The effect of molecular target drug, erlotinib, against endometrial cancer expressing high levels of epiderma l growth factor receptor

（上皮成長因子受容体の過剰発現を認める子宮体癌における分子標的薬エルロチニブの効果について）

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Introduction

Endometrial carcinoma (EC) is the most common gynecological malignant tumors in Japan, and over 8,000 women were diagnosed with it in 2012. Based on the clinicopathological findings, there are two subtypes of endometrial carcinoma, type I and type II EC (1, 2). Type I EC, accounting for about 80% of EC, is generally associated with better outcome than type II EC since it is composed of low grade endometrioid histology with less aggressive characteristics and favorable prognosis (3). However, the number of patients with advanced stage or recurrent low-grade tumor cannot be negligible since type I EC comprises about 80% of the newly diagnosed EC in western Europe, North America, and Japan (3, 4).

After staging surgery, adjuvant therapy is considered based on the pathological risk factors such as tumor grade, histological type, myometrial invasion, positive margin, lymphovascular space invasion, and positive node status (5). Radiotherapy has proved to reduce the risk of local recurrence, but no randomized study has shown benefit for overall survival (6, 7). In the last decades, there has been emerging evidence suggesting that systemic cytotoxic chemotherapy may have favorable prognosis in advanced EC (8, 9). Taxanes, platinum agents, and anthracyclines have been utilized in advanced or recurrent EC patients, with response rates to these drugs ranging from 33% to 57% (8, 10-14).

Recently, a better understanding of the molecular and genetic characteristics of EC has promoted clinical research that targets angiogenesis and cellular signaling pathways involved in cancer development and progression. Epidermal growth factor receptor (EGFR) has been shown to be overexpressed in human cancers, including lung (15, 16), central nervous system (17), head and neck (18), bladder (19),

pancreas (20), and breast (21), and have a correlation with poor prognosis (22). EGFR expression has been demonstrated in 43–67% of EC tissue and associated with patient outcomes (23-25). In type II EC, including serous carcinoma and clear cell carcinoma, EGFR and HER2, another member of the EGFR family, have been shown to be expressed. Targeted therapy against the signaling system of the tyrosine kinase family could beneficial for patients with type II EC (26, 27). However, there have been no promising therapies, including small molecule tyrosine kinase inhibitors and the anti-EGFR monoclonal antibody, for antagonizing EGFR functions (28, 29). Thus, in this study, we aimed to evaluate whether targeting the EGFR tyrosine kinase has a therapeutic effect against EC, by precisely analyzing the expression levels of EGFR in cancer cells.

Material and Method

Reagents

Erlotinib (Abcam, Tokyo, JAPAN) was dissolved in DMSO, and Pertuzumab (Tyugai, Tokyo, JAPAN) was dissolved in distilled water for the *in vitro* and *in vivo* study. EGF (Invitrogen, Carlsbad, CA) was dissolved in phosphate buffered saline (PBS) (stock solution: 20 ng/mL).

DMEM (without phenol red) and gentamicin sulfate (Geneticin®) were purchased from Invitrogen. (Carlsbad, CA). DMEM /Ham's nutrient mixture F-12 (1:1, vol/vol) (without phenol red) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and culture condition

Ishikawa cells were purchased from Japanese Collection of Research Bioresources

(JCRB) cell bank (Tokyo, JAPAN). HEC-1A and KLE cells were purchased from American Type Culture Collection (Manassas, VA).

Ishikawa cells were maintained in DMEM supplemented with 10% charcoal fetal bovine serum (FBS) and 50 μg/μL gentamicin sulfate. HEC-1A cells were maintained in McCoy`s 5A medium supplemented with 10% charcoal FBS. KLE cells were maintained in DMEM/nutrient mix F-12 Ham's supplemented with 5% charcoal FBS. All media used were phenol red free. Cells were incubated at 37℃ in a humidified atmosphere containing 5% CO2. All cells were harvested using trypsin/EDTA when confluence was less than 80%.

Tissues and patient

All carcinoma tissues (from 51 patients) were obtained from Gunma University Hospital. Quantitative RT-PCR and immunohistochemistry were conducted according to the ethical guidelines of Gunma University and approved by the Institutional Review Board of Gunma University. Tissue specimens were handled according to the guidelines of the local ethics committee.

Immunohistochemistry

Formalin fixed samples were embedded in paraffin, sectioned and dried, then deparaffinized and rehydrated. The sections were immunostained using DAKO ENVISION+ KIT/HRP (DAKO, Carpentaria, CA) and Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to manufacturers' protocols. Rabbit monoclonal anti-EGFR antibody (diluted 1:100, DAKO, Carpentaria, CA) and mouse monoclonal anti-HER-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunohistochemistry (IHC) to determine EGFR and HER-2 expression

levels. Positivity was defined as more than 50% of specific cell staining of any intensity.

Western blotting

Twenty-four hours before starting the analysis, all cells were changed to medium without FBS. For analysis of phosphorylated extracellular signal-regulated kinases (ERK) 1/2, cells were treated with EGF (range from 1 pg/mL to 1 ng/mL) for 10 min, then washed twice with cold PBS, and incubated on ice with RIPA buffer (pH 7.4, supplemented with protease inhibitors, 200 mM NaF, 200 mM sodium orthovanadate) for 30 min. Lysates was aspirated and centrifuged at 15000 rpm for 10 min at 4°C. Supernatant was collected and protein concentration was measured. Frozen patient samples were homogenized and lysed in RIPA buffer. Protein samples (10–20 mg) were diluted in equal volume sample buffer (pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl) and incubated for 30 min at 25°C. Protein samples were loaded on a 12% polyacrylamide/bisacrylamide SDS-PAGE gel and transferred onto PVDF membrane (BIO-RAD, Hercules, CA, USA). Membranes were blocked with 5% BSA or 5% skim milk in TBST (100 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. Membranes were incubated overnight at 4°C with the primary antibody (phosphor-ERK 1/2 at 1:2000, total-ERK at 1:1000, EGFR at 1:1500, rabbit anti-human HER-2 at 1:1000 [Cell Signaling Technology, MA, USA], and mouse anti-human beta-actin at 1:3000 [Sigma-Aldrich]). After incubation, the membranes were washed 5 times with TBST and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (anti-rabbit or mouse at 1:40000, BIO-RAD) for 1 h at room temperature. After washing 5 times more with

TBST, the membranes were incubated with Immobilon Western Detection reagent (Millipore, Billerica, MA) for 5 min and detected by an Image Quant Imager (GE Healthcare Bio Science). The expression levels of phosphorylated ERK were quantified by scanning the digital image and digitized data were analyzed with the Image J (NIH, USA).

RNA isolation and quantitative RT-PCR

RNA was extracted from the endometrial cancer cell lines and primary resected endometrioid adenocarcinoma tissues. Total cellular and tissue RNA were extracted using Isogen (WAKO, Osaka, Japan) and 2 μg total RNA was treated with DNase I (Isogen, De Meern, Netherlands) according to manufacturer's protocol. RNA was reverse transcribed using SuperScript III transcriptase (Invitrogen) with random primers (Invitrogen). The samples were incubated with RNAse at 37°C to remove RNA, and were diluted to 100 μL with distilled water. Each quantitative PCR consisted of 5 μL of cDNA template, 12.5 μL SYBR Green real-time PCR master mix (Toyobo, Osaka, JAPAN), 0.2 μL forward and reverse primers (50 μM), and 7.1 μL distilled water. The sequences for the forward and reverse primers are as follows: human EGFR: 5' –GGAGAACTGCCAGAAACTGACC- 3' and 5' –GCCTGCAGCACACTGGTTG- 3'; human HER-2: 5' –ATCTGGCGCTTTTGGCACAG- 3' and 5' –CACCAGCCATCACGTATGCT- 3'; human GAPDH: 5' -AATTCCATGGCACCGTCAAG- 3' and 5' –GGTGAAGACGCCAGTGGACT- 3'. The reactions were carried out in an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) for 40 cycles (95°C for 15 sec, 60°C for 1 min) after initial 1-min incubation at 95°C. The fold change in the expression levels of each gene was calculated using the standard

curve method, with GAPDH as an internal control.

siRNA transfection

SiRNA against human EGFR (siEGFR) or HER-2 (siHER-2), and siRNA for negative control (si cont) were obtained from Applied Biosystems. All cell lines were plated for 24 h to approximately 50% confluence, and were transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Ambion, Grand Island, NY, USA). The transfected cells were subjected to western blotting, quantitative RT-PCR, and growth inhibition assay.

Growth inhibition assay

Cells were plated at 5000 cells (Ishikawa, KLE) or 10000 cells (HEC-1A) per well in 96 well plates. After 12 h incubation at 37°C in a humidified atmosphere containing 5% CO2, the cells were treated with drugs(ErbB inhibitor: erlotinib(EGFR tyrosine kinase inhibitor) and trastuzumab(HER-2 monoclonal antibody)) or transfected with siRNA, and incubated for further 48 h in the same conditions. Erlotinib was dissolved in DMSO and added to the cell culture medium at a concentration not exceeding 0.1% (v/v). At the end of various treatments, 10 μL cell counting solution (WST-1, Dojindo Labs, Tokyo, Japan) was added. The absorbance was measured at a wavelength of 450–650 nm using a Microtiter Plate Reader (Becton Dickinson, Franklin Lakes, NJ).

Tumor xenograft model and treatment

Female mice, 4-weeks-old nude BALB/C nu/nu, were obtained from Charles River Japan (Tokyo, JAPAN). Mice were housed in suitable cages in a pathogen-free

condition in a room maintained at 23–26°C, 50% humidity, and 12-h light/12-h dark cycle. The mice were allowed to acclimatize for 2 weeks prior to the study. Regular health checks were done. Mice were implanted with tumor cells in a single subcutaneous (s.c.) site on the shoulder flank (5×10^5 HEC-1 and 1×10^6 Ishikawa per mice in a 0.1 mL growth factor reduced matrigel (Corning, Tewksbury, MA) and 0.1 mL culture medium. Tumor-bearing mice were randomized into erlotinib (1 mg, 3 mg, 10 mg, 30 mg/kg/day, intraperitoneal (i.p.) for 5 days per week), pertuzumab (1 mg, 3 mg, 10 mg/kg, i.p. twice per week), and vehicle (DMSO and distilled water, i.p.) groups when the mean tumor volume was 100–150 mm^3. Equal volume of the vehicle (0.1 mL) was injected in all animals. Tumor volume and body weight were determined twice weekly. The tumor volume was determined according to the following formula: tumor volume = (length) x $(width)^2$ /2. On day 28, mice were euthanized; tumor was excised, and fixed in formalin. Tumors were processed for hematoxylin and eosin (HE) staining.

Data analysis

The data represent the means ± SEMs from at least three independent experiments.

Comparisons between groups were performed by one-way ANOVA. The significance of the differences between the mean values of the control group and each treated group was determined by Dunnett's multiple-comparison test. A value of $P < 0.05$ was considered significant.

Results

Expression of EGFR and HER-2 in endometrial cancer

Fifty-one surgically resected endometrioid carcinoma samples, diagnosed as well (Grade 1, G1), moderately (G2), or poorly (G3) differentiated adenocarcinoma, were obtained from patients who had undergone surgery at Gunma University Hospital with their consent (Table 1). In our institution, 20.9% of patients with endometrial cancer with low-grade endometrioid histology have been diagnosed as stage III and IV. As a first step, IHC was carried out on endometrial carcinoma to confirm the expression of EGFR and HER-2 proteins (Fig. 1A). EGFR protein was highly expressed in G1 and G2 endometrioid carcinoma, whereas HER-2 was almost evenly expressed in G1, G2, and G3 tumors. We also evaluated the EGFR mRNA and HER-2 mRNA expression levels in EC tissues by RT-PCR (Fig. 1C). EGFR mRNA levels were higher in G1 and G2 ($P < 0.05$) than in G3, but there was no significant difference in HER-2 mRNA expression between the three grades.

EC cell line experiments

Cancer cell lines were utilized for further experiments to elucidate the roles of EGFR and HER-2 in EC cells. Three cell lines (Ishikawa, HEC-1A, and KLE) were evaluated by western blotting to determine protein expression levels of EGFR and HER-2. HEC-1A showed high EGFR and low HER-2 expression while Ishikawa had low EGFR and high HER-2 expression. In KLE, the expression levels of EGFR and HER-2 were intermediate between Ishikawa and HEC-1A. (Fig. 2A). These results were reconfirmed by quantitative RT-PCR experiments, which indicated that EGFR mRNA level were significantly the highest in HEC-1A ($P < 0.005$), but there was no significant difference in HER-2 mRNA expression between the three cell lines. (Fig. 2B)

The three cell lines were treated with EGF and were evaluated for downstream signaling of EGFR, by detecting phosphorylated ERK 1/2 by western blotting (Fig. 3). The phosphorylation of ERK 1/2 was induced in all three cell lines, but increased in HEC-1A at a lower concentration in comparison with the other two cell lines. This result suggested that the amount of EGFR expression was an important factor for the activation of mitotic-activated protein kinase (MAPK) pathway by EGF stimulation in endometrial carcinoma cells.

To investigate the significance of EGFR and HER-2 in the proliferation of endometrial cancer cells, all cells were transfected with siRNA to knock down EGFR or HER-2. After 48 h, EGF was added, and ERK 1/2 phosphorylation and proliferation were evaluated. When EGFR was knocked down (Fig. 4A and 4C), all cells showed decreased ERK 1/2 phosphorylation ($P < 0.05$). The viability of Ishikawa cells was reduced to 72%, HEC-1A to 76%, and KLE to 73%, compared with negative control ($P < 0.05$). When HER-2 was knocked down (Fig. 4A and 4C), ERK 1/2 phosphorylation was significantly decreased in Ishikawa, which highly expressed HER-2 ($P < 0.05$), but not in HEC-1A and KLE. Similarly, cell viability was reduced in Ishikawa (to 65% compared with negative control) ($P < 0.05$), but not in other cell types (HEC-1A: to 85% KLE: to 76% compared with negative control).

Growth inhibition assay following ErbB inhibitor treatment *in vitro*

The results in figures 1A and 2A prompted us to investigate whether ErbB inhibitors could effectively inhibit EC proliferation. In subsequent experiments, all cells were treated with erlotinib (ERL: EGFR tyrosine kinase inhibitor) or trastuzumab (TRA: HER-2 monoclonal antibody), and evaluated for ERK 1/2 phosphorylation and proliferation in EC cells. The result shown figure 5A

demonstrated that all cells treated with ERL showed decreased ERK 1/2 phosphorylation ($P < 0.001$). However, only HEC-1A treated with ERL showed reduction in cell viability to 38% compared with vehicle control ($P < 0.01$). In the case of TRA treatment (Fig 5A and 5B), only Ishikawa cells showed a decrease in ERK 1/2 phosphorylation ($P < 0.05$) and cell viability to 78% compared with vehicle control ($P < 0.05$).

Tumor growth inhibition assay following ErbB inhibitor treatment in mice xenograft model

Because the *in vitro* studies were examined for short periods, the long-term effect of either ERL or TRA was studied using an EC xenograft *in vivo* model. Tumor-bearing mice were treated with either ERL or TRA for 28 days. The results showed that only tumors in HEC-1A xenografted mice administered with ERL at a dose of 3 mg/kg or more (Fig. 6A and 6B) showed reduction, whereas TRA did not induce significant tumor growth inhibition in mice implanted with either HEC-1A or Ishikawa. The resected tumor from the xenograft model stained with HE, suggesting that clear fibrosis occurred in HEC-1A tumor treated with ERL (Fig. 6C).

Discussion

In the present study, we demonstrate that both mRNA levels and protein levels of EGFR were highly expressed in low-grade endometrioid carcinoma, but were expressed at low levels in high-grade endometrioid carcinoma. We examined the molecular differences that underlie the variable responsiveness to erlotinib in accordance with the expression levels of both mRNA and protein of EGFR in the

endometrial carcinoma cells, using quantitative RT-PCR and IHC. We found that erlotinib, a known potent selective inhibitor of the EGFR tyrosine kinase, significantly inhibits the proliferation of endometrial carcinoma cells, which express high levels of EGFR in xenograft mice models.

The degree of tumor differentiation is one of the prognostic factors in EC; low-grade endometrioid tumors tend not to progress to deep myometrial invasion or spread to distant sites (30). In contrast, high-grade endometrioid tumor is aggressive and diagnosed at advanced stages, and involved recurrent or metastatic tumors at high rate. On the other hand, overall prognosis for those who are diagnosed with low-grade tumor is positive, although the number of patients with recurrent or metastatic tumors is still quite large due to the corresponding amount of newly diagnosed type I EC patients (3, 4). In fact, in our institution, 20.9% of endometrial cancer patients with low-grade endometrioid histology have been diagnosed as stage III and IV (Table 1). We comprehensively analyzed EGFR and HER2 expression levels in endometrioid carcinoma (Fig. 1), demonstrating that EGFR mRNA and protein were highly expressed in low-grade endometrioid tumor as compared to high-grade endometrioid tumor. In contrast, HER2 was not significantly expressed at a varying level in any grade of endometrioid tumor. Collectively, these results prompted us to further investigate the significance of EGFR in the proliferation of low-grade endometrioid tumor.

To date, anti-EGFR antibody, anti-EGFR, or dual EGFR/HER2 tyrosine kinase inhibitors have been evaluated across a variety of disease types. For HER2-positive patients with breast cancer, trastuzumab has significantly reduced the rate of recurrence (31). In the subsequent study (32), lapatinib, the EGFR and HER2 dual kinase inhibitor, demonstrated a significant antiproliferative effects in

HER2 overexpressing breast tumor cell lines, suggesting that EGFR expression level has no association with the sensitivity to lapatinib. In contrast, both EGFR and HER2 expression has been found in patients of non-small-cell lung cancer with poor prognosis (33), and erlotinib was beneficial in those patients in an EGFR dependent way (34). In this study, trastuzumab did not reduce the tumor growth of Ishikawa cells in xenograft mice (Fig. 6B), which was in contrast to the *in vitro* results (Fig. 5B). On the other hand, the antitumor effects of erlotinib against HEC-1A cells clearly inhibited tumor growth both *in vitro* (Fig. 5B) and *in vivo* (Fig. 6A), whereas it reduced the tumor growth of Ishikawa cells in the xenograft mice to a less extent. Taken together, the current data indicate that the expression levels of EGFR is a key factor in the molecular targeted therapy against pathogenic tyrosine kinases in endometrial cancer, and suggest that EGFR inhibitor may be clinically useful in well-defined subgroups of endometrial cancer.

A phase II study (NCIC IND-148) has been largely referred to conclude that erlotinib is not a promising agent for recurrent or metastatic EC. However, in that study, tumors were regarded as EGFR positive if tumor cell membranes stained positively with anti-EGFR antibody in IHC in more than 10% of tumor cells. Thus, we speculate that this clinical study contained large cases of high-grade endometrioid tumors and type II EC, based on our finding that a majority of cell membranes were stained (Fig. 1).

Patients with risk factors such as tumor grade, deep myometrial invasion, and positive lymph nodes are recommended for systemic chemotherapy, although it is not unanimously accepted. Basic cancer research is conducted to identify the markers that determine patients to chemotherapy regimen according to the responses. In malignant tumors, it is unlikely that one signaling pathway is solely

engaged in its aggressive behavior including progression and metastasis. However, the present data shown in this study demonstrate that erlotinib has an efficacy for treatment of endometrial cancer, which highly express EGFR. We believe that further analysis of the molecular signature of the EC tumors will define patients who can be benefited by erlotinib therapy.

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Table.1 Characteristics of surgical cancer patients

	well differentiated (G1)	mediate differentiated (G2)	poorly differentiated (G3)
N	24	17	10
age(median)	57	59	58
Surgical Staging			
Ⅰ	16(66.7%)	10(58.8%)	5(50.0%)
Ⅱ	3(12.5%)	1(5.9%)	0(0.0%)
Ⅲ	4(16.7%)	6(35.3%)	3(30.0%)
Ⅳ	1(4.2%)	0(0%)	2(20.0%)

Fig. 1 Detection of EGFR and HER-2 proteins in endometrial adenocarcinoma (surgically resected endometrioid cancer sample)

A) We used tissue samples of well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) endometrial carcinoma for immunohistochemical study. The tissues were fixed in formalin and embedded in paraffin. Sections were taken from the paraffin-embedded tissue and stained with 1:200 anti-EGFR or 1:150 anti-HER-2. Primary antibody binding was detected through a biotin-conjugated secondary antibody. Top panels, HE stained; middle panels, stained with anti-EGFR; bottom panels, anti-HER-2. Magnification × 200. Bars =1000μm.

B) The expression status of EGFR and HER-2 in each grade of tumor were assessed by immunohistochemistry. The ratio of immunopositive cases for each protein is represented in the bar graph.

C) The carcinoma portions were excised, and RNA was isolated. EGFR and HER-2 mRNA levels were measured using quantitative RT-PCR, GAPDH mRNA levels were quantitated as an internal control. The amounts of EGFR and HER-2 mRNA were respectively normalized by the amounts of GAPDH mRNA.

*, Decrease in the expression level of EGFR mRNA in G3 compared to those in G1 and G2 cancers, $P < 0.05$

Fig. 2 EGFR and HER-2 protein and mRNA expression levels in EC cell lines

A) Cells were cultured, harvested, solubilized in detergent, and resolved by 12% reducing SDS-PAGE. Each sample was confirmed with anti-EGFR, anti-HER-2, and anti-β-actin antibody. The detection of β-actin protein served as a loading control. The blot is representative of three independent experiments. *, increased expression

levels of EGFR protein in HEC-1A compared to those in HEC293 and Ishikawa, $P < 0.001$ **, increased expression levels of EGFR protein in HEC-1A compared to those in KLE, and increased expression levels of HER-2 protein in Ishikawa and KLE compared to those in HEC-1A, $P < 0.05$.

B) EGFR and HER-2 mRNA levels were measured by quantitative RT-PCR. Data were normalized with GAPDH mRNA level in each sample. Data represent the means ± SEMs of five independent experiments. *, increased expression levels of EGFR mRNA in HEC-1A compared to those in HEC293 and Ishikawa, $P < 0.005$ **, increased expression level of EGFR mRNA in HEC-1A compared to those in KLE, and increased expression level of HER-2 mRNA in Ishikawa and KLE compared to those in HEC-1A $P < 0.05$.

Fig. 3 Phosphorylation of ERK treated EGF in EC cell lines

EC cells were incubated with epithelial growth factor (EGF) (1–1000 pg/mL), and cells were harvested at the 10 min for western blotting. Each sample was confirmed with either anti-phospho-ERK or anti-total-ERK.

Fig. 4 EGFR is involved in ERK phosphorylation in EC cell lines

All EC cells were transfected with 10 nM of siRNA (control, EGFR, or HER-2). Cells were harvested 48 h after transfection to evaluate ERK phosphorylation after knockdown of EGFR (A) or HER-2 protein (B). Cells were incubated with EGF (1 ng/mL) for 10 min and harvested for western blot analysis. The detection of ß-actin protein served as a loading control. The blot is representative of three independent experiments. The expression levels of phosphorylated ERK were quantified by scanning the digital image and digitized data were analyzed with the Image J. Data

represent the means ± SEMs of three independent experiments. *, decreased compared to siRNA control transfection (NC), $P < 0.05$.

C) All EC cells were transfected with 10 nM of siRNA (control, EGFR or HER-2), and cell proliferation was monitored after 48 h using WST-1 assay. *, decreased compared to siRNA control transfection (negative control), $P < 0.05$.

Fig. 5 Effect of inhibition of ERK phosphorylation by erlotinib (ERL) or trastuzumab (TRA) on proliferation in EC cell lines

A) All cells were treated with either ERL (3 μM, 30 μM) or TRA (100 μg/mL, 1000 μg/mL). After a 2-h incubation with the drug, cells were treated EGF (1 ng/mL) for 10 min and harvested for western blot analysis. The blot is representative of three independent experiments. The expression levels of phosphorylated ERK were quantified by scanning the digital image and digitized data were analyzed with the Image J. Data represent the means ± SEMs of three independent experiments. *, decreased with the drug treatment compared to control, $P < 0.001$

B) All cells were treated with either ERL (0.1–30 μM) or TRA (10–1000 μg/mL). After 2 h incubation with the drug, all cells were treated EGF (1 ng/mL). Cell proliferation was monitored after 96 h using WST-1 assay. *, decreased as compared to vehicle control, $P < 0.01$.

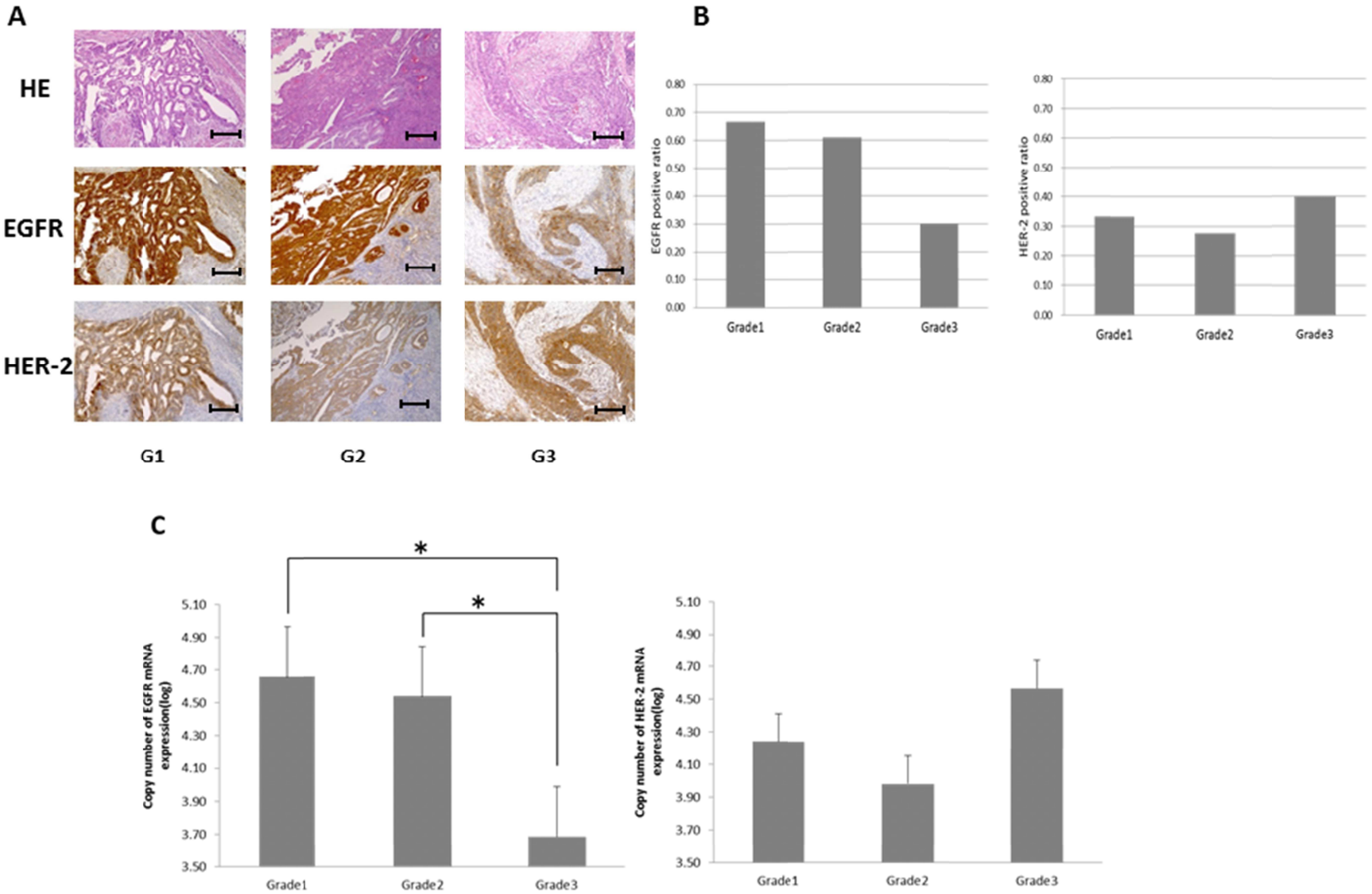
Fig. 6 Inhibition of tumor growth by Erlotinib (ERL) *in vivo*

Mice were implanted with Ishikawa (A) or HEC-1A (B) and treated with ERL or TRA for 28 days. Tumor volume was measured twice a week. Data represent the means ± SEMs of three independent tumor volumes. *, decreased as compared to vehicle control, $P < 0.05$.

C) On day 28 after starting treatment, mice were euthanized and tumor was excised. The tissues were fixed in formalin and embedded in paraffin. Sections were taken from the paraffin-embedded tissue and HE stained. Top and upper middle panels, HEC-1A tumor; lower middle and bottom panels, Ishikawa tumor. Magnification × 200. Bars =1000 μm.

Fig. 1

584


A
HE
EGFR
HER-2
G1
G2
G3
B
EGFR positive ratio
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0.60
0.50
0.40
0.30
0.20
0.10
0.00
Grade1
Grade2
Grade3
HER-2 positive ratio
0.80
0.70
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Grade1
Grade2
Grade3
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*
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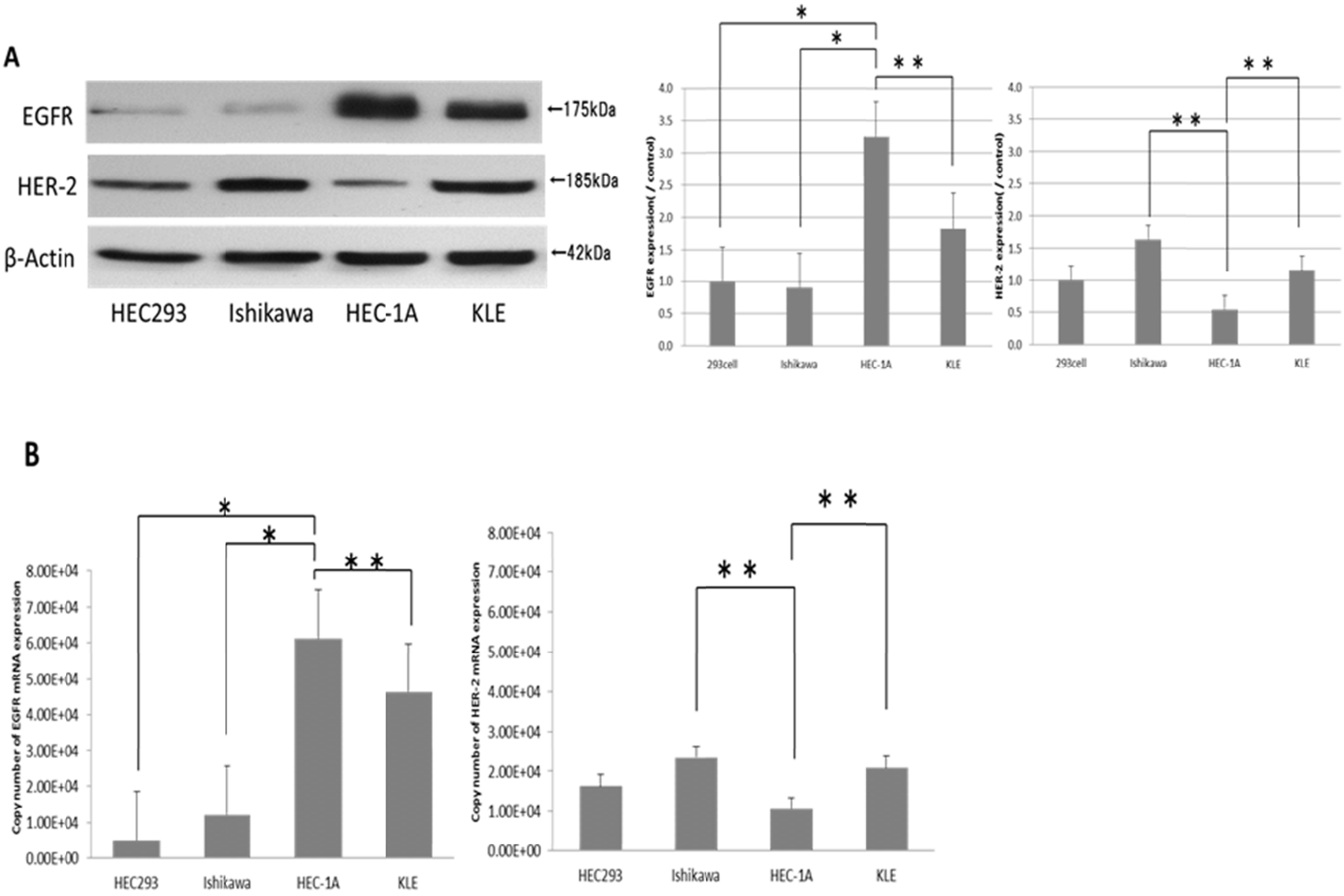
Grade3

Fig. 2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EGFR
HER-2
β-Actin
←175kDa
←185kDa
←42kDa
HEC293
Ishikawa
HEC-1A
KLE
EGFR expression(/ control)
HER-2 expression(/ control)
293cell
Ishikawa
HEC-1A
KLE
B
Copy number of EGFR mRNA expression
Copy number of HER-2 mRNA expression
8.00E+04
7.00E+04
6.00E+04
5.00E+04
4.00E+04
3.00E+04
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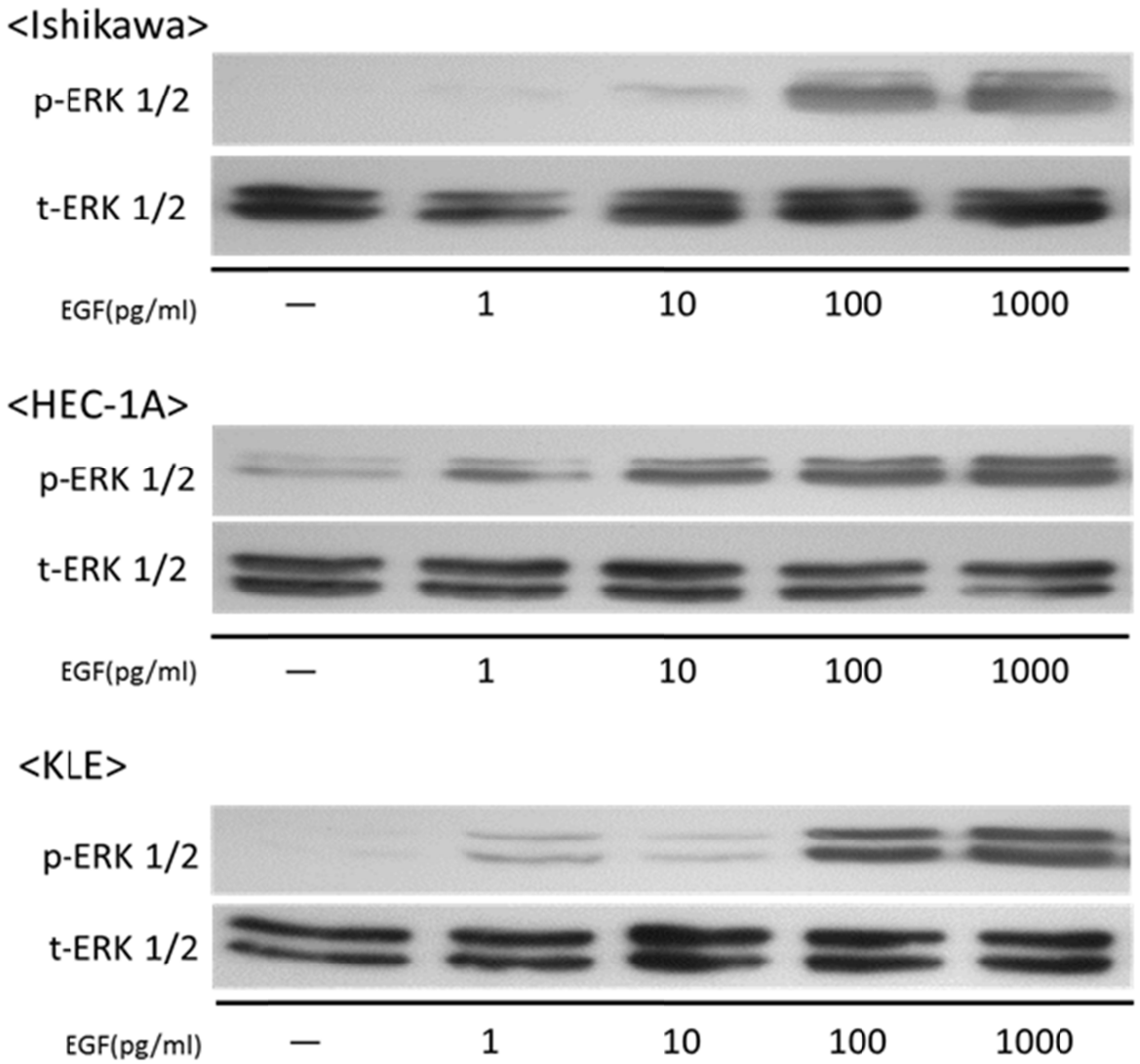
0.00E+00
HEC293
Ishikawa
HEC-1A
KLE

Fig. 3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t-ERK 1/2
EGF(pg/ml)
—
1
10
100
1000
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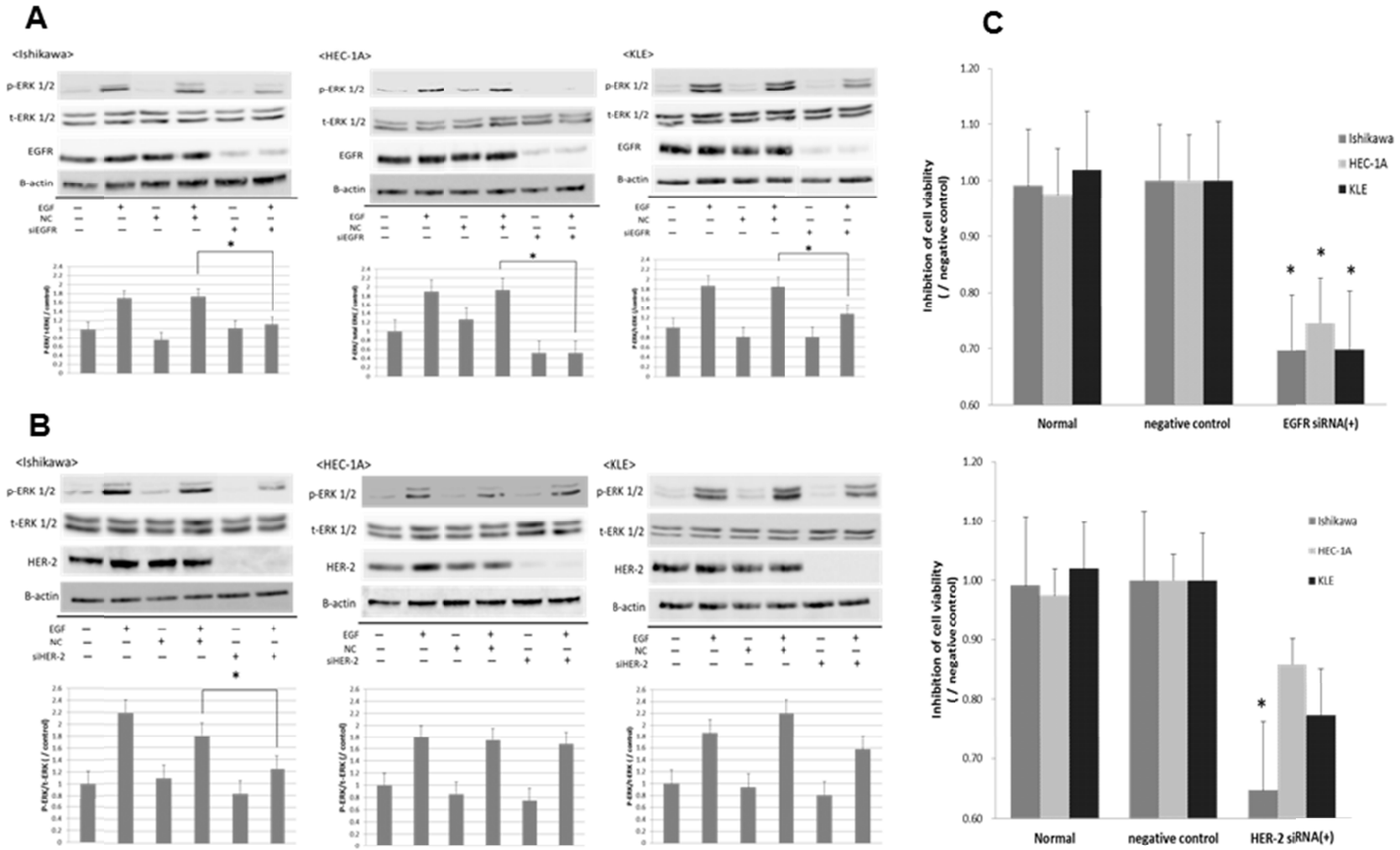
t-ERK 1/2
EGF(pg/ml)
—
1
10
100
1000
<KLE>
p-ERK 1/2
t-ERK 1/2
EGF(pg/ml)
—
1
10
100
1000

Fig. 4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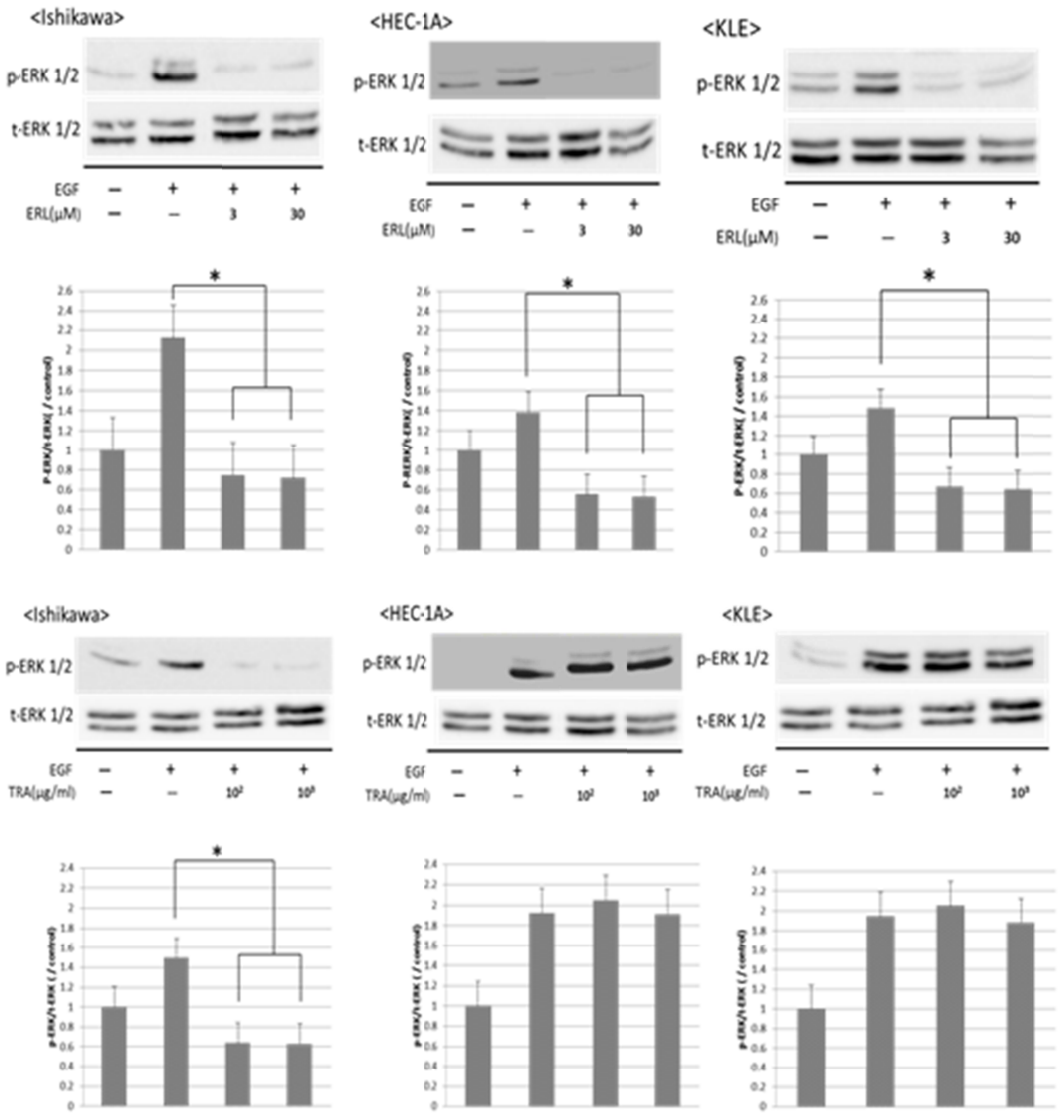
t-ERK 1/2
EGFR
B-actin
EGF
NC
siEGFR
B
HER-2
siHER-2
C
Inhibition of cell viability
(/ negative control)
Ishikawa
HEC-1A
KLE
Normal
negative control
EGFR siRNA(+)
HER-2 siRNA(+)

Fig. 5

A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<KLE>
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t-ERK 1/2
EGF
ERL(μM)
TRA(μg/ml)
*

B

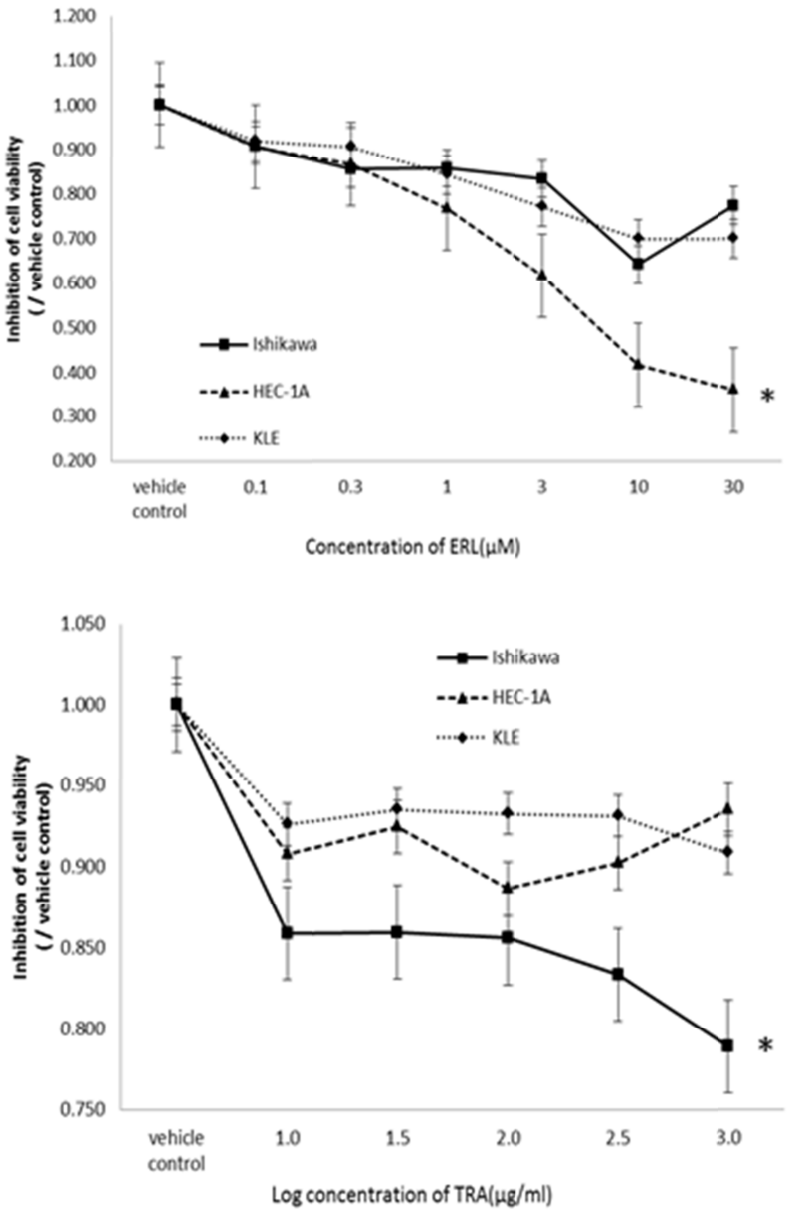
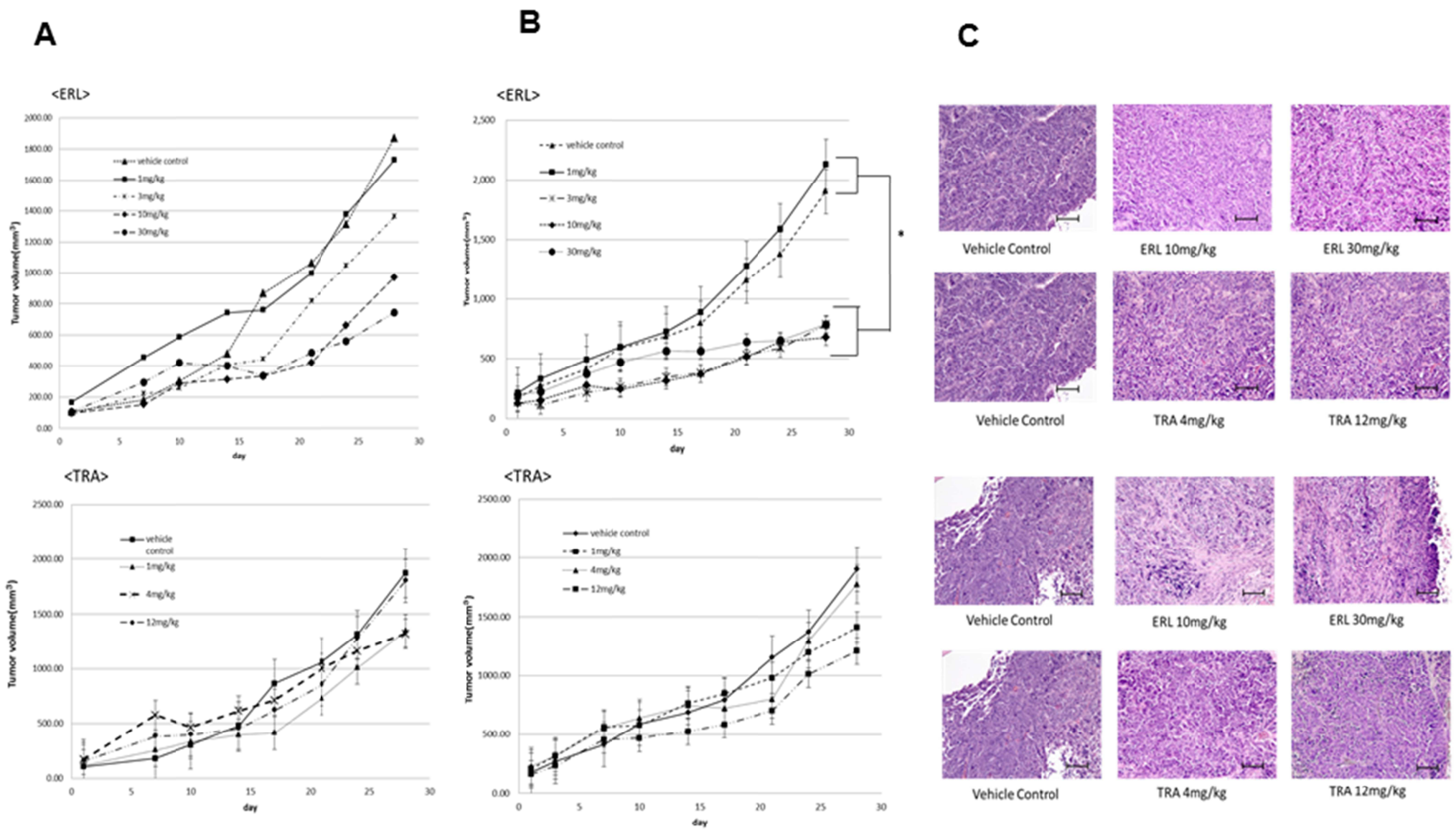

Inhibition of cell viability
(/ vehicle control)
Ishikawa
HEC-1A
KLE
vehicle control
Concentration of ERL(μM)
Log concentration of TRA(μg/ml)
*

Fig. 6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vehicle control
1mg/kg
3mg/kg
10mg/kg
30mg/kg
Tumor volume(mm³)
day
<TRA>
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Tumor volume(mm³)
day
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1mg/kg
3mg/kg
10mg/kg
30mg/kg
*
Tumor volume(mm³)
day
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vehicle control
1mg/kg
4mg/kg
12mg/kg
Tumor volume(mm³)
day
C
Vehicle Control
ERL 10mg/kg
ERL 30mg/kg
Vehicle Control
TRA 4mg/kg
TRA 12mg/kg
Vehicle Control
ERL 10mg/kg
ERL 30mg/kg
Vehicle Control
TRA 4mg/kg
TRA 12mg/kg

The effect of molecular target drug, erlotinib, against endometrial cancer expressing high levels of epiderma l growth factor receptor

Molecular Cancer Therapeutics（投稿中）

Toshio Nishimura, Kazuto Nakamura, Sadatomo Ikeda, Keiko Kigure, Soichi Yam ashita, Takashi Minegishi