Trastuzumab-Based Photoimmunotherapy Integrated with Viral HER2 Transduction Inhibits Peritoneally Disseminated HER2-Negative Cancer

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Abstract

Peritoneal dissemination is the most frequent metastasis in gastric cancer and is associated with poor prognosis. The lack of particular target antigens in gastric cancer other than HER2 has hampered the development of treatments for peritoneal dissemination of gastric cancer. We hypothesized that HER2-extracellular domain (HER2-ECD) gene transduction combined with trastuzumab-based photoimmunotherapy (PIT) might provide excellent and selective antitumor effects for peritoneal dissemination of gastric cancer. In vitro, adenovirus/HER2-ECD (Ad/HER2-ECD) efficiently transduced HER2-ECD into HER2-negative gastric cancer cells. Trastuzumab-IR700 (Tra-IR700)–mediated PIT induced selective cell death of HER2-ECD–transduced tumor cells. Ad/HER2-ECD also induced homogenous expression of HER2 in heterogeneous gastric cancer cells, resulting in uniform sensitivity of the cells to Tra-IR700–mediated PIT. Anti-HER2 PIT integrated with adenoviral HER2-ECD gene transfer was applied in mice bearing peritoneal dissemination of HER2-negative gastric cancer. Intrapertoneal administration of Ad/HER2-ECD and Tra-IR700 with PIT inhibited peritoneal metastasis and prolonged the survival of mice bearing MKN45. Furthermore, minimal side effects allowed the integrated therapy to be used repeatedly, providing better control of peritoneal dissemination. In conclusion, the novel therapy of molecular-targeted PIT integrated with gene transfer technology is a promising approach for the treatment of peritoneal dissemination in gastric cancer. Mol Cancer Ther; 15(3); 402–11. ©2016 AACR.

Introduction

Gastric cancer is one of the most common malignancies worldwide, making it the third leading cause of cancer-related death (1). Although the localized stage of gastric cancer can be treated curatively with surgical resection, it is frequently diagnosed as far advanced disease (2). In addition, even after an optimal curative surgery, cancer recurrence is sometimes inevitable (3, 4).

Peritoneal dissemination, which is the most frequent mode of metastasis in gastric cancer (2, 4), is associated with poor prognosis (5, 6). The lack of treatment success is due to the difficulty of selectively delivering anticancer drugs to peritoneal lesions while sparing normal tissue. Therefore, a novel therapeutic strategy that can provide highly selective antitumor effects against peritoneal dissemination is desired.

Photoimmunotherapy (PIT) is a novel cancer therapy employing a mAb conjugated to a photosensitizer, IRDye700DX (mAb-IR700). PIT provides a highly specific cytotoxicity to tumor cells expressing particular antigens (7). PIT could work only where mAb-IR700 binds to the targeted cell membrane following the irradiation of near-infrared (NIR) light. Therefore, its ability to target tumors fully depends on the nature of antibody conjugated to IRDye700DX. Trastuzumab, a humanized mAb for HER2, is the only molecular-targeting anticancer drug that improves the survival of patients with HER2-positive advanced gastric cancer (6, 9). Hence, trastuzumab-based PIT might be an ideal candidate as a novel targeted therapy even for peritoneally disseminated gastric cancer (10).

Mitsunaga and colleagues showed that Tra-IR700–mediated PIT has promising antitumor effects on HER2-positive cancer (7). However, before Tra-IR700–mediated PIT is widely applied to the treatment of peritoneal dissemination of gastric cancer, some problems remain to be addressed. Only 12.2% to 22.1% of gastric cancers are HER2 positive (9, 11–13). Moreover, peritoneal dissemination of gastric cancer has an extremely low HER2 expression rate of 2.9% as HER2-positive status is more common among intestinal-type cells than diffuse-type cells that often cause peritoneal dissemination (14, 15). In addition, gastric cancer itself exhibits intratumoral and intertumoral HER2 heterogeneity (16–18). Thus, even a HER2-positive tumor contains cells with different levels of HER2 expression, which leads to the inaccurate assessment of HER2 status, leading to therapeutic resistance of HER2-targeted therapy (19–21). The lack of particular target antigens in gastric
cancer also hampers the success of molecular-targeted therapy (22). Consequently, cancer-targeted therapy cannot be simply applied as a therapy of choice for peritoneal dissemination of gastric cancer.

To overcome these limitations, we previously developed an adenoviral vector that expresses the HER2-extracellular domain (HER2-ECD) on the cancer cell membrane, Ad/HER2-ECD. Ad/HER2-ECD induced exogenous HER2-ECD overexpression on HER2-negative cancer cells and successfully sensitized them to trastuzumab (23). In addition, we demonstrated that the integrated therapy of Ad/HER2-ECD and Tra-IR700–mediated PIT effectively and selectively killed HER2-negative breast cancer cells in vitro, suggesting that the integration of gene transduction with PIT expands molecular-targeted therapy even for target-negative cancer (24).

Here, we investigated the therapeutic effects of this integration therapy for peritoneal dissemination of HER2-negative gastric cancer in vivo. The approach used in the present study overcomes the limitations of HER2-mediated therapy and provides a novel therapeutic strategy for the peritoneal dissemination of gastric cancer.

Materials and Methods

Cell lines and cell cultures

The human gastric adenocarcinoma cell lines MKN1 and MKN45 were obtained from Human Science Research Resources Bank (Osaka, Japan). The HER2-expressing human gastric adenocarcinoma cell N87 was obtained from the American Type Culture Collection. MKN45/Luc and SKOV-3/Luc, a human ovarian cancer cell N87 was obtained from the American Type Culture Collection. MKN45 were obtained from Human Science Research Resources Bank (Osaka, Japan) and Cell Biolabs, Inc., respectively. The authentication was not performed by the authors. All gastric cancer cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich), and the ovarian cancer cell line was cultured in DMEM (high glucose) with 0.1 mmol/L nonessential amino acids (MP Biomedicals). The media were supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 10% FBS. Cells were maintained at 37°C in a humidified incubator at an atmosphere of 95% air and 5% CO2.

Recombinant adenovirus

A replication-deficient adenoviral vector expressing the extracellular and transmembrane domains of HER2 (Ad/HER2-ECD) was constructed, expanded, and purified as described previously (23). The titer of virus vector was determined by a plaque assay using HEK 293 cells. Adenovirus/GFP (Ad/GFP), which expressed GFP, was used as control adenovirus (25).

Synthesis of IR700-conjugated trastuzumumab

A water-soluble, IRDye 700DX NHS ester was obtained from LI-COR Bioscience. Trastuzumumab was purchased from Chugai Pharmaceutical Co. Conjugation of IR700 with trastuzumumab was performed according to previous reports (7, 10). Briefly, trastuzumumab was incubated with IR700 in Na2HPO4 at room temperature for 15 minutes. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare UK Ltd.). The protein concentration was determined with a BIORAD protein assay kit (Bio-Rad).

Western blotting analysis

MKN1, MKN45, and N87 cells (2 \times 10^5 cells) were seeded into 6-well plates. Cells were washed with cold PBS and lysed with the SDS buffer. Equivalent amounts of protein from whole-cell lysates were loaded into each lane of an 8% SDS–polyacrylamide gel and electrophoretically transferred to Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare UK Ltd.). Membranes were incubated with primary antibodies against HER2-ECD (Thermo Scientific) overnight at 4°C and visualized using an Amersham ECL chemiluminescence system (GE Healthcare UK Ltd.) according to the manufacturer’s protocol. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-β-actin antibody (Sigma-Aldrich). In this experiment, MKN1 and MKN45 cells were infected with Ad/HER2-ECD at a different multiplicity of infection (MOI; 0, 20, 50, and/or 100) for 24 and/or 48 hours. Ad/GFP was used as a control adenovirus.

Flow cytometric analysis

Cells were fixed with 4% paraformaldehyde for 10 minutes without permeabilization and labeled with the primary antibodies at room temperature for 45 minutes. APC-conjugated anti-human ErbB2 (HER2) monoclonal mouse antibody (R&D Systems Inc.) and phycoerythrin (PE)-conjugated anti-human epithelial cell adhesion molecule (EpCAM) monoclonal mouse antibody (BD Biosciences) were used for the confirmation of HER2-ECD expression and the identification of MKN45 cells, respectively. APC-conjugated mouse IgG antibody (Milenyi Biotec, K.K.) and PE-conjugated mouse IgG antibody (BD Biosciences) were also used as each isotype control antibody. After cells were washed with PBS and trypsinized, flow cytometry was performed with a FACS instrument (BD Biosciences). The intensity of staining was calculated by using the BD-FACS Software (FlowJo 7.6.1; BD Biosciences). MKN1 and MKN45 cells were infected with Ad/HER2-ECD at an MOI of 50 for 48 hours.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 30 minutes and then washed with PBS. After blocking with blocking one reagent (Nacalai Tesque, Inc.), cells were labeled with the primary antibodies for 1 hour. The secondary antibodies were also reacted for 1 hour. To detect HER2 expression, the anti-HER2 extracellular domain monoclonal mouse antibody (R&D Systems Inc.) with FITC-conjugated polyclonal goat secondary antibody to mouse IgG (abcam) was used. APC-conjugated mouse monoclonal anti-human HER2-ECD antibody (R&D Systems Inc.) was also used for HER2 detection. PE-conjugated anti-human EpCAM monoclonal mouse antibody (BD Biosciences) was used to identify gastric cancer cells. DAPI was used for nuclear and chromosomal counterstaining. Tra-IR700 could be detected by IR700 fluorescence. Cells were subsequently photographed, and the merged images were overlaid using a confocal laser scanning biological microscope (FV10i; Olympus).

In vitro PIT with adenovirus/HER2-ECD

MKN1 and MKN45 cells were seeded on plates (4 plates or wells per group) for 24 hours. The cells were infected with Ad/HER2-ECD or Ad/GFP at an MOI of 50 for 48 hours. Trastuzumumab and Tra-IR700 (10 μg/mL) were added for 6 hours, and cells were irradiated with NIR light at 10 J/cm2 (20 mW/cm2, 500 s). The irradiation was performed by an irradiator using a light emitting diode (LED) light with a peak at
690 nm. The irradiation power density was measured by energy meter console, PM100D (Thorlabs, Inc.). The morphologic cell changes after treatment were observed under a fluorescence microscope (IX71; Olympus). The time-lapse movies were taken serially at hourly intervals for 60 hours just after PIT using a confocal laser scanning biological microscope with built-in culture incubator (FV10i; Olympus).

Cell death and viability assay
To assess the HER2 target selectivity cell death, N87 cells labeled with Cell Tracker Blue CMAC dye (Life Technologies) were cocultured with unlabeled MKN1 cells. Cocultured cells were infected with Ad/HER2-ECD at an MOI of 50 for 48 hours and incubated with Tra-IR700 (10 μg/mL) for 1 hour. The irradiation of NIR light was performed at 5 J/cm². After the irradiation, the cells were stained with propidium iodide (PI; 1 μg/mL) to identify dead cells. Cell viability for quantitative evaluation was determined using an XTT Cell Proliferation Kit II (Roche Life Science) according to the manufacturer's protocol.

Ex vivo experiments
Athythic female BALB/c nu/nu nude mice were purchased from CLEA. The animal care and experimental procedures were conducted in accordance with the regulations of the Animal Care and Use Committee of Okayama University. Normal mouse peritoneal cells were collected from 6-week-old nude mice. Briefly, 5 cc of RPMI1640 medium (Sigma-Aldrich) was injected into the abdominal cavity, and then the abdomen was massaged. The ascites was collected, and cells were isolated by a centrifugal separator. The cells collected from normal mice were seeded into two culture conditions, a single culture (normal mouse peritoneal cells. 2 × 10⁶ cells) and a coculture (mixed normal mouse peritoneal cells with MKN45 cells, 1 × 10⁶ cells of each type). We analyzed the cells using two-color flow cytometry (BD Biosciences) with APC-conjugated anti-human HER2 monoclonal mouse antibody (R&D Systems Inc.) and PE-conjugated anti-human EpCAM monoclonal mouse antibody (BD Biosciences). We confirmed the HER2 expression and Tra-IR700 conjugation on mouse cells by light microscopy (IX71; Olympus). The time-lapse movies were taken serially at hourly intervals for 60 hours just after PIT using a confocal laser scanning biological microscope with built-in culture incubator (FV10i; Olympus).

Evaluation of antitumor effects in the peritoneal dissemination mouse model
We established the peritoneal dissemination xenografted mouse model by i.p. administration of MKN45 cells (1 × 10⁷ cells) into 6- to 8-week-old nude mice using a 22-gauge catheter needle. To assess the efficiency of adenoviral gene transfer to the peritoneally disseminated tumors, mice were injected with Ad/HER2-ECD at a dose of 1 × 10⁸ pfu in 500 μL PBS into the peritoneal cavity 14 days after injection of MKN45 cells. These mice were sacrificed 48 hours later. Immunohistochemical analysis of paraffin-embedded tissues was performed using HER2/ErbB2 (D8F12) XP rabbit monoclonal antibody (Cell Signaling Technology, Inc.), according to the manufacturer's protocol. To evaluate the antitumor effects, the mice were randomly divided into the following four groups (each group: n = 5): no treatment (control), treated with Tra-IR700–mediated PIT (IR + PIT), treated with Ad/HER2-ECD–mediated PIT (Ad + PIT), and treated with Ad/HER2-ECD with Tra-IR700–mediated PIT (Ad + IR + PIT). Ad/HER2-ECD was i.p. administered 5 days after tumor cell injection into the peritoneal cavity at a dose of 1 × 10⁸ pfu in 500 μL PBS. Tra-IR700 was also i.p. administered at 80 μg in 500 μL PBS 48 hours after Ad/HER2-ECD administration. One day after Tra-IR700 administration, irradiation was performed with 50 J/cm² of NIR light using an LED light source (L690-66-60 with Lens550; EPITEX, Inc.) at 690 nm as the peak wavelength. Subcutaneous anesthesia was used for all procedures. All mice were sacrificed, and the disseminated peritoneal tumors were resected. The total weight of tumors per mouse was measured on day 28 after tumor cell injection.

Evaluation of luciferase activity in peritoneal dissemination mouse model
We assessed the tumor-inhibitory effects of PIT by using the peritoneal dissemination mouse model bearing luciferase-expressing cells, SKOV-3/Luc cells, and MKN45/Luc cells. SKOV-3/Luc and MKN45/Luc cells (5 × 10⁶ of each cell type) were injected into the peritoneal cavity of 6- to 8-week-old nude mice. Seven days after tumor injection, the fluorescence intensities of the peritoneal dissemination in the mice were measured. The mice bearing SKOV3/Luc cells that exhibited luciferase fluorescence were randomly assigned to three groups: no treatment (control), only Tra-IR700 (IR), and Tra-IR700–mediated PIT (IR + PIT). After the randomization, Tra-IR700 (80 μg) was i.p. administered, and irradiation with NIR light was performed 2 days after Tra-IR700 administration. The mice bearing MKN45/Luc cells were also randomly assigned to one of 7 groups as follows: no treatment (control); a single treatment of Tra-IR700–mediated PIT (IR+PIT×1), Ad/HER2-ECD–mediated PIT (Ad+PIT×1), or Ad/HER2-ECD with Tra-IR700–mediated PIT (Ad+IR+PIT×1); and three repeated treatments of Tra-IR700–mediated PIT (IR+PIT×3), Ad/HER2-ECD–mediated PIT (Ad+PIT×3), or Ad/HER2-ECD with Tra-IR700–mediated PIT (Ad+IR+PIT×3). The schedule of the integrated therapy regimen is shown in Fig. 5A. For analyzing fluorescence intensities, the mice were intraperitoneally injected with Xenolight Redject D-luciferin (Caliper Life Sciences) at 150 mg and imaged under isoflurane anesthesia after 4 minutes. The in vivo fluorescence and bioluminescence images were obtained with an IVIS Lumina imaging system (Xenogen IVIS Lumina II; Caliper Life Sciences), and the image analysis and bioluminescent quantification were performed by using Living Image software. In MKN45/Luc mice, the survival rate at 50 days after tumor injection was also assessed.

Statistical analysis
Data analysis was performed using the two-sided Student t test or one-way repeated measures ANOVA followed by a Dunnett test for multiple comparisons. Survival curves were estimated using the Kaplan–Meier method, and survival differences between subgroups were analyzed using the log-rank test. All statistical analyses were performed with SPSS for Windows, version 19.0 (SPSS, Inc.). P values <0.05 were considered to indicate a statistically significant difference.
Results

HER2-ECD expression induced by Ad/HER2-ECD and the distribution of Tra-IR700 in gastric cancer cells

HER2 protein expression in the parental gastric cancer cell lines MKN1, MKN45, and N87 was evaluated. The expression of HER2-wild type (wt) protein (185 kDa) was undetectable in MKN1 and MKN45 cells, whereas N87 cells were HER2-positive. To examine functionality of HER2 protein, the sensitivity to trastuzumab was tested in these cells. MKN1 and MKN45 cells were resistant to trastuzumab, whereas the growth of N87 cells was efficiently inhibited by trastuzumab (Supplementary Fig. S1). These findings indicate that MKN1 and MKN45 cells were HER2-negative and did not express functional HER2.

To transduce extracellular HER2 protein in these MKN1 and MKN45 cells, they were infected with Ad/HER2-ECD. Infection with Ad/HER2-ECD markedly increased the expression of the HER2-ECD protein in MKN1 and MKN45 cells in a time- and dose-dependent manner (Fig. 1A and B), and the expression of HER2-ECD proteins was specific to Ad/HER2-ECD (Fig. 1C). Based on these pilot experiments, infection with Ad/HER2-ECD at an MOI of 50 for 48 hours was used as the optimal conditions for all subsequent experiments.

To further examine whether transduced HER2-ECD was expressed on the cell surface, Ad/HER2-ECD–infected cells were subjected to flow cytometric analysis. The increased HER2 expression was confirmed in the infected cells, as compared with the parental cells (Fig. 1D). The transduced HER2-ECD was also detected on the cellular membrane by immunocytochemistry (Fig. 1E). In HER2-ECD–transduced and Tra-IR700–treated cells, the fluorescence signal of IR700 conjugated to trastuzumab was distributed in the same pattern of HER2-ECD expression. These findings indicated that Ad/HER2-ECD could successfully transduce the HER2-ECD proteins on the cellular membrane of HER2-negative gastric cancer cells, and Tra-IR700 could target and bind to it efficiently in vitro.

Microscopic observations of Ad/HER2-ECD–infected gastric cancer cells after treatment with Tra-IR700–mediated PIT

PIT provokes its cytotoxicity on Tra-IR700–bound cells through exposure to NIR light. Next, we irradiated gastric cancer cells with NIR light and observed the effects of PIT on the cells. After PIT, only the cells that were treated with Ad/HER2-ECD and Tra-IR700 showed morphologic changes within a few minutes after irradiation with NIR light. The cells swelled and ballooned with bleb formations of the cell membranes (red arrows), whereas cells in the other conditions continued to proliferate without damage even after PIT (Fig. 2A). Furthermore, the time lapse imaging of the MKN45...
cells confirmed that the morphologic cell changes were irreversible (Supplementary Movie). These findings indicate that Tra-IR700–mediated PIT directly injures the membrane of cancer cells and that the morphologic cell changes are rapid and crucial.

To further verify the target specificity of cell death induced by Tra-IR700–mediated PIT, PIT was exposed on the N87 cells labeled with Cell Tracker dye together with unlabeled MKN1 cells cocultured on the same plate. Staining the treated cells with PI, which stains dead cells with disrupted cell membranes, showed that PI stained only labeled N87 cells bound by Tra-IR700 (middle row). The cocultured plate was infected with Ad/HER2-ECD at an MOI of 50 for 48 hours and treated with Tra-IR700–mediated PIT. PI stained labeled N87 cells as well as unlabeled MKN1 cells (bottom row). C, a comparison of the cell viability after the treatment of the Ad/HER2-ECD with Tra-IR700–mediated PIT. MKN1 and MKN45 cells were seeded on 96-well plates (n = 4 per group) for 24 hours. Ad/HER2-ECD were infected at an MOI of 50 for 48 hours. After 24 hours of treatment with trastuzumab and Tra-IR700, the cells were irradiated with NIR light (10 J/cm²). An XTT assay demonstrated the statistically significant loss of cell viability in only the integration of Ad/HER2-ECD to the Tra-IR700–mediated PIT group as compared with other groups (n = 4; *, cell viability; P status: MKN1, 19%, P < 0.01; MKN45, 26%, P < 0.01, using the Student t test).
and Tra-IR700–mediated PIT has the potential to eradicate HER2-negative gastric cancer cells with low cytotoxicity.

In vivo experiments with Ad/HER2-ECD and Tra-IR700

To apply the integrated therapy for the treatment of peritoneal dissemination in vivo, we assessed the influence of Ad/HER2-ECD on normal mouse peritoneal cells ex vivo. The normal mouse peritoneal cells including the mesothelium cells and lymphocytes were collected from the peritoneal cavities of mice. The single culture of normal mouse peritoneal cells and the coculture of normal mouse peritoneal cells and MKN45 cells were infected with Ad/HER2-ECD. In flow cytometric analysis, the cells with low expression levels of anti-human EpCAM indicated normal mouse peritoneal cells, and the cells with high expression levels of anti-human EpCAM indicated MKN45 cells. The normal mouse peritoneal cells did not show a difference in HER2 expression levels between before and after Ad/HER2-ECD infection. On the other hand, the cocultured cells showed increased HER2 expression levels after Ad/HER2-ECD infection, especially the cells with high expression levels of anti-human EpCAM; these results indicate that the MKN45 cells had increased HER2 expression levels (Fig. 3A).

We further assessed the efficacy of HER2-ECD gene transfer to the peritoneal dissemination of MKN45 cells in vivo. Ad/HER2-ECD was i.p. administered 14 days after tumor cell injection, and the peritoneal tumors and organs were subjected to HER2 staining 48 hours later. Immunohistochemistry revealed that the HER2-ECD gene was transferred diffusely into the peritoneal tumors and not normal organs, including the liver and the small intestine (Supplementary Fig. S2). Together with the ex vivo results, Ad/HER2-ECD appeared to transduce the HER2-ECD gene preferentially on cancer cells in the peritoneal cavity.

At last, we confirmed the HER2-ECD expression levels and the distribution of Tra-IR700 on the peritonally disseminated cells in vivo. Ad/HER2-ECD and Tra-IR700 were i.p. administered. Peritonally disseminated cells were collected by peritoneal lavage. Immunocytochemistry demonstrated that HER2 expression and Tra-IR700 conjugation could be identified in only the anti-human EpCAM-positive cells (MKN45 cells; Fig. 3B). These findings indicated that Ad/HER2-ECD administered into the peritoneal cavity could infect the cancer cells and change them to HER2-ECD-positive cells. In addition, Tra-IR700 could diffuse in the peritoneal cavity and bind to cells that express HER2-ECD.

In vivo antitumor effects of the integrated therapy in a peritoneal dissemination mouse model

At first, to confirm the effect of Tra-IR700–mediated PIT in vivo, we also established a peritoneal dissemination mouse model bearing luciferase-expressing HER2-positive ovarian cancer cells, SKOV-3/Luc cells (Supplementary Fig. S3). Luciferase-expressing cells enable therapeutic evaluation with noninvasive bioluminescence imaging. The mice bearing SKOV-3/Luc were treated with Tra-IR700–mediated PIT, and the fluorescence intensities were measured. The bioluminescence imaging showed that total
fluorescence of the mice treated with Tra-IR700–mediated PIT was lower than that of other mouse groups (Supplementary Fig. S4). These findings indicated that the NIR light applied by external LED light irradiation could reach the mouse peritoneal cavity and that Tra-IR700–mediated PIT could control the progression of the peritoneal dissemination of HER2-positive cells.

Next, we investigated the effect of the integrated therapy of Ad/HER2-ECD and Tra-IR700–mediated PIT in the peritoneal dissemination mouse model with MKN45 cells. We sacrificed the mice 28 days after treatment and resected the disseminated peritoneal tumors. The tumor weight of the integrated therapy group \((n = 4; \text{Ad} + \text{IR} + \text{PIT}; 0.50 \pm 0.06 \text{ g})\) was significantly lower than that of the other groups (Fig. 4A). In addition, the survival curve showed that the integrated therapy significantly prolonged the survival of mice bearing peritoneal dissemination as compared with controls (Fig. 4B). We also recorded the weight of mice every week after treatment and calculated the median weight change based on the weight on treatment start day. No significant difference in weight change among the groups was identified (data not shown).

**In vivo luciferase activity in peritoneal dissemination mouse model**

We monitored the bioluminescence intensity of the peritoneal dissemination after the treatment in a mouse model xenografted with luciferase-expressing gastric cancer cells, MKN45/Luc (Fig. 5A and B). We compared the total fluorescence of the integrated treatment mice with that of untreated control mice in single and repeat integrated therapy groups, respectively (Fig. 5C and D). Both the single and repeated integrated therapy groups had remarkably suppressed tumor growth as compared with the controls. In particular, the repeated integrated treatment group exhibited significantly lower fluorescence during the observation period. The survival rate on the last day of the observation period (50 days after tumor injection) was as follows: control, 0%, IR + PIT × 1; 0%, Ad + IR + PIT × 1; 20%, Ad + IR + PIT × 3; 25%, Ad + PIT × 3; 0%, and Ad + IR + PIT × 3, 60%. These results indicate that the repetitive therapy of Tra-IR700–mediated PIT integrated with Ad/HER2-ECD can provide better control of peritoneal dissemination and has the potential to improve the prognosis of gastric cancer.

**Discussion**

Peritoneal dissemination of gastric cancer is an intractable disease entity to which no effective treatments have been established (26–28). Recent efforts to categorize gastric cancer into various molecular subtypes (29) have not yet elucidated specific therapeutic targets. In the present study, we described a novel strategy for peritoneally disseminated gastric cancer that integrates HER2-targeted PIT and virus-mediated HER2 gene transfer, exploiting the high specificity for target, the compartmentalized nature of the peritoneal disease, deeply reachable but harmless NIR light, and the efficient *in vivo* gene transfer capability of adenovirus vector. In a series of *in vivo* experiments using mouse models with xenografted human gastric cancer cells, HER2-targeted PIT integrated with Ad/HER2-ECD demonstrated robust therapeutic activity even in HER2-negative gastric cancer without overt adverse effects.

Among numerous molecular targeting therapies, thus far, the anti-HER2 strategy is the most successful in breast and gastric cancer (8, 9, 30); but, its usefulness is limited by low expression rates and the heterogeneous nature of HER2 expression (20, 21). A critical advantage of the present integrated strategy is that it addresses the general hurdles of cancer-targeting therapy, including low frequency of cancer target antigen and its intra- or intertumor heterogeneity. The development of resistance due to the loss of antigen during the course of treatment and acquired mutations in its signal unit are problems that need to be addressed (31–33).

Against these issues, we employed gene transfer technology using adenovirus. HER2 antigen transduced by Ad/HER2-ECD consisted of extracellular and transmembrane domains that were successfully expressed on the surface membrane of gastric cancer cells without oncogenic activation, as previously proven (23). We have further shown that in coculture of HER2-negative cells and...
HER2-positive cells, HER2-negative gastric cancer cells can be transformed to be HER2-positive cells by Ad/HER2-ECD. Transduced HER2-ECD can be engaged by Tra-IR700, and both HER2-negative and HER2-positive cells were evenly killed by PIT, indicating that any untargeted cancer cells can be modified to be targeted cancer cells as long as viral vector can transduce a gene of interest, and the nature of heterogeneity might be overcome by this strategy.

Another advantage of PIT is its mode of action. In contrast with conventional photodynamic therapy (PDT) which requires the uptake of photosensitizer into tumor cells and the production of reactive oxygen species (ROS; ref. 34), the phototoxic effect of PIT does not require the internalization of IR700 to the tumor cells. PIT directly provokes structural damage to the tumor cell membrane by exposure to NIR light and leads to necrotic cell death (7). Thus, any resistance due to the loss of antigen or acquired mutations in its signal unit is theoretically negligible in this PIT integrated with Ad/HER2-ECD. In addition, although PDT necessitates tissue oxygen for its action (35), the phototoxic phenomenon of PIT works even under hypoxia because the ROS formation is a minor cause of the cell death for PIT (36).

Although brisk and substantial tumor suppression was achieved with this integrated therapy, complete eradication was not observed in a series of in vivo experiments. There are two potential hurdles for this experimental therapy. One is uneven dispersion of Ad/HER2-ECD in the peritoneal cavity, and another is uneven distribution of extracorporeally irradiated NIR light to the disseminated tumors. Despite the complex shape of the peritoneal cavity, we have administered an adenovirus directly into it rather than into the blood vessels. Several studies demonstrated that i.p. administration of anticancer drugs is effective for peritoneal dissemination of gastric cancer, maintaining high concentrations of the drug in the peritoneal cavity (37). We and others also had previously demonstrated that gene therapy with i.p. administration of adenoviral vector expressing a therapeutic gene suppressed the growth of disseminated gastric cancer (38, 39). Although unintended, Ad/HER2-ECD worked mainly on tumor cells in the peritoneal cavity. Because adenoviral infection greatly depends on receptor-mediated integration, the preference for tumor cells is probably due to the difference in susceptibility to adenoviral infection between the implanted tumor cells and the other cells including lymphocytes and mesothelial cells. The report by Tomko and colleagues showing that the tissue distribution of adenosine receptor was detected mainly in epithelial cells (40) might support this speculation, although the precise underlying mechanism is not fully understood.

On the other hand, there is concern that the i.p. administered mAb-IR700 might be taken up into the liver and that there is a risk of losing a large amount of its efficacy (41). However, because we targeted floating peritoneal tumor cells in our mouse model, i.p. administration of Tra-IR700 was chosen as a more optimal route than intravenous administration.

Because PIT necessitates sufficient NIR light to kill Tra-IR700-engaged cancer cells, the target lesions must be within its reach. NIR light travels at least several centimeters through tissues (42–44). Thus, extracorporeally irradiated NIR light was expected to penetrate the murine bodies, reach the whole peritoneal cavity, and elicit photo toxicity (10, 41). To apply this therapy to clinical situations, however, the source of NIR light needed to be devised to reach intra-visceral gastric cancer lesions. Nowadays, laparoscopy is widely used in gastric cancer surgery (45) as the peritoneal cavity can be fully inspected through the scope (46, 47).
Accordingly, as long as the disseminated cancerous lesions are compartmented in the peritoneal cavity, NIR light can reach them through an endoscope. Based on the advantages of HER2-targeted PIT integrated with viral gene transfer, including its tremendous specificity, fast-acting property, and minimal side effects, repetitive administration is expected to enhance the therapeutic activity of a single treatment. In the present study, we have tested up to three courses of treatment, which significantly improved the survival rate at 50 days after tumor inoculation. Although the treatment schedule may need more optimization, repetitive administration of PIT integrated with Ad/HER2-ECD appeared to be a feasible and effective option.

Even after multiple courses of PIT, however, eradication of peritoneally disseminated gastric cancer remained incomplete; thus, we still need further improvement of the system. In the present study, we have employed an adenovirial vector that was engineered not to replicate after infection. However, to address incomplete gene transduction by adenovirus, a conditionally replicating viral vector might be a solution as an alternative approach. We had previously developed a telomerase-targeted replicating adenovirus, which can replicate only in telomerase-active malignant cells (48). Kishimoto and colleagues previously demonstrated that, when intraperitoneally administered, replicating adenovirus carrying the GFP gene efficiently infected the disseminated cancer nodules and expressed GFP in the lesions in the peritoneal cavity of mice (49). These data suggested that viral vectors with cancer-specific replication would facilitate the expression of the transduced gene and improve transduction efficiency by further infection of viral progeny to the uninfected adjacent cancer cells in the peritoneal cavity.

In conclusion, our data demonstrated that PIT integrated with adenovirus-mediated HER2-ECD gene transfer could overcome the lack of tumor-targeted antigen and its heterogeneity and efficiently inhibit the growth of peritoneal dissemination of gastric cancer and prolong mouse survival. The novel integration therapy of gene transfer technology and antibody-based PIT is a promising approach for breaking the limitations of and resistance to cancer therapy.

Disclosure of Potential Conflicts of Interest

T. Fujiwara is a consultant/advisory board member for Onconys BioPharma, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Ishida, S. Kagawa, H. Kobayashi, T. Fujiwara Development of methodology: M. Ishida, K. Shimoyama, K. Takehara, K. Noma, H. Tazawa, H. Kobayashi Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Ishida Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ishida, S. Kagawa, Y. Shirakawa, H. Tazawa, T. Fujiwara Writing, review, and/or revision of the manuscript: M. Ishida, S. Kagawa, H. Kobayashi, T. Fujiwara Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kagawa, S. Tanabe, H. Kobayashi Study supervision: S. Kagawa, H. Kobayashi, T. Fujiwara

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