer.[10] The rate of HER2 over-expression in epithelial ovarian cancer has been reported to be around 20%.[32–34] In our experiments, HER2 over-expression was observed in 32 of 111 cases (28.8%), which is only slightly higher. In serous tumor, which accounts for most epithelial ovarian cancer, the rate of HER2 over-expression was 20.8%, whereas in CCA, over-expression was 42.9% in our study (P=0.026). Thus, CCA patients have a higher over-expression rate of HER2. A similar observation was reported in endometrial cancer. Rolitsky *et al.* reported high HER2/*neu* amplification and a high over-expression rate in endometrial clear cell adenocarcinoma compared with other types of endometrial adenocarcinoma.[35] One reason why HER2 is a poor prog-

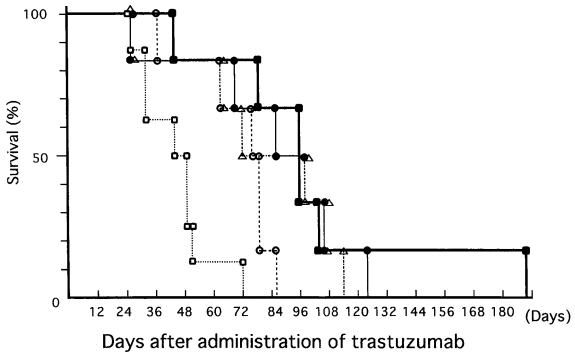

Fig. 6. Survival of SCID mice bearing CCA (RMG-1) after administration of trastuzumab. Significant survival prolongation was observed in trastuzumab-administered mice compared with the control group (control vs. trastuzumab 0.01 mg/kg and 10 mg/kg, P<0.01; control vs. trastuzumab 0.1 mg/kg and 1.0 mg/kg, P<0.05) (□ control, ○ trastuzumab 0.01 mg/kg, △ trastuzumab 0.1 mg/kg, ● trastuzumab 1.0 mg/kg, ■ trastuzumab 10 mg/kg).

nostic factor in breast cancer could be that HER2-positive tumors do not respond to hormone therapy such as tamoxifen.[36–38] A low response rate to hormone therapy in HER2-positive metastatic breast cancer patients has been reported.[36, 39] HER2-positive cancer cells were also reported not to respond to estrogen and tamoxifen *in vitro*.[40, 41] This finding can be explained in terms of the ability of HER2 over-expressing cells to grow without estrogen stimulation. Also, we demonstrated the absence of ERα in CCA clinical specimens and CCA cell lines KK, RMG-1 and HAC-II in a previous study.[5] The growth of those cell lines was not influenced by addition of 17β-estradiol (data not published). Based on these findings, we hypothesize that ovarian CCA over-expressing HER2 loses ERα expression and acquires the ability to grow without estrogen stimulation.

Because HER2 is over-expressed in various human cancer tissues, and predicts a poor outcome, HER2 is considered to be a good target for the treatment of those tumors. HER2/*neu* anti-sense DNA oligomer was found to inhibit the growth of breast cancer cells.[42, 43] Mouse monoclonal HER2/*neu* antibody, 4D5, has a potent inhibitory effect on the growth of HER2 over-expressing breast cancer cells,[44] and is thought to be a good candidate to treat HER2 over-expressing cancer. Humanized recombinant monoclonal antibody against HER2, which is commercially available as trastuzumab, was developed from this background, and was recently introduced for breast cancer treatment.[45] When trastuzumab was administered together with paclitaxel in a BT474 xenograft model, which over-expresses HER2, *in vivo* growth of the xenograft was strongly suppressed.[46] Also trastuzumab plus paclitaxel treatment demonstrated significant efficacy and resulted in longer survival in HER2 over-expressing metastatic breast cancer patients.[14] Based on our current study, ovarian CCA which over-expresses HER2 should be a good candidate for treatment with trastuzumab. Our *in vitro* and *in vivo* data clearly support the potential value of treatment with trastuzumab for chemo-resistant ovarian CCA. Trastuzumab should be considered for remission induction in the clinical setting, because it was administered intra-abdominally after the tumor had reached an appropriate size in our experiments. Also our *in vitro* data showed that the more strongly the cell lines expressed HER2, the more effectively trastuzumab inhibited tumor growth. The combination of trastuzumab and paclitaxel or anthracyclines was reported to show a significant effect in breast cancer cells.[14, 46] The growth-inhibitory effect of several anti-cancer drugs in combination with trastuzumab should be further examined. Trastuzumab did not cause significant body weight loss or disturbance of activity in mice in the current study (data not shown). A recent report demonstrated that HER2 over-expressing cells are also potential targets of epidermal growth factor receptor (EGF-R) tyrosine kinase inhibitors, such as ZD1839.[47]

Because trastuzumab is thought to inhibit signal transduction through HER2, it is expected to induce apoptosis. Cuello *et al.* suggested that the growth-inhibitory mechanism of trastuzumab involves inhibition of AKT kinase activity by down-regulation of HER2 receptors, leading to apoptosis.[48] Our current study revealed that trastuzumab induced apoptosis in a CCA cell line, RMG-1, during 24 h of exposure. Cuello *et al.* observed induction of apoptosis in 2% of a cultured HER2 over-expressing breast cancer cell line, SKBR3, by 1 μM trastuzumab.[48] The apoptosis induction rate after exposure to trastuzumab seemed to be very high in our experiment compared with Cuello's result, but the long incubation time (96 h) in their experiments might be a factor. Although mechanisms of growth suppression other than induction of apoptosis, such as antibody-dependent, cell-mediated cytotoxicity (ADCC), might operate in the *in vivo* situation, trastuzumab-induced apoptosis is likely to play a role in the growth inhibition of xenografted RMG-1 cells.

Our findings strongly indicate that trastuzumab is a good candidate for a novel treatment modality for ovarian CCA.

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