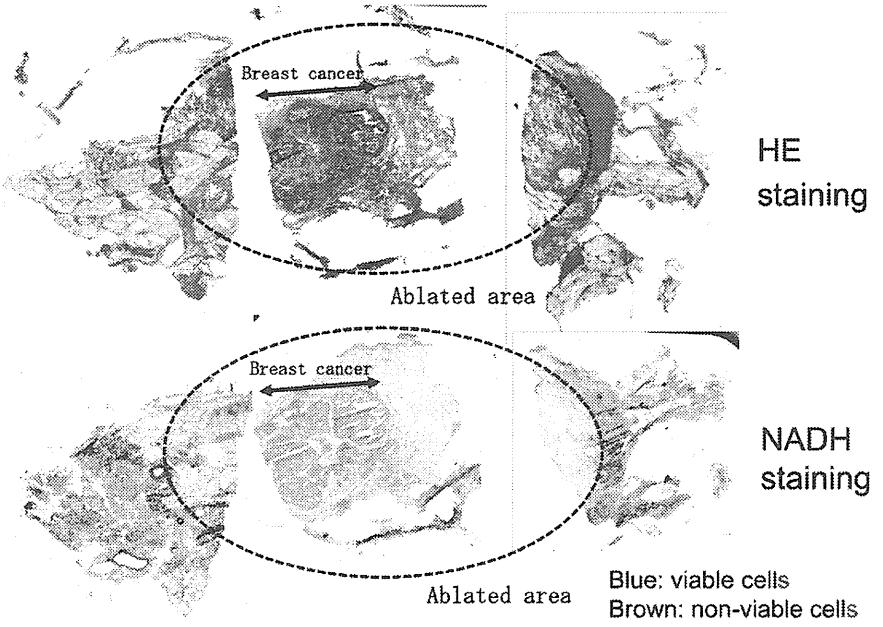

Fig. 3. NADH staining showed pathological complete cell death in the entire ablated area including surgical margin. HE staining of ablated lesion removed immediately after RFA could not demonstrate complete tumour cell death.

Table 4
Feasibility studies on radiofrequency ablation followed by surgical resection.

First Author (year) Ref.	No. of cases	T	Electrode	Application time	Power (W)	Time (min)	Complete ablation	Complications
Jeffrey (1999)[12]	5	T2-T3	LeVeen	2 times	20–60	12–28	80%	None
Izzo (2001)[13]	26	T1-T2	LeVeen	2 times	25–80	7–25	96%	Skin burn (1case)
Burak (2003)[11]	10	T1	LeVeen	Over 2 times	–	7–21	90%	None
Singletary (2003)[14]	29	T1-T2	RITA	1 time	–	30–45	86%	skin burn (1 case), Wound infection (4 cases)
Hayashi (2003)[15]	22	T1	RITA	1 time	–	–	64%	skin burn (1 case)
Noguchi (2006)[16]	10	T1	RITA	Over 2 times	–	–	100%	None
Khatri (2007)[17]	15	T1	Cool-Tip	Over 2 times	14–53	7–36	93%	Skin puckering (2 cases), Wound infection (1 case)
Medina-Franco (2008)[18]	25	T1-T2	Elektrotom	1 time	30–55	–	76%	Skin burn (3 cases), Wound infection (1 case)
Imoto (2009)[19]	30	T1	LeVeen	2 times	7–89	5–42	87%	Skin burn (2 cases), Muscle burn (7 cases)
Wiksell (2010)[20]	31	T1	NeoDynamics AB	1 time	–	6.5–11	84%	Skin burn (1case), Muscle burn (1 case), Pneumothorax (1 case)
Kinoshita (2011)[21]	49	T1-T2	Cool-Tip	2 time	5–118	–	85%	Skin burn (2 cases), Muscle burn (3 cases)
Present study	41	T1	Cool-Tip	1 time	50–110	6–15	88%	Skin burn (1 case)
Total	293	T1-T3	Various	1-over 2 times	–	–	84%	Skin burn (12 cases), Muscle burn (11 cases) Pneumothorax (1 case), Miscellaneous (8 cases)

surgery. Theoretically, one application of RFA by a 3 cm electrode can ablate 3 cm in the shorter diameter; our technique ablated 1.8–5.5 cm (median, 2.5 cm) in the shorter diameter. If the greater diameter of the tumour is 1.5 cm and the surgical margin is 0.5 cm, at minimum, the zone of ablation must therefore be 2.5 cm, and so localized tumours less than 1.5 cm are indicated for RFA.

Our findings demonstrated that a single RFA session can eradicate breast cancer less than 1.5 cm by greater diameter with no EIC (extra-intraductal component); 2 or more sessions may be effective in tumours over 1.5 cm. Paradoxically, patient selection remains a very important factor in determining the suitability of RFA for breast cancer. NADH staining demonstrated complete cell death within the ablated area (the degenerative protein ring) by MRI scanning. Thus, when performing RFA without resection, if the tumour is located within this area, core needle biopsy is unnecessary to confirm cell viability. A post-ablation MRI scan can predict the therapeutic effect by RFA for breast cancer.

RFA represents a minimally invasive treatment option for the local therapy of early breast cancer instead of breast-conserving surgery. Despite the use of various RFA procedures, the overall total ablation rate in the literature is 84% (Table 4).[11–21] RFA followed by whole breast irradiation may be a promising protocol for the local control of breast cancer. To confirm that RFA is an alternative to breast-conserving surgery, a randomized control clinical trial is indispensable for comparing the 2 treatments.

Conclusion

We found that RFA for breast cancer could be safely applied in an outpatient setting with good patient tolerance, and that only 1 RFA session could achieve total tumour cell death within the whole ablated area. RFA is a promising, minimally invasive alternative to breast-conserving surgery for local treatment in women with small ($\leq$1.5 cm) breast cancer.

Conflict of interest statement

None declared.

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E-cadherin expression on human carcinoma cell affects trastuzumab-mediated antibody-dependent cellular cytotoxicity through killer cell lectin-like receptor G1 on natural killer cells

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Trastuzumab is a recombinant antibody drug that is widely used for the treatment of HER2-overexpressing breast carcinoma. Despite encouraging clinical results, many HER2-overexpressing carcinomas are primarily resistant to trastuzumab. We attempted to explain trastuzumab resistance and search for solutions. Since the killer cell lectin-like receptor G1 (KLRG1), an inhibitory receptor expressed on subsets of natural killer (NK) cells recognizes E-cadherin as ligands and may inhibit immune responses by regulating the effector function of NK cells, we used HER2-overexpressing carcinoma cells which were expressing E-cadherin to investigate the role of antibody-dependent cellular cytotoxicity (ADCC) through KLRG1 on NK cells in vitro and vivo. The results indicated that HER2-overexpressing carcinoma cells were killed by trastuzumab-mediated ADCC and the ADCC activity was reflected the degree of E-cadherin expression on carcinoma cells. We found that expression of E-cadherin was shown to be a predictor of response to trastuzumab-based treatment for HER2-overexpressing carcinomas, furthermore, trastuzumab-mediated ADCC was markedly enhanced by KLRG1-negative peripheral blood mononuclear cells (PBMCs$^{KLRG1(-)}$).

The HER2 receptor tyrosine kinase is one of the four members of the epidermal growth factor receptor (HER) family of transmembrane receptors that have an intracellular tyrosine kinase domain, and phosphorylation of the tyrosine kinase domain by homodimerization or heterodimerization induces both cell proliferation and survival signaling.[1–3] Overexpression of HER2, primarily as a result of gene amplification, is found in 20–30% of human invasive breast carcinomas, and it is associated with an aggressive clinical course and shortened disease-free and overall survival.[2,4] There have been several similar reports of HER2 overexpression being associated with aggressive clinical behavior by colon carcinoma, ovarian carcinoma, gastric carcinoma, and esophageal carcinoma. Trastuzumab is a key drug in the treatment of HER2-overexpressing breast carcinoma.[5] Overall response rates (complete responses plus partial responses) ranging from 15 to 30% have been reported when used as monotherapy and response rates of 50–80% have been reported when used in combination with taxanes or vinorelbine.[6–11] Multiple new combination therapies that include trastuzumab are being developed and undergoing clinical testing. However, for the remaining patients trastuzumab has no beneficial effect even though HER2 is overexpressed in the primary tumor. Trastuzumab is a humanized monoclonal antibody of the immunoglobulin G1 type. Trastuzumab is manifested its mechanism of action in multiple ways, but its mechanism of action is not fully understood.[12] The effects of trastuzumab in relation to HER2 include direct downregulation of HER2 and activation of antibody-dependent cellular cytotoxicity (ADCC) in the presence of human peripheral blood mononuclear cells (PBMCs).[13–15] One of main antitumor effects of trastuzumab is mediated by ADCC, and the results of studies in preclinical models have led to the hypothesis that trastuzumab recruits immune effector cells that are responsible for ADCC. The finding that animals deficient in immune-cell-activating Fc receptors do not respond to trastuzumab supports this hypothesis. Preoperative administration of trastuzumab has been reported to increase tumor infiltration by lymphoid cells and modulation of ADCC in vitro.[16–18] The uncertaining in defining the ways trastuzumab exerts its effect on carcinoma cells is mirrored by the difficulty of explaining the mechanism of carcinoma cell

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resistance to trastuzumab. Nevertheless, several mechanisms,[19–27] including loss of PTEN function, activation of alternative pathways (IGF-I), expression of EGFR family ligands, receptor masking or epitope inaccessibility, and impaired ADCC response have been hypothesized to explain trastuzumab resistance. ADCC is mainly attributable to the activation of natural killer cells (NK cells), which are large granular lymphocytes that participate in innate immune responses to neoplastic cells.[28] Although most innate immune cells express both inhibitory and activating receptors,[28,29] killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor that is expressed on subsets of NK cells. KLRG1 is a transmembrane inhibitory receptor and member of the C-type lectin-like superfamily.[30–34] Recently, E-cadherins have been identified as ligands of KLRG1.[35–38] E-cadherins comprise a family of transmembrane glycoproteins that mediate cell-cell adhesion. Down-regulation of E-cadherin represents a crucial step in epithelial tumor development and metastasis. E-cadherin binding to KLRG1 prevents the lysis of E-cadherin-expressing carcinoma cells by KLRG1-mediated NK cells cytotoxicity. These findings led us to hypothesize that tumor cells expressing E-cadherin inhibit ADCC activity, meaning that tumor cells lacking E-cadherin may be more susceptible to NK cell attack. To test this hypothesis, in the present study we investigated whether E-cadherin expression in HER2 overexpressing breast carcinoma cells affects trastuzumab-mediated ADCC through KLRG1 on NK cells *in vitro*, *in vivo*, and in clinical specimens.

Tumor Immunology

Material and Methods

Cell lines

We used two HER2-overexpressing breast carcinoma cell lines, SKBR3 and HCC1569, and two gastric carcinoma cell lines, NCI-N87 and MKN-7, in our study. All cell lines were obtained from the American Type Culture Collection (Manassas, VA). SKBR3 cells and MKN-7 cells were propagated in DMEM medium supplemented with 10% heat-incubated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). HCC1569 cells and NCI-N87 cells were propagated in RPMI 1640 medium supplemented as described above.

Reagents and antibodies

Trastuzumab was obtained from Chugai Pharmaceutical (Tokyo, Japan). Mouse IgG_1 antibody (BioLegend, San Diego, CA) was used as a control. Antibody to KLRG1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to the following were used for immunopathological analysis: c-erbB-2, CD45 (Ventan, Kanagawa, Japan), E-cadherin, N-cadherin (DAKO, Tokyo, Japan), and Pan-cadherin (Cell signaling, Tokyo, Japan).

Isolation of peripheral blood mononuclear cells

PBMCs were isolated from healthy volunteer, having taken informed consent. The PBMCs were isolated directly from fresh peripheral blood by using the BD vacutainer CPT (Becton Dickinson, Mansfield, MA) on density-gradient centrifugation. Highly purified KLRG1-negative mononuclear cells ($PBMCs^{KLRG1(-)}$) were obtained by magnetic cell sorting (MACS) by using the system from Miltenyi Biotec according to the manufacturer's instructions. The purity of the KLRG1-negative mononuclear cells was greater than 95% as determined by flow cytometry analysis (Figure S1).

Cell viability assay

HER2-overexpressing carcinoma cells in medium were seeded into six-well cell plates (1×10^5 per well). After 24 hours, target cells (T) and PBMCs (E) as effector cells (T:E ratios, 1:0, 1:1, 1:20) were cocultured at 37C in medium containing either trastuzumab or mouse IgG_1 isotype antibody in concentrations of 0 mg/ml, 0.021 mg/ml, and 0.105 mg/ml under a 5% CO2 atmosphere. Cell viability was assayed after 4 or 24 hr later by counting living target cells. Cell viability assays were performed in triplicate.

ADCC assays

To investigate the effect on ADCC activity, after the target HER2 overexpression breast carcinoma cells were labeled with 100 μCi ^{51}Cr (Perkin-Elmer Life and Analytical Sciences, Boston, MA) for 60 min, target cells (T) (1×10^4 per well) and PBMCs (E) at various T:E ratios were coincubated in 200 μL DMEM or RPMI 1640 in a 96-well U-bottomed plate in triplicate for 4 hr at 37°C with trastuzumab or control antibody. Next, the amount of radioactivity in the supernatant liquid was measured by a gamma counter. The percentage of specific cytolysis was calculated using the formula: percentage specific lysis = [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100. ADCC assays were performed in triplicate.

Small interfering RNA (siRNA)

Commercially available siRNAs against CDH1 (Silencer® select Pre-designed siRNA, Ambion, Austin, TX) was used. A possible nonspecific gene silencing effect was assessed by using a control siRNA (Silencer® select Negative Control #1, Ambion, Austin, TX). Transfections were performed in 6-well plates according to manufacturer's protocols. The level of transcripts of the silenced genes was measured 48, 72 and 96 hr post-transfection by the quantitative real-time polymerase chain reaction (qRT-PCR). Relative gene expression was quantified on a Smart Cycler II System (TaKaRa, Shiga, Japan) by the calibration curve method, and the data were normalized against GAPDH and expression of E-cadherin protein was investigated by flow cytometory (FACSCalibur, Becton Dickinson, Mansfield, MA).

Mouse model

Six-week-old female severe combined immunodeficiency (NOD/SCID) mice were purchased from CLEA JAPAN (Tokyo, Japan). All animals were maintained and treated in accordance with institutional guidelines under approved protocols.

NOD/SCID mice were subcutaneously injected (s.c.) with HCC1569 cells (5.0×10^6) suspended in 0.2 ml PBS. Tumor-bearing mice were divided into five groups of at least three mice each: (*i*) an untreated control group, (*ii*) a group treated with human $PBMCs^{KLRG1(-)}$, (*iii*) a group treated with trastuzumab, (*iv*) a group treated with human PBMCs + trastuzumab and (*v*) a group treated with human $PBMCs^{KLRG1(-)}$ + trastuzumab. Trastuzumab (0.005 mg/g, intraperitoneally [i.p.]) and human PBMCs (5.0×10^6 cells/mouse, i.p.) injections were started 4 weeks after cell implantation and continued once per a week. Tumor volume (mm^3) was calculated as the product of the length, width and height of the tumor measured in millimeters once a week with a caliper. Tumor volume was calculated by using the formula: Tumor volume = length × width × depth (mm^3).

Patients

Patients who received trastuzumab (Herceptin®, Chugai Pharmaceutial Co., Tokyo Japan)-based treatment for metastatic HER2-overexpressing breast carcinoma at the National Cancer Center Hospital East between July 2001 and March 2007 were registered in our study. In accordance with practice at our institution, eligibility for treatment with trastuzumab was determined by IHC HER2 grade 3+ overexpression (as determined by Herceptest, Ventana medical systems,) or confirmation of c-erbB-2 amplification by fluorescence *in situ* hybridization in IHC 2+ cases. The initial dose of trastuzumab was 4 mg/kg administered as an i.v. infusion and it was followed by weekly doses of 2 mg/kg or triweekly doses of 6 mg/kg i.v. Response to trastuzumab therapy was evaluated based on the results of a clinical and radiological examination of the tumor before the start of treatment and every 6 months after the start of treatment. All patients were treated after obtaining their informed consent in accordance with institutional guidelines. To determine whether the expression of the selected markers could be used to predict response or resistance to trastuzumab, two binary outcomes were considered: clinical benefit and overall survival. Clinical benefit (response group) was defined as the cases with a complete response or partial response, or stable disease that lasted at least a year and cases that stopped trastuzumab-based treatment for the patient's own reasons or reduced cardiac function, in spite of last partial response status for longer than six months. All patients gave written informed consent to use of their medical data for scientific purposes.

Immunohistochemistry

All immunohistochemical analyses were performed on paraffin-embedded tissues obtained from the primary tumor in the surgical specimen obtained during initial mastectomy. The surgically resected specimens were fixed in 10% formalin and embedded in paraffin for routine pathological examination. We prepared and used 4-μm-thick paraffin sections cut from a paraffin block containing histological findings that were representative of the tumor. Antigen retrieval was performed in citrate buffer solution (pH 6.0). All slides were heated to 95°C by exposure to microwave irradiation for 20 min and after allowing them to cool for 1 hr at room temperature, they were washed in PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min, and after incubation overnight at 4°C with the primary antibodies on the slides were incubated for 40 min with a labeled polymer EnVision TM+, Peroxidase Mouse or Rabbit (Dako, Tokyo, Japan). The chromogen used was 2% 3, 3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen.

All samples were evaluated and scored independently without any knowledge of the patients' history.

Statistical analysis

The GraphPad Prism4 (GraphPad Software, CA) statistical software package was used to perform the statistical analyses. We used ANOVA to statistically evaluate differences in cytotoxicity. The correlations between each group and the clinicopathological variables and results of immunohistochemical staining were evaluated by the χ^2-test or Fisher's exact test, as appropriate. Overall survival distributions were estimated by the Kaplan-Meier method and compared by using log-rank tests. p values < 0.05 were considered significant.

Results

Trastuzumab enhances cell cytotoxicity against HER2-overexpressing breast carcinoma cell lines through human PBMCs

We measured the level of expression of HER2 and E-cadherin protein on the surface of SKBR3 and HCC1569 cell lines. Figure 1*a* shows the results of immunocytochemical staining for HER2 and E-cadherin. The expression of E-cadherin protein on the surface of cancer cells were also confirmed by FACS analysis (Fig. 1*a*). HER2 overexpression in SKBR3 and HCC1569 cell lines were also recognized by FACS analysis. The expression of E-cadherin was very low in SKBR3 and high in HCC1569. We investigated the efficacy of trastuzumab in SKBR3 and HCC1569 cells. Firstly, we examined whether trastuzumab directly inhibited the cell viability of SKBR3 and HCC1569 cells by counting viable carcinoma cells. Treatment with either 0 to 0.105 mg/ml trastuzumab had no effect on cell viability of any of the cells. Next, to examine the cytotoxic ability of trastuzumab through human PBMCs indirectly, SKBR3 or HCC1569 cells as target (T) cells and human PBMCs as effector (E) cells were mixed in different ratios, and trastuzumab was added to different concentrations. As shown in Figure 1*b*, Trastuzumab elicited cell cytotoxicity for SKBR3 with very low expression of E-cadherin when compared to the control mAb ($p < 0.01$). Cytotoxicity tended to be higher at higher T:E ratios. However, trastuzumab exhibited no cytotoxicity for HCC1569 cells with high expression of E-cadherin, regardless of higher T:E ratios. We performed ADCC assay by ^{51}Cr release assay, similar results were obtained (Fig. 1*c*). The similar results were obtained with the other HER2-overexpressing breast carcinoma cell lines (data

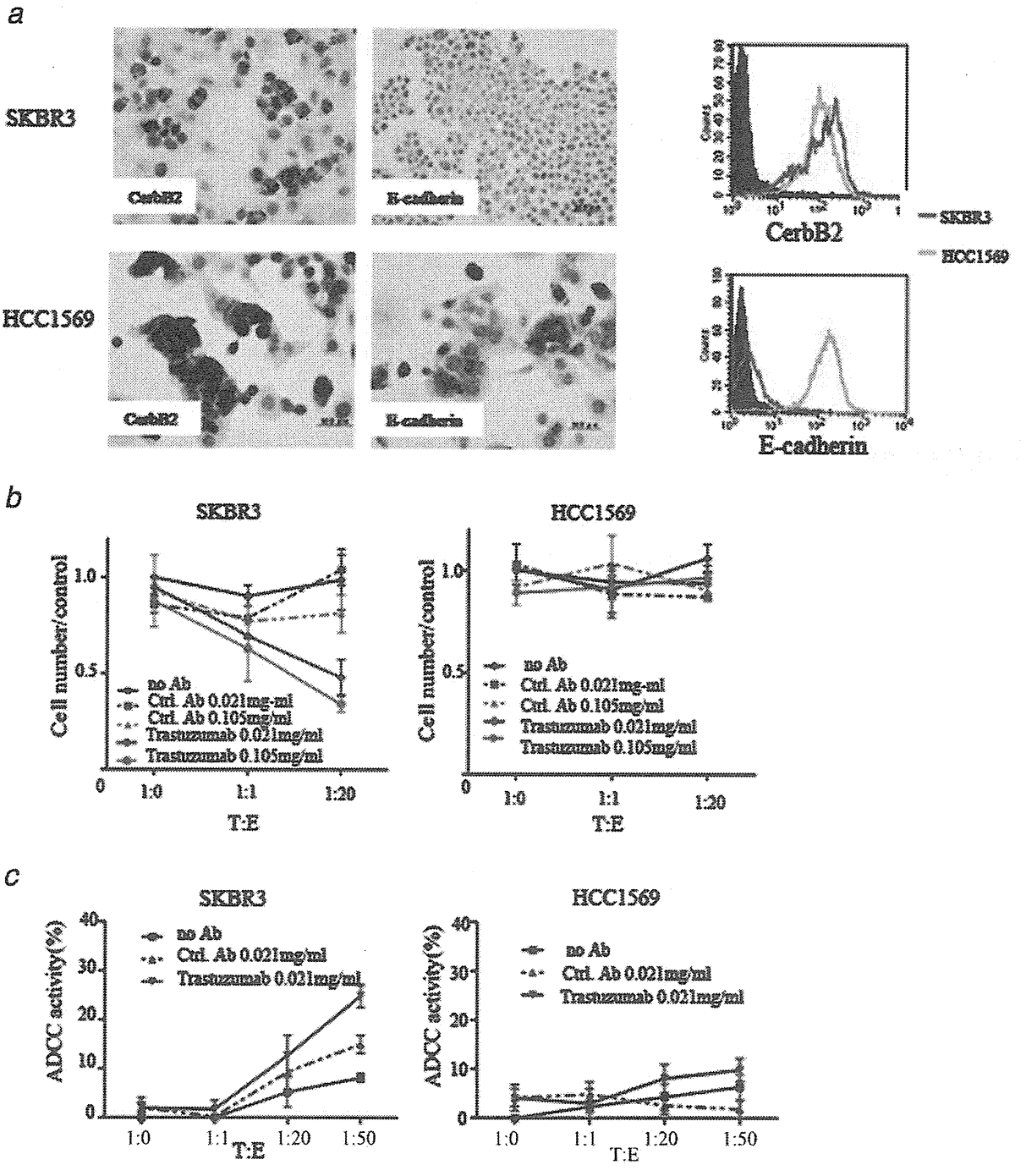

Figure 1. The level of HER2, E-cadherin expression on the surface of carcinoma cell lines and cell viability. (*a*) HER2 expression and E-cadherin expression were evaluated immunocytochemically and by flow cytometry using SKBR3 cells and HCC1569 cells. (*b*) Very low E-cadherin expressing SKBR3 and high E-cadherin expressing HCC1569 breast carcinoma cells (T) in medium were seeded into six-well cell plates (1×10^5 per well each). After 24 hr, PBMCs (E) as effector cells, and target cells (T:E ratios, 1:0, 1:1, 1:20) were cocultured in medium containing either trastuzumab or control mAb (mouse IgG_1 antibody) at concentrations of 0.021 mg/ml or 0.105 mg/ml. At 24 hr after incubation, cell viability was assessed by counting living target cells. Cell viability assays were performed in triplicate. (*c*) Breast carcinoma cells (1×10^4 per well each) were analyzed for ADCC in the presence of Trastuzumab 0.021 mg/ml or control mAb in the various T:E ratios by 4h-51Cr release assay. Assays were performed in triplicate to confirm the reproducibility. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

not shown). The indirect cytotoxic ability of trastuzumab through human NK cells or NK cell depleted-PBMCs was examined using HCC1569 cells as target cells (T), and human NK cells or NK cell depleted-PBMCs as effector cells (E). NK cells (CD3-negative and CD56-positive cells) were purified by FACS (Figure S1). Those cells were mixed (T:E =1:20) and trastuzumab was added. As a result, trastuzumab was able to elicite cell cytotoxicity against SKBR3 and HCC1569 with NK cells as well as PBMCs, however, trastuzumab was not able to elicit cell cytotoxicity with NK cell depleted-PBMCs (Figure S3). Therefore, we decided to perform the following experiments using PBMCs as a effector cells (E). It is well-known that NK cells mediated target cell-killing works commonly within 4 hr, the result of the treatment for 4 hours was same as one of 24 hr (Figure S2).

Knockdown of E-cadherin in HER2-overexpressing breast carcinoma enhances the cytotoxicity of trastuzumab

We investigated whether down-regulation of E-cadherin by small interfering RNAs (siRNAs) would overcome resistance to trastuzumab. HCC1569 cells treated with CDH1 siRNA transfection showed an 80% decreased expression of E-cadherin protein 48 hr after transfection. The knockdown level of E-cadherin mRNA and protein was confirmed by RT-PCR and FACS analysis (Figure S4). We examined whether trastuzumab directly inhibited the viability of CDH1-siRNA HCC1569 cells by counting viable carcinoma cells. Treatment with either 0 to 0.105 mg/ml trastuzumab had no effect on cell viability of any of the cells. To examine the cytotoxic ability of trastuzumab through human PBMCs indirectly, CDH1-siRNA HCC1569 cells as target (T) cells and human PBMCs as effector (E) cells were mixed in different ratios, and trastuzumab was added to different concentrations. As shown in Figure 2*a*, trastuzumab elicited cytotoxicity for HCC1569 cells with decreased expression by CDH1 siRNA transfection when compared to the cells treated with control siRNA ($p < 0.01$). We performed ADCC assay by ^{51}Cr release assay, similar results were obtained (Fig. 2*b*). HCC1569 was resulted in increasing of sensitivity to trastuzumab by knockdown of CDH1. These findings indicate that E-cadherin-expressing HER2-overexpressing breast carcinoma cells were induced resistance to treatment with trastuzumab, because the cells were inhibited ADCC activity.

Furthermore, we investigated whether transduced expression of E-cadherin caused resistance of trastuzumab against breast cancer cells, SKBR3 cells with transduced expression of E-cadherin by transfection with hE-cadherin pHβ Apr-1-neo vector (hE-cadherin SKBR3) were used for cell viability assay. As a result, trastuzumab exhibited no cytotoxicity for human E-cadherin-expressing SKBR3 cells trandeuced, regardless of higher T:E ratios (Figure S5). These results suggest that expression of E-cadherin is involved in ineffectiveness of trasutuzumab.

The cytotoxicity against HER2-overexpressing breast carcinoma cells treated with Trastuzumab is enhanced by human PBMCs$^{KLRG1(-)}$

Because KLRG1 on NK cells were inhibitory receptor and E-cadherin was a ligand with KLRG1, we investigated the efficacy of trastuzumab through KLRG1-nonexpressing mononuclear cells (PBMCs$^{KLRG1(-)}$). We obtained highly purified PBMCs$^{KLRG1(-)}$ by MACS (Figure S1). SKBR3 and HCC1569 as target (T) cells, and human PBMCs$^{KLRG1(-)}$ as effector (E) cells were mixed in different ratios, and trastuzumab was added with different concentrations. Trastuzumab elicited cytotoxicity for SKBR3 with very low expression of E-cadherin when compared to the treatment with control mAb ($p < 0.01$). Cytotoxicity tended to be higher at higher T:E ratios. Similar results were obtained with PBMCs (Figs. 3*a* and 3*b*). And, trastuzumab also elicited cell cytotoxicity for HCC1569 cells with high expresseion of E-cadherin, unlike results with PBMCs (Figs. 3*c* and 3*d*). KLRG1 is only expressed by a minority of NK cells (Figure S1). However, trastuzumab exhibited no cytotoxicity for HCC1569 cells treated with KLRG1-positive cells, while, trastuzumab showed remarkable cytotoxicity for HCC1569 cells treated with KLRG1-negative cells (Figure S6). Antibody blocking experiments using anti-E-cadherin antibody directly showed that inhibition of target cell killing is mediated by KLRG1/E-cadherin interaction (Figure S7). Taken together, these results suggest that ADCC activity was inhibited by interaction with E-cadherin and KLRG1. The response of KLRG1-positive and of KLRG1-negative NK cells towards the various target cells in the presence of trastuzumab was confirmed by flow cytometry-based assays using anti-CD69 antibody as a NK cell activating marker. About 50% of NK cells from KLRG1-negative PBMCs showed higher expression of CD69 irrespective of target cells (HCC1569 cells) and the ratio of the number of nonactivated NK cells to that of activated NK cells did not change by co-culture with target cells. However, the number of activated NK cells increased by co-culture using media containing trastuzumab (Figure S8). Therefore, E-cadherin expressing and HER2-overexpressing breast carcinoma cells were induced resistance to treatment with trastuzumab. Similar results were obtained for the other HER2-overexpressing breast carcinoma cell lines (data not shown).

Trastuzumab enhances the cytotoxicity against HER2-overexpressing gastric carcinoma cells

We used two gastric carcinoma cell lines, MKN-7 cells which were not expressed E-cadherin and NCI-N87 cells which were expressed E-cadherin. We examined whether trastuzumab directly inhibited the cell viability of MKN-7 and NCI-N87 cells by counting viable carcinoma cells. Treatment with either 0 to 0.105 mg/ml trastuzumab had no effect on cell viability of any of the cells. Next, to examine the cytotoxic ability of trastuzumab through human PBMCs indirectly, MKN-7 or NCI-N87 cell lines as target (T) cells

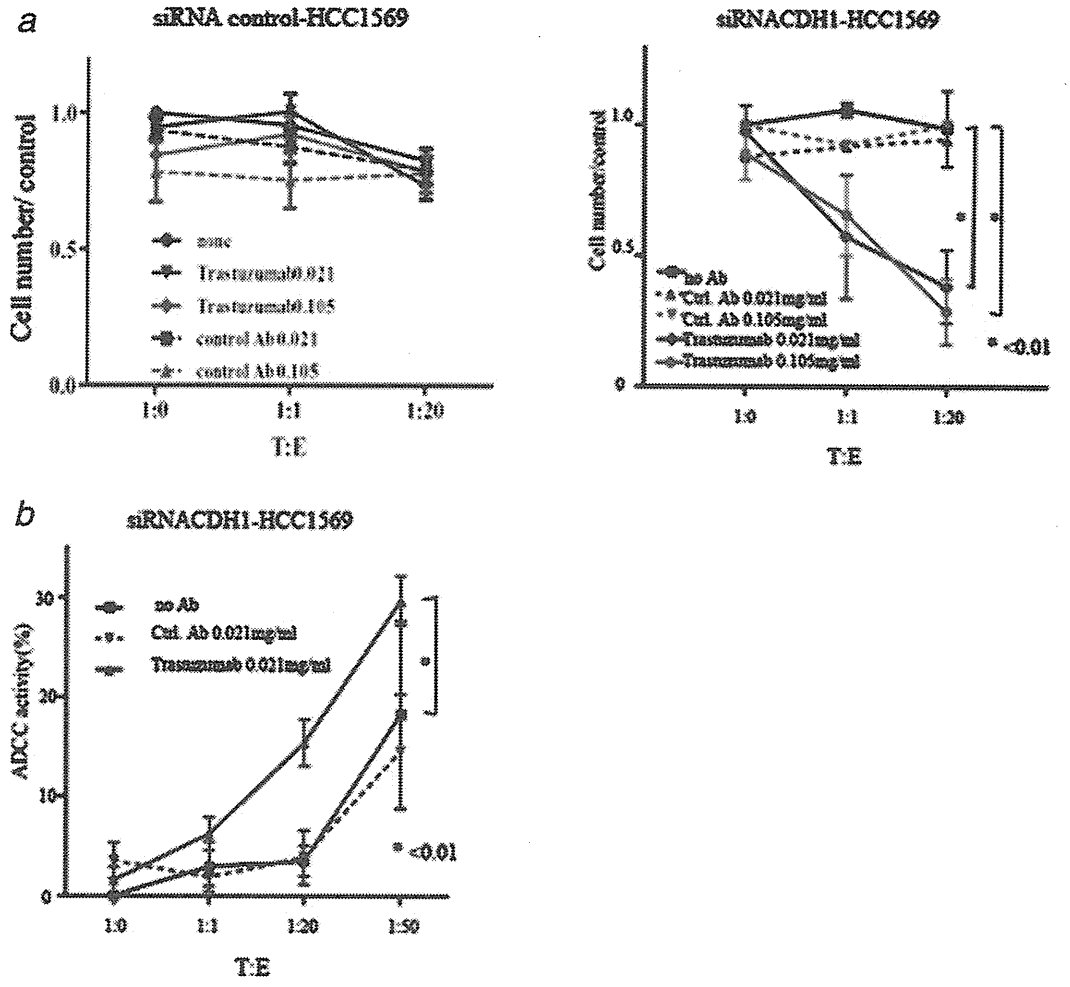

Figure 2. Knockdown of E-cadherin in HER2-overexpressing breast carcinoma enhances the cytotoxicity of trastuzumab. (*a*) HCC1569 cells transfected with control siRNA (upper) and CDH1 siRNA (lower) in medium were seeded into six-well cell plates (1×10^5 per well each). After 24 hr, PBMCs (E) as effector cells, and target cells (T:E ratios, 1:0, 1:1, 1:20) were co-cultured in medium containing either trastuzumab or control mAb (mouse IgG_1 antibody) at concentrations of 0.021 mg/ml or 0.105 mg/ml. At the end of the 24-hr incubation period cell viability was assessed by counting living target cells. Cell viability assays were performed in triplicate. (*b*) HCC1569 cells transfected with CDH1 siRNA (1×10^4 per well each) were analyzed for ADCC in the presence of Trastuzumab 0.021 mg/ml or control mAb in the various T:E ratios by 4h-51Cr release assay. Assays were performed in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and human PBMCs as effector (E) cells were mixed in different ratios, and trastuzumab was added to different concentrations. As shown in Figure 4*a*, Trastuzumab elicited cytotoxicity for MKN-7 which were not expressed E-cadherin when compared to the isotype control ($p < 0.01$). Cytotoxicity tended to be higher at higher T:E ratios. However, trastuzumab exhibited no cytotoxicity for NCI-N87 cells which expressed E-cadherin. Furthermore, we investigated the efficacy of trastuzumab through $PBMCs^{KLRG1(-)}$. Trastuzumab elicited cytotoxicity for both MKN-7 and NCI-N87 cells, regardless of expression of E-cadherin. These results are the same as that was obtained with the breast carcinoma cell lines.

Trastuzumab induces potent antitumor activity in the mouse model of human PBMCs-engrafted NOD/SCID mice

HCC1569 cells were implanted into the flank of severe combined immunodeficiency (NOD/SCID) mice. The tumor-bearing mice were divided into five groups each for treatment. As a results, injection of trastuzumab together with human $PBMCs^{KLRG1(-)}$ showed significant therapeutic efficacy as demonstrated by the effect on the tumor volume at day 28 after starting treatment (mean ± S.D.: 281.5 ± 213.8 mm^3), compared to the control group (878.4 ± 204.9 mm^3), human $PBMCs^{KLRG1(-)}$ group (1098.0 ± 427.1 mm3), trastuzumab alone group (868.7 ± 204.9 mm^3), and trastuzumab and

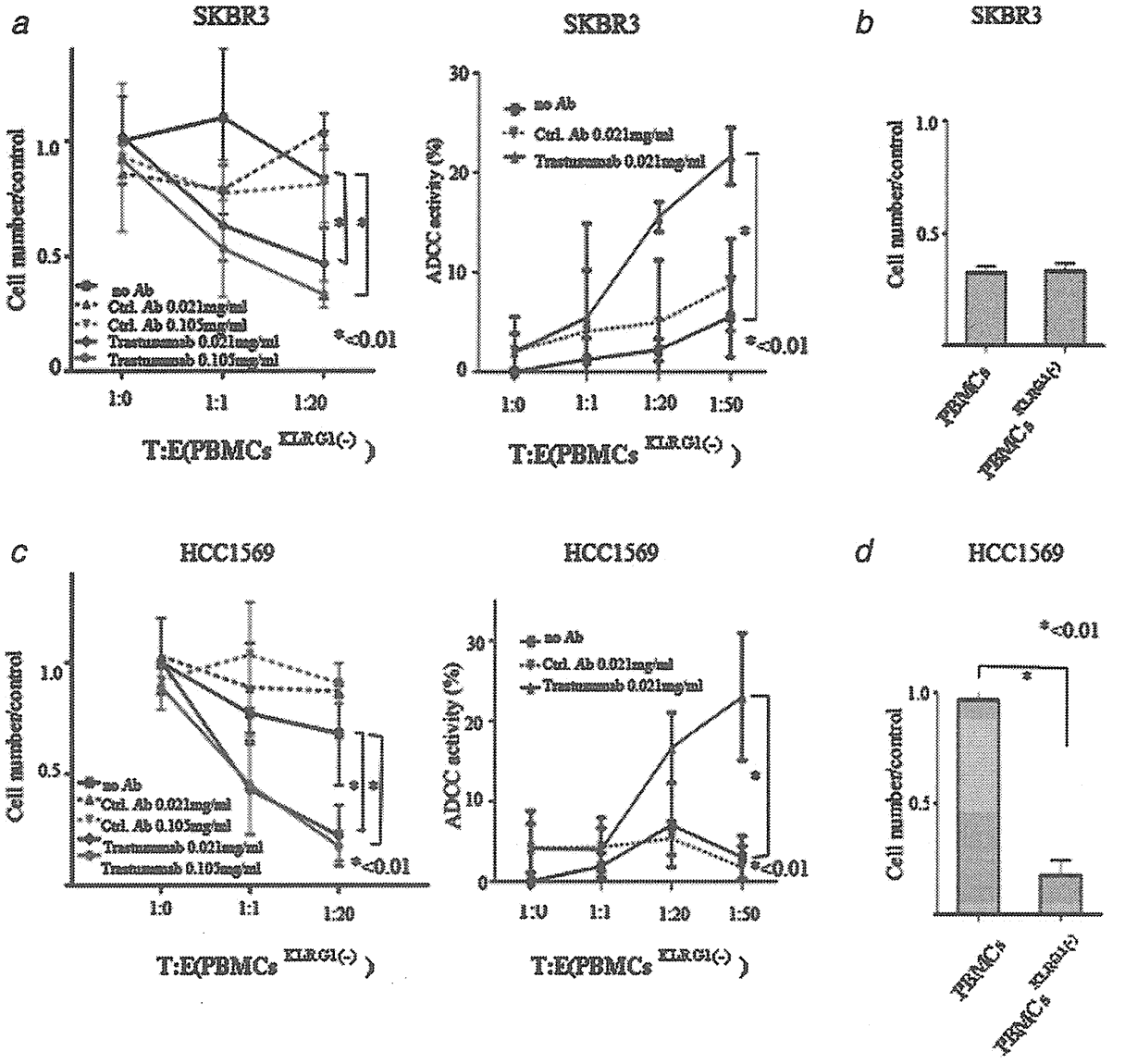

Figure 3. Trastuzumab enhances cytotoxicity against HER2-overexpressing breast carcinoma cells through human PBMCs$^{KLRG1(-)}$. (*a* and *c*, left) Breast carcinoma cells in medium were seeded into six-well cell plates (1×10^5 per well each). After 24 hr, PBMCs$^{KLRG1(-)}$ as effector cells (E), and target cells (T) (T:E ratios, 1:0, 1:1, 1:20) were cocultured in medium containing either trastuzumab or control mAb (mouse IgG_1 antibody) at concentrations of 0.021 mg/ml or 0.105 mg/ml. At the end of the 24-hr incubation period cell viability was assessed by counting living target cells. Cell viability assays were performed in triplicate. (*a* and *c*, right) Breast carcinoma cells (1×10^4 per well each) were analyzed for ADCC in the presence of Trastuzumab 0.021mg/ml or control mAb in the various T:E ratios by 4h-51Cr release assay. (*b* and *d*) We compared cell viability with PBMCs and PBMCs$^{KLRG1(-)}$ by counting living target cells in the presence of trastuzumab 0.105mg/ml in the T:E ratios =1:20. Assays were performed in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

human PBMCs group (686.9 ± 326.5 mm^3). As demonstrated by the lack of effect on tumor volume trastuzumab alone did not exert any therapeutic efficacy (Figs.5*a* and 5*b*). We showed effect of each treatment on the characteristics. Photographs of each mouse were taken at day 56 after the HCC1569 cell implantation (Fig. 5*c*, top). Tissue from each tumor stained with H.E (middle) and anti-human CD45 (bottom) is shown (scale bar, 1 cm). The tumor size was bigger growth and central lesions of tumor tissue were showed with necrosis or fibrosis for cases which control, trastuzumab alone and human PBMCs$^{KLRG1(-)}$. Trastuzumab and Human PBMCs or PBMCs$^{KLRG1(-)}$-engrafted mice cases showed that

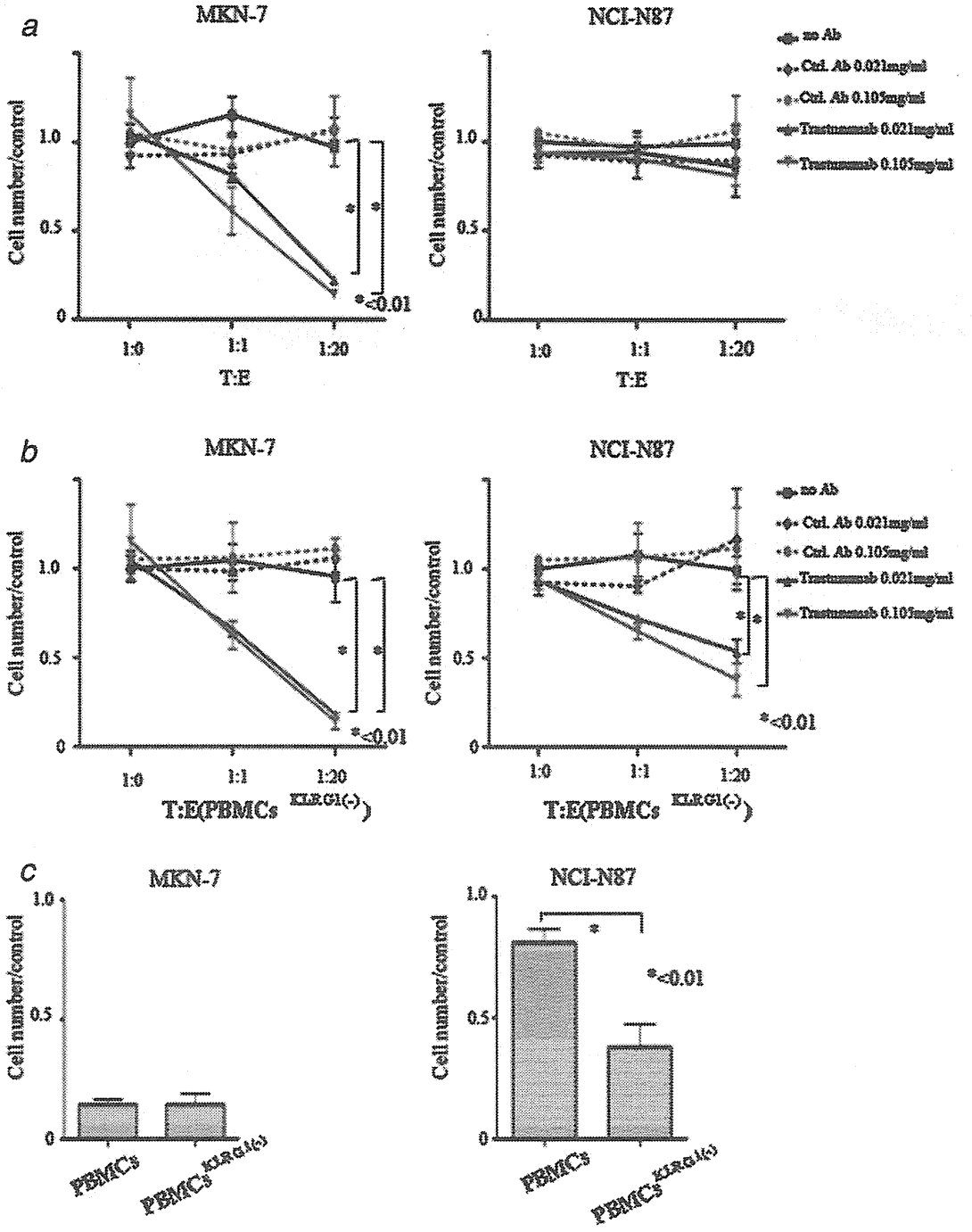

Figure 4. Trastuzumab enhances cytotoxicity against HER2-overexpressing gastric carcinoma cells. Gastric carcinoma cells in medium were seeded into six-well cell plates (1×10^5 per well each). After 24 hr, (*a*) PBMCs or (*b*) PBMCs$^{KLRG1(-)}$ as effector cells and target cells (T:E ratios, 1:0, 1:1, 1:20) were cocultured in medium containing either trastuzumab or control mAb (mouse IgG_1 at antibody) concentrations of 0.021 mg/ml or 0.105 mg/ml. At the end of the 24-hr incubation period cell viability was assessed by counting living target cells. Cell viability assays were performed in triplicate. (*c*) We compared cell viability with PBMCs and PBMCs$^{KLRG1(-)}$by counting living target cells in the presence of trastuzumab 0.105 mg/ml in the T:E ratios = 1:20. Assays were performed in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

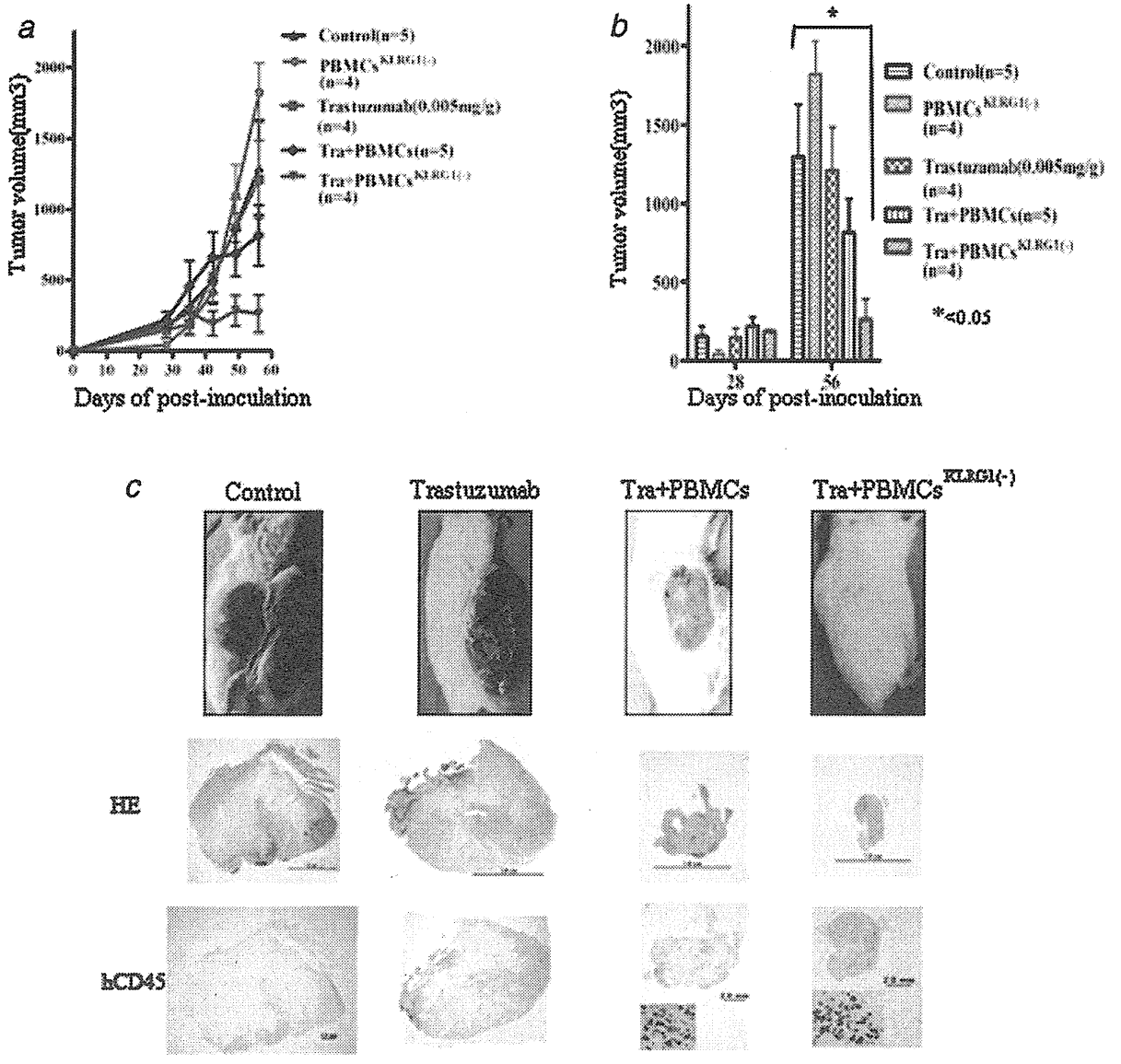

Figure 5. Trastuzumab induces potent antitumor activity as demonstrated by its effect on tumor volume in a mouse model. Antitumor activity of trastuzumab against pre-established subcutaneous HCC1569 tumors. Treatments was started when tumor volume was about 300–500 mm^3 (28 days after HCC1569 cell inoculation). (*a*) Tumor volume was calculated by using the formula: Tumor volume = length × width × depth (mm^3). (*b*) Tumor volume was compared day 28 with day 56 after HCC1569 cell inoculation. Significant differences between the groups are indicated by asterisks, $p < 0.05$. (*c*) Effect of each treatment on the characteristics. The photographs of each mouse were taken at day 56 after HCC1569 cell inoculation (day 28 from the start of treatments) (upper). Tissue from each tumor stained with H.E (middle, scale bar, 1cm) and anti human CD45 antibody (lower) is shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

human lymphocytes were invaded intercellular and stroma in tumor tissue.

Patients with E-cadherin-expressing breast cancer were resistant with trastuzumab-based treatment

Out of a total of 1,344 patients, 115 patients had tumors that overexpressed HER2, and 28 of 115 patients relapsed. A total of 22 patients were treated with trastuzumab for HER2-overexpressing metastatic breast carcinoma (Fig. 6*a*). The characteristics of the patients and the tumors are summarized in Table S1. The data revealed that a clinical benefit was achieved in 50% (11/22). Clinicopathological variable was not significantly different. Figures 6*a* and 6*b* shows markers expression. E-cadherin immunoreactivity was found in 27%

a Results of immunohistochemical analysis

Antibodies	HER2 positive	HER2 positive	HER2 positive Trastuzumab therapy	
		recurrence(+)	Response	Resistance
	n=115(%)	n=28(%)	n=11	n=11
Pan-cadherin				
negative	42(37)	12(43)	8	1
positive	73(63)	16(57)	3	10
E-cadherin				
negative	48(42)	12(43)	8	2
positive	66(58)	16(57)	3	9
N-cadherin				
negative	113(98)	27(96)	11	10
positive	2(2)	1(4)	0	1

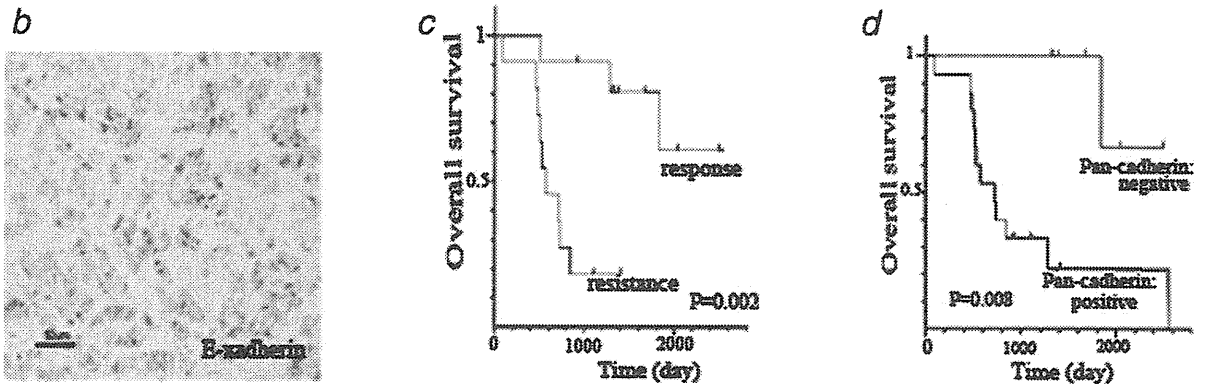

Figure 6. The tumors of patients with Pan-cadherin-positive breast carcinoma were resistant to trastuzumab-mediated therapy. (*a*) Results of immunohistochemical analysis (Pan-cadherin, E-cadherin, N-cadherin) with HER2-overexpressing breast carcinomas are shown (*b*) Typical photographs of the results of immunohistochemical analysis of E-cadherin are shown. (*c*) Kaplan-Meier analysis of overall survival is shown according to the response to trastuzumab-based therapy. (*d*) The Kaplan-Meier analysis of overall survival is shown according to Pan-cadherin expression. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the tumors in of the responder group and in 81% of the tumors in resistant group, and the difference was significant ($p = 0.01$). Kaplan-Meier survival curves were plotted based on clinical follow-up data regarding overall survival. Figure 6*c* shows the overall survival data for the responder group and the resistant group, and the difference between the two groups was significant ($p = 0.002$). A comparison between the survival curves revealed longer overall survival in the group with E-cadherin negative tumors ($p = 0.008$, Fig. 6*d*). We could not identify other predictors that had been implicated as trastuzumab-resistance factors such as PTEN, IGF-I and $p27^{kip}$ (Table S2).

Discussion

We demonstrated that E-cadherin-expressing HER2-overexpressing breast carcinoma cells were induced resistance to treatment with trastuzumab, because the cells were blocked

ADCC activity that was inhibited by interaction with E-cadherin and KLRG1.

It was reported previously that the loss of PTEN in cells occurs commonly in breast carcinomas, and that leads to activation of the PI3K/AKT pathway and has been suggested to cause trastuzumab resistance. However, we demonstrated that although HCC1569 is a HER2-overexpressing and loss of PTEN breast carcinoma cell line, CDH1-siRNA HCC1569 cells were sensitive to trastuzumab with PBMCs, and HCC1569 cells were sensitive to trastuzumab with $PBMCs^{KLRG1(-)}$.

We thought that ADCC activity was blocked by interaction with E-cadherin and KLRG1. KLRG1 is an inhibitory receptor that is expressed on subsets of NK cells and T cells, and 30–80% of NK cells obtained from healthy adult donors express KLRG1. KLRG1 is rapidly up-regulated on NK cells and CD8+ T cells when mice are infected with virus, and in humans it is up-regulated during chronic infection with some virus.[39–41] KLRG1 is a transmembrane inhibitory receptor and member of the C-type lectin- like superfamily, and it contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. E-, N- and R-cadherins have recently been identified as ligands of KLRG1.[35–38] Cadherins comprise a family of transmembrane glycoproteins that mediate $Ca2^{+}$-dependent cell-cell adhesion, and they contribute to tissue morphogenesis during embryonic development and to the maintenance of tissue type and architecture. E-cadherin is found at epithelial cell junctions and on Langerhans cells. The malignancy of epithelial tumors or infection with virus is often associated with down-regulation of E-cadherin, which makes tumor cells invasive and likely to metastasize.[42,43] E-cadherin and KLRG1 interactions triggers an inhibitory pathway that viral infection. A higher threshold for activation for NK cells expressing KLRG1 are imposed when their targets express E-cadherin. These finding shows that KLRG1-mediated inhibition of effector cell function may help to prevent collateral damage to healthy cells. The results of a study by Ito *et al.*[35,44,45] indicate that tumor cells lacking of E-cadherin expression would be more prone to killing by NK cells expressing KLRG1. So down-regulation of E-cadherin on carcinoma cells were a poor prognosis factor, however, down-regulation of E-cadherin on HER2-overexpressing carcinoma cells was a good factor for trastuzumab-mediated treatment. Moreover, our present results suggest that the increase of ADCC activity may be attributed to activated NK cells from KLRG1-negative PBMCs in the case of removal of KLRG1-positive mononuclear cells from PBMCs (Fig. 3) and the ratio of the number of PBMCs to that of $PBMCs^{KLRG1(-)}$ may not affect ADCC activity. It is suggested that KLRG1-positve cells may have some effect on the activation of KLRG1-negative cells, however, further analyses should be performed to conclude about this point.

It increased ADCC activity markedly not less than ratio of PBMCs: $PBMCs^{KLRG1(-)}$, when we removed KLRG1-positive mononuclear cells from PBMCs, we require more researches.

NK cells recognize the absence of self MHC class I as a way to discriminate normal cells from cells in distress. In humans, this "missing self" recognition is ensured by inhibitory receptors such as killer cell immunoglobulin-like receptors (KIR), which dampen NK cell activation upon interaction with their MHC class I ligands.[46] MHC class I expression has been lost or down-regulated in about 80% of HER2-overexpressing breast carcinomas. With our data, about 40% of cases who relapsed HER2-overexpressing breast carcinoma showed down-regulation E-cadherin and MHC class I (Fig. 6*d*), and this ratio is the same as response ratio for trastuzumab-based treatment.

Based on these data, we assume that interactions of E-cadherin and KLRG1 on NK cells block the ADCC activity for other molecular target antibodies besides trastuzumab. In other words, if we are able to remove KLRG1-positive mononuclear cells from PBMCs, response cases for trastuzumab-mediated treatment and other molecular target antibody drugs would increase, greatly.

In conclusion, HER2-overexpressing human carcinoma cells were mainly killed by trastuzumab-mediated ADCC and the ADCC activity was reflected the degree of E-cadherin expression on carcinoma cells. Furthermore, trastuzumab-mediated ADCC activity was markedly increased by $PBMCs^{KLRG1(-)}$, and expression of E-cadherin was shown to be a predictor of response by human HER2-overexpressing carcinomas to trastuzumab-based treatment.

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乳癌

Utility of frozen section diagnosis of surgical margin and sentinel lymph nodes for operable breast cancer patients

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【ポイント】

◆ 乳房温存手術は，整容性を考慮して画像診断による適切な切除範囲を決めることが重要である．

◆ 乳腺の切除断端における術中病理診断は，画像診断において乳管内に進展する方向を中心に検索する．

◆ センチネルリンパ節の術中病理診断は 2 mm 間隔での検索が望ましいが，少なくとも数割面において転移の検索を行うべきである．

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はじめに

乳癌は集学的治療の時代にあり，手術は主に局所コントロールの目的で行われている．1980年代に導入された乳房部分切除は，放射線治療を併用した乳房温存療法として行われている[1]．現在は，画像診断によって乳管内進展を予測して，適切な範囲の乳腺を部分切除している．一方，従来見逃されていた乳管内進展巣が描出されるようになり，広範囲の乳房部分切除が必要な場合が増えている．また，乳房温存療法はその整容性が重視される時代にある．そこで，部分切除に伴う乳房の変形が予想される場合には，あえて乳房は温存せず，乳頭乳輪温存あるいは合併切除を伴う乳腺全切除を行い，筋皮弁あるいは tissue expander などの人工物による乳房再建を行う場合も増えている．いずれにせよ，乳房部分切除における術中病理診断は，現在も可及的に切除断端を陰性にすべく行われている．

センチネルリンパ節生検は，癌の転移を見張るリンパ節を検索してリンパ節郭清の有無を決定するリンパ節転移診断法である．しかし，微小なリンパ節転移を術中に病理診断することが困難な場合もある．

本稿では，乳腺の切除断端とセンチネルリンパ節に関する術中病理診断について述べる．

乳管内進展と温存療法後の予後

Holland ら[2]は，乳癌の主病巣からどこまで腫瘍縁を越えて乳管内を癌が進展しているか検討した．その結果，主病巣の乳管内進展の有無によって，主病巣から 2 cm 離れた範囲で33%と2%の症例で乳管内に癌を認めた（図1）．以上から，画像診断上，限局した腫瘍でも 2 cm の範囲を部分切除した場合に断端が陽性になる可能性がある．Komoike ら[3]は，3 cm 以下の乳癌1,901 例を対象とした乳房温存療法に関する多施設共同研究について報告した．その結果，10 年生存率と健存率は 84%と 78%であった（観察期間中央値は 107 か月）．同側乳房内の 10 年累積再発率は乳房照射群で 8.5%，乳房非照射群で 17.2%であった．同側乳房内再発に関する危険因子は多変量解析の結果，若年，断端陽性と乳房非照射であった．また，同側乳房内再発群は非再発群に比べて有意に遠隔転移再発を認めた．EBCTCG（Early Breast Cancer Trialists' Collaborative Group）の早期乳癌における乳房切除範囲と放射線治療に関するメタアナリシス[4]では，乳房照射の有無による 15 年生存率について，リンパ節転移陰性群 6,097 例で 5%，リンパ節陽性群 1,214 例で 7%の予後の改善が認められた．以上から，乳房温存療法では最終的な乳腺の切除断端の陰性化が望ましいが，切除断端の乳

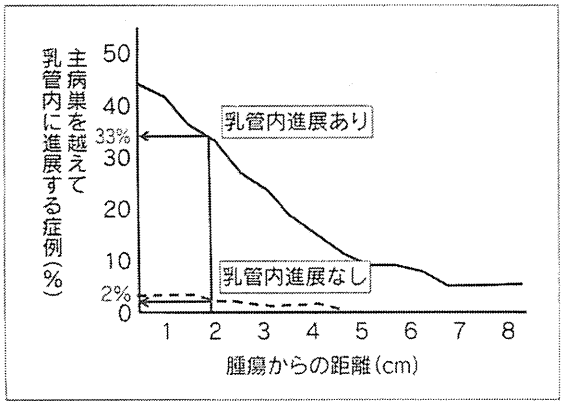

図1　腫瘍内の乳管内進展の有無と腫瘍を越えて存在する乳管内進展の範囲（文献2より引用して改変）

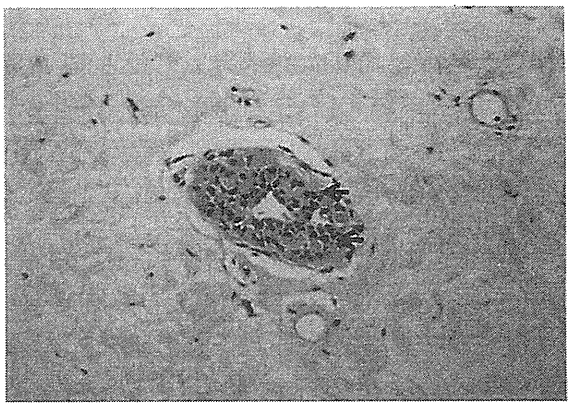

図2　切除断端に露出した乳管の迅速病理標本（×200）
乳管上皮細胞の下へ進展する小葉癌細胞（矢印）.

管内進展の有無にかかわらず乳房照射が必要である.

乳房部分切除における術中病理診断

主病巣からの乳管内進展巣は乳頭方向に進展することが多い．しかし，MRI画像などから水平方向あるいは垂直方向に進展する場合も稀ではない．筆者は乳頭方向の切除断端を術中病理診断に供しているが，以前所属していた国立がんセンター（現・国立がん研究センター）東病院の乳腺外科での乳房部分切除における断端陰性率は83%であった（2004年 日本乳癌学会総会抄録集O-208）．乳頭方向の切除断端をほぼ陰性化しても，側方，頭側（尾側），あるいは深部側の断端の陰性化を術中病理診断ですべて陰性化することは困難である.

切除断端を術中にどう調べるか？

温存乳房の整容性を考慮して，かつ少なくとも切除断端ぎりぎりで乳管内進展巣の露出を防ぐということであれば，切除標本の側面ならびに胸筋側をすべて術中に調べるということになる．しかし，実地臨床では全く不可能である．また，凍結切片標本による術中病理診断の敏感度と特異度は，それぞれ65～78%と98～100%と報告されている[5]．さらに，非浸潤性乳管癌の乳房部分切除において，切除断端から腫瘍までの距離を少なくとも2 mm以上離すことで同側乳房内再発を最小限に抑えられたと報告された[6]．以上から，まず画像診断による乳管内進展巣の予測から切除範囲を決定し，次に乳管内進展巣が最も近接すると考えられる乳頭側，頭側，尾側，あるいは外側の切除断端を術中病理診断に供することが実際的である．しかし，切除断端に浸潤癌が露出する場合や，多発する乳管内進展巣が認められる場合は，追加部分切除あるいは全切除すべきである．特に，画像診断で乳管内進展が広範に存在する場合は，術中追加切除あるいは術中または2期的乳腺全切除の可能性について術前に説明すべきである．一方，小葉癌の術中病理診断では偽陰性になる場合もあるので，術前の組織診断で小葉癌の場合は，術者が病理医にその情報を事前に伝えて慎重に術中病理診断を行うべきである（図2）.

センチネルリンパ節の術中病理診断

2010年4月より乳癌のセンチネルリンパ節生検は保険収載された．センチネルリンパ節に転移がなければリンパ節郭清は不必要である．そこで，センチネルリンパ節を術中に詳細に病理診断することは重要である．Veronesiら[7]は，I期乳癌を対象としたセンチネルリンパ節生検の第III相臨床試験において詳細な術中病理診断を行った．5 mm以上のリンパ節をその長軸方向で半割し，各々50 μm間隔で15組の4 μm切片（合計60切片）を作製した．さらに残ったリンパ節は100 μm間隔で切片を作製した．それぞれHE染色で診断し，診断が曖昧な場合はサイトケラチン抗体（MNF116）を用いた迅速法による免疫染色診断を行った．登録された516例中175例（34%）がセンチネルリンパ節転移陽性であった．しかし，ここまで迅速病理診断を行う施設あるいは臨床試験は稀である.

センチネルリンパ節のOSNA法による術中診断

One-step nucleic acid amplification（OSNA）法は，サイトケラチン19のmRNAの増幅測定によってリン

パ節を転移診断する方法である[8]．その結果，2 mmより大きいマクロ転移と0.2～2 mmのミクロ転移とを98%の診断精度をもって区分し診断することができた．偽陽性例はなく，短時間にリンパ節のマクロ転移を診断する方法として優れている．しかし，サイトケラチン19 mRNA発現のカットオフ値の設定から0.2 mm以下のisolated tumor cells（ITC）とミクロ転移との検出率は低かった．いずれにせよ，病理医の精度と遜色のない優れた術中のリンパ節転移診断法である．

センチネルリンパ節を術中どう調べるか？

術中病理診断には，人的資源，切片作製と染色の技術，手術症例数，診断までの時間制限，病理医の診断精度など，数多くの制約がある．実地臨床において，少なくともミクロ転移を見逃さないように可及的に2 mm間隔で割面を作製して，HE染色で診断することが望ましい．もし困難であれば，少なくとも数割面を作製して診断することが望まれる．

センチネルリンパ節転移陽性乳癌の予後

米国大学外科腫瘍グループ（ACOSOG）では，Ⅰ-ⅡA期乳癌で乳房温存療法を予定した患者を対象に，HE染色で診断されたセンチネルリンパ節転移陽性乳癌におけるリンパ節郭清の意義について，第Ⅲ相臨床試験を1999年に開始した[9]．Primary endpointは郭清の有無による生存率の差であり，1,900例の登録予定であった．しかし，症例集積の遅延と再発率が低かったため，2004年に試験は中止された．その結果，登録された郭清群445例中97例（22%）で非センチネルリンパ節に転移を認めた．しかし，驚くべきことに6年の観察期間において非郭清群446例の所属リンパ節再発はわずか4例（1%）であった．多変量解析では，腫瘍の悪性度のみが，局所あるいは所属リンパ節再発に関する有意な因子であった．非郭清群に所属リンパ節再発が少なかった原因として，9割以上の症例で補助薬物療法が行われたこと，温存乳房の照射野の一部に腋窩が含まれていた可能性が高いこと，癌の生物学的特性によって再発が顕性化していないことが挙げられた．いずれにせよ，精度の高いセンチネルリンパ節の同定を行い，そのリンパ節を詳細に検討することは重要であるが，迅速病理診断によってセンチネルリンパ節転移陽性であった場合に郭清を一期的に行うかどうかは，今後の重要な課題である．

おわりに

乳癌手術における迅速病理診断の意義について考察した．乳癌の診断と治療の体系において，迅速病理診断の意義も変化している．ただし，各施設における外科，病理，臨床検査の人的資源が有効に活用され，病理検体を提出する側と診断する側の相互信頼が質の高い病理診断に不可欠であることは変わらない．

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Combination treatment with fulvestrant and various cytotoxic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) has a synergistic effect in estrogen receptor-positive breast cancer

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Patients with estrogen receptor (ER)-positive breast cancers have a better prognosis than those with ER-negative breast cancers, but often have low sensitivity to chemotherapy and a limited survival benefit. We have previously shown a combination effect of taxanes and fulvestrant and suggested that this treatment may be useful for ER-positive breast cancer. In this study, we evaluated the effects of combinations of hormone drugs and chemotherapeutic agents. *In vitro*, the effects of combinations of five chemotherapeutic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) and three hormone drugs (fulvestrant, tamoxifen, and 4-hydroxytamoxifen) were examined in ER-positive breast cancer cell lines using CalcuSyn software. Changes in chemoresistant factors such as Bcl2, multidrug resistance-associated protein 1, and microtubule-associated protein tau were also examined after exposure of the cells to hormone drugs. *In vivo*, tumor sizes in mice were evaluated after treatment with docetaxel or doxorubicin alone, fulvestrant alone, and combinations of these agents. Combination treatment with fulvestrant and all five chemotherapeutic agents *in vitro* showed synergistic effects. In contrast, tamoxifen showed an antagonistic effect with all the chemotherapeutic agents. 4-Hydroxytamoxifen showed an antagonistic effect with doxorubicin and 5-fluorouracil, but a synergistic effect with taxanes and vinorelbine. Regarding chemoresistant factors, Bcl2 and microtubule-associated protein tau were downregulated by fulvestrant. *In vivo*, a combination of fulvestrant and docetaxel had a synergistic effect on tumor growth, but fulvestrant and doxorubicin did not show this effect. In conclusion, fulvestrant showed good compatibility with all the evaluated chemotherapeutic agents, and especially with docetaxel, *in vitro* and *in vivo*. (*Cancer Sci* 2011; 102: 2038–2042)

The malignant grade of estrogen receptor (ER)-positive breast cancer is generally low, thus patients with this cancer have a better prognosis than those with ER-negative breast cancer. However, ER-positive breast cancers have low sensitivity to chemotherapy and the survival benefit of chemotherapy is limited.[1–3] Estrogen receptors play a crucial role in the development and progression of breast cancers and modulate many genes including of chemoresistant factors. The low sensitivity to chemotherapy may be caused by ER itself or by ER modulation of the levels of factors that cause resistance to chemotherapy.[4–11]

Tamoxifen has significant efficacy for ER-positive breast cancer. However, the mechanism is not completely understood. Tamoxifen is metabolized and the metabolites (4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen) have different affinities for ER and exert a variety of agonist and antagonist properties.[1,12–14] Fulvestrant, a selective ER inhibitor, is an active hormone drug for advanced breast cancer, and a randomized phase II comparative study of a fulvestrant high-dose regimen for advanced hormone receptor-positive breast cancer in postmenopausal women found that fulvestrant was at least as effective as anastrozole in terms of clinical benefit and objective response, and was associated with a significantly longer time to progression.[15,16]

Previous *in vitro* studies have shown that hormone drugs have an antagonistic effect on anticancer drugs. Several clinical studies have found similar antagonistic effects on concurrent chemotherapeutic agents, with combined use of tamoxifen and these agents being inferior to use of the drugs sequentially.[17–22] Therefore, the current view is that concomitant chemotherapy and endocrine therapy should be avoided. However, most studies have examined the compatibility of tamoxifen and anthracycline regimens. There have also been a few reports on combination therapy of fulvestrant with chemotherapeutic agents (taxanes, vinca alkaloids, and doxorubicin) *in vitro*, but the number of studies is limited and combination treatment using modern hormone therapy with chemotherapeutic agents has not been examined thoroughly.[11,23–25]

Our previous study showed that expression of microtubule-associated protein tau (MAPT), a taxane-resistant factor, is modulated by ER; and that tamoxifen, a selective ER modulator, and fulvestrant, a selective ER downregulator, had different effects on MAPT expression through the ER. We also showed that tamoxifen upregulated MAPT expression through its estrogen-like agonist activity and decreased sensitivity to taxanes, whereas fulvestrant downregulated ER and MAPT expression and increased sensitivity to taxanes.[11]

In the current study, we tested the following hypotheses. (i) As fulvestrant downregulates anticancer drug-resistant factors modulated by ER, combination treatment of fulvestrant and chemotherapeutic agents should be beneficial. (ii) As tamoxifen and its metabolites have different effects on ER, combination treatment of these molecules with chemotherapeutic agents should show different results. We examined combinations of chemotherapeutic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) with hormone drugs (tamoxifen, its active metabolite, 4-hydroxytamoxifen, and fulvestrant) based on the combination index (CI) in ER-positive human breast cancer cell lines. We also evaluated the effects of hormone drugs on the levels of chemoresistant factors such as Bcl2, multidrug resistance-associated protein 1 (MRP1), and MAPT, and assessed the compatibility of doxorubicin and docetaxel with fulvestrant *in vivo*.

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Materials and Methods

Cell culture and agents. MCF-7 and ZR75-1 cell lines were obtained from ATCC (Rockville, MD, USA). Cells were maintained at 37°C in a 5% CO_2 incubator in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 10% heat-inactivated FBS and 1% penicillin–streptomycin. Paclitaxel, docetaxel, 17-β estradiol, tamoxifen, and 4-hydroxytamoxifen (Sigma-Aldrich), vinorelbine and doxorubicin (Kyowa Hakkoh, Tokyo, Japan), and fulvestrant (Sigma-Aldrich and Astra Zeneca, London, UK) were purchased from commercial sources. Cells were cultured in a phenol-free medium containing 10% dextran-coated, charcoal-treated FCS (Hyclone, Logan, UT, USA), then treated with 17-β estradiol, fulvestrant, tamoxifen, and 4-hydroxytamoxifen alone or in combination.

Western blot analysis. Samples from cultured cells were prepared for Western blot analysis, as previously described.[26,27] The samples were separated on a NuPAGE Bis-Tris Gel 4–12% (Invitrogen, Carlsbad, CA, USA) and electroblotted onto a PVDF membrane. Primary antibodies were used to identify MAPT (T1029; US Biologicals, Swampscott, MA, USA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), P-glycoprotein (MDR1) (Calbiochem, San Diego, CA, USA), and actin (Sigma-Aldrich). The blots were exposed to the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology) with development using the enhanced chemiluminescence detection method (ECL kit; Amersham Pharmacia Biotech, Chandler, AZ, USA).

Cell viability assay. Cells were seeded on 96-well plates at concentrations of 2×10^3–5×10^3 cells/well. After incubation for 24 h, the cells were cultivated in the presence of various concentrations of a chemotherapeutic agent (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) alone, a hormone drug (fulvestrant, tamoxifen, and 4-hydroxytamoxifen) alone, or combinations of these agents for 72 h at 37°C in 5% CO_2. An 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was then carried out using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA), according to the manufacturer's protocol. The assays were carried out independently four times and mean IC_{50} values are reported.

Combination effect. The combination effect of two drugs was evaluated using the CI, which was calculated using Calcusyn software (Biosoft, Cambridge, UK) and is defined as follows:

$$CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2,$$

where: (Dx)1 is the dose of Drug 1 alone required to produce an X% effect; (D)1 is the dose of Drug 1 required to produce the same X% effect in combination with Drug 2; (Dx)2 is the dose of Drug 2 alone required to produce an X% effect; and (D)2 is the dose of Drug 2 required to produce the same X% effect in combination with Drug 1. The combination effect is defined as follows: CI < 1 is a synergistic effect; CI = 1, an additive effect; and CI > 1, an antagonistic effect.

Xenograft model and treatments. Female athymic (nu/nu) mice (5–6 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). 17β-Estradiol pellets (SE-121; Innovative Research of America, Sarasota, FL, USA) were implanted s.c. into the back of mice 3 days before tumor transplantation. Xenografts were created using MCF-7 cells transplanted into both sides of the back of BALB/c nu/nu mice. The tumors were allowed to reach 3–6 mm in size before the mice were randomized into several treatment groups. The size of each tumor was measured twice a week. The tumor volume was calculated using the formula: $1/6\pi r_1 r_{22}$, where r_1 is the largest tumor diameter and r_2 is the smallest tumor diameter. The study protocol was approved by the Animal Care and Use Committee, Okayama University (Okayama, Japan).

Results

Combination effect of hormone drugs and chemotherapeutic agents *in vitro*. The combination effects of chemotherapeutic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) and hormone drugs (fulvestrant, tamoxifen, and 4-hydroxytamoxifen) were evaluated using the CI in two ER-positive human breast cancer cell lines (MCF-7 and ZR75-1). A cell viability assay was carried out and IC_{50} values were determined for the antiproliferative activity of each drug. The study was carried out using a constant ratio design, with the molar ratios for drug combinations determined based on individual IC_{50} values (Table 1). Fulvestrant showed a synergistic effect with all five chemotherapeutic agents, regardless of the fulvestrant dose. In contrast, tamoxifen had an antagonistic effect with all the chemotherapeutic agents, with the strongest antagonism occurring at a low dose of tamoxifen. 4-Hydroxytamoxifen showed an antagonistic effect with doxorubicin and 5-fluorouracil, but a synergistic effect with taxanes and vinorelbine. The antagonistic effects of 4-hydroxytamoxifen with doxorubicin and 5-fluorouracil were weaker than those of tamoxifen. These trends were similar in the two cell lines (Fig. 1, Table 2).

Influence of hormone drugs on the expression of chemoresistant factors. To explain the results of the combination effects of hormone drugs and chemotherapeutic agents, we examined the protein levels of Bcl2, MRP1, and MAPT, which are well-known chemoresistant factors. MCF-7 cells were seeded in a serum-free medium, incubated for 24 h, then cultivated in a medium containing 17-β estradiol, fulvestrant, tamoxifen, or 4-hydroxytamoxifen alone or various combinations of these agents, followed by Western blot analysis. The levels of MAPT and Bcl2 were increased by 17-β estradiol and decreased

Table 1. Molar ratios of chemotherapeutic agents and hormone drugs used in combination treatment for breast cancer

Drugs	MCF-7 Ratio	ZR75-1 Ratio
Doxorubicin		
Doxo:Ful	1:30	1:50
Doxo:TAM	1:40	3:200
Doxo:4OH-TAM	1:30	3:100
Paclitaxel		
Pacli:Ful	4:5	1:1
Pacli:TAM	3:5	3:4
Pacli:4OH-TAM	4:5	3:2
Docetaxel		
Doce:Ful	4:5	1:1
Doce:TAM	3:5	3:4
Doce:4OH-TAM	4:5	3:2
Vinorelbin		
Vino:Ful	1:30	1:600
Vino:TAM	1:40	1:800
Vino:4OH-TAM	1:30	1:400
5-Fluorouracil		
5-FU:Ful	1:3	1:6
5-FU:TAM	1:4	1:8
5-FU:4OH-TAM	1:3	1:4

Cell viability assay was carried out and IC_{50} values were determined for the antiproliferative activity of each drug. Molar ratios for drug combinations were determined based on individual IC_{50} values. 4OH-TAM, 4-hydroxytamoxifen; 5-FU, 5-fluorouracil; Doce, docetaxel; Doxo, doxorubicin; Ful, fulvestrant; Pacli, paclitaxel; TAM, tamoxifen; Vino, vinorelbine.