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Effects of gefitinib treatment on cellular uptake of extracellular vesicles in EGFR-mutant non-small cell lung cancer cells

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Abstract

Extracellular vesicles (exosomes, EVs) are cell membrane particles (30-200 nm) secreted by virtually all cells. During intercellular communication in the body, secreted EVs play crucial roles by carrying functional biomolecules (e.g., microRNAs and enzymes) into other cells to affect cellular function, including disease progression. We previously reported that the macropinocytosis pathway contributes greatly to the efficient cellular uptake of EVs. The activation of growth factor receptors, such as epidermal growth factor receptor (EGFR), induces macropinocytosis. In this study, we demonstrated the effects of gefitinib, a tyrosine kinase inhibitor of EGFR, on the cellular uptake of EVs. In *EGFR*-mutant HCC827 non-small cell lung cancer (NSCLC) cells, which are sensitive to gefitinib, macropinocytosis was suppressed by gefitinib treatment. However, the cellular uptake of EVs was increased by gefitinib treatment, whereas that of liposomes was reduced. In accordance with the results of the cellular uptake studies, the anti-cancer activity of doxorubicin (DOX)-loaded EVs in HCC827 cells was significantly increased in the presence of gefitinib, whereas the activity of DOX-loaded liposomes was reduced. The digestion of EV proteins by trypsin did not affect uptake, suggesting that the cellular uptake of EVs might not be

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Appendix A. Supplementary data

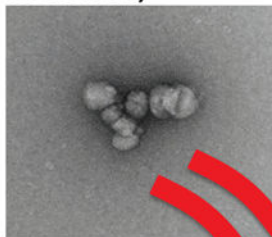
Supplementary data to this article can be found online at ~.

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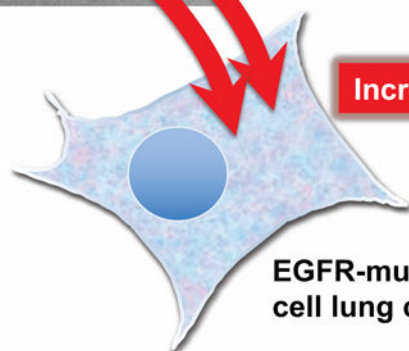
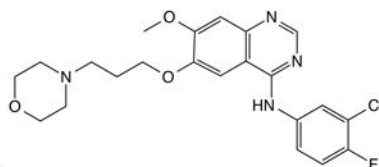
mediated by EV proteins. These results suggest that gefitinib can enhance cell-to-cell communication via EVs within the tumor microenvironment. In addition, EVs show potential as drug delivery vehicles in combination with gefitinib for the treatment of patients harboring *EGFR*-mutant NSCLC tumors.

Graphical Abstract

Extracellular vesicles (Exosomes)



Gefitinib



Increased cellular uptake

EGFR-mutant non-small cell lung cancer

Keywords

Gefitinib; extracellular vesicles; exosomes; EGFR; non-small cell lung cancer

1. Introduction

Extracellular vesicles (exosomes, EVs) are cellular membrane-derived nanosized particles (30-200 nm) that are secreted by virtually all cells. In cell-to-cell communication, EVs, such as exosomes, play crucial roles by delivering functional biomolecules that are encapsulated in EVs (e.g., microRNAs and proteins) to other cells to effect cellular biological functions (Tan et al., 2013). Particularly in the research field of cancer, EV-mediated cellular communication has been actively investigated over the last decade, and EVs have been shown to influence cancer-related signaling pathways, including hypoxia-driven epithelial–mesenchymal transition (EMT), cancer stemness, angiogenesis, and metastasis (Azmi et al., 2013; Maia et al., 2018). Based on experimental findings, novel strategies to inhibit cancer metastasis via the blockade of cancer-derived EVs have been developed (Nishida-Aoki et al., 2017; Kosaka et al., 2013). However, detailed assessment and mechanism elucidation in terms of the influence of clinical cancer therapeutics on EV-based cell-to-cell communications is urgently needed.

Various mechanisms of cellular EV uptake have been proposed, including clathrin-mediated endocytosis, phagocytosis, and membrane fusion (Mulcahy et al., 2014). In addition, our research group recently reported that macropinocytosis contributes greatly to the efficient cellular uptake of EVs (Nakase et al., 2015). Macropinocytosis is a unique mode of endocytosis in which extracellular fluid is internalized in a clathrin- and caveolin-independent manner and relies on actin-dependent ruffle (lamellipodia) formation (> 1 μm in diameter) (Zwartkruis et al., 2013). Macropinocytosis can be induced by the expression of oncogenic K-Ras and the activation of growth factor receptors such as epidermal growth factor receptor (EGFR) (Swanson 2008). Macropinocytosis is mediated by appropriate signals when reorganizing the actin cytoskeleton that include the activation of Rho proteins, such as the small G-protein Rac1, and upstream effectors such as Ras (Zwartkruis et al., 2013; Maltese et al., 2015).

Gefitinib (Fig. 1a) is a tyrosine kinase inhibitor (TKI) of EGFR and has been approved for the treatment of non-small cell lung cancers (NSCLCs) with *EGFR* mutations (Berardi et al., 2013; Watanabe et al., 2015). Patients with a sensitizing exon 19 deletion or an exon 21 substitution mutation in *EGFR* are highly responsive to gefitinib (Wang et al., 2016). EGFR phosphorylation triggers the activation of the anti-apoptotic Ras signaling cascade, eventually leading to uncontrolled cell proliferation in *EGFR*-mutant cancer cells (Seshacharyulu et al., 2012; Oda et al., 2005). Gefitinib selectively binds to the kinase domain and inhibits the phosphorylation of EGFR, resulting in the suppression of downstream signals and the induction of apoptotic cell death (Wang et al., 2016; Seshacharyulu et al., 2012; Kobayashi, Ji et al., 2005; Kobayashi, Boggon et al., 2005; Campiglio et al., 2004).

In this study, we assessed the effects of gefitinib treatment on EV-mediated intercellular communication. We hypothesized that blockade of the Ras signaling cascade would lead to the decreased cellular uptake of EVs via the suppression of macropinocytosis. As expected, gefitinib suppressed macropinocytosis in *EGFR*-mutant (exon 19 deletion) HCC827 cells that were sensitive to gefitinib but did not have any influence on *EGFR* wild-type A549 cells that were resistant to gefitinib. Unexpectedly, the cellular uptake of EVs increased following gefitinib treatment in HCC827 cells, whereas the cellular uptake of liposomes decreased following gefitinib treatment, in agreement with the results of the inhibition of micropinocytosis by gefitinib. Subsequently, we evaluated the influence of gefitinib pretreatment on the *in vitro* anti-cancer efficacy of doxorubicin (DOX)-loaded EVs and DOX-loaded liposomes. Moreover, we investigated the effect of EV membrane proteins on cellular uptake in the presence of gefitinib.

2. Materials and Methods

2.1. Materials

The following reagents were used in this study: gefitinib (Cell Signaling Technology, Inc., Danvers, MA, USA), human epidermal growth factor (EGF), penicillin-streptomycin, FITC-dextran (70 kDa) (Sigma-Aldrich Co., Inc., St. Louis, MO, USA), FITC-transferrin (Rockland Immunochemicals Inc., Limerick, PA, USA), RPMI-1640, minimum essential medium- α (α -MEM), fetal bovine serum (FBS) (Gibco, Life Technologies Corporation,

Grand Island, NY, USA), exosome-free FBS (EXO-FBS, ATLAS Biological, Fort Collins, CO, USA), Dulbecco's phosphate-buffered saline (PBS) and trypsin (0.5 g/L)/ ethylenediaminetetraacetic acid (EDTA) (0.53 mmol/L) solution with phenol red (Nacalai Tesques Inc., Kyoto, Japan), Hoechst 33342 (Invitrogen, Austin, TX, USA), dimethyl sulfoxide (DMSO), doxorubicin (Wako Pure Chemical Co., Inc., Osaka, Japan), the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), the Premix WST-1(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan), FITC-labeled liposomes (DOPC:CHOL:FITC-DHPE at a molar ratio of 54:45:1), doxorubicin-loaded liposomes (HSPC:CHOL:mPEG2000-DSPE at a molar ratio of 56.2:38.5:5.3) (FormuMax Scientific Inc., Sunnyvale, CA, USA), SDS-PAGE gel plates (Bio Craft Co., Tokyo, Japan), and silver stain reagent (Cosmo Bio Co., Tokyo, Japan).

2.2. Cell culture

HCC827 and A549 cell lines (American Type Culture Collection, Manassas, VA, USA), which are human NSCLC cell lines, were cultured in RPMI-1640 media containing 10% FBS and penicillin-streptomycin (100 units/mL and 100 µg/mL) in 100-mm cell culture dishes (Iwaki, Tokyo, Japan) and incubated at 37 °C in 5% CO₂. Human cervical cancer-derived HeLa cells (Riken BRC Cell Bank, Ibaraki, Japan) were cultured in α -MEM containing 10% FBS in 100-mm dishes and incubated at 37 °C in 5% CO₂.

2.3. Isolation of EVs

HeLa cells (2×10^6 cells) were seeded into 100-mm dishes in α -MEM (10 mL) containing 10% FBS and incubated for 1 day at 37 °C in 5% CO₂. The cells were washed with serum-free α -MEM (five times, 5 mL) and incubated in α -MEM (10 mL) containing 10% exosome-free FBS for 2 days. The cell culture medium was collected, and the secreted EVs were isolated by the ultracentrifugation method. The collected cell culture medium was centrifuged ($300 \times g$) for 10 min at 4 °C to remove the cell debris. The supernatant was centrifuged ($2,000 \times g$) for 10 min at 4 °C and centrifuged again ($10,000 \times g$) for 30 min at 4 °C to remove the microvesicles. The supernatant was then centrifuged twice ($150,000 \times g$) for 70 min at 4 °C using an ultracentrifuge (Himac CP65 β , Hitachi Koki, Tokyo, Japan), and the pellet was collected in PBS. The concentrations of the isolated EVs are described in terms of their protein concentrations, which were determined by BCA protein assays. The particle size distribution of the EVs was measured by a NanoSight LM10 with NTA2.3 Analytical Software (Malvern Panalytical, Malvern, UK). The encapsulation of FITC-labeled dextran into EVs was conducted as previously described (Nakase et al., 2015).

2.4. Western blotting analysis

To detect EV (exosomal) marker proteins, the isolated EVs were added to SDS sample buffer and boiled. The exosome samples were separated via 10% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA, USA), and treated with anti-CD9 (EPR2949, Abcam, Cambridge, UK) or anti-CD63 antibody (TS63, Abcam, Cambridge, UK). Donkey anti-rabbit HRP-linked IgG (GE Healthcare), for CD9, or anti-mouse IgG HRP NA931V secondary antibody (GE Healthcare), for anti-CD63, was used. The immunoreactivity was detected using an Enhanced Chemiluminescence

(ECL) Plus western blotting detection system (GE Healthcare) with an Amersham Imager 600 (GE Healthcare).

2.5. Electron microscopy

The exosomes were resuspended in PBS (30 µg/mL) and subsequently applied onto a carbon-coated grid (400 mesh) and washed with distilled water. Uranyl acetate was applied to the grid, which was incubated for 10 s at room temperature. Next, the reagent was removed with filter paper, and the grid was dried prior to imaging with a transmission electron microscope (TEM) (JEM1200EX, JEOL, Tokyo, Japan).

2.6. Encapsulation of DOX into EVs

To load DOX into EVs, isolated EVs (25 µg) were mixed with DOX (33 µg) in PBS (100 µL). The electroporation was performed as follows: two initial poring pulses (100 V, 5 msec) followed by five transfer pulses (20 V, 50 msec) were performed in a 1-cm electroporation cuvette at room temperature using a super electroporator NEPA21 TypeII (NEPA Genes, Tokyo, Japan). The removal of unencapsulated DOX was accomplished by washing and filtration using Amicon Ultra centrifugal filters (100 K device, Merck Millipore; triple washing with 500 µL PBS, 18,000 × g, 4 °C, 10 min). The concentration of DOX in the DOX-loaded EVs was confirmed using a spectrofluorometer (RF-5300PC, Shimadzu, Kyoto, Japan).

2.7. Cell viability

For the cytotoxicity studies using gefitinib, HCC827 or A549 cells (7.2×10^4 cells/well (600 µL/well) each) were incubated in 24-well microplates for 24 h at 37 °C. After the removal of the medium, the cells were treated with experimental sample (200 µL/well) containing EGF (100 nM) and penicillin-streptomycin (100 units/mL and 100 µg/mL) for 24 h or 72 h at 37 °C. After sample treatment, the cells were washed with PBS (triple washing, 200 µL) and treated with trypsin (0.1 g/L)-EDTA (0.11 mmol/L) (200 µL/well) at 37 °C for 10 min. Following the addition of RPMI-1640 medium (200 µL), the cell viability was determined using a OneCell Counter (Bio Medical Science Inc. Tokyo, Japan).

For the cytotoxicity studies using DOX-loaded EVs or liposomes, HCC827 or A549 cells (1.2×10^4 cells/well, 100 µL/well) were incubated in 96-well microplates for 24 h at 37 °C. After the removal of the medium, the cells were then treated with experimental sample (50 µL/well) containing EGF (100 nM) and penicillin-streptomycin (100 units/mL and 100 µg/mL) for 24 h at 37 °C. After the medium was replaced with fresh medium containing 10% FBS and penicillin-streptomycin (100 units/mL and 100 µg/mL), the cells were incubated for 48 h at 37 °C. The cells were then washed with PBS (three washes, 50 µL/wash) and treated with trypsin (0.1 g/L)-EDTA (0.11 mmol/L) at 37 °C for 10 min. Following the addition of RPMI-1640 medium (50 µL), the living cell number was counted using a OneCell Counter. The cell viability was calculated based on the ratio of the number of living cells in each well relative to that of the control. The experiments were repeated four times, and the average 50% growth inhibitory concentrations (IC₅₀) were calculated.

2.8. Confocal microscopy

HCC827 or A549 cells (2.8×10^4 cells/well each (200 μ L)) were plated on a μ Slide 8 Well (Ibidi) and incubated for 24 h at 37 °C. After complete adhesion, the cells were treated with FITC-dextran-loaded EVs (10 μ g/mL protein concentration) or FITC-labeled liposomes (5 μ M lipid concentration, 200 μ L/well) containing EGF (100 nM) and penicillin-streptomycin (100 units/mL and 100 μ g/mL) for 24 h at 37 °C in the presence or absence of gefitinib (10 nM). The cells were stained with Hoechst 33342 (5 μ g/mL) for 15 min at 37 °C prior to cell washing. The cells were then washed with fresh cell culture medium (three times, 200 μ L) and analyzed using a FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.9. Flow cytometry

HCC827 or A549 cells (7.2×10^4 cells, 1 mL) were plated into 24-well microplates (Iwaki) and incubated for 24 h at 37 °C. After the removal of the medium, the cells were treated with experimental sample (200 μ L/well) containing EGF (100 nM) and penicillin-streptomycin (100 units/mL and 100 μ g/mL) for 24 h at 37 °C. After sample treatment, the cells were washed with PBS (three washes, 200 μ L/wash) and treated with trypsin (0.1 g/L)-EDTA (0.11 mmol/L, 200 μ L/well) at 37 °C for 10 min. After the addition of PBS (200 μ L), the cells were centrifuged at $400 \times g$ for 5 min at 4 °C. After the removal of the supernatant, the cells were suspended in PBS (400 μ L) and subjected to fluorescence analysis with a Guava EasyCyte flow cytometer (Merck Millipore, Billerica, MA, USA) using 488-nm laser excitation and a 525-nm emission filter. Living cells (10,000 cells/sample) were identified based on forward and side scatter analyses. The experiments were repeated three times, and the average cellular fluorescence intensity was calculated.

2.10. Protein digestion of the EV membrane by trypsin

FITC-dextran-loaded EVs (200 μ g/mL) were incubated with trypsin (0.25 g/L)-EDTA (0.27 mmol/L) at 37 °C for 30 min. The digestion of proteins was confirmed by SDS-PAGE. The particle size distribution of the EVs was measured by a NanoSight LM10. The residual trypsin and EDTA were removed by washing and filtration using Amicon Ultra centrifugal filters (100 K device; washed twice with 500 μ L PBS, $18,000 \times g$, 4 °C, 10 min).

2.11. Zeta potential

The zeta potential of each sample was detected in PBS according to the manufacturer's instructions using an ELSZ-DN2 zeta potential analyzer (Otsuka Electronics, Osaka, Japan).

2.12. Statistical analyses

All statistical analyses were performed using GraphPad Prism software (v5, GraphPad, San Diego, CA, USA). For comparisons of two groups, an unpaired Student's t-test was used for the verification of equal variances via an F-test. Welch's correction was performed when the variances between groups were assumed to be unequal. For multiple comparison analyses, a one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparison test was used. Differences were considered significant when the calculated *p*-value was < 0.05 .

3. Results

3.1. Suppression of macropinocytosis by gefitinib treatment

As described above, it has been reported that macropinocytosis plays a crucial role in the efficient cellular uptake of EVs. Macropinocytosis is induced by activation of EGFR and, to the best of our knowledge, the effects of EGFR inhibitors such as gefitinib on macropinocytosis induction have not been studied. We evaluated the cellular uptake of FITC-dextran (70 kDa), a known marker of macropinocytosis (Falcone et al., 2006; Nakase et al., 2009). HCC827 is an NSCLC cell line with an activating EGFR mutation (del E746-A750) that renders the cells sensitive to gefitinib. A549 harbors wild-type EGFR and is resistant to gefitinib. In fact, cytotoxicity studies have revealed that the viability of HCC827 cells is affected by 100 nM gefitinib following 24 h and 72 h of exposure (Fig. 1b and c). On the other hand, the viability of A549 cells was not affected by gefitinib concentrations as high as 10 μ M treatment following 72 h of exposure (Supplementary Fig. S1). Next, HCC827 and A549 cells were treated with FITC-dextran in the presence of gefitinib (0, 10, and 100 nM) for 24 h, and then the cellular uptake of FITC-dextran was evaluated by flow cytometry analysis (Fig. 1d and e). In the case of the HCC827 cells, the cellular uptake of FITC-dextran was reduced by gefitinib treatment (Fig. 1d), which indicates that gefitinib suppressed macropinocytosis. On the other hand, the cellular uptake of FITC-dextran was not affected by gefitinib treatment in A549 cells (Fig. 1e). These results suggest that gefitinib inhibits macropinocytosis in *EGFR*-mutant HCC827 cells.

3.2. Effects of gefitinib treatment on the cellular uptake of EVs and liposomes

Next, we compared the effects of gefitinib treatment on the cellular uptake of EVs to that of liposomes, which are artificial lipid nanoparticles. EVs were isolated from HeLa cells by differential ultracentrifugation. A NanoSight particle tracking system was used to evaluate the size distribution of the isolated EVs, which was determined to be 173 ± 9 nm (Supplementary Fig. S2a and b). Transmission electron microscopy (TEM) observations of isolated exosomes showed the vesicular structures (Supplementary Fig. S2c). The EV marker proteins CD63 and CD81 were detected in isolated EVs by western blot analysis (Supplementary Fig. S2d). FITC-dextran was encapsulated into the isolated EVs by electroporation, followed by ultrafiltration to remove the unencapsulated FITC-dextran. The zeta potential of the FITC-dextran-loaded EVs was -13.3 mV. In this study, we also used FITC-labeled liposomes, and their zeta potential was -10.2 mV.

HCC827 or A549 cells were treated with FITC-dextran-loaded EVs (2 μ g/mL) or FITC-labeled liposomes (5 μ M) in the presence or absence of gefitinib (10 nM). After 24 h of incubation, the cellular uptake of FITC was evaluated by flow cytometric analysis (Fig. 2a and b, Supplementary Fig. S3 and S4). Surprisingly, the cellular uptake of EVs was increased following gefitinib treatment in HCC827 cells, despite the suppression of macropinocytosis (Fig. 2a). On the other hand, the cellular uptake of liposomes was reduced following gefitinib treatment (Fig. 2b). In A549 cells, there was no significant change in uptake regardless of the presence or absence of gefitinib treatment for both EVs and liposomes (Fig. 2a and b). Following flow cytometric analysis, confocal microscopic observation was conducted to visualize the cellular uptake of FITC-dextran-loaded EVs or

FITC-labeled liposomes. When HCC827 cells were treated with FITC-dextran-loaded EVs, increased endosome-like dot signals from FITC in cells exposed to gefitinib could be observed (Fig. 2c). However, when HCC827 cells were heated with FITC-labeled liposomes, gefitinib treatment reduced the number of endosome-like dot signals from FITC (Fig. 2d). These results are in agreement with the results of the flow cytometry analysis. In A549 cells, FITC uptake was minimal under all experimental conditions (Supplementary Fig. S5).

3.3. Effects of gefitinib treatment on the *in vitro* cytotoxicity of anti-cancer reagent-encapsulated EVs or liposomes

Gefitinib demonstrated distinct and opposing effects on the cellular uptake of EVs and liposomes in HCC827 cells. To evaluate the biological activities of EVs and liposomes following gefitinib treatment, we analyzed the *in vitro* cytotoxicity of EVs and liposomes with the encapsulated chemotherapeutic doxorubicin (DOX). DOX was encapsulated into EVs by electroporation, followed by ultrafiltration to remove unencapsulated DOX. The efficiency of DOX encapsulation into EVs was calculated to be 0.5% based on the quantification of DOX using a spectrofluorometer. The recovery rate of free DOX (without electroporation) not encapsulated into EVs through the purification process was less than 0.1%. These results indicate that DOX was loaded in EVs efficiently.

HCC827 and A549 cells were treated with DOX-loaded EVs and liposomes in the presence or absence of gefitinib (10 nM). After 24 h of exposure and 48 h of incubation, the cell viability was evaluated by living cell counting analysis (Fig. 3). The cell viability was decreased in a DOX concentration-dependent manner in each cell line. In HCC827 cells treated with DOX-loaded EVs, the cell viability was significantly reduced in the presence of gefitinib, whereas treatment with gefitinib alone had no effect on cell viability (Fig. 1b and c). In contrast, the cell viability of HCC827 cells treated with DOX-loaded liposomes was increased following gefitinib treatment. The IC_{50} values of DOX-loaded EVs in the presence of gefitinib were approximately five times lower than the IC_{50} values in the absence of gefitinib (0.08 μ M vs. 0.42 μ M) (Supplementary Table S1). On the other hand, the IC_{50} value of DOX-loaded liposomes in the presence of gefitinib was more than three times greater than the IC_{50} value in the absence of gefitinib (0.95 μ M vs. 0.28 μ M). The cell viability of A549 cells was not affected by gefitinib treatment (Supplementary Table S1). These results were consistent with the results of the cellular uptake analysis by flow cytometry.

3.4. Effect of EV protein digestion on cellular uptake

To elucidate the mechanism underlying the increased cellular uptake of EVs following gefitinib treatment, we evaluated the effect of the trypsin digestion of EV proteins on cellular uptake. FITC-dextran-loaded EVs were first incubated with trypsin to digest the proteins present in the EVs. SDS-PAGE analysis demonstrated an absence of higher molecular weight proteins following trypsin treatment, which indicates EV surface protein degradation (Fig. 4a). NanoSight measurements revealed that the average size and distribution of EVs was unaltered following trypsin digestion (Fig. 4b and c). In addition, trypsin digestion did not affect the zeta potential of the EVs (before trypsin digestion: -13.3 mV, after trypsin digestion: -13.4 mV). HCC827 cells were treated with trypsin-treated and

trypsin-untreated FITC-dextran-loaded EVs (2 µg/mL) in the presence or absence of gefitinib. After 24 h of incubation, the cellular uptake of FITC-dextran-loaded EVs was increased after gefitinib treatment, regardless of protein digestion (Fig. 4d). The degree of increased cellular uptake was greater in cells treated with digested EVs relative to that in untreated cells.

4. Discussion and conclusion

In the current study, we determined whether gefitinib inhibits macropinocytosis in a gefitinib-sensitive, *EGFR*-mutant HCC827 NSCLC cell line. The *EGFR* wild-type A549 cells were shown to be insensitive to gefitinib-mediated macropinocytosis inhibition via the evaluation of the cellular uptake of FITC-dextran, a known macropinocytosis marker (Fig. 1d and e). This was thought to be due to the gefitinib-mediated inhibition of *EGFR* phosphorylation and the blockage of subsequent Ras signaling activation. The Ras signaling pathway is known to be involved in macropinocytosis induction (Zwartkruis et al., 2013; Swanson, 2008; Maltese et al., 2015). This blocking effect was observed even at concentrations of gefitinib (10 nM) that did not affect cell viability (Fig. 1b and c).

Next, we evaluated the effects of gefitinib treatment on the cellular uptake of EVs, since it has been reported that EVs communicate within the tumor microenvironment and can induce cancer malignancy via the acceleration of tumor growth, angiogenesis, and metastasis (Azmi et al., 2013). In the current study, we compared EVs to liposomes, which were considered a control lipid nanoparticle. Liposomes are also suitable as a drug delivery vehicle control. EVs have attracted attention as drug delivery carriers due to a number of advantageous properties, including reduced immunogenicity, low cytotoxicity, and their intrinsic targeting potential (Mulcahy et al., 2014; Ha et al., 2016; Hoshino et al., 2015). Unexpectedly, the cellular uptake of EVs was increased following gefitinib treatment in HCC827 cells (Fig. 2a and c), whereas that of liposomes was reduced (Fig. 2b and d). In addition, we compared the *in vitro* cytotoxicity of DOX-loaded EVs and liposomes. Here, we evaluated cell viability by counting the living cell number, since we reported in a previous paper that the cell proliferation reagent WST-1, a tetrazolium salt that detects activation of succinate-tetrazolium reductase, did not reflect the living cell number when HCC827 cells were treated with gefitinib at a high cell density because of the enhancement of mitochondrial biological activity, including the possible increased activation of succinate-tetrazolium reductase, by gefitinib treatment (Takenaka et al., 2017). In agreement with the results of the uptake study, the cell viability of HCC827 cells treated with DOX-loaded EVs decreased significantly in the presence of gefitinib (Fig. 3a). In the case of DOX-loaded liposomes, cell viability was increased in the presence of gefitinib, although the difference was not significant (Fig. 4c). Interestingly, the IC_{50} value of DOX-loaded EVs was more than ten times lower than that of DOX-loaded liposomes in HCC827 cells in the presence of gefitinib (0.08 µM vs. 0.95 µM) (Supplementary Table S1). These results indicate the following: first, cellular uptake pathways specific to EVs might be activated by gefitinib treatment despite the inhibition of macropinocytosis, which suggests the possibility that cell-to-cell communication via EVs might be enhanced within the tumor microenvironment. Second, gefitinib influences the cellular uptake of EVs and liposomes as well as the intracellular activity of bioactive agents loaded in the carriers. Finally, EVs may represent a superior delivery vehicle for doxorubicin

compared to liposomes. For example, an EV-based drug delivery system might overcome the clinical inefficiencies of combinational gefitinib treatment with standard chemotherapies such as paclitaxel, carboplatin, and cisplatin (Giaccone et al., 2004; Herbst et al., 2004).

Next, we evaluated the effect of the protein digestion of EVs on cellular uptake. It has been reported that the cellular uptake mechanism of EVs may depend on the proteins and glycoproteins found on the surfaces of both vesicles and target cells (Mulcahy et al., 2014; Christianson et al., 2013; Morelli et al., 2004). We found that the cellular uptake of trypsin-digested EVs was enhanced by gefitinib treatment (Fig. 4d). This result indicates that EV-membrane proteins do not contribute to the increased cellular uptake of EVs in the presence of gefitinib. Toda *et al.* reported that the cellular uptake of glioblastoma-derived EVs into parent cells was not affected by the protein digestion of EVs, and lipid molecules were critical for the effective incorporation of EVs (Toda et al., 2015). Similarly, EV membrane components other than proteins, such as lipids, might have influenced our results. Moreover, Fig. 4d demonstrates that the cellular uptake of digested EVs is further enhanced by gefitinib treatment. A potential explanation for this phenomenon is that the trypsin-mediated digestion of EVs leads to the exposure of amino acid sequences that are favorable for cellular uptake or the degradation of proteins that contribute to the suppression of cellular uptake. However, further research is needed to understand the precise requirements for the gefitinib-mediated enhancement of EV cellular uptake.

In conclusion, our results demonstrate that gefitinib increases the cellular uptake of EVs in *EGFR*-mutant NSCLCs despite the suppression of macropinocytosis. Gefitinib may enhance EV-mediated intercellular communication within the tumor microenvironment, thereby influencing cancer malignancy. From a drug delivery perspective, we have provided evidence to support the use of EVs as a drug delivery vehicle in combination with gefitinib.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

EVs	extracellular vesicles
EGFR	epidermal growth factor receptor
NSCLC	non-small cell lung cancer
DOX	doxorubicin

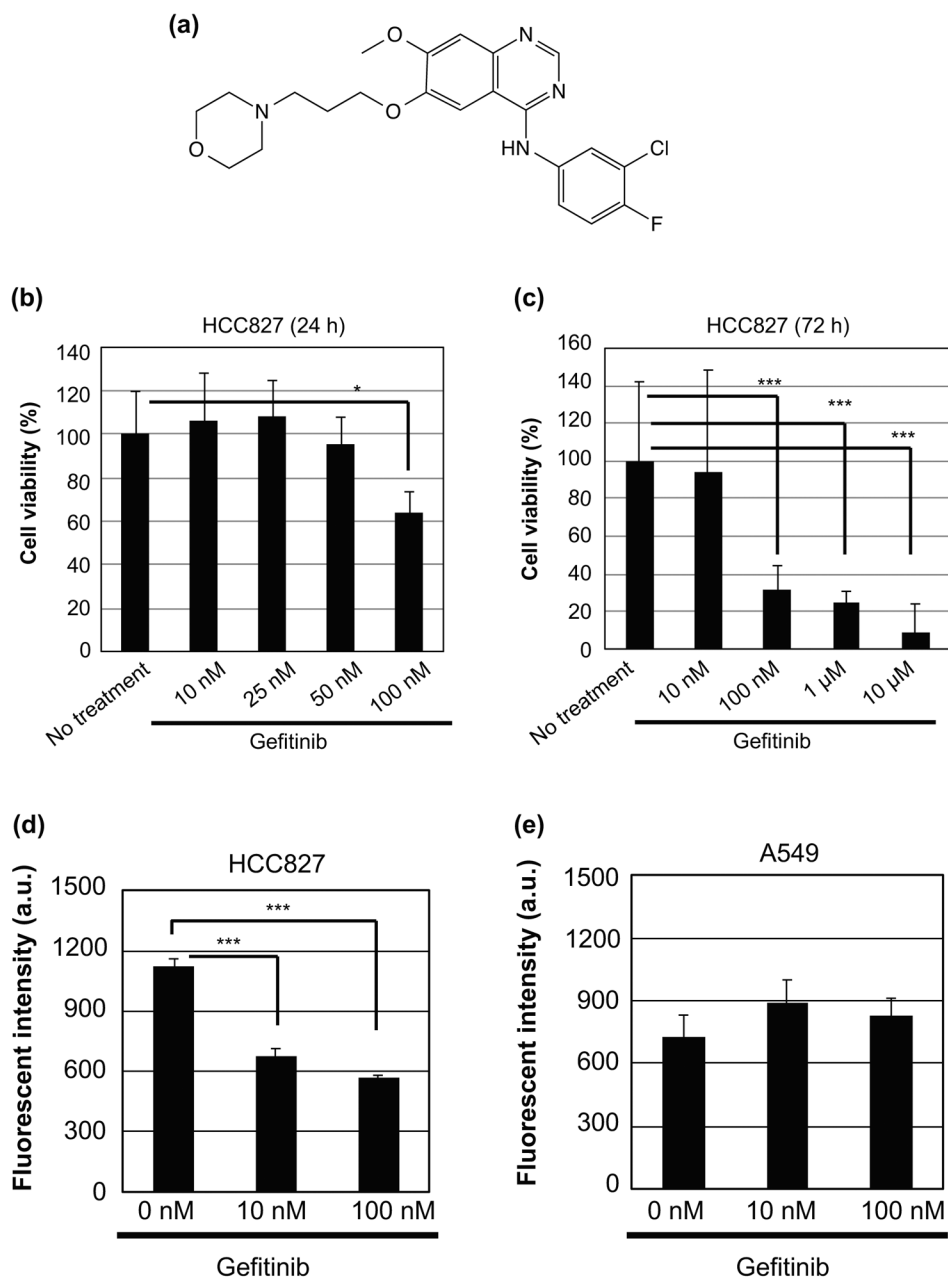
EMT	hypoxia-driven epithelial–mesenchymal transition
TKI	tyrosine kinase inhibitor
α-MEM	minimum essential medium α
FBS	fetal bovine serum
PBS	phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
DMSO	dimethyl sulfoxide
PVDF	polyvinylidene fluoride
HRP	horseradish peroxidase
TEM	transmission electron microscope
IC₅₀	50% growth inhibitory concentrations
ANOVA	one-way analysis of variance

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**Figure 1.**

Effects of gefitinib treatment on cell death and micropinocytosis. (a) Chemical structure of gefitinib. (b, c) Viability of HCC827 cells treated with gefitinib (0 ~ 10 μM) for 24 h (b) and 72 h (c) at 37 °C. Data are presented as the mean (± SD) of four experiments. (d, e) Relative cellular uptake of FITC-dextran (70 kDa), a macropinocytosis marker, in HCC827 (d) and A549 (e) cells treated with gefitinib (0 ~ 100 nM) for 24 h at 37 °C. The uptake was determined using a flow cytometer. Data are presented as the mean (± SD) of three experiments. * $p < 0.05$, *** $p < 0.001$.

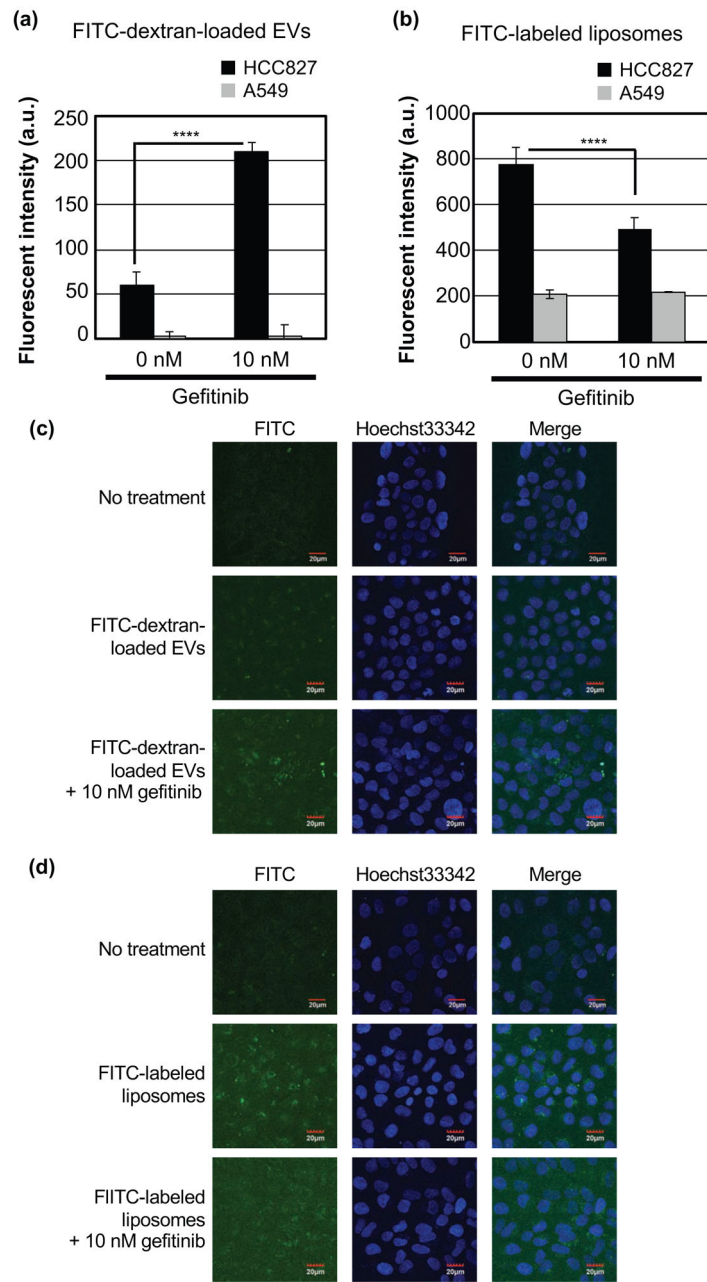


Figure 2. Effects of gefitinib treatment on the cellular uptake of EVs and liposomes. (a, b) Relative cellular uptake of FITC-dextran-loaded EVs (a) and FITC-labeled liposomes (b) in HCC827 or A549 cells in the presence or absence of gefitinib (10 nM) for 24 h at 37 °C using flow cytometry. Data are presented as the mean (\pm SD) of three experiments. (c, d) Confocal microscopic observations of HCC827 cells treated with FITC-dextran-loaded EVs (c) or FITC-labeled liposomes (d) in the same experimental conditions as those used in (a) and (b). **** $p < 0.0001$.

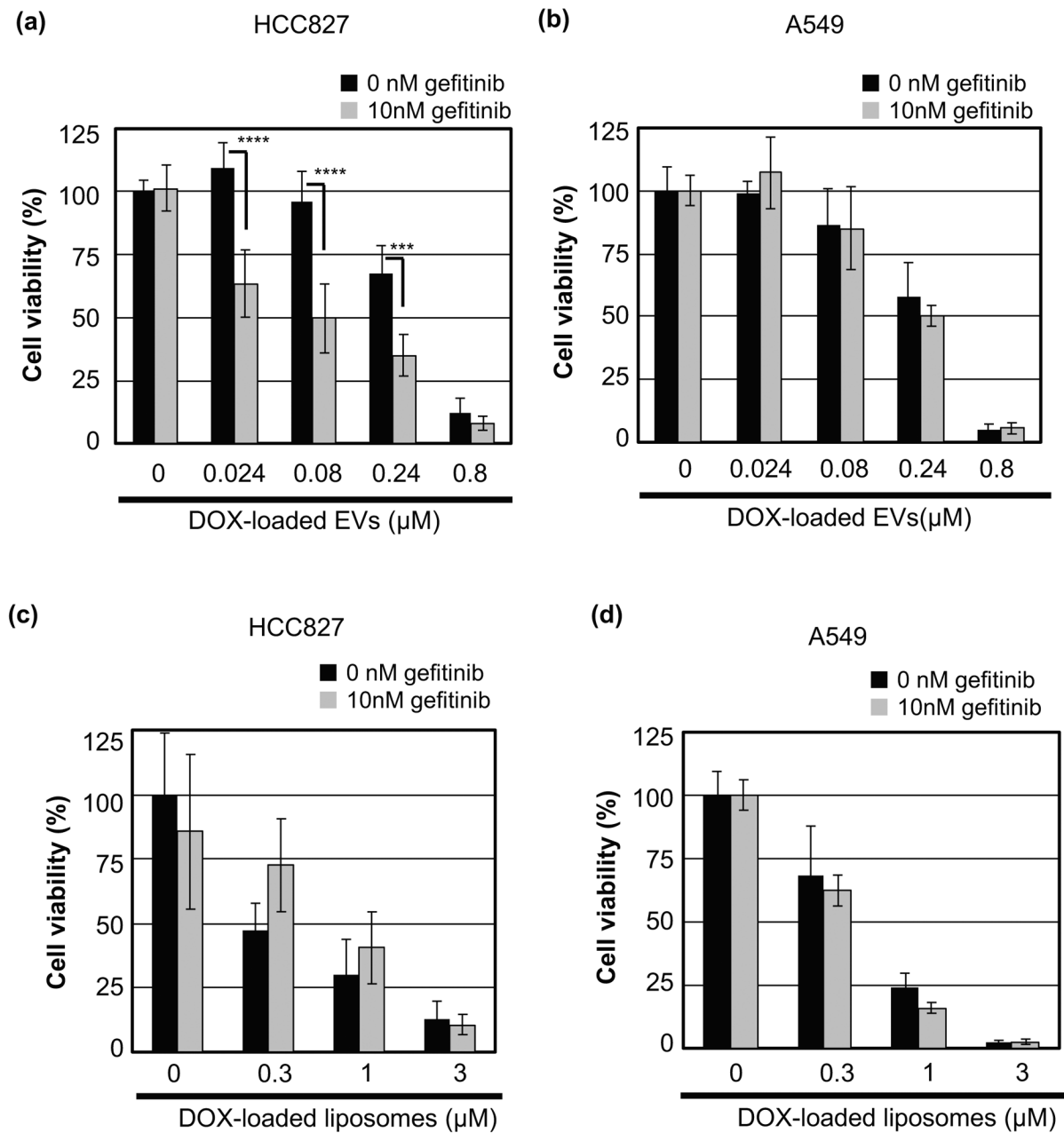


Figure 3.

Gefitinib treatment affects the *in vitro* cytotoxicity of DOX-loaded EVs and liposomes. (a, b) Viability of HCC827 (a) or A549 cells (b) treated with DOX-loaded EVs in the presence or absence of gefitinib (10 nM) for 24 h and 48 h post incubation at 37 °C. (c, d) Viability of HCC827 (c) or A549 cells (d) treated with DOX-loaded liposomes in the same experimental conditions as those used in (a) and (b). The data are the averages (\pm SD) of four experiments. ***p<0.001, ****p<0.0001.

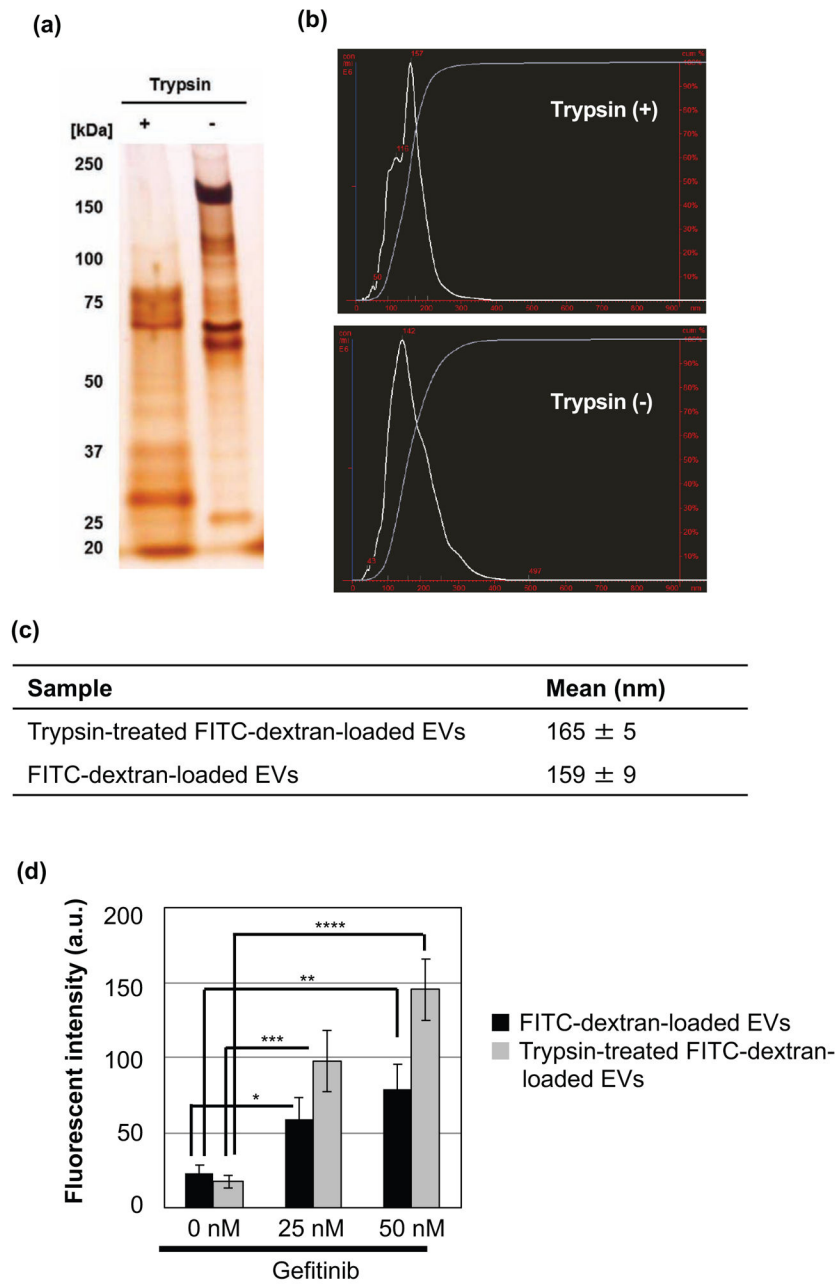


Figure 4. Effects of trypsin digestion on the cellular uptake of EVs following gefitinib treatment. (a) SDS-PAGE analysis of FITC-dextran-loaded EVs before and after treatment with trypsin for 30 min at 37 °C. (b) Particle size distribution of FITC-dextran-loaded EVs before and after treatment with trypsin. (c) Table summarizing the average sizes of EVs. Data are presented as the mean (\pm SD) of three measurements. (d) Relative cellular uptake of FITC-dextran-loaded EVs with or without trypsin treatment in HCC827 cells in the presence or absence of gefitinib (25 and 50 nM) for 24 h at 37 °C using a flow cytometer. The data are the averages (\pm SD) of three experiments. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.