Beneficial effect of erlotinib and trastuzumab emtansine combination in lung tumors harboring EGFR mutations

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Abstract

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) is the standard therapy for non-small cell lung cancer (NSCLC) harboring EGFR mutations, but the resistance is inevitable. The drug-tolerant persister cancer cells are thought to be involved in the resistance. We recently reported that HER2 expression had a negative impact on time-to-treatment-failure in patients with EGFR mutant NSCLC. In this study, we hypothesized that HER2 might be a potential target for alternative combination therapy in NSCLC harboring EGFR mutations. *In vitro* study showed that the level of HER2 expression had no correlation with the sensitivity to EGFR-TKI, erlotinib but showed some correlation with HER2-inhibitor, ado-trastuzumab emtansine (T-DM1) in multiple EGFR-mutant lung cancer cell lines. In addition, HER2 expression was increased in persister cancer cells in 11-18 cell line harboring EGFR L858R or HCC827 cell line harboring EGFR exon 19 deletion after the exposure to erlotinib *in vitro* and *in vivo*. The combination of erlotinib and T-DM1 showed a superior inhibitory effect on cell proliferation compared with those of the erlotinib or T-DM1 alone in either 11-18 or HCC827 cells *in vitro*. The combination therapy also induced a significantly greater inhibitory effect on tumor growth in xenograft model in mice transplanted with either 11-18 or HCC827 cells compared with erlotinib alone or T-DM1 alone. No body weight loss was observed in these mice. These results suggested that the combination therapy with EGFR-TKI and T-DM1 might be a potentially promising strategy for treating lung cancer harboring EGFR mutations.

Keywords: Non-small cell lung cell cancer, EGFR mutations, HER2, ado-trastuzumab emtansine

Introduction

Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) is a standard therapy for non-small cell lung cancers (NSCLC) harboring EGFR mutations[1]. However, the magnitude of tumor regression induced by EGFR-TKI is insufficient and the resistance is inevitably developed[2–4]. Pre-clinical studies suggested that drug-tolerant persister cells may be responsible for the process that hampers the pathological complete remission in the tumors treated with EGFR-TKI[5–9].

To develop an alternative treatment strategy, several clinical trials showed the beneficial effect of combination therapies such as EGFR-TKI plus anti-vascular endothelial growth factor or anti-vascular endothelial growth factor receptor antibody[10][11], or EGFR-TKI plus cytotoxic chemotherapy[12,13]. Several preclinical studies showed the promising effect of combination therapies of EGFR-TKI and novel therapeutic agents such as inhibitors for IGF-1 receptor, AXL and β-catenin, which targeted the drug-tolerant persister cells[5,8,9]. However, an ideal combination therapy still has not been established.

We have recently reported that the expression of HER2 protein may have a negative impact on the time-to-treatment failure (TTF) during the treatment of NSCLC harboring EGFR mutations with EGFR-TKIs[14]. HER2 is an established molecular target in breast and gastric cancers. In lung cancers as well, HER2 targeting agents have been actively developed[15-18], and subsequently ado-trastuzumab emtansine (T-DM1) has been recommended by the National

Comprehensive Cancer Network guideline as a therapy for lung cancer harboring HER2 mutations.

In this preclinical study, we assessed the HER2 expression in EGFR-mutant lung cancer cells after EGFR-TKI exposure and tested combination effect of EGFR-TKI plus T-DM1 in NSCLC cell lines harboring EGFR mutations *in vitro* and *in vivo*.

Materials and Methods

Cell culture

PC-9 (*EGFR Ex19 del E746_A750*), H1650(*EGFR Ex19 deletion*) and H1975 (*EGFR L858R + T790M*) cell lines were purchased from the European Collection of Cell Cultures in 2014. The following cell lines: 11–18 (*EGFR L858R*), H3255 (*EGFR L858R*), HCC827 (*EGFR Ex19 del E746_A750*), HCC4006 (*EGFR Ex19 del E746_A750*) and HCC4011 (*EGFR Ex19 del E746_A750*) were kindly provided by Dr. William Pao (Vanderbilt University, Nashville, TN, USA)[3]. ABC-6 (*EGFR Ex19 deletion*)[19] and OU-LU-26(*EGFR Ex19 deletion*) lines were established in our laboratory. Calu-3 (HER2 amplification) was kindly provided by Dr. Shinichi Toyooka (Okayama University, Okayama, Japan)[20].

Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% of penicillin/streptomycin in a humidified tissue culture incubator at 37°C under 5% CO_2.

Reagents and MTT assay

T-DM1 and erlotinib were purchased from Eveleth (Eveleth, Hiroshima, Japan). Growth inhibition was determined using a modified MTT assay[21]. Cells were plated in 96-well plates at a density of 2000–4000 cells per well and continuously exposed to each drug for 96 hours. Absorbance values were expressed as percentages relative to those of untreated cells.

The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was used to evaluate the effect of each drug on cell proliferation. Each assay was performed in duplicate or more.

Antibodies and immunoblotting

The following antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA): phospho-HER2, HER2, phospho-EGFR, EGFR, phospho-MET, GAPDH, and horseradish peroxidase (HRP)-conjugated anti-rabbit. For immunoblotting, cells were harvested, washed with a phosphate-buffered saline, and lysed in a radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol-phosphate, 10 mM NaF, and 1 mM sodium orthovanadate) containing a protease inhibitor tablet (Roche Applied Sciences, Penzberg, Germany). Lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to membranes and incubated with the indicated antibodies, and detected using enhanced chemiluminescence plus reagents (GE Healthcare Biosciences, Pittsburgh, PA, USA). Bands and dots were detected using an ImageQuant LAS-4000 imager (GE Healthcare Biosciences).

Fluorescence-activated cell sorting (FACS)

Single-cell suspensions were first incubated with the following antibodies (obtained from BD Biosciences) for 20 minutes at 4°C: anti-HER2 antibody (#340552) and isotype control (#551438). Samples were analyzed using a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany), and data were analyzed using a FlowJo software (Ashland, OR, USA).

Reverse transcription (RT)-quantitative PCR (qPCR) and mRNA expression analysis

HER2 mRNA expression was analyzed using RT-qPCR on the complementary DNA (cDNA) using primer and probe sets and the TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. The primer and probe set information is provided in a Supplementary Table 1. RNA was extracted from cells using a QIAamp RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and the cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using a LightCycler Real-Time PCR System (Roche Applied Science), and gene dosage was calculated using a standard curve analysis. The copy number ratio of the target gene to GAPDH was calculated to obtain relative expression values.

Xenograft mouse model

Female BALB/c nu/nu mice (7 weeks old) were purchased from Charles River Laboratories, Japan. All mice were provided with sterilized food and water, and were housed in a barrier facility under a 12-hour light/dark cycle. 11–18 cells (4×10^6) and HCC827 cells (2×10^6) were injected subcutaneously into the backs on both sides of the mice. When the average tumor volume reached approximately 200 mm^3, the mice were randomly assigned to one of four groups (4–5 mice per group) that received either a vehicle, erlotinib (20 mg/kg once daily 5 days per week for 11–18 cells or 10 mg/kg, once daily 5 days per week by gavage for HCC827 cells), T-DM1 (30 mg/kg per week, intraperitoneal administration), or a combination of the two drugs, in which T-DM1 was added 2 weeks after the erlotinib initiation with same dosage. The vehicle was administered once daily, 5 days per week by gavage. Tumor volume ($width^2 \times length/2$) was determined periodically. Statistical data were analyzed on day 56. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research. The experiments were performed under the Policy on the Care and Use of the Laboratory Animals, Okayama University and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions, Ministry of Education, Culture, Sports, Science and Technology—Japan. The experimental protocol was approved by the Animal Care and Use Committee, Okayama University, Okayama, Japan (OKU- 2014557).

Statistical analysis

Statistical analyses were performed using STATA software (ver. 16; StataCorp, College Station, TX, USA). Student's *t* test was used to compare the mean variables between the two groups. One-way ANOVA (analysis of variance) was used to compare the mean variables among multiple groups. In the box plots, the center line is the median and whiskers show minimum to maximum values. In all analyses, $p < 0.05$ was considered to indicate statistical significance.

Results

HER2 expression and the effect of trastuzumab emtansine treatment in lung cancer cell lines harboring *EGFR* mutations.

Firstly, we assessed the expression of HER2 in multiple NSCLC cell lines harboring *EGFR* mutations. Western blot analysis showed that HER2 expression varied among different lung cancer cell lines (Figure 1A). The sensitivity to EGFR-TKI, erlotinib, was not correlated with the level of HER2 expression in these cell lines (Figure 1B). This was consistent with the results of our clinical study showing that the response to EGFR-TKIs based on the Response Evaluation Criteria in Solid Tumors was not affected by the expression of HER2 protein in lung cancer harboring *EGFR* mutations[14].

We also assessed the correlation of HER2 expression and the effect of HER2 inhibitor, T-DM1 *in vitro* (Figure 1C). Consisted with the previous report, T-DM1 treatment inhibited cell proliferation in Calu-3 cell line, which are characterized by HER2 amplification and protein over-expression[22]. T-DM1 treatment showed a modest inhibitory effect on cell proliferation in H3255 cells harboring *EGFR* mutation and with high HER2expression level. In contrast, T-DM1 treatment had a lower inhibitory effect on cell proliferation in lung cancer cell lines harboring *EGFR* mutation and with low expression of HER2 protein such as 11-18 or HCC827 lines compared with Calu-3 or H3255.

EGFR-TKI induced HER2 expression in lung cancer cells harboring *EGFR* mutations *in vitro* and *in vivo*.

Next, we assessed the effect of EGFR-TKI on HER2 expression *in vitro*. Interestingly, FACS analysis showed that the expression of HER2 protein was increased in 11–18 cells 48 hours after the exposure to EGFR-TKI, erlotinib (Figure 2A and Supplementary Fig. 1A). The increased expression of HER2 protein was observed not only in 11–18 cells but also in other cell lines such as HCC827 (Figure 2A). In contrast, HER2 protein expression was not increased in response to erlotinib exposure in H3255 cells, in which the baseline HER2 expression level was relatively high (Supplementary Figure 1B). The increase in HER2 expression was not observed in erlotinib resistant H1975 cells harboring *EGFR* L858R + T790M mutations (Supplementary Figure 1C). The increase in HER2 expression was also confirmed using Western blot or RT-PCR analysis in both 11–18 and HCC827 cells (Figure 2B and 2C). To ask whether the increased expression of HER2 was temporary, we compared the HER2 expression in 11–18 cells among the following conditions: before erlotinib treatment, 72 hours after erlotinib exposure, and 24 or 72 hours after the cessation of erlotinib. As a result, HER2 expression was returned to the original level after the cessation of erlotinib (Figure 2D). Furthermore, we tested the HER2 expression in residual cancer cells after the erlotinib exposure; thus, non-viable cells were eliminated, and only viable cells were tested for HER2 expression (Supplementary Figure 2A). Interestingly, the increase in HER2 protein expression

was also observed in the residual cancer cells 5 days (120 hours) or 15 weeks after the erlotinib exposure (Supplementary Figure 2B and Figure 2E).

Additionally, we also asked whether expression of HER2 protein was increased in response to EGFR-TKI administration *in vivo*. We treated xenograft tumors derived from 11–18 cells with erlotinib (10 mg/kg, 20 mg/kg and 40 mg/kg) for two weeks. FACS analysis revealed that HER2 expression differed significantly among the residual cancer cells after initiation of the erlotinib treatment and those treated with the vehicle (Figure 3A). Immunohistochemistry analysis also showed the increased expression of HER2 protein in the residual tumors treated with erlotinib compared with that in tumors treated with the vehicle (Figure 3B). We also tested the HER2 expression in xenograft tumors obtained from HCC827 cells. We administered erlotinib (5 mg/kg, 10 mg/kg and 20 mg/kg) in mice bearing the xenograft tumors derived from HCC827 cells for two weeks. Consistent with the results obtained with 11–18 cells, the HER2 protein expression differed significantly among these tumors (Figure 3C).

Beneficial effect of erlotinib and T-DM1 combination *in vitro* and *in vivo*

Based on the result showing that HER2 expression was increased after EGFR-TKI exposure, we tested the effect of erlotinib plus HER2 inhibitor, T-DM1 combination in lung cancer cell lines harboring *EGFR* mutations *in vitro* and *in vivo*. The inhibitory effects of the following therapies were compared in 11-18 cells: erlotinib monotherapy, switching therapy from

erlotinib to T-DM1, or a combination therapy, in which erlotinib monotherapy was initially administered for 48 hours and after that T-DM1 was added for the next 96 hours (Supplementary Figure 3A). As expected, the combination of erlotinib and T-DM1 showed a superior inhibitory effect on cell proliferation compared with those of either erlotinib monotherapy or erlotinib-T-DM1 switching therapy (Figure 4A). In addition, we tested the effect of erlotinib and T-DM1 combination *in vivo*. The mice harboring xenograft tumors derived from 11–18 or HCC827 cells were treated with erlotinib monotherapy (20 or 10 mg/kg, 5 days per week p.o.), T-DM1 monotherapy (30 mg/kg, once a week i.p.) for 8 weeks or the combination therapy, in which erlotinib monotherapy was initially administered for 2 weeks and T-DM1 was added for the next 6 weeks (Supplementary Figure 3B). The combination therapy showed a superior inhibitory effect on tumor growth in both of xenograft tumors (Figures 4C and 4E)). No body weight loss was observed in either group (Figures 4D and 4F).

Discussion

In this study we have demonstrated the beneficial effect of and tolerability to EGFR-TKI, erlotinib and HER2-inhibitor, T-DM1 in lung cancer cells harboring *EGFR* mutations *in vivo*. Based on the findings that HER2 protein level was increased in the residual lung cancer cells after erlotinib exposure, an addition of T-DM1 to EGFR-TKI could be a reasonable strategy to achieve deep remission in *EGFR* mutant lung cancers.

EGFR mutant lung cancers are heterogeneous, thus the magnitude of treatment effect induced by EGFR-TKI is varied[23,24]. Some lung cancers may be suitable for EGFR-TKI monotherapy, while the others might be more suitable for intensive combination therapy with EGFR-TKI and other agents. Currently, there is no established biomarker which predicts positive or negative effect of EGFR-TKI monotherapy. We recently reported an observational study suggesting that the expression of HER2 protein might have a negative impact on TTF in response to EGFR-TKI treatment in lung cancer harboring *EGFR* mutations[14]. In addition, *HER2* amplification was reported as one of the secondary resistance mechanisms to EGFR-TKIs[25]. Altogether with current preclinical study and previous reports, the increased expression of HER2 protein in EGFR-mutant lung cancers after the exposure to EGFR-TKI, may play a role in the maintenance of residual cancer cells and could be an alternative target for the drug-tolerant persister cancer cells. Consistent with our results, other groups reported the effect of EGFR-TKI and T-DM1 combination in a preclinical model[26,27] and another clinical study which assesses the effect of third generation EGFR-TKI, osimertinib plus T-DM1, is ongoing (NCT03784599).

Our study has several limitations. First, we have not used clinical samples to test whether the expression of HER2 protein was increased in the residual tumors after EGFR-TKI administration. Second, we have not assessed the combination effect using anti-HER2 antibody, trastuzumab or EGFR/HER2-TKI such as afatinib or dacomitinib. Considering that several

studies showed no additional effect of trastuzumab combined with cytotoxic chemotherapy[28] or a limited effect of dacomitinib in HER2 overexpressing NSCLCs[29], these agents might have insufficient capability to inhibit lung cancer with HER2 overexpression.

In conclusion, the expression of HER2 protein was increased in EGFR mutant lung cancer cells after the EGFR-TKI exposure and the combination therapy with EGFR-TKI and T-DM1 showed a beneficial effect in lung cancer harboring *EGFR* mutations *in vitro* and *in vivo*. Further investigation is warranted with clinical samples and clinical trials.

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Conflicts of interest

All authors declare no conflicts of interest regarding this study.

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Figure 1. HER2 protein expression in EGFR-mutant lung cancer cell lines and their sensitivity to erlotinib or T-DM1

A. Expression of HER2 protein in lung cancer cell lines harboring EGFR mutations

B. Expression of HER2 protein and the sensitivity to erlotinib. IC_{50} of H1975 is > 1.0 μM

C. Expression of HER2 protein and the sensitivity to T-DM1

EGFR: epidermal growth factor receptor, Wt: wild type, Ex19: EGFR exon19 deletions, ex21: EGFR L858R, IC_{50}: half maximal (50%) inhibitory concentration MFI: median fluorescence intensity,

Figure 2. Increased expression of HER2 in vitro in lung cancer cells harboring EGFR mutations

A. FACS shows the increased expression of HER2 protein 48 hours after erlotinib exposure in a dose depending manner in 11-18, andHCC827 cells.

B. Western blot analysis shows the increased expression of HER2 protein 48 hours after erlotinib exposure in 11-18 cells and HCC827 cells.

C. Quantitative PCR indicates the increased HER2 mRNA expression 48 hours after erlotinib exposure in 11-18 cells and HCC827 cells. **, *** $p<0.05$ (Student t-test)

D. The expression of HER2 protein is returned to the original level after erlotinib cessation in 11-18 cells

E. The expression of HER2 protein was increased in the residual cancer cells of 11-18 cells

15 weeks after erlotinib exposure. * $p<0.05$ (Student t-test)

FACS: Fluorescence assisted cell sorting, EGFR: epidermal growth factor receptor, MFI: median fluorescence intensity

Figure 3. Increased expression of HER2 *in vivo* in lung cancer cells harboring EGFR mutations.

A. FACS shows the expression of HER2 protein differed significantly in response to erlotinib exposure in xenograft tumors of 11–18 cells. FACS analysis was performed using cell populations from which apoptotic 7-ADD positive cells were eliminated. Error bars, SE. $p<0.05$ (One-way ANOVA)

B. Immunohistochemistry shows the increase expression of HER2 protein in response to erlotinib exposure in xenograft tumors of 11-18 cells.

C. FACS shows the expression of HER2 protein differed significantly in response to erlotinib exposure in xenograft tumors of HCC827 cells. FACS analysis was performed using cell populations from which apoptotic 7-ADD positive cells were eliminated. Error bars, SE. $p<0.05$ (One-way ANOVA)

EGFR: epidermal growth factor receptor, FACS: Fluorescence assisted cell sorting, MFI: median fluorescence intensity, SE: standard error, ANOVA: analysis of variance, 7-AAD （7-Amino-Actinomycin D）

Figure 4. Effect of erlotinib and T-DM1 combination treatment *in vitro* and *in vivo*

A. Inhibitory effect of the combination of erlotinib (1.0 μM) with T-DM1 (3 μg/ml on cell proliferation in 11–18 cells. Erlotinib monotherapy was initially administered for 48 hours and after that each of the drugs were applied for 96 hours. The treatment schedule is shown in supplementary Figure 3A. *p<0.05 (Student-t with Bonferroni correction)

B. Effects of erlotinib and T-DM1 combination on tumor growth in xenograft model in mice transplanted with 11–18 cells. Mice were treated with vehicle, erlotinib (20 mg/kg, five times per week p.o.), T-DM1 (30 mg/kg, once per week i.p.) for 8 weeks, or the combination therapy, in which erlotinib monotherapy was initially administered for 2 weeks and T-DM1 was added for the next 6 weeks. The treatment schedule is shown in supplementary Figure 3B. Error bars, SE. *p<0.05 (Student-t with Bonferroni correction)

C. Effects of erlotinib and T-DM1 combination on tumor growth in xenograft model mice transplanted with HCC827 cells. Mice were treated with vehicle, erlotinib (10 mg/kg, five times per week p.o.), T-DM1 (30 mg/kg, once per week i.p.) for 8 weeks, or the combination therapy, in which erlotinib monotherapy was initially administered for 2 weeks and T-DM1 was added for the next 6 weeks. The treatment schedule is shown in supplementary Figure 3B. Error bars, SE. *p<0.05, **p<0.01 (Student-t with Bonferroni correction)

D. Body weight in mice transplanted with 11–18 cells.

E. Body weight in mice transplanted with HCC827 cells.

Erlo: erlotinib, Combo: combination of erlotinib and T-DM1, SE: standard error, ANOVA:

analysis of variance, p.o.: per oral, i.p.: intraperitoneal