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The Synergistic Antitumor Effect of Combined Anti-Human Epidermal Growth Factor Receptor 2 Antibody and Gamma Interferon Therapy on Antibody-resistant Breast Cancer Cells

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Abstract: The anti-human epidermal growth factor receptor 2 (HER2) antibody (Ab) is a molecularly targeted Ab for cancer therapy. In the field of breast cancer, approximately 20% overexpress HER2 protein. However, the recurrence rate is 30% and the metastasis rate is 18% one year after treatment of anti-HER2 Ab for HER2 positive breast cancer. The resistance to Ab treatment is a major problem for patients. We previously reported that anti-HER2 Ab and Gamma Interferon (IFN-γ) combined therapy show a higher anti-tumor effect than typical therapy in *in vitro* and *in vivo* mouse experiments. In this study, we evaluated whether anti-HER2 Ab and IFN-γ combined therapy shows a good synergistic effect against drug-resistant HER2 positive breast cancer cells and a higher antitumor effect than chemotherapy as a conventional clinical treatment. Further, we evaluated a synergy effect with the PD-L1 as a new check point inhibitor. The resistant cell lines were made under the continuous presence of Ab until cell growth was not affected by the drug. The resistant cells were divided into the appropriate number of groups, and then treated with anti-cancer therapy. We evaluated the antitumor effect for both the *in vitro* study and *in vivo* mouse xenograft model prepared with the same immunogenicity. The differences of immunofluorescence staining of CD8, Gr-1 and PDL-1 in tissues were investigated, especially in relation to the immune system. The combined therapy showed a significantly higher antitumor effect than other groups in *in vitro* and *in vivo* experiments. The combined therapy affected anti-tumor immunity in this immunofluorescence experiment. Taken together, we showed the possibility that combined therapy could be an effective treatment option for anti-HER2 Ab resistant breast cancer, thus helping patients suffering from cancer progression after developing treatment resistance.

Key words: HER2, gamma interferon, resistant tumor

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Introduction

Human epidermal growth factor receptor 2 (HER2) belongs to the HER family of receptors with tyrosine kinase activity. HER2 affects cell proliferation, cell differentiation, apoptosis, and cell survival through signal transduction systems such as PI3K, MAPK, RAS, and SRC[1]. The overexpression of HER2 is associated with carcinogenesis[2,3], with overexpression and genetic amplification of HER2 found in many cancer types including 20–30% of breast cancers, 10–20% of stomach cancers, and others (uterine, head and neck, and esophageal cancers). The expression level of HER2 correlates with the malignancy of the cancer and is associated with a poor prognosis[3,4].

Trastuzumab is a monoclonal anti-HER2 Ab used to treat breast and gastric cancer via attachment of the ectodomain of HER2. The treatment outcome for HER2-positive cancer has improved since the appearance of Trastuzumab[5].

Herceptin® with chemotherapy is currently recommended as the primary therapy for HER2-positive breast cancer and gastric cancer[6,7]. Trastuzumab + Pertuzumab + chemotherapy is a standard therapy for HER2-positive unresectable breast cancer and distant metastasis breast cancer, and Herceptin + capecitabine or 5-fluorouracil plus cisplatin is the standard therapy for HER2-positive gastric cancer, extending median overall survival : 13.8 months vs. 11.1 months ; hazard ratio (HR) 0.74 ; 95% confidence interval (CI), 0.60–0.91 ; $P = 0.0046$, vs chemotherapy alone[5].

Despite these advances, cancer cells still develop resistance to medical treatment. In fact, nearly all HER2-positive metastatic patients eventually succumb to their disease, and while the 5-year survival rate of progressive and recurrent breast cancer is 5–10%, the overall survival rate of HER2-positive gastric cancer treated with Herceptin® + chemotherapy is only 13.8 months[5,8]. At present, few studies on cancers that develop resistance to anti-HER2 Ab therapy have been conducted, thus the next best alternative therapy remains to be established[8,9].

Cancer immunotherapy has attracted attention in recent years. In fact, cancer immunotherapy was chosen as the "breakthrough of the year" in 2013 in the journal *Science*. Checkpoint inhibitors, including nivolumab and ipilimumab, have already shown some promising results for unresectable cancers[10,11]. New cancer immunological therapies are therefore anticipated for the treatment of unresectable and metastatic cancer that is resistant to conventional therapy, with encouraging results from a variety of ongoing clinical trials[12,13]. In this study, we therefore evaluated and investigated the anti-tumor effect of anti-HER2 Ab + IFN-γ + PD-L1 treatment.

Previously, we showed that there was a very high antitumor effect for combination therapy of monoclonal Ab and IFN-γ using an experiment system that fixed the same immunogenicity for mouse, cancer cell line, and the Ab. In that work, the Ab and IFN-γ combined therapy ① acted in cancer cells to change the malignancy itself, ② changed the signal transduction, ③ affected the cell cycle and inhibited cell proliferation, ④ accelerated CD8T cell cytotoxicity, and ⑤ decreased the number of Myeloid Derived Suppressor Cells (MDSCs) that inhibit the antitumor effect and raise immunoreactivity within the tumor tissue[14].

Therefore, we hypothesized that the anti-HER2 Ab and IFN-γ combined therapy might be

useful for cancers that developed resistance to anti-HER2 Ab. Thus, the present study evaluated whether Trastuzumab and IFN-γ combined therapy could act synergistically against drug-resistant HER2-positive human breast cancer cells.

Materials and methods

Cell lines and culture condition

H2N113

The H2N113 tumor cell line was generated from female Balb/c MMTV-ErbB-2 / neu transgenic mice with spontaneous breast cancer. The H2N113 cell lines were cultured in RPMI-1640 medium containing L-Glutamine (5 ml / 500 ml) (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Biosera, Kansas City, MO, USA), sodium pyruvate (5 ml / 500 ml) (Gibco Life Technologies), Glutamax (5 ml / 500 ml) (Gibco Life Technologies), MEM NEAA (5 ml / 500 ml) (Gibco Life Technologies), Gibco penicillin-streptomycin liquid (5,000 units/ml penicillin / 5,000 μg/ml streptomycin) (Gibco Life Technologies) at 37ºC with 5% carbon dioxide in 95% air.

H2N113R

We developed the resistant cancer cell line treatment by a widely published method. Briefly, the resistant cell line (H2N113R) was made under the continuous presence of 7.16.4 Ab, and 1×10^5 H2N113 cells were seeded on 10-cm petri dishes; 7.16.4 Ab was added every 3 days as the cells were passaged until confluent. The concentration of 7.16.4 Ab was gradually increased from 2 μg/ml to 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml, and 100 μg/ml finally.

Drugs

The 7.16.4 was used as the anti-mouse ErbB2 monoclonal Ab and was purified from a clone 7.16.4 hybridoma kindly provided by Mark Greene (John W. Eckman Professor of Medical Science, University of Pennsylvania Perelman School of Medicine, Director, Immunobiology and Experimental Pathology Division).

Mouse IFN-γ was purchased from PROSPEC (PROSPEC Protein Specialists, Rohovot, Israel). Docetaxel (DTX) was purchased from LC Laboratories (LC Laboratories, Woburn, MA, USA) and diluted with dimethyl sulfoxide (DMSO). Anti-mouse PD-L1 Ab was purchased from BioXcell (mouse PD-L1 (B7-H1), BE0101, inVivoMab, Lebanon, NH, USA). For the Ab and IFN-γ combined treatment, IFN-γ was added 30 minutes after Ab treatment in all experiments.

Cell Growth in vitro Proliferation Assay

We set up three groups as follows: control group, 7.16.4 group, and 7.16.4 + IFN-γ group. We seeded 1×10^5 H2N113R cells onto three 6-well plates containing normal culture media and these were divided equally into the three groups described above. Drug treatments were initiated after eight hours from seeding the cells. The cell culture was analyzed at two separate times (3 and 7 days), the cell number was recorded, and the cell growth curve was developed. Nothing was added to the control group. The 7.16.4 group was treated with 10 μg/ml of 7.16.4 Ab and the 7.16.4 + IFN-γ group was treated with 10 μg/ml of 7.16.4 Ab and 100 IU/ml of IFN-γ. Cell

number was counted by automated cell counting (Bio-Rad Laboratories, In c., Hercules, CA.).

Mouse in vivo Experiment

Eight-week-old female Balb/c mice were bought and bred for one week to adjust the environment for this experiment. Then 1×10^6 H2N113R cells were injected subcutaneously into both sides of the back of the mice. We distributed the mice into 4 groups to adjust the tumor size among the four groups and started drug treatment at 14 days after tumor inoculation.

We treated the groups as follows: control with PBS 100 μl (n = 9), 7.16.4 Ab 100 μg / 100 μl PBS to the 7.16.4 group (n = 11), IFN-γ 10,000 IU / 100 μl PBS to the IFN-γ group (n = 8), and 7.16.4 Ab 100 μg / 100 μl PBS and IFN-γ 10,000 IU / 100 μl PBS to the 7.16.4 + IFN-γ combined therapy group (n = 11). Drugs were delivered by intraperitoneal injection. In the combined therapy group, IFN-γ was added 30 minutes after the 7.16.4 injection. The drugs were given three times a week. Tumor size was measured three times a week with a digital caliper carbon fiber and calculated using a simple algorithm (length × width × height).

Mouse in vivo Comparative Experiment in Clinical use

We did the same *in vivo* experiment using H2N113R cells, dividing the cells this time into 6 groups as follows: administered PBS 100 μl to the control group (n = 5), 7.16.4 Ab 100 μg / 100 μl PBS to the 7.16.4 group (n = 8), DTX 100 μg / 100 μl DMSO to the DTX group (n = 7), 7.16.4 Ab 100 μg / 100 μl PBS and IFN-γ 10,000 IU / 100 μl PBS and DTX 100 μg / 100 μl DMSO to the 7.16.4 + IFN-γ + DTX combined therapy group (n = 10), anti-PD-L1 Ab.

100 μg / 100 μl PBS to the aPD-L1 group (n = 7), and 7.16.4 100 μg / 100 μl PBS and IFN-γ 10,000 IU / 100 μl PBS and aPD-L1 Ab 100 μg / 100 μl PBS to the 7.16.4 + IFN-γ + aPD-L1 combined therapy group (n = 9). Drugs were delivered by intraperitoneal injection. In the combined therapy group, IFN-γ was added 30 minutes after 7.16.4 injections. Docetaxel and aPD-L1 were given three times a week. Tumor size was measured three times a week with a digital caliper carbon fiber (19978, Sink, Niigata, Japan) and calculated using a simple algorithm (length × width × height). Tumors were removed from the mice on day 21 of the drug treatment. Specimens were fixed in 10% formaldehyde for 24 hours.

Fluorescent Immunohistochemistry

Tumors were removed from the mice on day 16 of the drug treatment for the *in vivo* experiments and from the mice on day 21 of the drug treatment for the *in vivo* experiments with chemotherapy. Specimens were fixed in 10% formaldehyde for 24 hours.

Tumor specimens were cut at a thickness of 3 μm and fixed in 10% formaldehyde. Sections were stained by hematoxylin and eosin (H & E). Immunostaining was performed by incubating these sections again in 0.3% H_2O_2 for 5 minutes to remove endogenous peroxidase activity and then in the Dako nonspecific blocking reagent for 5 minutes. The sections were then incubated with a CD8 (1 : 100) (LEAF™Purified anti-mouse CD8a, 100715, BioLegend®, San Diego, CA, USA), Gr-1 (1 : 100) (Purified anti-mouse Ly-6G / Ly-6c, 108401, BioLegend®), PDL-1 (1 : 1,000)

(mouse PDL-1 (B7-H1), or BE0101, inVivoMab) for 1 hour at room temperature, followed by a secondary, TRITC-conjugated anti-rabbit IgG (A21428, Life Technologies) and bisbenzimide H33342 (DojinDO Molecular Technologies, Inc., Kumamoto, Japan) in a humid chamber at 37°C for 30 min. The images were captured on a BZ-X800 fluorescent microscope (Keyence, Osaka, Japan) and were quantitated with Hybrid Cell Count BZ-H4C software (Keyence).

Statistical analysis

Statistical analysis was carried out using YSAT 2013 (Igakutosho-shuppan Ltd., Toda, Japan). Differences in mean values were statistically analyzed using the non-parametric repeated measures Friedman's test followed by the Wilcoxon test *in vitro* study. Non-repeated measures ANOVA followed by Dunnett's test was done for the *in vivo* studies. A P-value under 0.05 was used to determine statistically significant differences in all experiments. We set the 7.16.4 + IFN-γ levels as a statistical control to analyze in Figures 1, 2, 5, 6, 7 and 7.16.4 + IFN-γ + PD-L1 in Figures 3, 9, 10, 11.

Results

Combined 7.16.4 + IFN-γ therapy suppressed the H2N113R Cell Growth in an in vitro proliferation assay

The average cell numbers on day 3 and day 7 were 4.40×10^5 and 68.1×10^5 cells, respectively, in the control group, 3.8×10^5 and 62.6×10^5 cells, respectively, in the 7.16.4 group, and 3.04×10^5 and 51.6×10^5 cells, respectively, in the 7.16.4 + IFN-γ group.

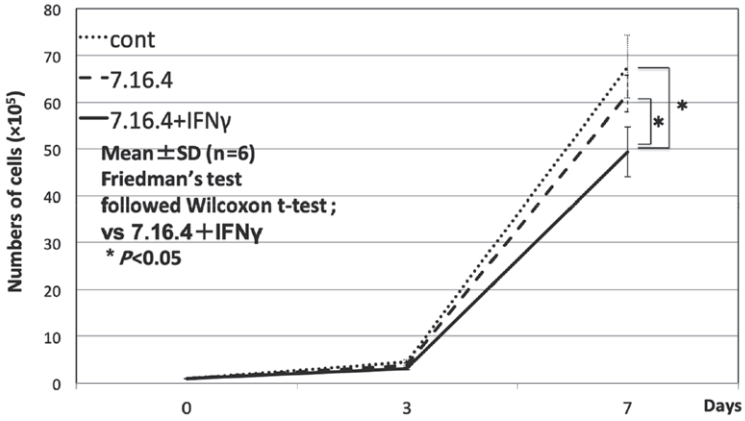

Fig. 1. 7.16.4 + IFN-γ therapy suppressed H2N113R cell growth *in vitro*

H2N113R cells (1×10^5) were seeded onto 6-well plates for the *in vitro* proliferation assay: Control, 7.16.4 (10 μg/ml), 7.16.4 + IFN-γ (10 μg/ml + 100 IU/ml). Treatment was started after 8 hours from seeding and continued during 7 days of culture. The cell numbers were counted on days 3 and 7 to construct a cell growth curve. Cell number was established using an automated cell counter (Bio-Rad Laboratories, Inc., Hercules, CA).

The 7.16.4 + IFN-γ combined treatment significantly decreased the cell number compared to the control group ($P < 0.05$), whereas the 7.16.4 group alone showed no significant difference to control cells.

The curves show the mean + standard deviation of the number of cells from three independent culture experiments (Friedman's test followed by Wilcoxon t-test $^*P < 0.05$; n = 6).

Thus on day 7, the 7.16.4 + IFN-γ group cell number was 75.8% of that in the control group, compared to 91.9% for the 7.16.4 group, indicating that the 7.16.4 + IFN-γ combined treatment significantly decreased the cell number compared to controls, whereas the 7.16.4 group showed no significant difference to controls (Figure 1).

The 7.16.4 Ab did not suppress growth in cell number of the H2N113R line, indicating that these cells acquired resistance after continuous presence of the antibody and that we can use this cell line as a resistant cell line. In addition, 7.16.4 + IFN-γ combined treatment significantly decreased the cell number, thus the combined therapy effectively suppresses tumor growth *in vitro* without the effect in immune cells.

The 7.16.4 + IFN-γ therapy showed an antitumor effect against resistant H2N113R cells in a mice Xenograft model

We set up an *in vivo* mice Xenograft experiment to investigate the antitumor effects of 7.16.4 + IFN-γ under the condition of the effective immunity including the effect of Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). We used the same immunogenicity condition to investigate the real immunoreaction without immune response to invasion by foreign substances. For example, we used the Balb/c mice, H2N113R cells from the Balb/c mice, 7.16.4 that is mouse IgG, mouse IFN-γ, and mouse anti-PD-L1 Ab. The 7.16.4 + IFN-γ treatment significantly suppressed tumor volumes compared with the other groups from

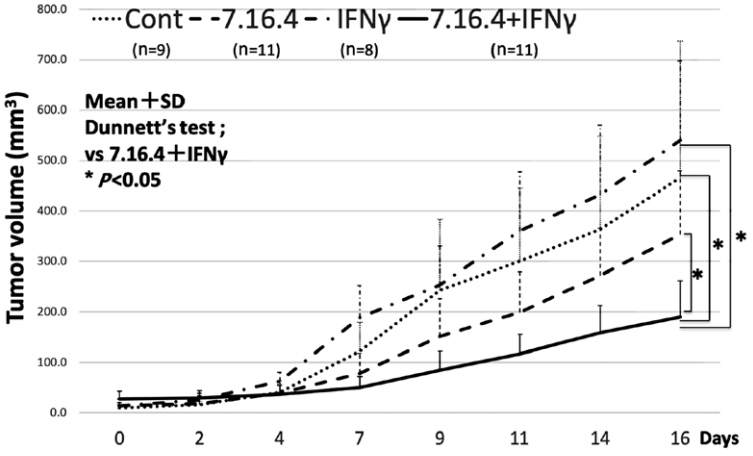

Fig. 2. 7.16.4 + IFN-γ therapy elicited an antitumor effect against resistant H2N113R cells in a Xenograft mouse model

H2N113R cells (1×10^6) were injected subcutaneously into both sides of the backs of Balb/c mice. We started drug treatment at day 14 post-injection (day 0 in the figure) after dividing the mice into 4 groups: control (PBS 100 µl), 7.16.4 (100 µg / 100 µl PBS), IFN-γ (10,000 IU / 100 µl PBS), 7.16.4 + IFN-γ (100 µg / 100 µl PBS + 10,000 IU / 100 µl). Tumor volumes were calculated as length × width × height. Measurements and drug treatments were performed three times a week.

7.16.4 + IFN-γ treatment significantly suppressed tumor volumes compared with the other groups from day 7 until the end ($^*P < 0.05$). The curve shows the mean + standard deviation of tumor volumes; the numbers of tumors shown as below (Cont: n = 9, 7.16.4: n = 11, IFN-γ: n = 8, 7.16.4 + IFN-γ: n = 10). Non-repeated measures ANOVA followed by Dunnett's test were done for the *in vivo* study. We set 7.16.4 + IFN-γ as the statistical control.

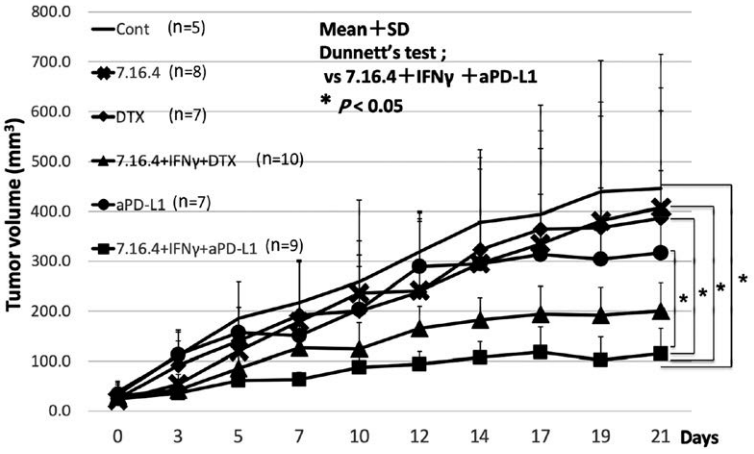

Fig. 3. 7.16.4 + IFN-γ therapy shows potential for clinical antitumor treatment against Ab treatment-resistant tumors

Balb/c mice were injected subcutaneously into both sides of the back with 1×10^6 H2N113R cells. We started drug treatment at day 14 post-injection (day 0 in the figure) after dividing the mice into 6 groups: control (PBS 100 µl), 7.16.4 (100 µg / 100 µl PBS), DTX (DTX 100 µg / 100 µl DMSO), 7.16.4 + IFN-γ + DTX (100 µg / 100 µl 7.16.4 / PBS + 10,000 IU / 100 µl IFN-γ / PBS + 100 µg / 100 µl DTX / DMSO), aPD-L1 (aPD-L1 100 µg / 100 µl PBS), 7.16.4 + IFN-γ + aPD-L1 (100 µg / 100 µl 7.16.4 / PBS + 10,000 IU / 100 µl IFN-γ / PBS + 100 µg / 100 µl aPD-L1 / PBS). Tumor volumes were calculated as length × width × height. Measurements and drug treatments were performed three times a week. The curve shows the mean + standard deviation of the tumor volumes; the numbers of tumors were as shown below (Cont: n = 5, 7.16.4: n = 8, DTX: n = 7, 7.16.4 + IFN-γ + DTX: n = 10, aPD-L1: n = 7, 7.16.4 + IFN-γ + aPD-L1: n = 9). Non-repeated measures ANOVA followed by Dunnett's test were used for the *in vivo* study. We set 7.16.4 + IFN-γ + PD-L1 as the statistical control.

7.16.4 + IFN-γ + aPD-L1 treatment showed the most tumor suppression compared with the other groups on day 7 and from day 12 to the end significantly ($^*P < 0.05$).

Treatment with 7.16.4 + IFN-γ + DTX or aPD-L1 significantly suppressed tumor volumes compared with the control, aPD-L1, DTX, and 7.16.4 alone treatment ($^*P < 0.05$).

day 7 to day 16 (Figure 2). Treatment with 7.16.4 alone showed the tendency for tumor regression, but not a significantly suppressed tumor volume compared with the other groups. On the other hand, IFN-γ alone showed no anti-tumor effect, but rather, the tumor volume actually increased.

The 7.16.4 + IFN-γ combined therapy showed potential for future clinical antitumor treatment against Ab treatment-resistant tumors

We did a similar mice Xenograft experiment using six groups this time and used the drugs that are clinically used.

The 7.16.4 + IFN-γ + aPD-L1 and 7.16.4 + IFN-γ + DTX groups showed significant levels of tumor suppression compared with the other groups from day 7 to day 21 (Figure 3). Although there was not significant difference between the 7.16.4 + IFN-γ + DTX and aPD-L1 group, the 7.16.4 + IFN-γ + aPD-L1 group showed a tendency toward being the most tumor-suppressive treatment. On the other hand, treatment with aPD-L1, DTX and 7.16.4 alone showed no significant difference in tumor suppression. This indicates that 7.16.4 + IFN-γ therapy is a key component in treating Ab-resistant tumors.

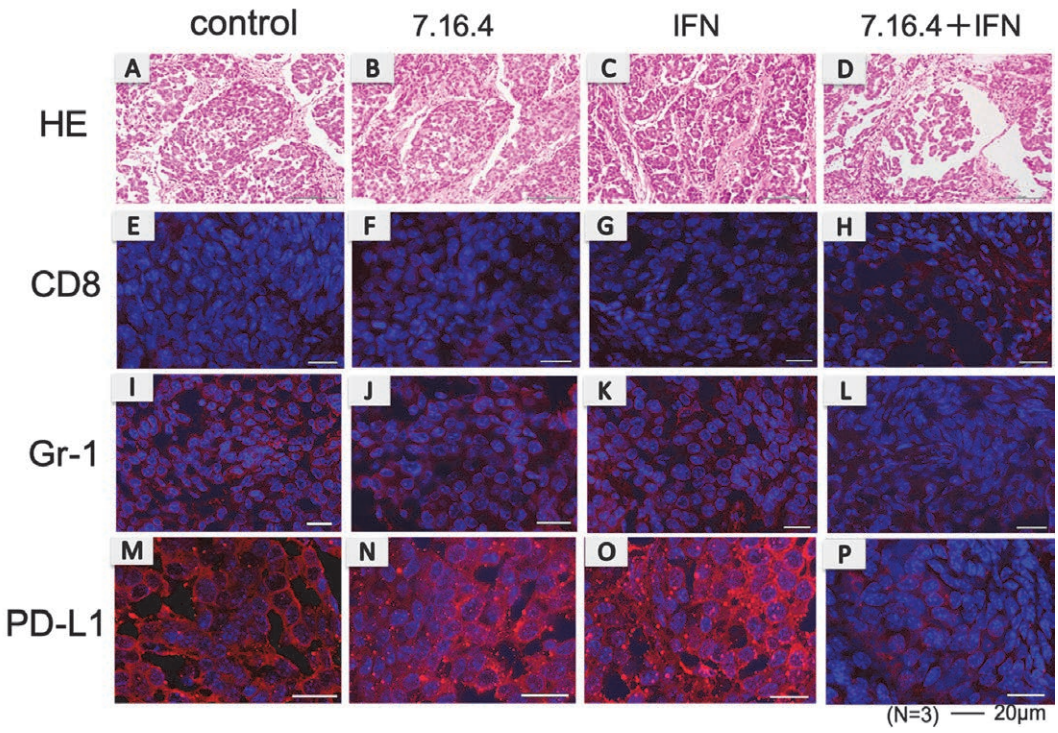

Fig. 4. Histopathological examination of mice Xenograft model
Hematoxylin/Eosin (H&E) staining was performed to compare the histopathological changes in tumor tissue among the different treatment groups.
Tumors were removed from the mice on day 16 of the drug treatment in the *in vivo* experiment, and images were captured by fluorescence microscopy (BZ-X800; Keyence, Osaka, Japan).
In the 7.16.4 + IFN-γ group, the tumor showed atrophy, vacuolisation, and interstitial lymphocyte proliferation. In the other group, a large number of tumors appeared swollen.

Histopathological examination of mice Xenograft model

H & E staining was performed to detect differences in histopathological changes in tumor tissue among the different treatment groups. In the 7.16.4 + IFN-γ group, the tumor was characterized by atrophy, vacuolization, and interstitial lymphocyte proliferation, whereas the other groups showed a large number of tumors with a swollen appearance (Figure 4).

To investigate the immunity differences among the different treatments tested for resistant breast cancer, we ascertained the expression of surface antigen proteins, CD8, Gr-1, and PD-L1 by immunofluorescence staining.

Based on fluorescence intensity, the protein expression levels of CD8 in the 7.16.4 + IFN-γ groups were significantly greater than in the other groups (Figure 5), while there was significantly reduced Gr-1 and PD-L1 expression (Figures 6, 7). There was no significant difference in the expression levels of CD8 and Gr-1 among the control, 7.16.4, and IFN-γ groups. Compared with the control group, PD-L1 expression was significantly greater in the Ab and IFN-γ alone groups.

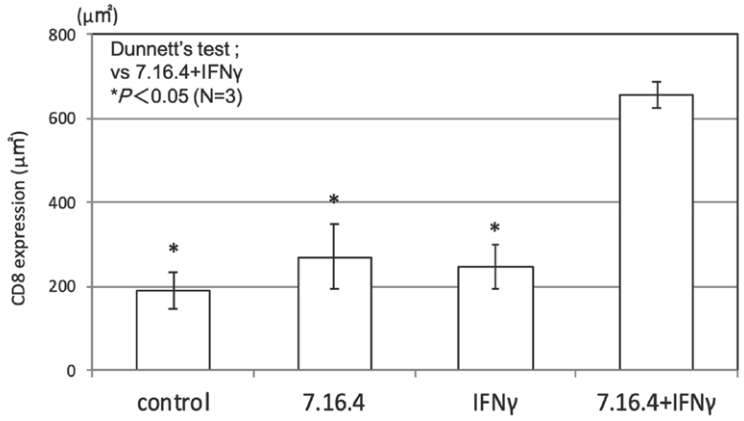

Fig. 5. Fluorescence intensity of CD8

The fluorescence intensity of CD8 in Figure 4 was quantified using Hybrid Cell Count BZ-H4C software (Keyence, Osaka, Japan).

The protein expression levels of CD8 in the 7.16.4 + IFN-γ groups were significantly greater compared to the other groups ($^*P < 0.05$). Non-repeated measures ANOVA followed by Dunnett's test. We set 7.16.4 + IFN-γ as the statistical control.

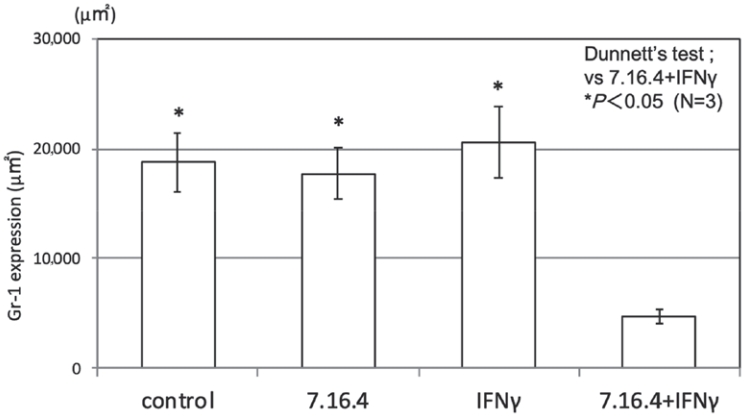

Fig. 6. Fluorescence intensity of Gr-1

The fluorescence intensity of Gr-1 in Figure 4 was quantified as described for Figure 5.

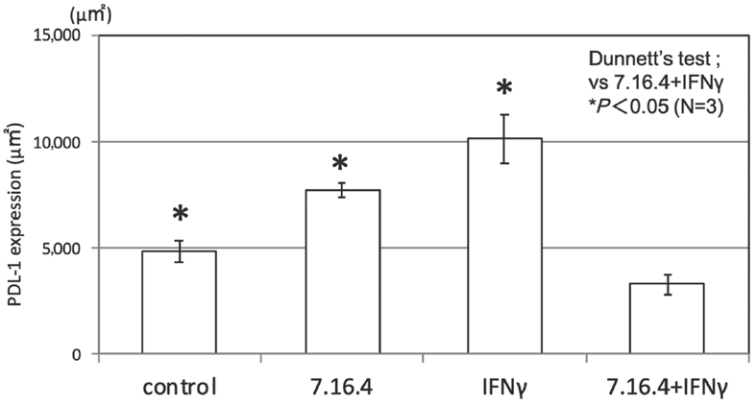

Fig. 7. Fluorescence intensity of PD-L1

The fluorescence intensity of PD-L1 in Figure 4 was quantified as described for Figure 5.

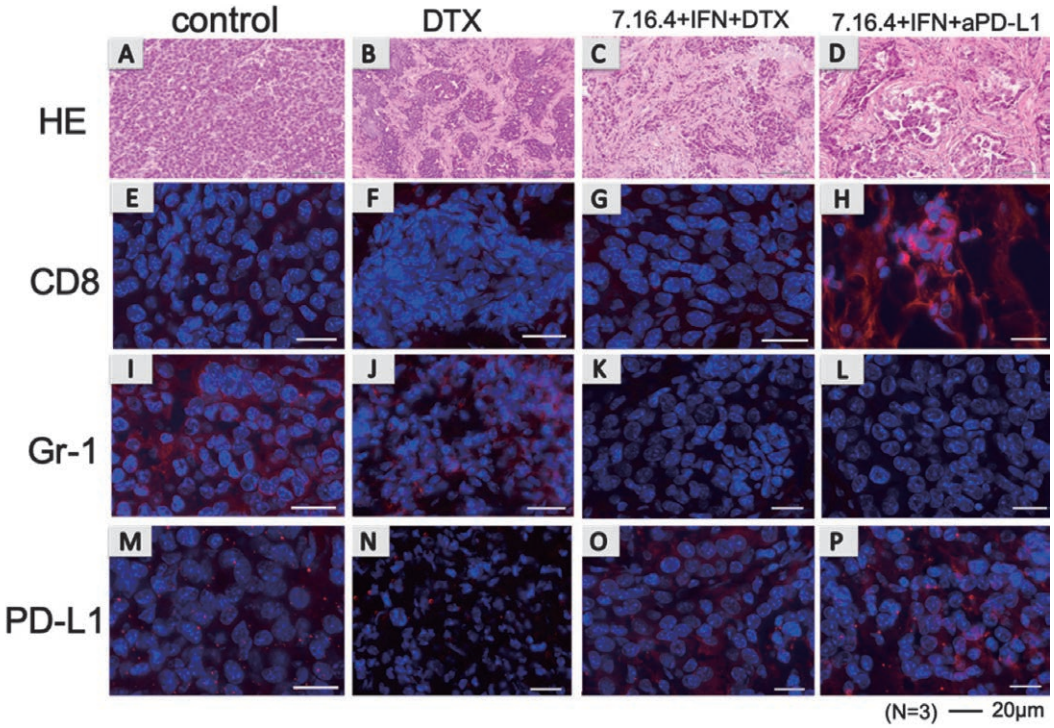

Fig. 8. Histopathological examination of mice Xenograft model using DTX, aPD-L1
H & E staining and the fluorescence intensity of CD8, Gr-1, and PD-L1 was performed in the clinical use mouse model as described for Figure 4.

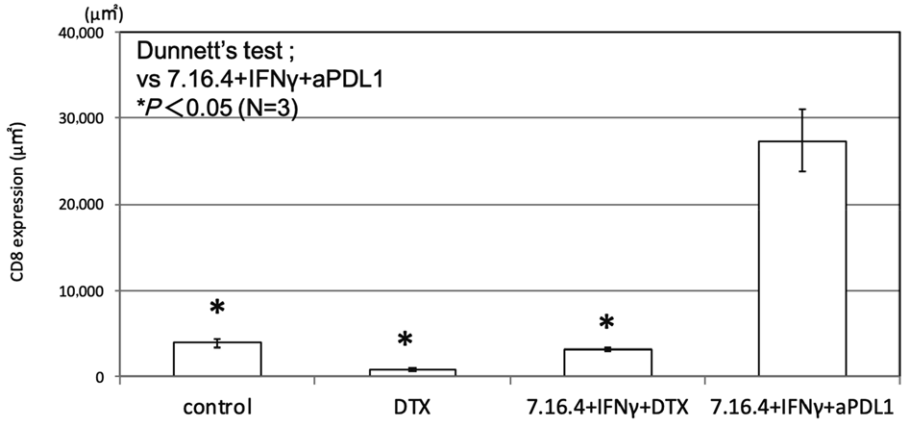

Fig. 9. Fluorescence intensity of CD8
The fluorescence intensity of CD8 in Figure 8 was quantified as described for Figure 5.
Non-repeated measures ANOVA followed by Dunnett's test. We set 7.16.4 + IFN-γ + PD-L1 as the statistical control.

Histopathological examination in clinical treatment

In the 7.16.4 + IFN-γ + DTX and 7.16.4 + IFN-γ + aPD-L1 groups, the tumor was characterized by atrophy and interstitial lymphocyte proliferation (Figure 8). The immunofluorescence staining also showed significantly higher expression of CD8 in the 7.16.4 +

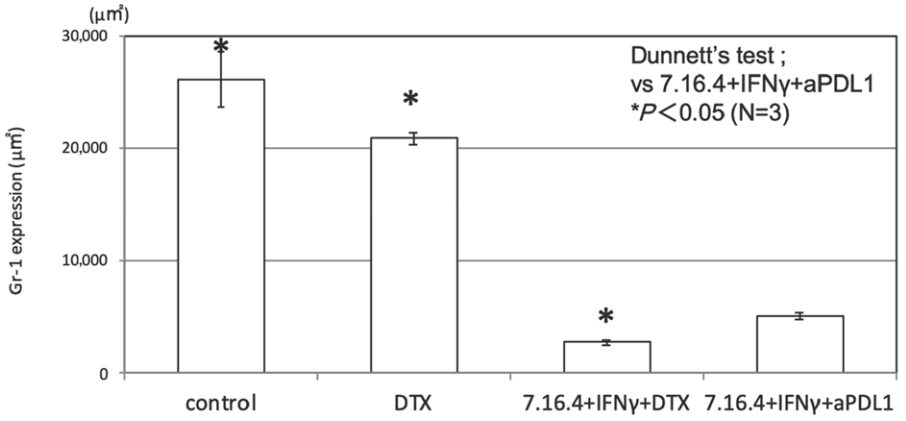

Fig. 10. Fluorescence intensity of Gr-1
The fluorescence intensity of Gr-1 in Figure 8 was quantified as described for Figure 9.

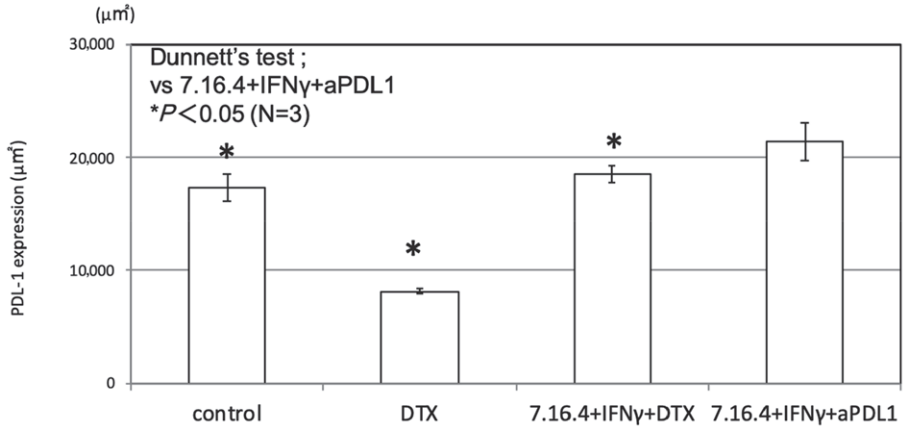

Fig. 11. Fluorescence intensity of PD-L1
The fluorescence intensity of PD-L1 in Figure 8 was quantified as described for Figure 9.

IFN-γ + aPD-L1 group compared to the other groups (Figure 9), and significantly lower Gr-1 expression in the 7.16.4 + IFN-γ + aPD-L1 and 7.16.4 + IFN-γ + DTX groups (Figure 10). In the DTX alone group, PD-L1 expression was significantly lower than that in the control group, but significantly higher in the 7.16.4 + IFN-γ + PD-L1 group (Figure 11).

Discussion

The combined Ab + IFN-γ therapy directly acted on cancer cells and inhibited cell proliferation against a resistant cancer cell line

Few studies to date have used the same immunogenicity of mouse, Ab and tumor cell lines. In particular, we cannot obtain the Ab resistant tumor cells from humans. Therefore we cannot know which immunological mechanism is taking place in patients. Thus for this study we made a cell line resistant to Ab therapy based on H2N113 cells derived from Balb/c mice to maintain

consistent immunogenicity.

The mechanism of drug resistance in cancer cells extends further than the issue of altered signal transduction in cells. It also relates to cells' ability to avoid the immune system. Overall, it is a complicated process that many have tried to elucidate and decipher[15–17].

We do not know what kind of resistant mechanism is working for the resistant cell developed herein, but we propose that there are various complex resistant mechanisms at play, just as in the clinical setting. We evaluated whether the combined anti-HER2 Ab and IFN-γ therapy shows an antitumor effect *in vitro*, utilizing the resistant cell line to test whether the combined therapy has a direct effect on malignant cells without effects from immune cells.

The lack of antitumor effects with the anti-HER2 Ab therapy alone proved that the cancer cells had acquired resistance to Ab therapy. Combination therapy with IFN-γ also significantly inhibited cell proliferation, indicating that the combined therapy has a direct effect on cancer cells by affecting cellular proliferation. In addition, the cell number was not decreased, which is consistent with our previous *in vitro* experiments[14]. It thus seems probable that the Ab therapy supresses cell proliferation without decreasing the cell number.

We previously demonstrated that the combined Ab and IFN-γ therapy changes the malignancy of cancer cells[14]. We also showed that this combination therapy acts on P27kip1 in RAJI and the antiCD20 Ab, inhibiting cellular proliferation[18]. Based on these prior results, we thought that the combined therapy would have an inhibitory effect on the malignant features of cancer cells, in correlation with the problem of therapeutic resistance and cellular proliferation. No studies have discussed the interaction of intracellular signal transduction by EGFR and EGFR2 with IFNGR and STAT1, although Shi *et al*[19] proposed that transduction could be at play with the antitumor effect of the anti-HER2 Ab therapy. Specifically, they showed that the activation of STAT1 by IFN-γ, which is secreted by immune cells, plays an important role to diminish the intracellular signal transduction of HER2.

In this *in vitro* study, we thought that signal transduction would be diminished by the combined Ab and IFN-γ therapy against the resistant cell line.

Combination therapy for the resistant strain showed antitumor effects in the mouse Xenograft model

Next, we evaluated whether the combined anti-HER2 Ab and IFN-γ therapy showed antitumor effects even in an *in vivo* experiment with constant immunogenicity. The combined therapy significantly inhibited the tumor volume, thus we concluded that the antitumor immunity also worked *in vivo*, and not only the direct action to the cancer cell was shown *in vitro*.

Combination therapy and CD8T cells (Cytotoxic T cell)

There have been many reports linking the invasive ability of cytotoxic T cells to tumor tissue and treatment outcomes in many kinds of cancer[20, 21]. Many researchers have reported on the role of the tumor infiltrating lymphocytes, with positive correlations shown between CD8T cells and the prognosis, while there is a reverse correlation between Th2 and Treg cells and prognosis.

As in these previous studies, the accumulation of CD8T cells in tumor tissue was significantly increased herein in the combined therapy group compared to other groups using the resistant cancer cells.

CD8T cells accumulation and MDSCs

It is well known that tumor cells use various methods to escape from the immune system. Immunosuppressive cells, such as regulatory T cell (Treg) and MDSCs, are very important for cancer progression because these cells inhibit CTL, NK-cell, and NKT cells from accumulating in the tumor tissue[22].

In the immunostaining of the tumor tissue, accumulation of MDSCs was significantly decreased in the combined-therapy group, while Gr-1 showed an inverse correlation with tumor size. This result is consistent with our prior report of secreted cytokines from cancer tissue being inhibited by the combination therapy. Although we could not prove a direct association between the increased accumulation of CD8T cells and the decreased accumulation of MDSCs, we propose that the combination therapy induced the decreased accumulation of MDSCs and that contributed to the increased accumulation of infiltrating CD8T cells. Nevertheless, CD-8 expression was not high and we could not find evidence to explain this finding.

Tumor-specific T cells, as part of adaptive immunity, present a molecular MHC Class I on the cell surface and show a strong antitumor effect, through the entry of cancer antigenic peptide and co-stimulation. HER2 Ab and the stimulation by IFN-γ also raises the MHC class I expression of APC's, leading us to speculate that adaptive immunity is promoted by our combination therapy.

Conflicting function of IFN-γ and PD-L1

It is known that ADCC (antibody-dependent cell cytotoxicity) plays an important role in the antitumor effect of molecular targeted medicine[23,24]. NK-cells, macrophages, and some neutrophils possess ADCC activity[24]. In addition, some researchers reported that IFN-γ often increases the FcγR expression of NK cells, while Motohashi and Nakayama[25] reported that IFN-γ from NK-cells reinforces the direct cell-damaging action through NKT cells. Thus, IFN-γ generally works to enhance host immunity; however, when IFN-γ is used in monotherapy, tumor growth is actually increased in *in vivo* experiments. Therefore, IFN-γ also has a negative effect on host immunity, such as raising the PD-L1 expression of tumors[26].

In this study, only IFN-γ treatment raised the expression of PD-L1 in the tumor tissue, thus the tumor size was larger than with any other treatment. On the other hand, the PD-L1 expression of the combination therapy was decreased. We could not find any previous data about this type of effect, but we propose that only double signals from Ab and IFN-γ could elicit a new phenomenon in the cancer cells. To this end, Hou *et al*[27] reported an association between the CD8T cells and tumor tissue of a patient with breast cancer and between the expression of PD-L1 and outcome.

Combination therapy and NKT cells

Recently, some researchers reported the contribution of NKT cells to antitumor immunity. For example, Motohashi and Nakayama[25] reported on immunotherapy using NKT cells, which also play a role in innate immunity, allowing targeting of all cancer cells. The NKT cells strongly activate the above NK-cell and CD8T cells by secreting IFN-γ.

Herein, we did not examine NKT cell accumulation in the tumor tissue ; however, we propose that the IFN-γ in combination therapy worked as if it was from NKT cells and showed the same antitumor effect in this experiment. In the future, we hope to examine the contribution of NKT cells in our combination therapy.

On the clinical application of the combination therapy with IFN-γ

Next, we evaluated which therapy would show the best antitumor effect *in vivo*, in order to determine if this combined therapy could be applied in the clinical setting. The current recommended first-line pharmacotherapy for HER2-positive cancer is chemotherapy with anti-HER2 Ab[5, 7, 9, 28]. A similar regimen of chemotherapy is administered for breast cancer treatment. When cancer cells acquire pharmacotherapy resistance, typically within one year, the response to therapy halts.

The median overall survival in the GBG 26 / BIG 3-05 phase Ⅲ study of breast cancer is 24.9 months[29], while the progression-free survival time in the Toga study of gastric cancer is 6.7 months, and the median duration of overall survival is 13.8 months. With such poor prognosis, further improvement is urgently required in the clinical setting[5].

When progression is found during first line chemotherapy, we have an option to change the chemo drug or choose TDM-1, and various RCT are now ongoing regarding the next choice[30].

From our prior results, the 7.16.4 + DTX therapy showed an antitumor effect equivalent to 7.16.4 + IFN-γ, and triple therapy with 7.16.4 + DTX + IFN-γ showed the highest antitumor effect[14]. Thus, we evaluated the next therapy for cancers that had progressed while on first-line chemotherapy. And we tested the immuno-check point inhibitor (CPI), which has attracted attention recently. We used the antiPD-L1 Ab for the immuno-check point inhibitor, because that showed greater antitumor effect than antiPD-1 Ab in previous experiments. The combination therapy utilizing 7.16.4 + IFN-γ and DTX or antiPD-L1 Ab treatment also showed a high antitumor effect. CD8T cells did not accumulate in the 7.16.4 + DTX + IFN-γ therapy, while a significantly greater number of CD8T cells accumulated in the 7.16.4 + IFN-γ + antiPD-L1 Ab treatment.

We proposed that the DTX has a direct cytotoxic effect as well as an antitumor immune effect by the host, and that such immunotherapy is more useful for the patient with recurrent cancer than chemotherapy. Typically, the physical condition is poor, due to recent chemotherapy use, in addition to the progression of the disease itself. Accordingly, we showed herein that anti-HER2 Ab + IFN-γ + CPI showed a significantly higher anti-tumor effect than single-agent treatment utilizing CPI alone.

Conclusion

In this study, we demonstrated an antitumor effect on resistant cancer cells following combined anti-HER2 Ab and IFN-γ therapy. The results also suggested that this antitumor effect was improved over that achieved with current conventional therapy and that this combination therapy would be useful in deciding on the optimal treatment for the many patients who suffer from cancer progression due to the development of treatment resistance.

Conflict of interest disclosure

The authors have no conflict of interest to declare.

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Showa University (Permit Number : 70230), which operates in accordance with the Japanese Government for the care and use of laboratory animals.

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