Breast Cancer Res Treat
DOI 10.1007/s10549-015-3265-y

PRECLINICAL STUDY

Viral transduction of the HER2-extracellular domain expands trastuzumab-based photoimmunotherapy for HER2-negative breast cancer cells

Kyoko Shimoyama · Shunsuke Kagawa · Michihiro Ishida · Shinichiro Watanabe · Kazuhiro Noma · Kiyoto Takehara · Hiroshi Tazawa · Yuuri Hashimoto · Shunsuke Tanabe · Junji Matsuoka · Hisataka Kobayashi · Toshiyoshi Fujiwara

Received: 19 September 2014 / Accepted: 26 December 2014

Abstract The prognosis of HER2-positive breast cancer has been improved by trastuzumab therapy, which features high specificity and limited side effects. However, trastuzumab-based therapy has shortcomings. Firstly, HER2-targeted therapy is only applicable to HER2-expressing tumors, which comprise only 20–25 % of primary breast cancers. Secondly, many patients who initially respond to trastuzumab ultimately develop disease progression. To overcome these problems, we employed virus-mediated HER2 transduction and photoimmunotherapy (PIT) which involves trastuzumab conjugated with a photosensitizer, trastuzumab-IR700, and irradiation of near-infrared light. We hypothesized that the gene transduction technique together with PIT would expand the range of tumor entities suitable for trastuzumab-based therapy and improve its antitumor activity. The HER2-extracellular domain (ECD) was transduced by the adenoviral vector, Ad-HER2-ECD, and PIT with trastuzumab-IR700 was applied in the HER2-negative cancer cells. Ad-HER2-ECD can efficiently transduce HER2-ECD into HER2-negative human cancer cells. PIT with trastuzumab-IR700 induced direct cell membrane destruction of Ad-HER2-ECD-transduced HER2-negative cancer cells. Novel combination of viral transduction of a target antigen and an antibody-based PIT would expand and potentiate molecular-targeted therapy even for target-negative or attenuated cancer cells.

Keywords Photoimmunotherapy · HER2 · Breast cancer · Adenovirus

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
ECD	Extracellular domain
HER2	Human epidermal growth factor receptor type 2
MOI	A multiplicity of infection
NIR	Near infrared
PBS	Phosphate-buffered saline
PI	Propidium iodide
PIT	Photoimmunotherapy
SDS	Sodium dodecyl sulfate
SQ	Self-quenched
Tra-IR700	Trastuzumab-IR700

Electronic supplementary material The online version of this article (doi:10.1007/s10549-015-3265-y) contains supplementary material, which is available to authorized users.

K. Shimoyama · S. Kagawa (✉) · M. Ishida · S. Watanabe · K. Noma · K. Takehara · H. Tazawa · Y. Hashimoto · S. Tanabe · T. Fujiwara
Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmacological Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
e-mail: skagawa@md.okayama-u.ac.jp

H. Tazawa
Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

J. Matsuoka
Department of Palliative Care, Okayama University Hospital, Okayama, Japan

H. Kobayashi
Molecular Imaging Program, Center for Cancer Research, National Cancer Institute, US National Institutes of Health, Bethesda, MD, USA

Published online: 24 January 2015

XTT The sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate

Introduction

HER2 is a well-known oncogene, the overexpression of which is strongly associated with more aggressive tumors and poorer overall survival [1]. Strategies such as trastuzumab that target HER2 have greatly improved the prognosis of HER2-positive breast cancer through their high specific targeting ability [2]. However, the clinical efficacy of HER2-targeted therapy is limited to breast cancers that overexpress HER2, which only account for 20–30 % of all breast cancer [3, 4] and which are also inclined to develop resistance [5]. In addition, although trastuzumab is known to have few side effects apart from cardiac toxicity, trastuzumab is usually used together with an anti-tumor drug [6, 7], because its effect is not strong enough when used as a sole agent.

Photoimmunotherapy (PIT) was developed as a new type of molecular-targeted cancer therapy. PIT uses specific monoclonal antibodies that are targeted toward cell surface receptors and that are conjugated to the photosensitizer phthalocyanine dye, IR700, which is cytotoxic upon irradiation with near-infrared (NIR) light [8]. Trastuzumab-IR700 (Tra-IR700) was designed to specifically target HER2-expressing cells. Cell death is only induced when the cells are bound by Tra-IR700 and irradiated with NIR light. Death is presumably induced by cell membrane damage due to local expansion of heated water [8].

Modified adenovirus type 5 vectors have been widely used as a platform for the delivery of genes of interest into various types of human cells. In a previous study, we constructed a replication-deficient adenoviral vector containing a gene that encodes the HER2 extracellular domain (Ad-HER2-ECD) [9]. We demonstrated that infection with Ad-HER2-ECD resulted in expression of the HER2-ECD on the surface of HER2-negative or trastuzumab-resistant HER2-attenuated cancer cells. The expressed HER2-ECD did not trigger HER2 signaling pathways because of the lack of the HER2 intracellular kinase domain [9].

We hypothesized that if the Ad-HER2-ECD-mediated gene transduction technique was used together with molecular-targeted cancer therapy using PIT, the range of tumor entities suitable for trastuzumab-based therapy would be expanded and the problem of resistance and narrow indication could be overcome.

Materials and methods

Cell lines and cell cultures

Three human mammary gland adenocarcinoma cell lines, SKBR3, MDA-MB-231, and MCF7, were obtained from the American Type Culture Collection. SKBR3, MDA-MB-231, and MCF7 were cultured in McCoy's 5A medium, Leibovitz's L-15 Medium, and Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/ml L-glutamine, respectively. The human osteosarcoma cell line Saos-2 was kindly provided by Dr. Kyo (Kanazawa University, Ishikawa, Japan), and the cells were propagated as monolayer cultures in DMEM. Penicillin (100 units/ml), streptomycin (100 μg/ml), and 10 % fetal bovine serum were added to the medium. The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

Recombinant adenovirus

A replication-deficient adenoviral vector expressing the extracellular and trans-membrane domains of HER2 (Ad-HER2-ECD) was constructed [9]. Briefly, the HER2-ECD expression cassette, which contains the human cytomegalovirus promoter, HER2-ECD cDNA, and the SV40 early polyadenylation signal, was inserted between the XbaI and ClaI sites of pXCJL.1. The HER2-ECD shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells. The culture supernatant of 293 cells that showed the complete cytopathic effect, which contained virus progenies, was collected and used for subsequent production. The resultant virus was purified by ultracentrifugation in cesium chloride step gradients, and its titer was determined by a plaque-forming assay using 293 cells. The virus was stored at −80 °C until use. Ad-GFP was used as a control adenovirus [10].

Western blotting analysis

Primary antibodies against HER2-ECD (Ab-20, Thermo Scientific) and β-actin (AC-15, Sigma Chemical, Co.) were used. Proteins were electrophoretically transferred to Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare Life Science), incubated with primary antibody and then with peroxidase-linked secondary antibody according to the manufacturer's protocol. The Amersham ECL chemiluminescence system (GE Healthcare Life Science) was used to detect the peroxidase activity of the bound antibody. In experiments with replication-deficient adenoviral vector, cells were infected with Ad-HER2-ECD or Ad-GFP at a multiplicity of infection (MOI) of 50 for 48 h.

Flowcytometric analysis

To measure the expression of HER2-ECD in cells infected with Ad-HER2-ECD, cells were labeled with APC-conjugated mouse monoclonal anti-HER2-ECD antibody (R&D Systems Inc.) or APC-conjugated IgG2b as control (Miltenyi Biotec, Inc.) on ice for 45 min and were then analyzed using a FACS instrument (BD Biosciences). The intensity of staining was determined using the BD-FACS Software (Flow Jo 7.6.1).

Immunocytochemistry

Cells cultured on 4 chamber glass slides were fixed with 4 % paraformaldehyde in PBS for 15 min and blocked with 3 % bovine serum albumin for 30 min. Slides were then labeled with APC-conjugated mouse monoclonal anti-HER2-ECD antibody (R&D Systems Inc.) on ice for 45 min and were subsequently photographed using a Confocal Laser Scanning Biological Microscope with a 647-nm excitation filter (FV10i, Olympus, Tokyo, Japan).

Synthesis of IR700-conjugated trastuzumab

Trastuzumab was obtained from Chugai Pharmaceutical Co. The IR700 dye (LI-COR Biosciences) was conjugated to trastuzumab following the manufacturer's instructions. Briefly, trastuzumab (1 mg) was incubated with IR700 (66.8 mg, 34.2 nmol, 5 mmol/L in DMSO) in 0.1 mol/L Na_2HPO_4 (pH 8.6) at room temperature for 1 h. Conjugated Tra-IR700 was purified using a Sephadex G50 column (PD-10; GE Healthcare). Protein concentrations were determined using a Coomassie Plus Protein Assay Kit (Pierce Biotechnology) by measurement of light absorption at 620 nm (8453 Value System; Agilent Technologies).

Fluorescence microscopy

To detect specific antigen-mediated localization of IR700 on the surface of MCF7 and MDA-MB-231 cells, the cells were seeded at 2×10^4/well on cover glass-bottomed dishes, incubated for 24 h, and infected with Ad-HER2-ECD at an MOI of 50 for 48 h. Tra-IR700 (10 μg/ml) was then added to the culture medium and incubated for 6 h at 37 °C. The cells were washed with PBS and fluorescence was observed under a Confocal Laser Scanning Biological Microscope (FV10i, Olympus, Japan) with a 647-nm excitation filter.

In vitro Photoimmunotherapy

Cells were seeded on 35 mm cell-culture dishes at 2×10^4/well or 96-well black plates at 5×10^3/well, and were infected with Ad-HER2-ECD or Ad-GFP at an MOI of 50 for 48 h. The medium was then replaced with fresh culture medium containing 10 μg/ml of Tra-IR700 and was further incubated for 6 h at 37 °C. After washing with PBS, the cells were then irradiated with light from a red light-emitting diode at wavelengths of 670–690 nm for 35 mm cell-culture dishes and at a wavelength of 625 nm for 96-well black plates. The filter set to detect IR700 when observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) consisted of a 590–650-nm excitation filter and a 665–740-nm band pass emission filter. Power density ranged from 40 to 100 mW/cm^2, as measured using an optical power meter (PM 100, Thorlabs, Inc.).

Cell viability assay

Cell viability was determined 3 days after PIT using the Cell Proliferation Kit II (Roche Molecular Biochemicals) with the sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, according to the manufacturer's protocol. Propidium iodide (PI), which is plasma membrane impermeable, was used to stain membrane-disrupted dead cells [11]. PI was added to the medium at an approximate final concentration of 1 μg/mL and was incubated at 37 °C for 30 min.

Statistical analysis

A comparison of continuous variables between two groups for in vitro assays was performed using the two-sided Student's t test. Differences between groups were considered to be statistically significant when the p values were <0.05.

Results

HER2-ECD expression induced by Ad-HER2-ECD

MCF7 and MDA-MB-231 breast cancer cells were chosen as HER2 negative breast cancer lines since their expression of HER2 is known to be almost negative, or barely detectable [12]. Initially, Ad-GFP was used as a substitute for Ad-HER2-ECD in preliminary experiments aimed at finding the optimal dose of Ad-HER2-ECD to transduce HER2-ECD into these cells and at examining the transduction efficiency of the adenovirus. We found that an MOI of 50 was the optimal MOI, at which almost all cells were transduced with GFP proteins without cellular toxicity (Supplementary Fig. 1). Based on these data, HER2-negative breast cancer cells were infected with Ad-HER2-ECD at an MOI of 50 for 48 h, to transduce HER2-ECD.

Western blot analysis showed that Ad-HER2-ECD infection resulted in a marked increase in the expression of the 100-kDa HER2-ECD protein in HER2 low-expressing MCF7 and MDA-MB-231 breast cancer cells as compared to parental cells and Ad-GFP-infected cells (Fig. 1a). Flowcytometric analysis further demonstrated the expression of HER2-ECD on the cell surface (Fig. 1b). The cell surface expression of HER2-ECD in Ad-HER2-ECD-infected cells was also confirmed using immunocytochemistry (Fig. 1c).

Trastzumab-IR700 binds to the transduced HER2-ECD

We next examined whether Tra-IR700 bound to the HER2-ECD that was overexpressed on MCF7 and MDA-MB-231 cells by Ad-HER2-ECD transduction. Because IR700 is fluorescent, it can be directly detected by fluorescence microscopy allowing visualization of the cellular location of bound Tra-IR700 conjugates. MCF7 and MDA-MB-231 cells were transduced with HER2-ECD using Ad-HER2-ECD and the cells were then incubated with Tra-IR700 for 6 h. Fluorescent imaging confirmed that Tra-IR700 primarily bound to the cell surface of infected, HER2-ECD-transduced cells (Fig. 2).

Morphological changes in Ad-HER2-ECD-infected HER2-negative cells after trastuzumab-IR700 with PIT

The effects of Tra-IR700 and PIT treatment on the cell morphology of Ad-HER2-ECD-infected breast cancer cells over time were microscopically examined. Two different types of morphological changes were observed in MCF7 and MDA-MB-231 cells after irradiation with NIR light. In MCF-7 cells, bubbling at the cell surface

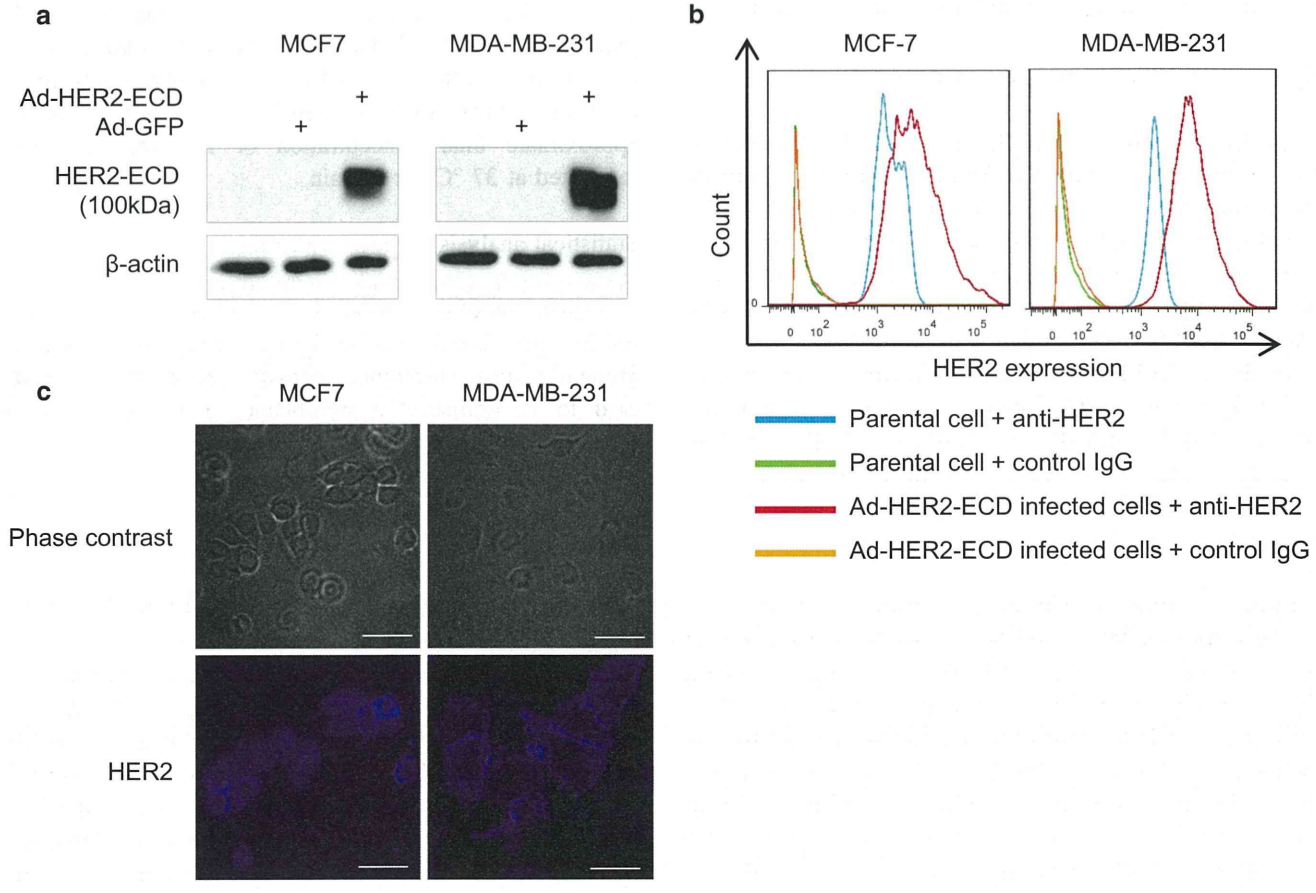

Fig. 1 HER2-extracellular domain (ECD) expression induced by Ad-HER2-ECD. **a** Western blot analysis showing expression of the 100-kDa HER2-ECD protein in Ad-HER2-ECD-infected cells but not in Ad-GFP-infected or parental cells. Actin was used as a loading control. **b** Flowcytometric analysis showing HER2-ECD expression on the cell membrane. Cells were labeled with APC-conjugated mouse monoclonal anti-HER2-ECD antibody (*blue* and *red*) or APC-conjugated IgG2b as a negative control (*green* and *yellow*). **c** Immunocytochemical analysis of the cell surface expression of HER2-ECD on Ad-HER2-ECD-infected cells using an APC-conjugated anti-HER2-ECD antibody. *Scale bars* 50 μm

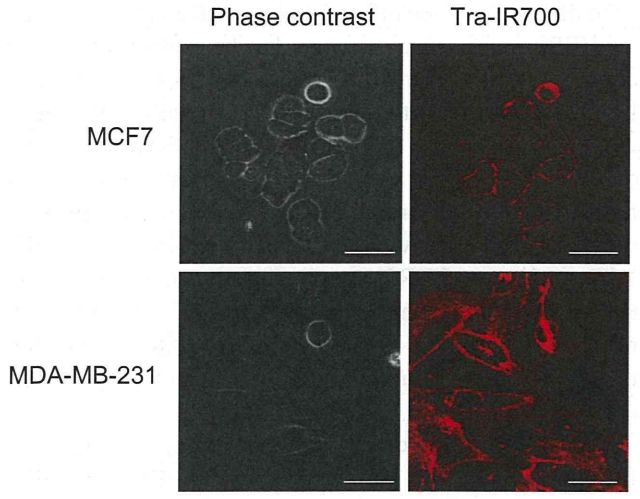

Fig. 2 Trastzumab-IR700 binds to the transduced cell surface HER2-ECD Immunofluorescent analysis of Trastzumab-IR700 (Tra-IR700) binding to HER2-ECD-transduced by Ad-HER2-ECD on the cell surface of breast cancer cells. *Scale bars* 50 μm

preceded total cell shrinkage. In MDA-MB-231 cells in contrast, instead of bubbling, many granules appeared in the cytoplasm, followed by shrinkage of the cells (Fig. 3a). Of note, these changes were observed within 1 min after irradiation with NIR light. A higher amount of light (18 J) resulted in more exaggerated changes in morphology than a lower amount of light (6 J) (Fig. 3a). Ad-HER2-ECD-infected cells died within 72 h after Tra-IR700 with PIT (6 J), while untreated cells and Ad-GFP-infected cells survived (Fig. 3b). These phenomena were captured as time-lapse movies (Supplementary movies 1, 2). We further confirmed cell death of the MCF7 and MDA-MD-231 cells at 72 h after irradiation with NIR light (18 J) by staining of cells whose membranes had been destroyed using PI staining (Fig. 3c). Morphological change and PI staining of cells immediately following PIT were also reproduced in Ad-HER2-ECD-infected and PIT-treated HER2 negative osteosarcoma Saos-2 cells (Supplementary Fig. 2).

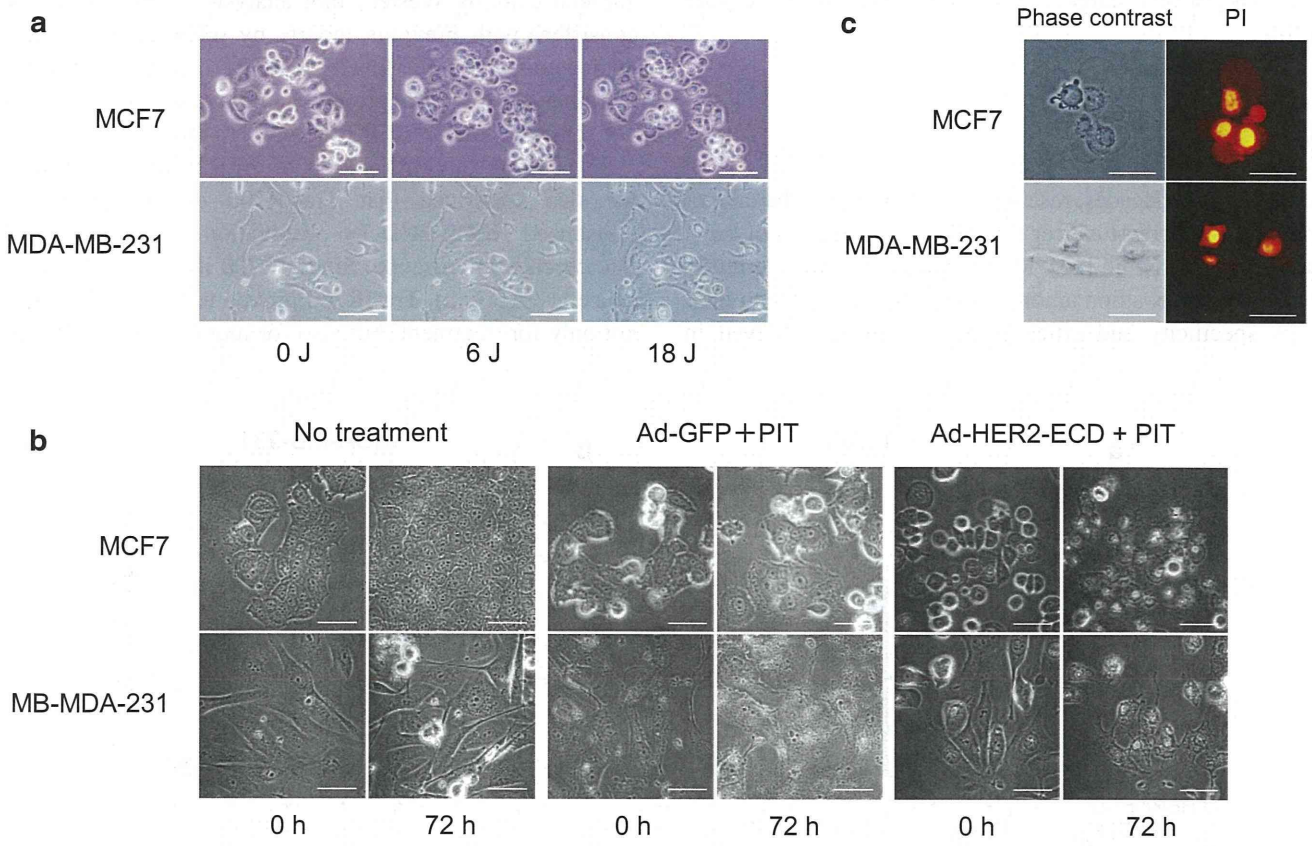

Fig. 3 Microscopic analysis of the effect of Tra-IR700-mediated PIT on the cell morphology and cell death of HER2-ECD-transduced HER2-negative cells. **a** Phase contrast analysis of the morphology of the breast cancer cells MCF-7 and MDA-MB-231 immediately following PIT using 0, 6, or 18 J. *Scale bars* 50 μm. **b** Phase contrast analysis of control, Ad-GFP-infected or Ad-HER2-ECD-infected MCF-7 and MDA-MB-231 cells before and after 72 h treatment with Tra-IR700-mediated PIT using 6 J. Ad-HER2-ECD-infected cells but not the other cells displayed cell shrinkage. *Scale bars* 50 μm. **c** Analysis of cell death of HER2-ECD-transduced cells following Tra-IR700-mediated PIT (12 J) 72 h after treatment. The PI staining indicates that plasma membrane destruction was induced by the treatment. *Scale bars* 50 μm

Quantitative evaluation of the cytotoxicity of Trastuzumab-IR700 with PIT for Ad-HER2-ECD-infected HER2 negative cells

The specific cytotoxicity of anti-HER2 PIT was quantitatively evaluated using the XTT assay of cell viability and HER2-transduced MCF7 and MDA-MB-231 cells (Fig. 4a, b). Treatment included seven control conditions in addition to Ad-HER2-ECD plus PIT. Only under the treatment condition that employed Ad-HER2-ECD together with Tra-IR700 mediated PIT (24 J for MDA-MB-231 and 36J for MCF-7), cell proliferation was significantly suppressed. In contrast, no significant cytotoxicity was observed under any other condition. Ad-HER2-ECD, Tra-IR700, or NIR irradiation did not harm the cells when each modality was given alone. HER2-specific cytotoxicity of Tra-IR700-mediated PIT was also reproduced in the HER2-positive breast cancer cell, SK-BR-3, and in Ad-HER2-ECD-infected Saos-2 cells (Supplementary Fig. 3a, b). These results indicated that Tra-IR700-mediated PIT selectively and specifically targets HER2-ECD-transduced cancer cells.

Discussion

Over the past decades, research has transformed the care of patients with breast cancer through the application of basic science to the clinic [13]. Monoclonal antibodies currently occupy a key position among anti-cancer drugs due to their high specificity and efficacy, which can be achieved in spite of their low side effects. The typical clinical approach for HER2-positive breast cancer has been altered since the approval of trastuzumab [13]. However, for such molecular-targeted therapy, the indication is limited to the populations which express the specific target antigen. In order to overcome this issue, we tried to artificially express the target antigen in target-negative cancer cells by viral transfection. We proved that viral transduction of HER2-ECD made even HER2-negative breast cancer cells sensitive to anti-HER2 photoimmunotherapy. Thus, this technique could expand the indication of antibody-directed therapy to not only target-positive but also to target-negative cancers.

In the present study, we have reproducibly expressed HER2-ECD on the cell membrane of HER2-negative MCF7 and MDA-MB-231 cells, as evidenced by immunocytochemistry as well as by flow cytometry. Although flow cytometric analysis suggested that both parental MCF7 and MDA-MB-231 cells express HER2, the 185 kDa HER2 protein could not be detected in these parental cells by Western blot analysis. These results are consistent with previous reports by other groups, which demonstrated that the levels of HER2 expression in these cell lines are low or negligible and that these cell lines are not sensitive to anti-HER2 therapy [14–16].

In addition to confirmation of HER2-ECD expression, we also confirmed that Tra-IR700 bound directly to transduced HER2-ECD by observation under a confocal fluorescence microscope. Since IR700 itself emits fluorescence [17] (Fig. 2), Tra-IR700 proved to be a useful agent not only for treatment, but also for monitoring [17–19]. In

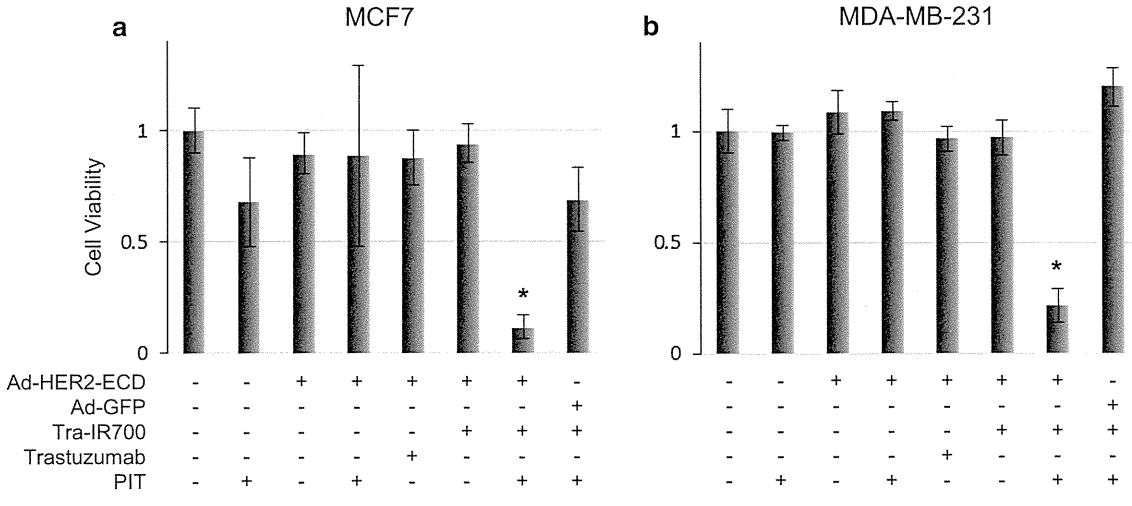

Fig. 4 Effect of Tra-IR700 mediated PIT treatment of HER2-ECD transduced HER2-negative cells on cell viability. Tra-IR700-mediated PIT was applied to the indicated cells along with seven control conditions and cell viability was quantified 72 h after PIT (24 J for MDA-MB-231 and 36 J for MCF-7) using the XTT assay. Only the group of Ad-HER2-ECD-infected cells treated with Tra-IR700-mediated PIT showed target-specific cell death. ($n = 4$, $^*p < 0.05$ for Tra-IR700-mediated PIT treatment compared to control groups using Student's t test.)

our previous study, we demonstrated that antibody-dependent cellular cytotoxicity (ADCC) activity against HER2-negative cells or HER2-downregulated cells could be recovered by forced expression of HER2-ECD using Ad-HER2-ECD. At the same time, we confirmed that the expressed HER2-ECD did not activate the HER2 signal transduction pathway and not induce malignant transformation due to the lack of an intracellular kinase domain [9].

There are two main mechanisms by which Trastuzumab stops abnormal cell proliferation; one is through ADCC activity and the other is by blocking signal transduction pathways [20, 21]. However, trastuzumab resistance is known to occur. The cause of trastuzumab resistance appears to be multifactorial [5]. Thus, multimodal treatment strategies are theoretically needed. Indeed, trastuzumab has been mainly used together with chemotherapy or with another monoclonal antibody [6, 7]. However, patients may ultimately suffer from the side effects of co-administered drugs [6, 7].

Cytotoxic agent-conjugated trastuzumab has been investigated to potentiate the anti-tumor activity of trastuzumab and to exploit the specific overexpression of HER2 on cancer cells [22–24]. One such agent is Tra-IR700, which was designed to induce cell death only when it is bound to the target cells and when the cells are exposed to NIR [8]. Although the mechanism of cell death induced by PIT remains to be fully elucidated, it assumed to differ from that in conventional photodynamic therapy or in antibody-mediated cytotoxicity [8, 25]. The mode of cytotoxicity in HER2-ECD-targeted Tra-IR700-mediated PIT is also different from ADCC activity or from a block in signal transduction pathways. In the present study, the cytotoxic effect of Ad-HER2-ECD plus Tra-IR700 with PIT was clearly shown by cellular morphological changes and by quantitative analysis of cell viability (Figs. 3, 4). Captured images of morphological change demonstrated that cell membrane injury occurred immediately after PIT, and time-lapse movies allowed continuous observation of morphological change (Supplemental movie 1, 2). Tra-IR700-mediated PIT directly affects the target cellular membrane and quickly damages it, suggesting that PIT for HER2-ECD can lead to target cell death regardless of intracellular conditions. This mechanism of cell death would suggest that this approach would overcome all modes of resistance known in HER2-targeted therapy including those mediated by altered signaling from receptor tyrosine kinases, constitutive phosphoinositide 3-kinase signaling, *PIC3CA* gene mutations, and amplification of signaling by HER family receptors [5, 26, 27].

Cell death was induced only in breast cancer cells infected with Ad-HER2-ECD and treated by Tra-IR700 with PIT. In contrast, none of the other incomplete treatment conditions could induce cell death. Thus, target-specificity was proved to be very high. Furthermore, we also reproduced this effect in HER2 negative, SaOS2 osteosarcoma cells, highlighting the possibility that this approach could be applied to cells irrespective of the tissue of origin, as long as the adenovirus can infect the target cell.

Although targeting a forcibly-transduced target seems to be an attractive approach, this approach might not be applicable to every anti-HER2 targeted therapy. Prior to employment of anti-HER2 PIT, we had exploited the trastuzumab-Saporin (Hum-ZAP: ADVANCED TARGETING SYSTEMS, San Diego, USA) conjugate, the so-called "immunotoxin". However, that strategy was unsuccessful, probably because of a defect in trans-membrane internalization of HER2-ECD (data not shown). Thus, any toxins or cytotoxic drugs such as Trastuzumab-DM1 [16, 28, 29], which require intracellular uptake to exert their toxicity, may not be suitable for anti-HER2-ECD-targeted therapy. In contrast, IR700 does not require intracellular trafficking because it can exhibit photo-toxicity at the surface of the membrane. This advantage of IR700 is therefore one of the reasons for the success of PIT in combination with ant-HER2-ECD-targeted therapy.

Since PIT necessitates sufficient NIR irradiation to kill the cancer cells, the actual target lesion must be within the reach of NIR. NIR light has been shown to travel at least several centimeters through human tissues [30]. Clinical potential of this therapy is therefore assumed for superficial cancer such as in a neoadjuvant setting for primary breast cancer, recurrences in the chest wall, and metastatic lesion in the skin from breast cancer. As long as the device that emits NIR light can gain access to the cancer, even an intravisceral approach though an endoscope might be possible [31]. Sano et al. recently reported that PIT enhanced local vascular permeability, which may enhance drug delivery through the so-called "enhanced permeability and retention" effect [32]. It is also expected that PIT might improve the efficacy of conventional chemotherapy. Furthermore, intrinsic ADCC activity can be expected simultaneously with PIT.

Next step will be proof of concept studies using HER2-negative breast tumors in mice. When this strategy is applied to a solid tumor model, success also depends on the penetration of adenoviral infection into tumor besides the reach of NIR light. Since Ad-HER2-ECD is a non-replicating adenoviral vector, intratumoral transduction might show at the less than ideal level [33]. Thus far, more appropriate viral and no-viral gene delivery systems have been extensively developed to be applied to preclinical and clinical studies [34, 35]. Therefore, in case of the application to in vivo study, the optimal vector such as a tumor-

specific replicating adenovirus [36] might be preferred to deliver the gene of the target antigen.

In conclusion, we demonstrated that the combination therapy of Ad-HER2-ECD and Tra-IR700 with PIT effectively and selectively killed HER2-negative cells in vitro. This result suggests that viral transduction technology will enable expansion of the indication of antibody-based therapy even to the target-negative group. Therefore, this strategy may also be applicable to other therapeutic antibodies that are currently being developed in rapid succession. The novel combination of gene transfer technology and antibody-based photoimmunotherapy is a promising approach for breaking the limitations of, and resistance to, cancer therapy.

Acknowledgments We thank Tomoko Sueishi for her technical support. This study was supported by grants-in-aid from the Ministry of Education Culture, Sports, Science and Technology, Japan (Toshiyoshi Fujiwara, Hiroshi Tazawa, Shunsuke Tanabe, and Shunsuke Kagawa) and grants from the Ministry of Health, Labor and Welfare, Japan (Toshiyoshi Fujiwara).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science (New York, N.Y.) 244:707–712
2. Dawood S, Broglio K, Buzdar AU, Hortobagyi GN, Giordano SH (2010) Prognosis of women with metastatic breast cancer by HER2 status and trastuzumab treatment: an institutional-based review. J Clin Oncol 28:92–98. doi:10.1200/jco.2008.19.9844
3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182
4. Carlsson J, Nordgren H, Sjostrom J, Wester K, Villman K, Bengtsson NO, Ostenstad B, Lundqvist H, Blomqvist C (2004) HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. Br J Cancer 90:2344–2348. doi:10.1038/sj.bjc.6601881
5. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ (2006) Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol 3:269–280. doi:10.1038/ncponc0509
6. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792. doi:10.1056/nejm200103153441101
7. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, Mackey J, Glaspy J, Chan A, Pawlicki M, Pinter T, Valero V, Liu MC, Sauter G, von Minckwitz G, Visco F, Bee V, Buyse M, Bendahmane B, Tabah-Fisch I, Lindsay MA, Riva A, Crown J (2011) Adjuvant trastuzumab in HER2-positive breast cancer. N Engl J Med 365:1273–1283. doi:10.1056/NEJMoa0910383
8. Mitsunaga M, Ogawa M, Kosaka N, Rosenblum LT, Choyke PL, Kobayashi H (2011) Cancer cell-selective in vivo near infrared photoimmunotherapy targeting specific membrane molecules. Nat Med 17:1685–1691. doi:10.1038/nm.2554
9. Yoshida R, Tazawa H, Hashimoto Y, Yano S, Onishi T, Sasaki T, Shirakawa Y, Kishimoto H, Uno F, Nishizaki M, Kagawa S, Fujiwara T (2012) Mechanism of resistance to trastuzumab and molecular sensitization via ADCC activation by exogenous expression of HER2-extracellular domain in human cancer cells. Cancer Immunol Immunother 61:1905–1916. doi:10.1007/s00262-012-1249-x
10. Umeoka T, Kawashima T, Kagawa S, Teraishi F, Taki M, Nishizaki M, Kyo S, Nagai K, Urata Y, Tanaka N, Fujiwara T (2004) Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene. Cancer research 64:6259–6265. doi:10.1158/0008-5472.can-04-1335
11. Kagawa S, He C, Gu J, Koch P, Rha SJ, Roth JA, Curley SA, Stephens LC, Fang B (2001) Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res 61:3330–3338
12. Papanikolaou V, Iliopoulos D, Dimou I, Dubos S, Tsougos I, Theodorou K, Kitsiou-Tzeli S, Tsezou A (2009) The involvement of HER2 and p53 status in the regulation of telomerase in irradiated breast cancer cells. Int J Oncol 35:1141–1149
13. Sledge GW, Mamounas EP, Hortobagyi GN, Burstein HJ, Goodwin PJ, Wolff AC (2014) Past, present, and future challenges in breast cancer treatment. J Clin Oncol. doi:10.1200/jco.2014.55.4139
14. Subik K, Lee JF, Baxter L, Strzepek T, Costello D, Crowley P, Xing L, Hung MC, Bonfiglio T, Hicks DG, Tang P (2010) The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. Breast Cancer 4:35–41
15. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A (1989) p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9:1165–1172
16. Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blattler WA, Lambert JM, Chari RV, Lutz RJ, Wong WL, Jacobson FS, Koeppen H, Schwall RH, Kenkare-Mitra SR, Spencer SD, Sliwkowski MX (2008) Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res 68:9280–9290. doi:10.1158/0008-5472.can-08-1776
17. Nakajima T, Sano K, Mitsunaga M, Choyke PL, Kobayashi H (2012) Real-time monitoring of in vivo acute necrotic cancer cell death induced by near infrared photoimmunotherapy using fluorescence lifetime imaging. Cancer Res 72:4622–4628. doi:10.1158/0008-5472.can-12-1298
18. Mitsunaga M, Nakajima T, Sano K, Kramer-Marek G, Choyke PL, Kobayashi H (2012) Immediate in vivo target-specific cancer cell death after near infrared photoimmunotherapy. BMC Cancer 12:345. doi:10.1186/1471-2407-12-345
19. Sano K, Mitsunaga M, Nakajima T, Choyke PL, Kobayashi H (2012) In vivo breast cancer characterization imaging using two monoclonal antibodies activatably labeled with near infrared fluorophores. Breast Cancer Res 14:R61. doi:10.1186/bcr3167
20. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA (1999) Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol 26:60–70
21. Hudis CA (2007) Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med 357:39–51. doi:10.1056/NEJMra043186
22. Berg K, Weyergang A, Prasmickaite L, Bonsted A, Hogset A, Strand MT, Wagner E, Selbo PK (2010) Photochemical internalization (PCI): a technology for drug delivery. Methods Mol Biol 635:133–145. doi:10.1007/978-1-60761-697-9_10

23. Berstad MB, Weyergang A, Berg K (2012) Photochemical internalization (PCI) of HER2-targeted toxins: synergy is dependent on the treatment sequence. Biochim Biophys Acta 1820:1849–1858. doi:10.1016/j.bbagen.2012.08.027
24. Owen SC, Patel N, Logie J, Pan G, Persson H, Moffat J, Sidhu SS, Shoichet MS (2013) Targeting HER2 + breast cancer cells: lysosomal accumulation of anti-HER2 antibodies is influenced by antibody binding site and conjugation to polymeric nanoparticles. J Control Release 172:395–404. doi:10.1016/j.jconrel.2013.07.011
25. Shirasu N, Yamada H, Shibaguchi H, Kuroki M, Kuroki M (2014) Potent and specific antitumor effect of CEA-targeted photoimmunotherapy. Int J Cancer. doi:10.1002/ijc.28907
26. Garrett JT, Arteaga CL (2011) Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: mechanisms and clinical implications. Cancer Biol Ther 11:793–800
27. Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, Xiong Y, Tseng LM, Li SH, Ding Z, Sahin AA, Esteva FJ, Hortobagyi GN, Yu D (2011) Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat Med 17:461–469. doi:10.1038/nm.2309
28. Burris HA 3rd, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, Limentani S, Tan-Chiu E, Krop IE, Michaelson RA, Girish S, Amler L, Zheng M, Chu YW, Klencke B, O'Shaughnessy JA (2011) Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. J Clin Oncol 29:398–405. doi:10.1200/jco.2010.29.5865
29. Junutula JR, Flagella KM, Graham RA, Parsons KL, Ha E, Raab H, Bhakta S, Nguyen T, Dugger DL, Li G, Mai E, Lewis Phillips GD, Hiraragi H, Fuji RN, Tibbitts J, Vandlen R, Spencer SD, Scheller RH, Polakis P, Sliwkowski MX (2010) Engineered thiotrastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. Clinical Cancer Res 16:4769–4778. doi:10.1158/1078-0432.ccr-10-0987
30. Weissleder R (2001) A clearer vision for in vivo imaging. Nat Non-viral Biotechnol 19:316–317. doi:10.1038/86684
31. Mitsunaga M, Nakajima T, Sano K, Choyke PL, Kobayashi H (2012) Near-infrared theranostic photoimmunotherapy (PIT): repeated exposure of light enhances the effect of immunoconjugate. Bioconjugate Chem 23:604–609. doi:10.1021/bc200648m
32. Sano K, Nakajima T, Choyke PL, Kobayashi H (2014) The effect of photoimmunotherapy followed by liposomal daunorubicin in a mixed tumor model: a demonstration of the super-enhanced permeability and retention effect after photoimmunotherapy. Mol Cancer Ther 13:426–432. doi:10.1158/1535-7163.mct-13-0633
33. Schuler M, Herrmann R, De Greve JL, Stewart AK, Gatzemeier U, Stewart DJ, Laufman L, Gralla R, Kuball J, Buhl R, Heussel CP, Kommoss F, Perruchoud AP, Shepherd FA, Fritz MA, Horowitz JA, Huber C, Rochlitz C (2001) Adenovirus-mediated wild-type p53 gene transfer in patients receiving chemotherapy for advanced non-small-cell lung cancer: results of a multicenter phase II study. J Clin Oncol 19:1750–1758
34. Smith E, Breznik J, Lichty BD (2011) Strategies to enhance viral penetration of solid tumors. Hum Gene Ther 22:1053–1060. doi:10.1089/hum.2010.227
35. Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG (2014) vectors for gene-based therapy. Nature reviews. Genetics 15:541–555. doi:10.1038/nrg3763
36. Kishimoto H, Kojima T, Watanabe Y, Kagawa S, Fujiwara T, Uno F, Teraishi F, Kyo S, Mizuguchi H, Hashimoto Y, Urata Y, Tanaka N, Fujiwara T (2006) In vivo imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. Nat Med 12:1213–1219. doi:10.1038/nm1404

PLOS | ONE

RESEARCH ARTICLE

Establishment of a Non-Invasive Semi-Quantitative Bioluminescent Imaging Method for Monitoring of an Orthotopic Esophageal Cancer Mouse Model

Shinji Kuroda[1]*, Tetsushi Kubota[1], Katsuyuki Aoyama[1], Satoru Kikuchi[1], Hiroshi Tazawa[2], Masahiko Nishizaki[1], Shunsuke Kagawa[1], Toshiyoshi Fujiwara[1]

1. Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, **2.** Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

*shinkuro@okayama-u.ac.jp

Citation: Kuroda S, Kubota T, Aoyama K, Kikuchi S, Tazawa H, et al. (2014) Establishment of a Non-Invasive Semi-Quantitative Bioluminescent Imaging Method for Monitoring of an Orthotopic Esophageal Cancer Mouse Model. PLoS ONE 9(12): e114562. doi:10.1371/journal.pone.0114562

Editor: Sunil Singhal, University of Pennsylvania, United States of America

Received: August 14, 2014

Accepted: November 11, 2014

Published: December 10, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports and Technology, Japan (TF) and Grants from the Ministry of Health, Labour and Welfare, Japan (TF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Orthotopic models of various types of tumors are widely used in anti-tumor therapeutic experiments in preclinical studies. However, there are few ways to appropriately monitor therapeutic effect in orthotopic tumor models, especially for tumors invisible from the outside. In this study we aimed to establish a non-invasive semi-quantitative bioluminescent imaging method of monitoring an orthotopic esophageal cancer mouse model. We confirmed that the TE8 esophageal cancer cell line implanted orthotopically into the abdominal esophagus of *nu/nu* mice (n=5) developed not only a main tumor at the implanted site, but also local lymph node metastases and peritoneal disseminations within 6 weeks after inoculation. We established a TE8 cell line that stably expressed the firefly luciferase gene (TE8-Luc). We showed that TE8-Luc cells implanted subcutaneously into *nu/nu* mice (n=5) grew over time until 5 weeks after inoculation. Tumor volume was strongly correlated with luminescent intensity emitted from the tumor, which was quantified using the IVIS imaging system. We then showed that TE8-Luc cells implanted orthotopically into the mouse abdominal esophagus (n=8) also formed a tumor and that the luminescent intensity of such a tumor, as detected by IVIS, increased over time until 7 weeks after inoculation and was therefore likely to reflect tumor progression. We therefore propose that this orthotopic esophageal cancer model, monitored using the non-invasive semi-quantitative IVIS imaging system, will be useful for in vivo therapeutic experiments against esophageal cancer. This experimental setting is expected to contribute to the development of novel therapeutic technologies for esophageal cancer in preclinical studies.