Antitumor effects of cytoplasmic delivery of an innate adjuvant receptor ligand, poly(I:C), on human breast cancer

Touko Inao[1,2], Nanae Harashima[1], Hiroyuki Monma[1,2], Shinji Okano[3], Masayuki Itakura[2], Tsuneo Tanaka[2], Yoshitsugu Tajima[2], Mamoru Harada[1]

[1] Department of Immunology and [2] Department of Surgery, Shimane University Faculty of Medicine, Shimane, Japan

[3] Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Running title: Antitumor effects of poly(I:C) transfection on breast cancer

Correspondence: Mamoru Harada, MD, PhD, Department of Immunology, Shimane University Faculty of Medicine, Izumo, Shimane 693-8501, Japan.

TEL & FAX; +81-853-20-2150, E-mail; haramamo@med.shimane-u.ac.jp

Abstract

Innate adjuvant receptors are expressed in immune cells and some types of cancers. If antitumor therapies targeting these receptors are established, it is likely that they will be therapeutically beneficial because antitumor effects and immune-cell activation can be induced simultaneously. In this study, we tested this possibility of using an innate adjuvant receptor ligand, polyinosinic-polycytidylic acid [poly(I:C)], to treat human breast cancer cell lines. Three breast cancer cell lines (MCF-7, MDA-MB-231, and BT-549) were utilized in this study. Poly(I:C) was transfected into these cancer cells to stimulate melanoma differentiation-associated gene (MDA) 5, which is a cytoplasmic adjuvant receptor. Poly(I:C) transfection significantly reduced the viability of all cell lines in a manner partially dependent on MDA5. Flow cytometeric analyses and immunoblot assays revealed that the antitumor effect depended on both caspase-dependent apoptosis and c-Myc- and cyclinD1-dependent growth arrest. Interestingly, poly(I:C) transfection was accompanied by autophagy, which is thought to protect cancer cells from apoptosis after poly(I:C) transfection. In a xenograft mouse model, local transfection of poly(I:C) significantly inhibited the growth of xenografted MDA-MB-231 cells. Our findings indicate that cytoplasmic delivery of poly(I:C) can induce apoptosis and growth arrest of human breast cancer cells, and that therapy-associated autophagy prevents apoptosis. The results of this study suggest that innate adjuvant receptors are promising targets and that their ligands could serve as antitumor reagents, which have the potential to simultaneously induce antitumor effects and activate immune cells.

Keywords: apoptosis, growth arrest, poly(I:C), MDA5, autophagy

Abbreviations:

CFSE	Carboxyfluorescein diacetate, succinimidyl ester
DC	dendritic cell
IFN	interferon
MDA	melanoma differentiation-associated gene
PI	propidium iodide
poly(I:C)	polyinosinic-polycytidylic acid
siRNA	small interfering RNA
TLR	Toll-like receptor
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt

Introduction

Innate adjuvant receptors, including toll-like receptors (TLRs), play a crucial role in many aspects of immune response [1]. These receptors are broadly expressed in immune cells, particularly in antigen-presenting cells, and recognize pathogen-associated molecular patterns such as lipopolysaccharide, viral double-stranded RNA, and unmethylated CpG islands. The initiation of adjuvant receptor signaling induces the release of inflammatory cytokines, the maturation of dendritic cells (DCs), and the activation of adaptive immunity [1]. Recent studies have revealed that signaling through TLRs influences the outcome of cancer-bearing hosts after anticancer therapies. When cancer-bearing hosts are treated with chemotherapy or radiotherapy, cancer cells undergoing cell death secrete high-mobility group box 1 as a danger signal to DCs, which blind to TLR4 or TLR2, resulting in the effective induction of antitumor T cell immunity [2, 3]. Signaling through TLR4 also affects the disease-free survival of human breast cancer patients after radiochemotherapy [2]. In addition, other recent reports have revealed that the immunological competence of cancer-bearing hosts is essential for better outcomes after anticancer therapies [4–6]. These lines of evidence imply that anticancer therapies should be conducted with careful attention to avoid the deterioration of immunological competence in cancer patients.

In addition to immune cells, various types of cancer cells have been reported to express functional innate adjuvant receptors, and the majority of these studies has been focused on TLRs. Lipopolysaccharide, a TLR4 ligand, activates TLR4-expressing human head and neck squamous cell carcinoma, ovarian cancer cells, and lung cancer cells to increase resistance to anticancer therapies [7–9]. TLR9 signaling can suppress tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in several types of cancers [10]. TLR5 signaling by flagellin can suppress the proliferation of human breast cancer cell lines

[11]. Among a panel of TLRs, TLR3 has been suggested to be therapeutically useful. TLR3 may be a potential therapeutic target in clear-cell renal cell carcinoma and melanoma [12, 13] and TLR3 signaling triggers apoptosis in human breast and prostate cancer cells [14]. In addition, it has been reported that TLR3 expression in human breast cancer tissues may be a biomarker for the therapeutic efficacy of adjuvant treatment with double-stranded RNA [15].

Polyinosinic-polycytidylic acid [poly(I:C)], which is a ligand for endosomal adjuvant receptor TLR3, can induce the expression of inflammatory cytokines and type I interferon (IFN) through the NF-κB, MAPK, and IFN regulatory factor 3 pathways [16], thus enhancing the antitumor immune responses. Additionally, poly(I:C)-induced type I IFN can augment antitumor immune surveillance while mitigating regulatory T cells in tumor-bearing hosts [15]. Alternatively, poly(I:C) can be a ligand for melanoma differentiation-associated gene (MDA) 5, which is another cytoplasmic adjuvant receptor [17]. In human melanoma, transfection of poly(I:C) into the cytoplasm can induce cell death through MDA5-mediated signaling [18, 19]. A previous study has shown that, poly(I:C) transfection is accompanied by autophagy [18] , which has been found to function cytoprotectively under starvation and stress conditions [20]. However, the precise roles of autophagy in treatment-associated cancer cell death have not yet been fully elucidated.

In the present study, we explored whether delivery of poly(I:C) into the cytoplasm of three human breast cancer cell lines could induce antitumor effects. Our results show that poly(I:C) transfection induced apoptosis and growth arrest in all examined breast cancer cell lines. We also found that autophagy played a cytoprotective role in apoptosis of human breast cancer cells after poly(I:C) transfection. Considering the potent agonistic effects on the immune system, our results suggest that innate adjuvant receptor ligands may be therapeutically useful reagents for simultaneously inducing antitumor effects and activating

the immune system.

Materials and Methods

Cell lines. Three human breast cancer cell lines (MCF-7, MDA-MB-231, and BT-549), which were kindly provided by Dr. K. Takenaga (Shimane University Faculty of Medicine), were maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Invitrogen, Grand Island, NY, USA) and 20 μg/ml gentamicin (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability assay. Cell viability was measured using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay (Nacalai Tesque, Kyoto, Japan). Briefly, cells were seeded in flat-bottomed 96-well plates. The next day, poly(I:C) (InvivoGen, San Diego, CA, USA) was added or transfected using X-tremeGENE transfection reagent (4.2 μl/ml; Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Two days later, WST-8 was added to each well, and plates were read at a wavelength of 450 nm after 3 h. For inhibition assays, z-VAD-fmk (R&D Systems, Minneapolis, MN, USA) was added at the time of poly(I:C) addition.

Flow cytometric analysis. Apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) and propidium iodide (PI). Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Fullerton, CA, USA). Expression of Ki-67 was examined using a Ki-67 staining kit (BD Biosciences, San Jose, CA, USA). Cell proliferation was estimated using a CellTrace™ carboxyfluorescein diacetate, succinimidyl ester (CFSE) Cell Proliferation Kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's procedure.

Immunoblot assay. Cells were lysed with a mammalian protein extraction reagent (M-PER; Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Nacalai Tesque). Equal amounts of protein were resolved on 4–12% gradient or 12% SDS-PAGE gels, followed by transfer to polyvinylidene fluoride membranes. After blocking membranes, blots were incubated with the indicated primary antibodies: anti-TLR3 (AnaSpec, San Jose, CA, USA), anti-MDA5 (ProSci Inc., Poway, CA, USA), RIG-I [Cell Signaling Technology (CST), Danvers, MA, USA], anti-caspase-3 (CST), anti-caspase-7 (CST), anti-caspase-8 [Medical Biological Laboratories Co., Ltd. (MBL), Nagoya, Japan], anti-caspase-9 (MBL), anti-LC3 (MBL), anti-yclinD1 (CST), anti-c-Myc (Epitomics, Burlingame, CA, USA), anti-$p21^{Waf1/Cip1}$ (CST), anti-$p27^{kip1}$ (CST), beclin-1 (CST), anti-β-actin (BioLegend, San Diego, CA, USA), and anti-α-tubulin [Santa Cruz Biotechnology (SCB), Santa Cruz, CA, USA]. Goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Invitrogen) were used to detect the primary antibodies.

Transfection of small interfering RNA (siRNA). Transfection of siRNA was performed using Lipofectamine^{TM} RNAiMAX (Invitrogen) according to the manufacturer's instructions. TLR3 siRNA (sc-36685), MDA5 siRNA (sc-61010), and Beclin-1 siRNA (sc-29797) were purchased from SCB. Control siRNA (#6568) was purchased from CST. Three days after siRNA transfection, cancer cells were used for the subsequent experiments.

Confocal imaging. LC3B (NM_022818) was amplified by PCR and inserted into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) in-frame with the GFP sequence. Transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were cultured on round microscope cover glasses in 24-well plates with the indicated reagents for 2 d. After incubation with Hoechst 33342 (5 μg/ml) for 30 min, cells were fixed with 3% formalin and placed on slide glasses with 4 μl

mounting medium for fluorescence (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA). Confocal imaging was performed using an Olympus FV1000-D laser scanning microscope (Olympus, Tokyo, Japan).

***In vivo* xenograft model.** BALB *nu/nu* female mice, purchased from CLEA Japan Inc. (Tokyo, Japan), were kept under specific pathogen-free conditions. Experiments were performed according to the ethical guidelines for animal experimental of the Shimane University Faculty of Medicine (approval number: IZ23-70). Mice were inoculated with MDA-MB-231 (5×10^6 cells) into the right mammary fat pad. On day 42, the mice were pooled and divided into four groups. For *in vivo* transfection, *in vivo*-jetPEI^{TM} transfection reagent (Polyplus-Transfection Inc., New York, NY, USA) was used according to the manufacturer's protocol. On days 1, 4, 8, and 11 after grouping, the mice were intratumorally injected with 5% glucose (control), poly(I:C) (10 μg/mouse), *in vivo*-jetPEI (1.4 μl/mouse) in 5% glucose), or poly(I:C) mixed with *in vivo*-jetPEI in a volume of 50 μl. Thereafter, tumor size was measured twice weekly.

Statistical analyses. Data were statistically evaluated using unpaired two-tailed Student's *t*-tests. A p-value < 0.05 was considered to be statistically significant.

Results

MDA5-dependent antitumor effect of poly(I:C) transfection on human breast cancer cells

First, we examined the expression of innate adjuvant receptors, including TLR3 and MDA5, which can be stimulated by poly(I:C) (Fig. 1A and supplementary data). Three breast cancer cell lines expressed both of these receptors. Because MDA5 belongs to the RIG-I family, we also examined the expression of RIG-I in these cell lines and found that

only MDA-MB-231 cells were positive for this receptor. Next, we examined the viability of human breast cancer cells following the addition of poly(I:C) or its delivery into their cytoplasm by transfection (Fig. 1B). Although a low level of antitumor effects was observed in both MCF-7 and BT-549 and a slight increase was observed in the cell viability of MDA-MB-231 when these cells were cultured with poly(I:C), poly(I:C) transfection drastically decreased the cell viability of the three cell lines in a dose-dependent manner. We further confirmed this finding using RNA interference. The protein expression of either TLR3 or MDA5 was selectively knocked down by transfection of TLR3 siRNA and MDA5 siRNA, respectively (Fig. 1C). Knockdown of MDA5, but not TLR3, partially but significantly restored the viability of all cell lines (Fig. 1D), indicating that the MDA5 was at least partially responsible for the antitumor effects following poly(I:C) transfection.

Caspase-dependent apoptosis of poly(I:C)-transfected breast cancer cells

Next, we observed morphological changes of breast cancer cell lines after poly(I:C) transfection (Fig. 2). Although treatment with either poly(I:C) or transfection reagent alone induced no morphological change, poly(I:C) transfection resulted in dramatic cell destruction and shrinkage, suggesting that poly(I:C) induced apoptosis. This finding suggests that apoptosis participated in the antitumor effects following poly(I:C) transfection. Although the addition of poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, 1000 ng/ml for MDA-MB-231 cells) or transfection reagent alone produced no change in the percentage of Annexin V^+ cells, poly(I:C) transfection significantly increased the percentages of PI^- Annexin V^+ (early apoptosis) and PI^+ Annexin V^+ (late apoptosis) cells of all breast cancer cell lines (Fig. 3A and B). The addition of z-VAD, a pan-caspase inhibitor, also significantly reduced the percentage of Annexin V^+ cells in three cell lines after the poly(I:C) transfection (Fig. 3C). Furthermore, apparent cleavages of caspase-3 and caspase-9 were detected in

both MDA-MB-231 and BT-549 cells (Fig. 3D). In contrast, caspase-3 was negative in MCF7, as previously reported [21], and cleaved caspase-8 and caspase-9 were faintly detected in poly(I:C)-transfected MCF-7 cells. However, cleaved caspase-7 was detected in poly(I:C)-transfected MCF-7 cells (Fig. 3E). These results indicate that caspase-dependent apoptosis participates in the antitumor effects on human breast cancer cells following poly(I:C) transfection.

Growth arrest of poly(I:C)-transfected breast cancer cells

In the studies described above, we examined the antitumor effects of breast cancer cells by measuring the viability 2 d after poly(I:C) transfection. Thus, changes in cell viability could reflect not only cell death but also cell growth. Therefore, we evaluated whether poly(I:C) transfection affected the proliferative capacity of surviving cancer cells. As shown in Fig. 4A, the expression level of Ki-67, a proliferation-related nuclear protein, in MCF-7 and MDA-MB-231 cells decreased after poly(I:C) transfection. The expression of Ki-67 in untreated BT-549 cells was relatively low and was further reduced following transfection of poly(I:C). We also directly examined the effect of poly(I:C) transfection on cell proliferation. In contrast to the ineffectiveness of poly(I:C) alone, poly(I:C) transfection decreased the proliferative capacity of three CFSE-labeled breast cancer cell lines (Fig. 4B). Additionally, in an immunoblot assay, poly(I:C) transfection resulted in decreased expression of c-Myc in all cell lines, and decreased expression of cyclinD1 in MCF-7 and MDA-MB-231 cells (Fig. 4C). Alternatively, poly(I:C) transfection increased the expression of $p21^{Waf1/Cip1}$ in BT549 cells, but resulted in slightly decreased expression of $p27^{Kip1}$ in MDA-MB-231 cells. Despite the slight decreases in the expression of CyclinD1 in MDA-MB-231 cells and $p21^{Waf1/Cip1}$ in BT-549 cells, the effects of poly(I:C) on these proteins were minimal (Fig. 4C). In total, these findings indicate that the proliferative

capacity of breast cancer cells that survived following poly(I:C) transfection was suppressed mainly through the inhibition of c-Myc and cyclinD1, and through the increased expression of $p21^{Waf1/Cip1}$.

Protective role of autophagy in apoptosis after poly(I:C) transfection

Recently, autophagy has received much attention in various fields of cell biology [22], and a recent report revealed that autophagy is induced in poly(I:C)-transfected human melanoma cells [18]. Thus, we explored the possibility that autophagy played a role in our experimental system. LC3 exists in two forms, LC3-type I, which is cytosolic, and its proteolytic derivative LC3-type II, which localizes to the autophagosomal membrane [23]. Poly(I:C) transfection increased expression of LC3-type II in MCF-7, whereas both MDA-MB-231 and BT-549 cell lines were positive for LC3-type II without poly(I:C) transfection (Fig. 5A). In MCF-7 cells, LC3-type II was induced after poly(I:C) transfection in a time-dependent manner (Fig. 5B). We also assessed autophagy using confocal imaging of LC3 foci in GFP-LC3 fusion protein-expressing cancer cells (Fig. 5C). The three cell lines were transiently transfected with the plasmid encoding GFP-LC3, and expression of GFP-LC3 foci was subsequently examined. Although no GFP-LC3 foci were detected in GFP/NT-transfected MCF-7 cells, faint but definite GFP-LC3 foci were detected in some (approximately one-third) GFP-LC3-transfected MCF-7 cells without poly(I:C) transfection. Upon transfection of GFP-LC3-expressing MCF-7 cells with poly(I:C) at a high dose of 500 ng/ml, GFP-LC3 foci were detected in the cytoplasm. In GFP/NT-transfected MCF-7 cells, GFP-LC3 foci were not observed and GFP proteins that were scattered from destroyed cancer cells were observed. When GFP-LC3-expressing MCF-7 cells were transfected with poly(I:C) at a low dose of 100 ng/ml, GFP-LC3 foci were detected in the cytoplasm. In MDA-MB-231 and BT-549 cells, GFP-LC3 foci were

observed in the majority of cells and without poly(I:C) transfection, consistent with the results of LC3-type II immunoblots (Fig. 5A).

We further attempted to determine the role of autophagy in poly(I:C)-transfected breast cancer cells. We then examined the effect of knocking down beclin-1, which is essential for autophagy [24], on apoptosis of breast cancer cells after poly(I:C) transfection. Transfection of beclin-1 siRNA selectively knocked down the expression of beclin-1 protein in the three cell lines (Fig. 6A). The knockdown of beclin-1 significantly increased the percentage of Annexin V^+/PI^+ (late apoptotic) cells in all breast cancer cell lines after poly(I:C) transfection, whereas an increase in the percentage of Annexin V^+/PI^- (early apoptotic) cells by beclin-1 knockdown was observed only in MDA-MB-231 cells (Fig. 6B and C). These results indicate that constitutively expressing or inducible autophagy plays a protective role in apoptosis of breast cancer cells after poly(I:C) transfection.

In vivo antitumor effect of poly(I:C) transfection in a xenograft mouse model

Next, we evaluated whether poly(I:C) transfection exerted an antitumor effect against established human breast cancer in a xenograft mouse model (Fig. 7). Nude mice were inoculated with MDA-MB-231 cells into the mammary fat pad, and the local injection of either poly(I:C), *in vivo*-jetPEI transfection reagent, or poly(I:C) mixed with *in vivo*-jetPEI transfection reagent [i.e., *in vivo* poly(I:C) transfection] was performed on days 1, 4, 8, and 11 after grouping. Although the local injections of poly(I:C) or transfection reagent alone moderately decreased tumor growth, mice that received *in vivo* poly(I:C) transfections significantly suppressed tumor growth compared with the other three groups. These results suggest that poly(I:C) transfection can inhibit the growth of established human breast cancer *in vivo*.

Discussion

Recent reports have revealed that the immunological competence of cancer-bearing hosts is essential for better outcomes after anticancer therapies [4-6], suggesting that anticancer therapies should be conducted with careful attention to avoid deterioration of immunological competence in cancer patients. In this study, we demonstrated that poly(I:C) transfection induced antitumor effects (i.e., apoptosis and growth arrest) in human breast cancer cells while activating DCs, which play an important role in innate and adaptive immunity against cancer [25, 26]. The efficacy of this strategy was also confirmed using a xenograft mouse model. These data indicate that, in addition to being a ligand for innate adjuvant receptors, poly(I:C) has the potential to exert antitumor effects on breast cancer cells.

Poly(I:C) is double-stranded RNA, with the ability to modulate immune responses. Poly(I:C) activates professional antigen-presenting cells, such as DCs [16, 27] which secrete type I IFN, resulting in the effective activation of NK cells [28]. Before the identification of TLR3 and MDA5 as endosome and cytoplasmic receptors of poly(I:C), respectively, poly(I:C) transfection had been reported to induce cell death in a panel of human cancer cell lines [29]. Notably, recent reports revealed that transfection of poly(I:C) into human melanoma cells could induce cell death through the cytosolic poly(I:C) receptor, MDA5 [18, 19]. In addition, poly(I:C) transfection was reported to induce apoptosis in human hepatoma cells [30]. While testing the antitumor effects of poly(I:C) against several types of human cancer cell lines, we found that poly(I:C) transfection could induce antitumor effects against human breast cancer cells. Therefore, we undertook this study.

In this study, we evaluated whether TLR3 or MDA5 was responsible for the antitumor effects in poly(I:C)-transfected breast cancer cells. We showed that the selective knockdown

of MDA5, but not TLR3, partially restored the viability of poly(I:C)-transfected cancer cells (Fig. 1D). In addition, we recently reported that TLR3-mediated signaling by poly(I:C) can induce apoptosis and growth arrest in human prostate cancer LNCaP cells and that the selective knockdown of TLR3 restored their decreased viability [31]. Given that the selective knockdown of TLR3 did not recover the decreased viability of three breast cancer cell lines transfected with poly(I:C), TLR3-mediated signaling does not appear to induce antitumor effects on human breast cancer cell lines. In MDA5 knockdown cells, there was incomplete recovery of cancer cell viability. We have no clear explanation for these results, but it should be considered that siRNA is double-stranded RNA and that consecutive transfection of poly(I:C) and siRNA may confound the results. Currently, we cannot exclude the possibility that other receptors or mechanisms are involved in the antitumor effects following poly(I:C) transfection.

The addition of poly(I:C) induced fluctuating effects on three cell lines (Fig. 1B): a suppressive effect on both MCF-7 and BT-549, and a stimulatory effect on MDA-MB-231. We observed by immunoblot anlyssis that co-incubation of these three cancer cell lines with poly(I:C) increased nuclear NF-κB expression (data not shown), which suggests that MDA-MB-231 cells were stimulated by activation of the NF-κB pathway. However, the reason for the suppression of both MCF-7 and BT-549 cells by the addition of poly(I:C) is unknown. Although we recently reported that poly(I:C) induces apoptosis and growth arrest in the human prostate cancer cell line LNCaP [31], we did not observe apoptosis and growth arrest in MCF-7 and BT-549 cells cultured with poly(I:C) (Fig. 3 and 4).

Poly(I:C) transfection showed dual antitumor effects on human breast cancer cells, including apoptosis and growth arrest. Regarding apoptosis, caspase activation seemed to explain poly(I:C) transfection-induced apoptosis. Poly(I:C) transfection induced cleavage of both caspase-3 and caspase-9 in MDA-MB-231 and BT-549 cells. Although MCF-7 lacks

caspase-3, cleaved caspase-7 and caspase-8 were observed (Fig. 3D and E). Regarding the underlying mechanism of apoptosis, we found no definitive change in the expressions of pro-apoptotic p53 and anti-apoptotic Bcl-2 following poly(I:C) transfection (data not shown). In contrast, poly(I:C) transfection induced growth arrest in the three cell lines. We examined cell cycle-related molecules and found that the expression of c-Myc apparently decreased in the three cell lines after poly(I:C) transfection. The decreased expression of cyclinD1 after poly(I:C) transfection was observed in MCF-7 and MDA-MB-231 cells. In contrast, the expression of $p21^{Waf1/Cip1}$, which is a negative regulator of the cell cycle [32], was upregulated in BT-549 cells. Our results are compatible with the growth arrest of breast cancer cell lines mainly through the decreased expression of c-Myc and cyclinD1, and through the increased expression of $p21^{Waf1/Cip1}$.

Autophagy has received much attention in various fields of cell biology [22]. Because autophagy works primarily as a cytoprotective mechanism under starvation and stress conditions [20], we explored the possibility that autophagy participated in our experimental system. LC3 type-II was observed faintly in untreated MCF-7 cells, but was significantly augmented after poly(I:C) transfection. In contrast, autophagy was constitutively observed in both MDA-MB-231 and BT-549 without poly(I:C) transfection. The formation of GFP-LC3 foci was observed in GFP-LC3-transfected breast cancer cells using confocal imaging. We also investigated the role of autophagy in apoptosis after poly(I:C) transfection by selectively blocking autophagy (Fig. 6). A representative result was observed in MDA-MB-231 cells. Knockdown of beclin-1, a molecule essential for autophagy [24], promoted early and late apoptosis of MDA-MB-231 cells after poly(I:C) transfection. In addition, late apoptosis was augmented in both MCF-7 and BT-549 cells after poly(I:C) transfection. Despite the primary role of autophagy as a cytoprotectant, many reports have described autophagic cell death [33–35]. Moreover, the relationship between autophagy and

cell death has been controversial [36]. However, we suppose that, at least within our experimental system, autophagy serves a cytoprotective function. Nevertheless, it is unknown how autophagy inhibits poly(I:C)-induced apoptosis of breast cancer cells. Interestingly, a recent report revealed that cancer cells showed resistance to extrinsic death signal-mediated apoptosis through autophagic degradation of active caspase-8 [37]. In addition, mitochondrial autophagy has been reported to protect human cancer cells from heat shock-induced intrinsic apoptosis by reducing cytosolic cytochrome c release and downstream caspase-3 activation [38]. These reports suggest that autophagy exerts an inhibitory effect on apoptosis through inhibition of caspase activation. We are now attempting to test this possibility.

In the present study, we investigated whether local transfection of poly(I:C) induced antitumor effects against human breast cancer cells using a xenograft mouse model. Compared with poly(I:C) or transfection reagent alone, poly(I:C) transfection significantly suppressed tumor growth of MDA-MB-231 cells. The marginal antitumor effects observed after local poly(I:C) injections might have been due to the stimulatory effects of poly(I:C) on the expression of TLR3 in murine immune cells. Activated innate murine immune cells, including natural killer cells and macrophages, may be cytotoxic toward human breast cancer cells. In addition, we must consider that the addition of poly(I:C) stimulated MDA-MB-231 cells compared with the two other cell lines (Fig. 1B). In the xenograft model (Fig. 7), we locally injected poly(I:C) mixed with the *in vivo*-jetPEI transfection reagent, but it is possible that MDA-MB-231 cells were stimulated by free poly(I:C). Therefore, the plateau size of MDA-MB-231 cells in mice treated by poly(I:C) transfection may be a balance between stimulation by free poly(I:C) and cell death by poly(I:C) transfection. Further studies are required to elucidate the mechanism.

Disclosures: None

Acknowledgements: We thank Ms. Yasuko Moriyama for her technical assistance. This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 18591449 to M. H., no. 23701074 to N.H.).

References

1. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Ann Rev Immunol 21:335-376
2. Apetoh L, Ghiringhelli F, Tesniere A et al (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13:1050-1059
3. Curtin JF, Liu N, Candolfi M, et al (2009) HMGB1 mediates endogenous TLR2 activation and brain tumor regression. PLoS Med 13:6:e10
4. Park SG, Jiang Z, Mortenson ED et al (2010) The therapeutic effect of anti-HER2/neu antibody dependes on both innate and adaptive immunity. Cancer Cell 18:160-170
5. Bumette BC, Liang H, Lee Y et al (2011) The efficacy of radiotherapy relies upon induction of type I interferon-dependent innate and adaptive immunity. Cancer Res 71:2488-2496
6. Stagg J, Loi S, Divisekara U et al (2011) Anti-ErbB-2 mAb therapy requires type I and II interferins and synergizes with anti-PD-1 or anti-CD137 mAb therapy. Proc Natl Acad Sci U S A 108:7142-7147
7. Kelly MG, Alvero AB, Chen R et al (2006) TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 66:3859-3868
8. Szczepanski MJ, Czystowska M, Szajnik M et al (2009) Triggering of toll-like receptor 4 expressed on human head and squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. Cancer Res 69:3105-3113
9. He W, Liu Q, Wang L, Chen W, Li N, Cao X (2007) TLR4 signaling promotes immune escapes of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. Mol Immunol 44:2850-2859
10. Chiron D, Pellat-Deceunynck C, Maillasson M, Bataille R, Jego G (2009)

Phosphorothioate-modified TLR9 ligands protect cancer cells against TRAIL-induced apoptosis. J Immunol 183:4371-4377

11. Cai Z, Sanchez A, Shi Z, Zhang T, Liu M, Zhang D (2011) Activation of toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth. Cancer Res 71:2466-2475
12. Morikawa T, Sugiyama A, Kume H et al (2007) Identification of toll-like receptor 3 as a potential therapeutic target in clear renal cell carcinoma. Clin Cancer Res 13:7503-7509
13. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P (2007) Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin Cancer Res 13:4565-4574
14. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T (2006) TLR3 can direcetly rigger apoptosis in human cancer cells. J Immunol 176:4894-4901
15. Salaun B, Zitvogel L, Asselin-Paturel C et al (2011) TLR3 as a biomarker for the therapeutic efficacy of double-stranded RNA in breast cancer. Cancer Res 71:1607-1614
16. Matsumoto M, Seya T (2008) TLR3: interferon induction by double-strand RNA including poly(I:C). Adv Drug Delivery Rev 60:805-812
17. Kato H, Takeuchi O, Sato S et al (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441:101-105
18. Tormo D, Checinska A, Alonso-Curbelo D et al (2009) Targeted activation of innate immunity for therapeutic induction of autophagy and apoptosis in melanoma cells. Cancer Cell 16:103-114
19. Besch R, Poeck H, Hohenauer T et al (2009) Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human

melanoma cells. J Clin Invest 119:2399-2411

20. Gewirtz DA (2009) Autophagy, senescence and tumor dormancy in cancer therapy. Authophagy 5:1232-1234

21. Buckley CD, Pilling D, Henriquez NV et al (1999) RGD peptides induce apoptosis by direct caspase-3 activation. Nature 397:534-539

22. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of diseases. Cell 132:27-42

23. Kabeya Y, Mizushima N, Ueno T et al (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19:5720-5728

24. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402:672-676

25. Ghiringhelli F, Apetoh L, Housseau F, Kroemer G, Zitvogel L (2007) Links between innate and cognate tumor immunity. Curr Opin Immunol 19:224-231

26. Ullrich E, Menard C, Flament C et al (2008) Dendritic cells and innate defense against tumor cells. Cytokine Growth Factor Rev 19:79-92

27. Barchet W, Wimmenauer V, Schlee M, Hartman G (2008) Accessing the therapeutic potential of immunostimulatory nucleic acids. Curr Opin Immunol 20:389-395

28. Ebihara T, Azuma M, Oshiumi H et al (2010) Identification of polyI;C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 207:2675-2687

29. Hirabayashi K, Yano J, Inoue T et al (1999) Inhibition of cancer cell growth by polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity. Cancer Res 59:4325-4333

30. Peng S, Geng J, Sun R, Tian Z, Wei H (2009) Polyinosinic-polycytidylic acid liposome induces human hepatoma cells apoptosis which correlates to the up-regulation of RIG-I like receptors. Cancer Sci 100:529-536

31. Harashima N, Inao T, Imamura R, Okano S, Suda T, Harada M (2012) Roles of the PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer cells. Cancer Immunol Immunother, in press

32. Muise-Hemericks RC, Grimes H, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N (1998) Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Ae-depndent pathway. J Biol Chem 273:29864-29872

33. Akar U, Chaves-Reyz A, Barrio M et al (2008) Silencing Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. Autophagy 4:669-679

34. Mujumdar N, Mackenzie TN, Dudeja V et al (2010) Triptolide induces cell death in pancreatic cancer cells by apoptotic and autophagic pathways. Gastroenterology 139:598-608

35. Bonapace L, Bornhauser BC, Schmitz M et al (2010) Induction of autophagy-dependent necrosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest 120:1310-1323

36. Dalby KN, Takedareli I, Lopez-Berestein G, and Ozpolat B (2010) Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy 6:322-329

37. Hou W, Han J, Lu C, Goldstein LA, Rabinowich H (2010) Autophagic degradation of active caspase-8. a crosstalk mechanism between autophagy and apoptosis. Autophagy 6:891-900

38. Yang Y, Xing D, Zhou F, Chen Q (2010) Mitochondrial autophagy protects against

heat shock-indcued apoptosis through reducing cytosolic cytochrome c release and downsteam caspase-3. BBRC 395:190-195

Figure legends

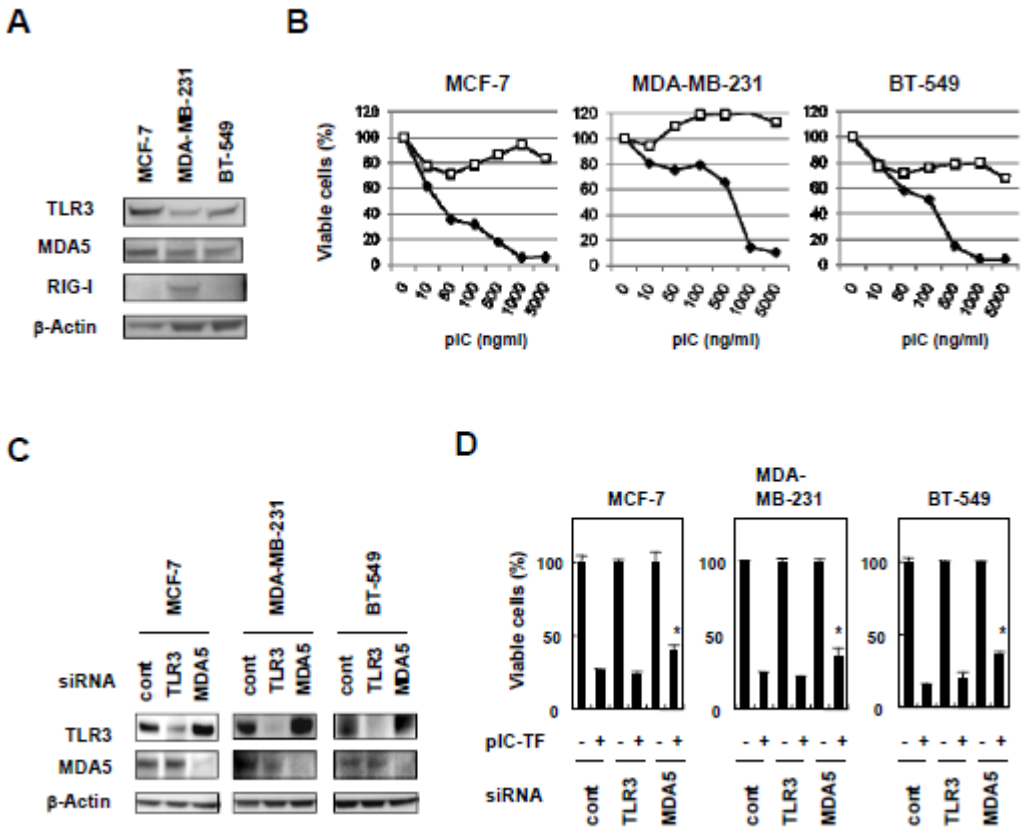

Figure 1 MDA5-dependent antitumor effect of poly(I:C) transfection on three breast cancer cell lines. (A) Immunoblot assays were performed to assess the expression of TLR3, MDA5, or RIG-I in three cell lines. (B) Three cell lines were cultured (open square) or transfected (closed diamond) with poly(I:C) at the indicated doses using X-tremeGENE transfection reagent (4.2 μl/ml). After 48 h, cell viability (%) was determined using the WST-8 assay. pIC, poly(I:C). (C) Three breast cancer cell lines were transfected with either TLR3, MDA5, or control siRNA using Lipofectamine RNAiMAX. After 3 d, cells were collected and immunoblot assays were performed. (D) siRNA-transfected breast cancer cell lines were transfected with or without poly(I:C) at doses of 62.5 ng/ml for MCF-7 cells, 125 ng/ml for MDA-MB-231 and BT-549 cells using X-tremeGENE transfection reagent. After

48 h, cell viability (%) was determined using the WST-8 assay. *$p < 0.05$ indicates statistical significance relative to cells that were transfected with control or TLR3 siRNA. pIC-TF, poly(I:C) transfection.

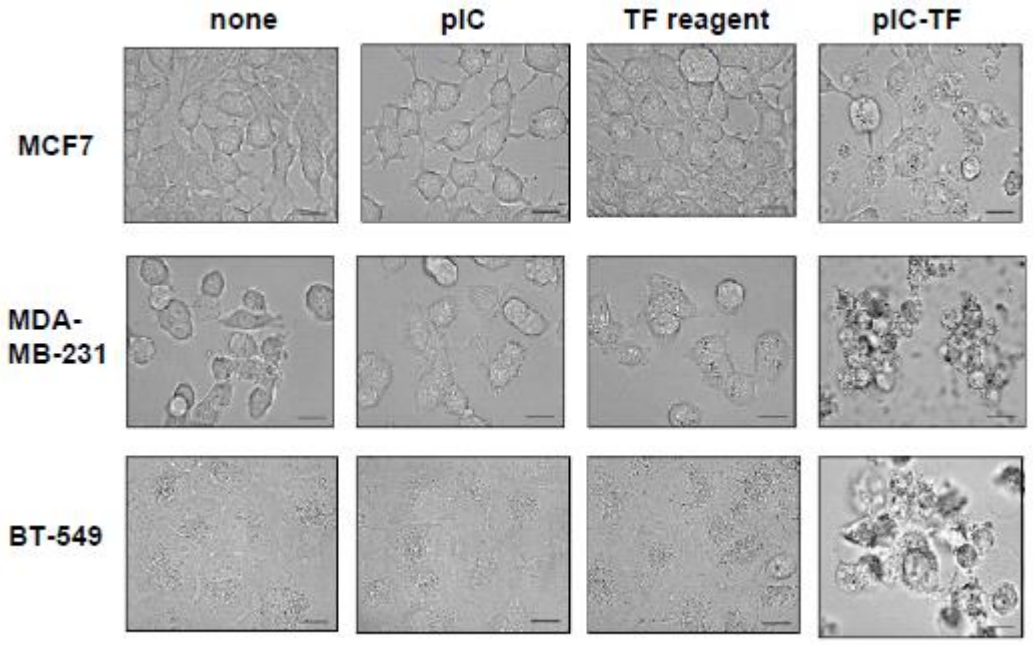

Figure 2　Morphological changes in breast cancer cell lines after poly(I:C) transfection. Three cancer cell lines were treated or transfected with or without X-tremeGENE transfection reagent and/or poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells). After 2 days, the treated cells were visualized microscopically. Scale bar; 20 μm. Magnification, ×60.

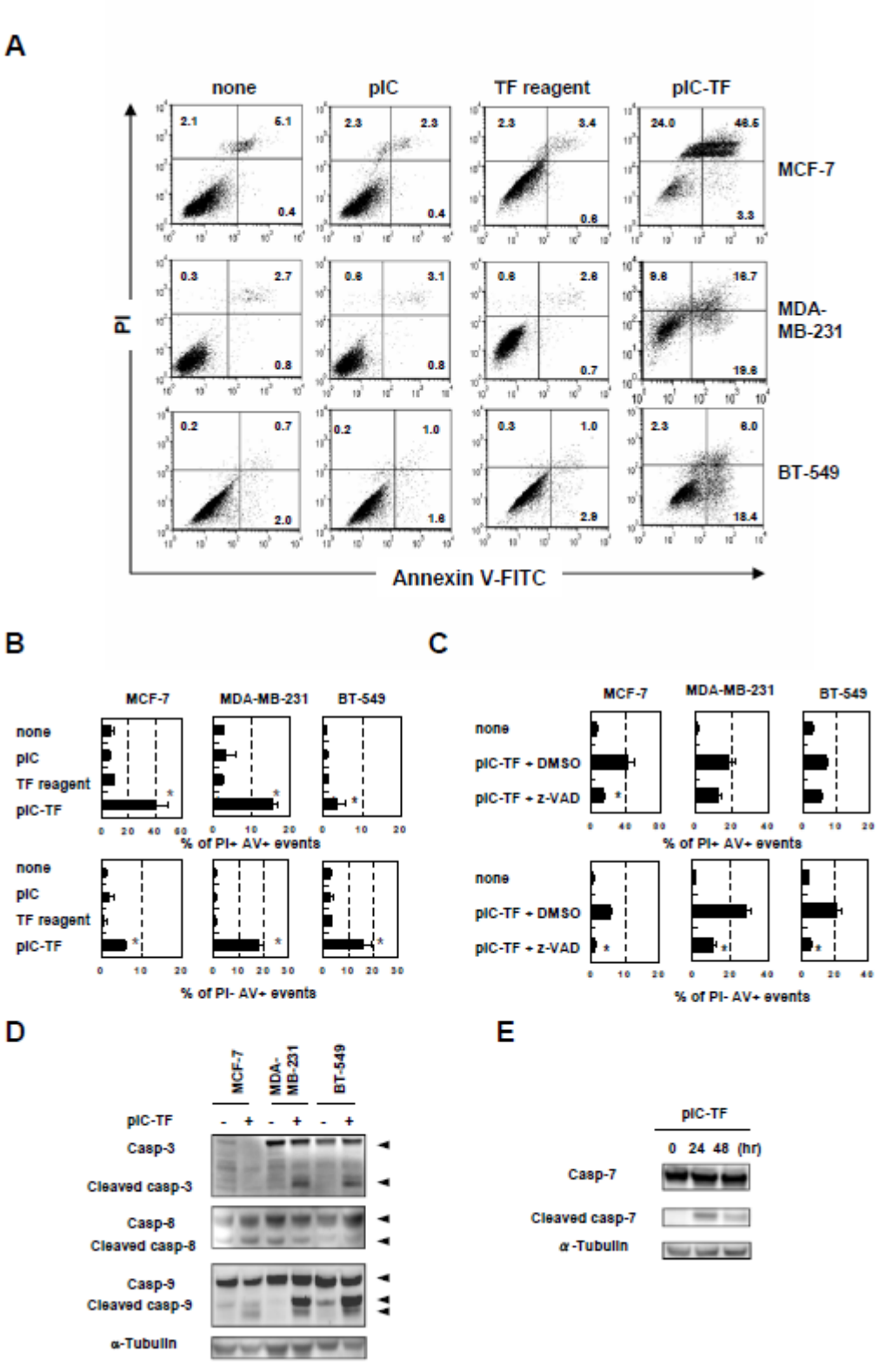

Figure 3 Caspase-dependent apoptosis of poly(I:C)-transfected breast cancer cells.

(A) Three cell lines were cultured or transfected with poly(I:C) (500 ng/ml for MCF-7 and

BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent (4.2 μl/ml). After 48 h, cells were stained with FITC-conjugated Annexin V and PI, and flow cytometry analysis was performed. The numbers represent the percentages of cells in each subset. pIC, poly(I:C); pIC-TF, poly(I:C) transfection. (B) Histogram summarizing the results of three flow cytometry experiments. *$p < 0.05$ indicates statistical significance. (C) A similar experiment was performed in the presence of DMSO or z-VAD (20 μM). The histogram summarizes the results of three flow cytometry experiments. *$p < 0.05$ indicates statistical significance. (D) Caspase expression was evaluated in three breast cancer cell lines, which were untreated or transfected with poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent. (E) MCF-7 cells were transfected with poly(I:C) (500 ng/ml) using X-tremeGENE transfection reagent and were examined for their expression of caspase-7.

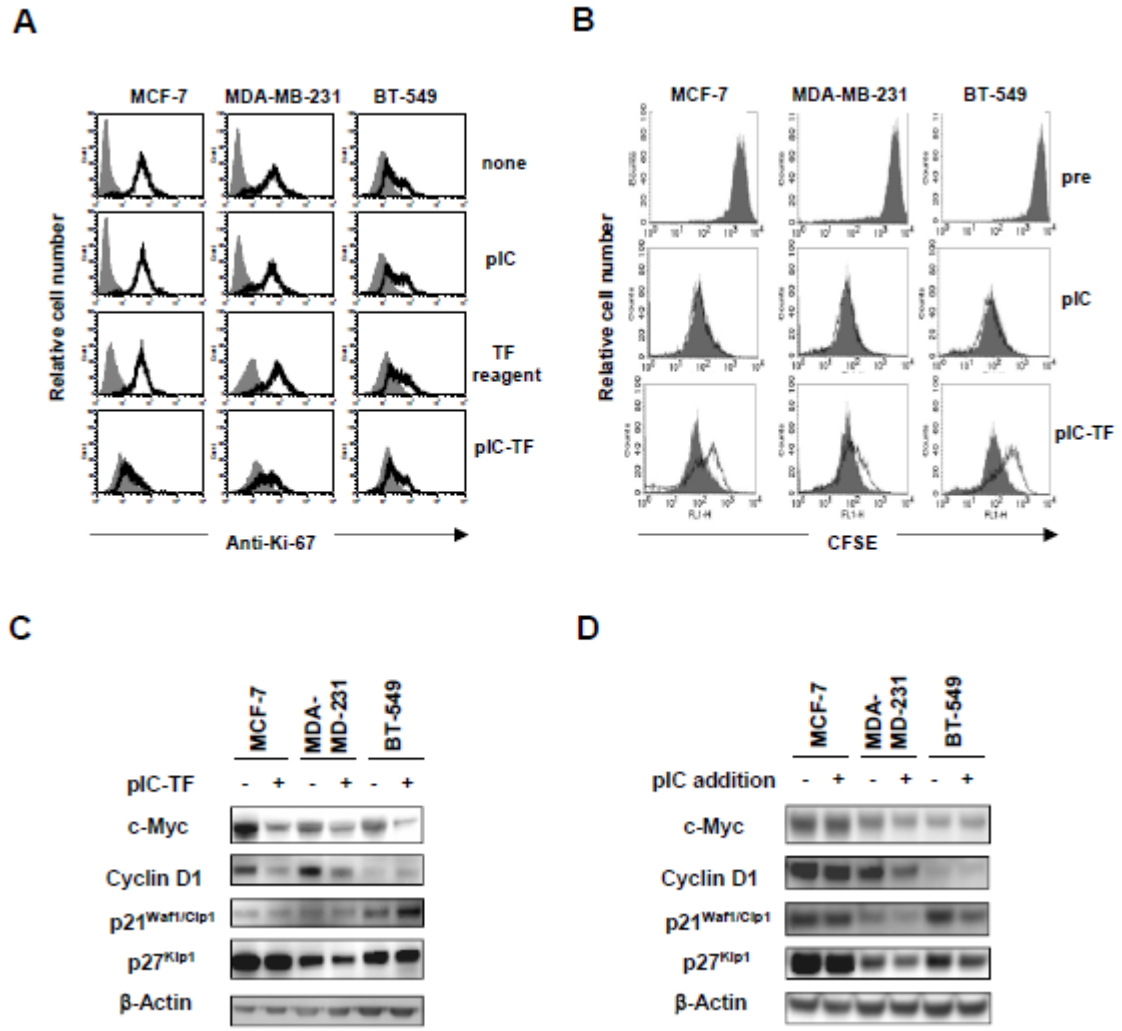

Figure 4 Decreased expression of proliferation-related protein levels in poly(I:C)-transfected breast cancer cells. (A) Three cell lines were cultured or transfected with poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent (4.2 μl/ml); and flow cytometry analysis was performed after staining with FITC-conjugated anti-Ki-67 antibodies. The gray shadow represents staining with an isotype-matched control antibody. pIC, poly(I:C); pIC-TF, poly(I:C) transfection. (B) The three cell lines, labeled with CFSE, were cultured or transfected with poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells; 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent. Two days (MCF-7) or four

days (MDA-MB-231 and BT-549) after treatment, flow cytometry analysis was performed. The gray shadow represents untreated control. (C and D) The expression of proliferation-related molecules in the three cell lines, transfected, using X-tremeGENE transfection reagent, or cultured with or without poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells; 1000 ng/ml for MDA-MB-231 cells) was examined using immunoblot analysis.

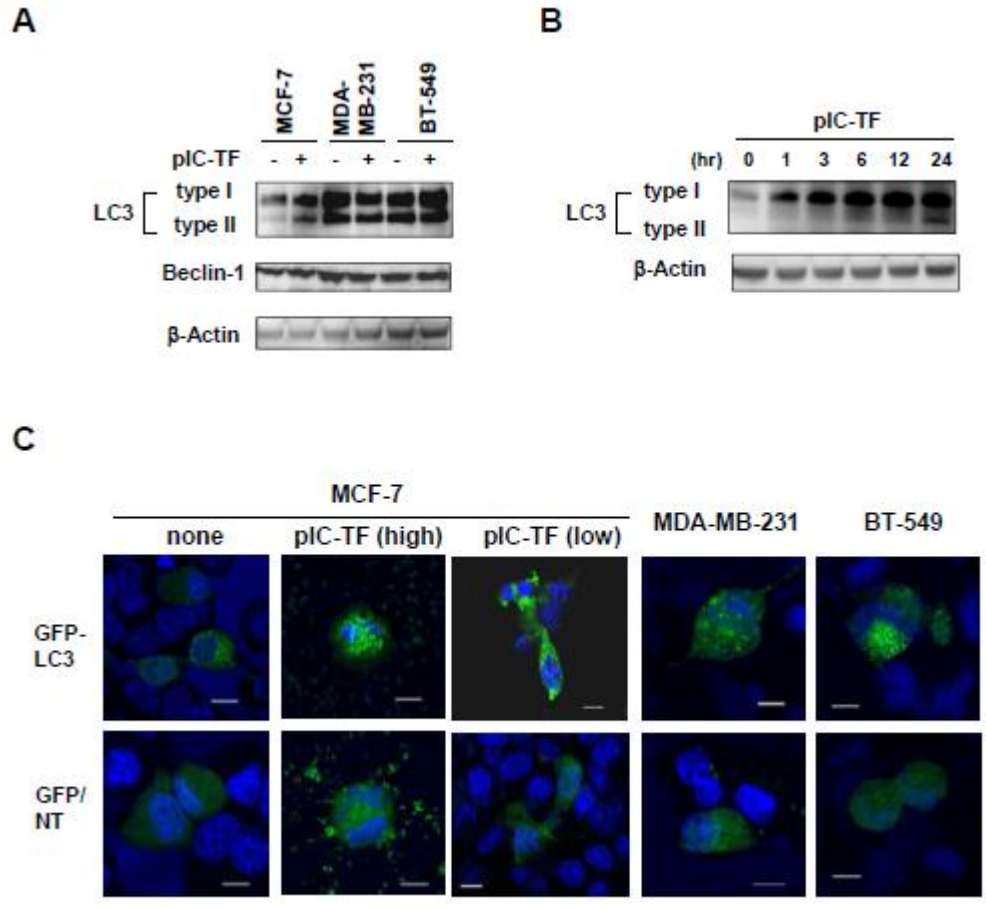

Figure 5 Autophagy in breast cancer cells after poly(I:C) transfection. (A) Three breast cancer cells were transfected with poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent (4.2 μl/ml),

and cultured for 48 h in the presence of pepstatin A (10 μg/ml). Thereafter, immunoblot analyses were performed. pIC-TF, poly(I:C) transfection. (B) MCF-7 cells were transfected with poly(I:C) (500 ng/ml) using X-tremeGENE transfection reagent and the expression of LC3 was examined. (C) MCF-7 cells, pre-transfected with the plasmid encoding GFP-LC3 or control GFP/NT using Lipofectamine 2000, were transfected with a high (500 ng/ml) or a low (100 ng/ml) dose of poly(I:C) using X-tremeGENE transfection reagent.
MDA-MB-231 and BT-549 cells, transfected with the plasmid encoding GFP-LC3 or GFP/NT, were not transfected with poly(I:C). In all cultures, 10 μg/ml pepstain A was added. After 48 h, the expression of LC3 (green) and nuclear staining with Hoechst 33342 (blue) were observed by confocal microscopy. Scale bar; 10 μm.

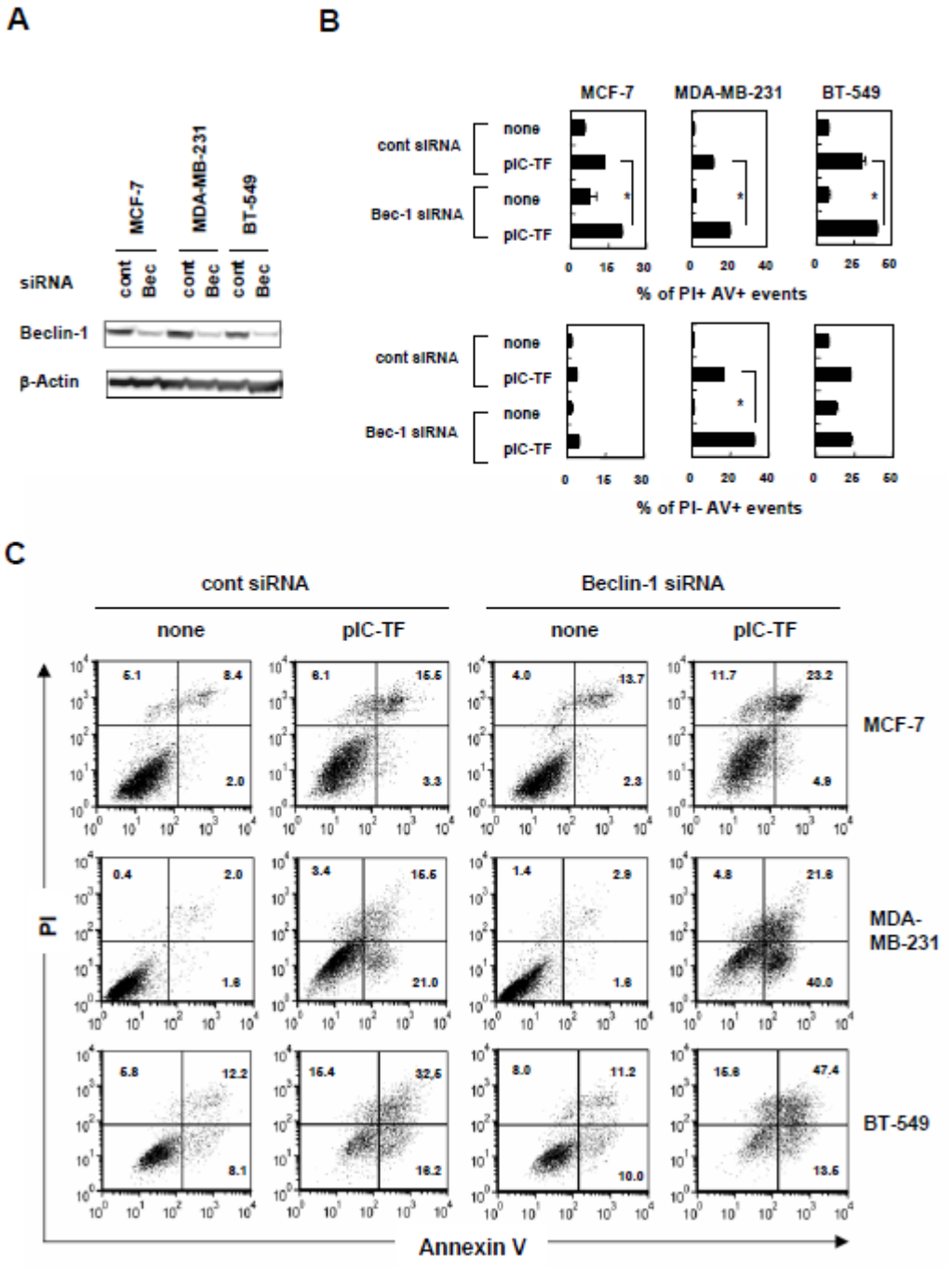

Figure 6 Protective role of autophagy in antitumor effects after poly(I:C) transfection.

(A) Three cell lines were transfected with beclin-1 siRNA or control siRNA using Lipofectamine RNAiMAX. After 3 d, the protein expression of beclin-1 was examined using immunoblot assays. (B) These breast cancer cells, which were pre-transfected with beclin-1 siRNA or control siRNA 3 d previously, were subsequently transfected with

poly(I:C) (500 ng/ml for MCF-7 and BT-549 cells, and 1000 ng/ml for MDA-MB-231 cells) using X-tremeGENE transfection reagent (4.2 μl/ml). After 48 h, these cells were stained with FITC-conjugated Annexin V and PI, and flow cytometry analysis was performed. The histogram summarizes the results of three flow cytometry experiments. *$p < 0.05$ indicates statistical significance. pIC-TF, poly(I:C) transfection. (C) A representative result is shown. Numbers represent the percentages of cells in each subset.

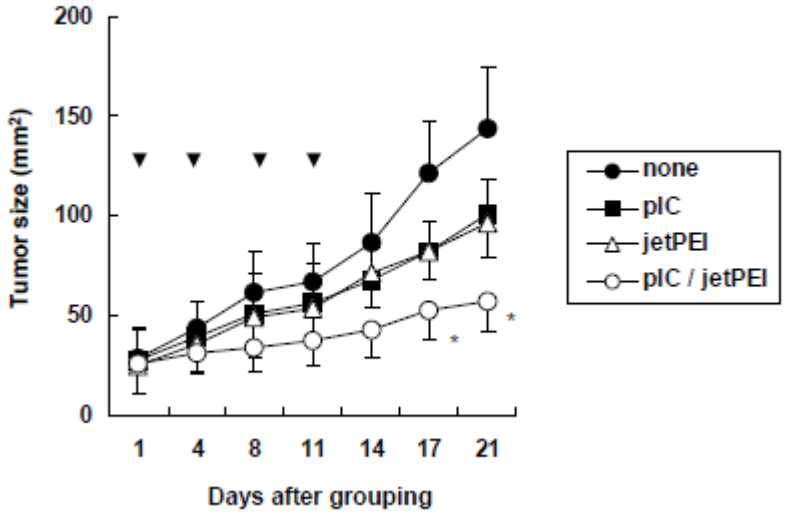

Figure 7 ***In vivo*** **antitumor effect of poly(I:C) transfection in a xenograft mouse model.** BALB *nu/nu* mice were inoculated with MDA-MB-231 cells (5×10^6) into the mammary fat pad. On day 42, the mice were pooled and divided into four groups. On days 1, 4, 8, and 11 after grouping, the mice were intratumorally injected four times with 5% glucose (control), poly(I:C) (10 μg/mouse), *in vivo*-jetPEI transfection reagent (1.4 μl reagent in 5% glucose), or poly(I:C) mixed with *in vivo*-jetPEI transfection reagent in a volume of 50 μl. Arrow heads represent the day of treatment. Each group contained five mice. Similar results were obtained in two independent experiments. *$p < 0.05$ indicates statistical significance

compared with the other three groups. pIC, poly(I:C).