Synergistic Blockade of EGFR and HER2 by New Generation EGFR Tyrosine Kinase Inhibitor Enhances Radiation Effect in Bladder Cancer Cells

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Abstract

Blockade of epidermal growth factor receptor (EGFR) has been proved useful in enhancing the effect of radiotherapy, but the advantages of new generation EGFR tyrosine kinase inhibitors in radiosensitization are not well known. We used two human bladder cancer cells with wild-type EGFR and HER2 to study the synergism between irradiation and afatinib (an EGFR/HER2 dual kinase inhibitor) or erlotinib (an EGFR kinase inhibitor). Here, we showed that afatinib has better radiosensitizing effect than erlotinib in increasing cancer cell killing, percentage of apoptotic cells and DNA damage. Afatinib is also superior to erlotinib in combining radiation to decrease tumor size, inhibit glucose metabolism, and enhance apoptotic proteins in vivo. Finally, erlotinib suppressed cell growth and induced more DNA damage in bladder cancer cells transfected with HER2 shRNA but not in control vector-treated cells. In conclusion, concomitant blockade of radiation-activated EGFR and HER2 signaling by a new generation EGFR tyrosine kinase inhibitor better inhibits the growth of bladder cancer cells both in vitro and in vivo. The absence of radiosensitization by EGFR or HER2 inhibition alone and the greater radiosensitizing effect of EGFR inhibitor in HER2 knocked-down cells suggests the synergism between HER2 and EGFR in determining radiosensitivity. The regained radiosensitizing activity of erlotinib implies that with proper HER2 inhibition, EGFR tyrosine kinase is still a potential target to enhance radiotherapy effect in these seemingly unresponsive bladder cancer cells.
Introduction

One strategy to enhance the effect of cancer radiotherapy is by utilizing signaling pathways activated by irradiation. Exposure of cells to ionizing radiation activates multiple signaling pathways such as phosphatidylinositol 3-kinases (PI3K)-Akt (1, 2) and mitogen-activated protein kinases (MAPK) pathways (3, 4) related to cell survival. These signaling pathways can abate the apoptotic cascade and enhance DNA repair. Inhibition of their upstream receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) can theoretically block these signaling pathways and increase radiosensitivity of cancer cells (5, 6). The role of anti-EGFR therapy as an effective radiosensitizing agent has been clearly settled by a large clinical trial of cetuximab in head and neck cancer patients (7).

Preclinical studies showed that cetuximab, an anti-EGFR monoclonal antibody, induces cell cycle arrest in the more radiosensitive G1 and G2-M phases, suppressing growth, radiation-induced DNA damage repair, and tumor angiogenesis (6). Meanwhile, although EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib enhance the radiation response in preclinical models (8), their clinical role in radiosensitization is not settled. It has been speculated that the radiosensitizing effect may differ between different classes of EGFR inhibitors (9). EGFR tyrosine kinase inhibition alone may be inadequate to overcome radioresistance.

Since EGFR heterodimerizes with other members of the ErbB family, a broader
inhibition of the receptors may enhance antitumor activity (10). EGFR inhibitors with intrinsic activity or combined with agents against human epidermal growth factor receptor 2 (HER2) (11, 12), HER3 (13), or insulin-like growth factor-1 receptor (IGF-1R) (14) have been reported to enhance radiosensitivity. Afatinib (BIBW2992) is an anilino-quinazoline TKI designed to irreversibly bind EGFR and HER2, and potently suppresses the kinase activity in wild-type and activated mutant cells (15, 16). We have previously demonstrated the \textit{in vitro} and \textit{in vivo} radiosensitizing activity of afatinib in a murine bladder cancer model (17).

It remains unclear whether new generation EGFR inhibitors with broader blockade of Erb-B family tyrosine kinase activities is superior to inhibitors blocking EGFR kinase activity alone in enhancing radiosensitivity of bladder cancer cells. In the present study, we used \textit{in vitro} and \textit{in vivo} models of human bladder cancer cells to test our hypothesis that in bladder cancer cells, the concomitant inhibition of EGFR and HER2 tyrosine kinase activity by afatinib has greater radiosensitizing activity than the inhibition of EGFR tyrosine kinase activity alone by erlotinib. To genetically verify the role of HER2, we also tested whether the radiosensitizing activity mediated by EGFR inhibition can be improved by downregulating HER2 expression.
Materials and Methods

Cell lines

The human bladder urothelial carcinoma cell line, T24, was purchased from the American Type Culture Collection / Bioresource Collection and Research Center (Hsinchu, Taiwan) in 2011. The cells were authenticated in BCRC by short-tandem repeat (STR)-PCR profiling. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Another human bladder carcinoma cell line, NTUB1, was established from human bladder cancer tissue and kindly provided by Dr. Hong-Jeng Yu (18). It was not authenticated. NTUB1 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Both cell lines were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. We sequenced the cDNA of both cell lines, and none of the common EGFR mutations was found.

Reagents

Afatinib and erlotinib were purchased from Selleck Chemicals (Houston, TX). For in vitro experiments, afatinib and erlotinib stock solutions were prepared in dimethyl sulfoxide (DMSO) and 50% acetonitrile, respectively. Both compounds were diluted in culture medium before dosing. For in vivo experiments, afatinib and erlotinib were suspended in a vehicle (0.5% methylcellulose [wt/vol] and 0.4% Tween 80 [vol/vol] in sterile water) for oral
administration to ICR nude mice (BioLASCO, Ilan, Taiwan) bearing tumor xenografts.

**Irradiation of cells and animals**

T24 and NTUB1 cells cultured in flasks were irradiated with various doses of ionizing radiation, using a 6-MV photon beam from a Siemens Primus linear accelerator (Siemens Oncology Medical Systems, Inc., Concord, CA). Mice were immobilized using a customized harness. With the body shielded, the thigh tumor was irradiated with a half-beam rectangular field. The distance from the radiation source to the bottom of the flask or the thigh tumor of nude mice was set at 100 cm.

**RTK signaling antibody array**

The PathScan® receptor tyrosine kinase (RTK) signaling antibody array kit from Cell Signaling Technology (Danvers, MA) contained 39 antibodies against phosphorylated forms of receptor tyrosine kinases or key signaling proteins. T24 cells were first treated with 100 nM afatinib or erlotinib for 30 min, and then with 10 Gy of radiation. After 24 h of incubation, the cells were processed for receptor tyrosine kinase (RTK) array analysis according to the manufacturer's instructions. The membrane was developed with LumiGLO® and Peroxide reagent (Cell Signaling Technologies), and RTK spots were visualized using a UVP imaging system and densitometrically quantified with ImageProPlus software. Each kinase array dot
was manually selected, and an average intensity for each kinase was calculated. For comparison of different stimulation conditions, sets were normalized to allow equal intensities of positive controls.

**Clonogenic assays**

T24 or NTUB1 human bladder cancer cells (1×10^3 per well) were cultured in 6-well plates, treated with different doses of radiation following 1-h pretreatment with afatinib or erlotinib on day 1, re-treated with the drugs on day 2 and day 3 using the same concentrations, incubated for 7 days, and stained with 0.5% crystal violet (Sigma-Aldrich; St. Louis, MO) in 10% methanol for 30 min at room temperature. Colonies with more than 50 cells were counted. At each drug concentration, the surviving fraction was determined by dividing the total number of colonies after irradiation by the number of colonies without irradiation. Each point on the survival curve represents the mean surviving fraction from 3 independent experiments.

**Cell-cycle analysis**

Cell cycle stages were analyzed using a BD FACSCan Flow Cytometer (Becton Dickinson; Franklin Lakes, NJ). In brief, T24 or NTUB1 bladder cancer cells were pretreated for 30 min with vehicle, 200 nM afatinib, or 200 nM erlotinib, irradiated (2.5 Gy), incubated 24 h, fixed
in 70% ethanol, and stained with a solution containing 50 μg/mL propidium iodide and 0.1 mg/mL RNAase (both from Sigma-Aldrich) in the dark for 30 min. Ten thousand events were examined for each determination. The relative proportions of cells in different cell cycle phases were determined using WinMDI software.

**Determination of apoptosis with fluorescence microscopy**

Apoptotic cells were detected using the annexin V/FITC apoptosis detection kit (AVK050, Strong Biotech, Taipei, Taiwan) according to the manufacturer’s instructions. The annexin V-positive cells were examined using a Zeiss Axio Imager A1 fluorescence microscope. Representative images from different treatment groups were taken into account and at least 50 cells were calculated in every group. The portion of annexin V-positive cells was calculated as the ratio of positively stained cells divided by the total cell numbers.

**Western blotting and immunoprecipitation**

Aliquots of T24 and NTUB1 bladder cancer cell lysates containing 50 μg of protein were separated by SDS-PAGE (8–15% polyacrylamide), and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with various antibodies. For immunoprecipitation experiments, we used the Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore) according to the manufacturer's
instructions. The immunoprecipitates (50 μg) of cells were eluted, resolved by 8% SDS-PAGE, electrotransferred to PVDF membranes and incubated with primary antibodies. For whole-cell preparations, tumor tissue from individual animals was homogenized with a motor driven pestle and then lysed in 0.2 ml of RIPA lysis buffer/20 mg tissue. The homogenate was then centrifuged (13,000 g) for 10 min and the supernatant was used as whole-cell extract. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by enhanced electrochemiluminescence (Roche Diagnostics; Basel, Switzerland). The antibodies used were EGFR, HER2, phosphor-Akt (Ser473), caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), cleaved PARP (Cell Signaling Technology), phospho-EGFR (pY1086), phospho-HER2 (pY1139) (Epitomics, Burlingame, CA), and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Chemical cross-linking**

Samples for the cross-linking analysis were obtained at 60 minutes after various treatments. The cells were washed with phosphate buffered saline (PBS) three times and incubated for 60 min at room temperature with 5 mM Suberic acid bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt (Sigma-Aldrich) in PBS, and the reaction was terminated using 20mM Tris-HCl, pH 8.5 for 15 min at room temperature. Subsequently, cells were washed with PBS and solubilized in lysis buffer. The collected proteins were subjected to western blotting using
EGFR or HER2 antibody.

**HER2 RNA interference and stable transfection**

To knockdown HER2 gene expression, we used a target-specific lentiviral vector plasmid encoding a 19–25 nt hairpin shRNA (Santa Cruz Biotechnology, cat. no. sc-29405-SH). One day before transfection, T24 and NTUB1 bladder cancer cells were seeded into 6-well culture plates and grown until 70–90% confluent for transfection. A mixture of HER2 shRNA plasmid or scramble plasmid (Santa Cruz Biotechnology, cat. no. sc-108083), blank Dulbecco's modified Eagle's medium, and lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) at a ratio of 1 μg:98 μl:2 μl was added to the T24 and NTUB1 cells, according to the manufacturer’s instructions. The cells were incubated 5–7 h at 37°C, harvested, and incubated with 2 μg/ml puromycin (Invitrogen) to select stable clones. The efficiency of HER2 knockdown in T24 and NTUB1 cells was confirmed by Western blot.

**Mouse xenograft models**

Male athymic ICR nude mice (6- to 8-weeks old; BioLASCO, Ilan, Taiwan) were housed in sterilized cages under 12 h light/dark cycles and given ad libitum access to food and water. NTUB1 human bladder cancer cells (2 × 10⁶ cells per site) were suspended in Matrigel (BD Biosciences, San Jose, CA) and injected into the right hind leg of mice. As the tumors
became established (mean starting tumor volume = 101 mm³), mice were randomized into 6 groups (n=6) to receive the following treatments: (1) methylcellulose/Tween 80 vehicle; (2) oral afatinib (daily 10 mg/kg of body weight) on days 1–7; (3) oral erlotinib (daily 50 mg/kg of body weight) on days 1–7; (4) vehicle plus 10 Gy of radiotherapy on day 4; (5) oral afatinib (daily 10 mg/kg of body weight) on days 1–7 plus 10 Gy of radiotherapy on day 4; (6) oral erlotinib (daily 50 mg/kg of body weight) on days 1–7 plus 10 Gy of radiotherapy on day 4. Tumors were measured twice a week using calipers, and tumor volume was calculated by the formula: Tumor volume (mm³) = 0.5 * tumor length (mm) * (tumor width [mm]²). Mice were sacrificed when tumor volume reached 1500 mm³. All experimental procedures using these mice were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee.

Animal imaging

Small animal positron emission tomography/computed tomography (PET/CT) scans with [18F]-2-fluoro-2-deoxy-D-glucose (FDG) were performed on day 0 and 14. The mice were intravenously injected with 14 MBq (378 Ci) of FDG in saline via the tail vein. After the PET/CT images, mice were sacrificed and tumors were excised for tissue Western blot or fixation in 10% neutral buffered formalin.
Statistical analysis

The paired-comparisons were assessed using the Student t test. Differences were considered significant at $p < 0.05$. 
Results

Concomitant EGFR and HER2 tyrosine kinase inhibition significantly suppresses radiation-activated signaling pathways

To understand the impact of irradiation on the phosphorylation of receptor tyrosine kinases (RTK) and key signaling proteins, we used an RTK signaling antibody array to determine the change in intensity of phosphorylated proteins 24 hours after irradiation in T24 bladder cancer cells. Figure 1A and 1B demonstrates that phosphorylation of EGFR, HER2, and downstream signals were enhanced after irradiation of these bladder cancer cells. Treatment with 100 nM afatinib 30 min before irradiation significantly suppressed the level of phosphorylated EGFR, HER2, Akt (Thr308 and Ser473), mitogen-activated protein kinases (MAPK), and S6 ribosomal proteins. In contrast, 100 nM erlotinib pretreatment followed by irradiation suppressed only levels of phospho-EGFR and phospho-MAPK. These results showed that afatinib (a tyrosine kinase inhibitor with activity against EGFR and HER2) inhibits radiation-activated signaling pathways and is a more efficient suppressor than erlotinib (a tyrosine kinase inhibitor blocking EGFR activity) in blocking the phosphorylation of HER2, Akt, and S6 ribosomal protein.

Dual blockade of EGFR and HER2 tyrosine kinases significantly radiosensitizes bladder cancer cells
The clonogenic assays of T24 and NTUB1 bladder cancer cells after treatment with afatinib or erlotinib once a day for three consecutive days and irradiation on the first day demonstrated that afatinib dose-dependently decreased the clonogenic survival of both cells (Fig. 2A and 2B) whereas erlotinib had no such effect, even at 1500 nM (Fig. 2C and 2D). The result indicated that EGFR/HER2 dual inhibitor (compared with EGFR inhibitor) was a better radiosensitizing agent of bladder cancer cells.

**Combined EGFR and HER2 tyrosine kinase inhibition promotes radiation-induced apoptosis**

To understand whether drug-mediated enhancement of radiosensitivity was due to synchronization of the cell cycle, propidium iodide staining and flow cytometry were used to determine the cell cycle phase distribution of bladder cancer cells 24 hours after the treatment. As shown in Figure 3A, irradiation (compared with afatinib or erlotinib treatment alone) significantly increased the proportion of T24 and NTUB1 cells in sub-G1 phase, and treatment with 200 nM afatinib 30 min before irradiation (compared with irradiation alone or irradiation plus 200 nM erlotinib) significantly increased the percentage of T24 cells in sub-G1 phase (Fig. 3A). The effects on NTUB1 cells are similar (Fig. 3B). Irradiation also significantly increased the proportion of both cells in G2-M phase. Treatment with afatinib, but not erlotinib, abolished this effect. To confirm the biological significance of combined
treatments in enhancing apoptosis, we performed fluorescence microscopic analysis of annexin V binding of both T24 and NTUB1 cells (Fig. 3C & 3D). The results showed the percentage of annexin V-binding cells increased dramatically after RT. The combined treatment of RT and afatinib, not the RT and erlotinib combination, further increased annexin V-positive cells. This indicated that radiation-induced apoptosis was enhanced by afatinib but not erlotinib. The representative pictures of annexin V-stained T24 and NTUB1 cells after different treatments were shown in supplemental Fig.1. These data suggested that this EGFR/HER2 dual inhibitor (in contrast to EGFR inhibitor alone) increases radiation-induced apoptosis in both T24 and NTUB1 cells.

**Effects of combining irradiation and afatinib on cell apoptosis**

We subsequently assessed the expression of apoptosis markers, cleaved forms of caspase 3 and PARP, in the two bladder cancer cell lines 24 hours after treatment. As shown in Fig. 3E, afatinib pretreatment combined with irradiation (when compared with irradiation or afatinib treatment alone) resulted in greater expression of cleaved forms of caspase-3 and PARP in both NTUB1 and T24 cells. The result further validates the finding that afatinib increases radiation-induced cell apoptosis.

**Combined EGFR and HER2 tyrosine kinase inhibition enhances radiation-induced**
DNA damage

Since radiation-induced DNA damage is a common cause leading to cancer cell apoptosis, we investigated whether tyrosine kinase inhibition by afatinib or erlotinib modulates the level of unrepaired DNA damage. We assessed the quantity of phosphorylated histone H2AX (γ-H2AX), which forms foci at DNA double-strand breaks and recruits double-strand break repair proteins. As shown in Fig. 4A to 4D, following 2.5 Gy of radiation, DNA damage in T24 and NTUB1 cells increased significantly as demonstrated by the mean number of γ-H2AX foci per cell 30 min after irradiation. Adding 1 h of 100 nM afatinib pretreatment (but not 100 nM erlotinib pretreatment) to irradiation resulted in an increased mean number of γ-H2AX foci per cell in both T24 and NTUB1 cells (25±0.7 vs 19±0.4, p<0.001, and 26±0.6 vs 17±0.4, p<0.001, respectively). These results suggest that increased DNA damage may account for the enhancement in cytotoxicity caused by adding this EGFR/HER2 dual inhibitor to irradiation.

Concomitant in vivo EGFR and HER2 tyrosine kinase inhibition results in improved tumor control

We next sought to examine whether concomitant EGFR and HER2 tyrosine kinase inhibition enhances the in vivo effect of radiation on bladder cancer cells. As shown in Fig. 5A, 7-day oral treatment with afatinib in combination with RT on day 4 suppressed the growth of tumor
xenografts to a greater extent than RT alone or erlotinib-RT combination. Afatinib itself had only a minor inhibitory effect on tumor growth. The tumor volume required 14.8, 16.3, 26.8, 34.9, 31.5, and 63.6 days, respectively, to reach 500 mm$^3$ in the sham treatment, erlotinib alone, afatinib alone, RT alone, erlotinib-RT combination, or afatinib-RT combination groups.

We then used 18F-FDG-micro-PET/CT to assess the effect of combining irradiation with dual blockade of EGFR and HER2 tyrosine kinases on glucose metabolism one week after the completion of drug treatment (day 14). The day-14 image (as compared to the pretreatment [day-0] image) showed that the metabolic tumor volume and level of glucose metabolism, representative of tumor viability, decreased after the afatinib-RT combination (Fig. 5B). In contrast, the metabolic tumor volume and level of glucose metabolism increased in mice treated with erlotinib-RT, while metabolic tumor volume increased even more in mice treated with sham, RT, erlotinib alone, or afatinib alone.

The phosphorylation of EGFR, HER2, and Akt in mouse tumor xenografts was then assessed by Western blot analysis of lysates of tumor tissue harvested on day 3. As shown in Fig. 5C, RT induced the activation of these proteins, and only the RT-afatinib combination treatment effectively suppressed all these signals. Similarly, RT combined with afatinib (but not RT combined with erlotinib) induced an increase in the level of cleaved PARP expression on day 7.
The superior radiosensitizing effect of afatinib over erlotinib is probably through HER2 inhibition

Given the \textit{in vitro} and \textit{in vivo} superiority of the EGFR/HER2 dual tyrosine kinase inhibitor (afatinib) over the EGFR inhibitor (erlotinib) in radiosensitizing bladder cancer cells, it is reasonable to suggest that HER2 synergizes with EGFR to determine the level of radiosensitivity. To prove this hypothesis, we used a target-specific lentiviral vector plasmid to silence HER2 expression by RNA interference. The expression of HER2 in T24 and NTUB1 bladder cancer cells decreased dramatically after the transfection with this plasmid (Fig. 6A and Supplemental Fig. 2A). Clonogenic assays showed that pretreatment with different concentrations of erlotinib had a radiosensitizing effect on T24 and NTUB1 cells transfected with HER2 shRNA (Fig. 6B and Supplemental Fig. 2B) but not on T24 and NTUB1 cells transfected with control vector (Fig. 6C and Supplemental Fig. 2C). Meanwhile, pretreatment with afatinib in control vector-transfected T24 and NTUB1 cells retained the radiosensitizing effect (Fig. 6D and Supplemental Fig. 2D).

The number of \(\gamma\)H2AX foci in T24 and NTUB1 cells transfected with HER2 shRNA was higher after treatment with the erlotinib-radiation combination than with radiation alone (Fig. 6E, 6F and Supplemental Fig. 2E, 2F), indicating that by inhibiting HER2 gene expression, bladder cancer cells treated with EGFR tyrosine kinase inhibitor increased their vulnerability to radiation-induced DNA damage.
Different approaches were used to confirm that receptor heterodimerization plays an important role in the radiosensitizing effect of afatinib. First, to investigate the influence of radiation and/or afatinib on the dimer formation of EGFR or HER2, we performed western blotting of cell lysates treated with chemical cross-linking. As shown in Supplemental Fig. 3A and 3B, T24 bladder cancer cells showed the increased dimer formation of both EGFR and HER2 at 60 minutes after radiation. However, the phenomenon was less prominent in T24 cells treated with the combination of radiation and afatinib (administered 30 minutes before radiation), suggesting the inhibition of afatinib in the radiation-induced dimer formation of both EGFR and HER2.

To further examine the effect of radiation and/or afatinib on EGFR-HER2 heterodimer, immunoprecipitation analysis of T24 bladder cancer cells was performed. As shown in Supplemental Fig. 4, western blotting of cell lysate precipitated with EGFR antibody showed the increased HER2 expression at 30 minutes after radiation, and the effect was decreased after the combined treatment of radiation and afatinib (administered 30 minutes before radiation). The results indicate that the level of heterodimerization between EGFR and HER2 was enhanced by radiation, but the enhancement was inhibited by the combined treatment of radiation and afatinib.

Taken together, these results emphasize that the superior radiosensitizing effect of afatinib over erlotinib is probably through HER2 blockade, and that the concomitant tyrosine
kinase inhibition of EGFR and HER2 may play a synergistic role in enhancing the radiosensitivity of bladder cancer cells.
Discussion

Bladder cancer is the most common urinary tract malignancy and is a major health threat to elderly patients (19). Because many bladder cancer patients cannot tolerate radical cystectomy (20), strategies to improve the effectiveness of radiotherapy are eagerly awaited. Previous reports showed that EGFR TKIs, including gefitinib and erlotinib, had only modest radiosensitizing effects on bladder cancer cells (21-23). In addition, the response to EGFR TKI was low in cells with low EGFR expression. In the current study, we tested T24 and NTUB1 cells with low baseline EGFR expression (21), a lack of EGFR activating mutations, and low probability of benefiting from EGFR tyrosine kinase inhibition. It is not surprising that erlotinib failed to enhance their radiosensitivity. Notably, the in vitro and in vivo radiosensitizing effect of an EGFR/HER2 dual inhibitor was demonstrated to surpass that of an EGFR TKI. Interestingly, the EGFR TKI showed improved radiosensitization in T24 cells with down-regulated HER2 expression as compared to vector-treated cells. The results indicate that EGFR is an important determinant of radioresponse in bladder cancer cells, provided the associated pathways like HER2 are concomitantly blocked.

It has been proposed that EGFR-mediated radioprotection occurs in three phases: 1) an immediate early phase involving DNA damage repair, 2) a later phase of suppressed DNA damage-induced apoptosis, and 3) a repopulation phase in which tumor cells can recover from radiation-induced cell cycle arrest (24). We clearly showed the advantage of
EGFR/HER2 blockade in enhancing radiation-induced DNA damage, promoting apoptosis, and inhibiting the recovery of cells from radiation. Our approach reversed all three phases of EGFR-mediated radioprotection, irrespective of baseline EGFR expression.

This study also underscores the importance of HER2 in EGFR-mediated radioprotection. It was shown that HER2 expression can be induced by radiation in breast cancer cells with a low basal level of HER2, and inhibition of HER2 resensitizes resistant cell lines to radiation (25). Toulany et al. reported that radiation, but not epidermal growth factor (EGF), enhances EGFR/HER2 heterodimerization and activates the downstream Akt signaling pathway (26). Given that HER2 is a prognostic factor of bladder cancer progression (27, 28), some may wonder whether HER2 inhibition itself accounts for the difference in radiosensitizing activity (29, 30) between afatinib and erlotinib. Our results in Fig. 6B and 6C failed to support this idea because clonogenic survival after radiation alone is basically the same in HER2 down-regulated and control (vector-treated) T24 cells. Interestingly, afatinib also overcomes resistance to cetuximab (an anti-EGFR monoclonal antibody) in T24 cells, probably through downregulation of 611-CTF (a C-terminal fragment of HER2) (31). Since 611-CTF is robustly expressed in T24 cells and perhaps afatinib could affect its expression, the role of 611-CTF in afatinib-enhanced radiosensitivity deserves further investigation.

Based on our RTK signaling antibody array data, afatinib inhibited both radiation-activated MAPK and Akt signals, but erlotinib inhibited MAPK signal only. It is
reasonable to assume that Akt and downstream molecules play important roles in HER2-augmented radioresistance by EGFR. Akt activation has been shown to affect the activation of the G2/M checkpoint induced by DNA damage and to enhance the survival of cells by blocking the function of proapoptotic proteins (32, 33). In contrast to radiation alone or radiation plus erlotinib, radiation plus afatinib induced a significant decrease in cells in G2/M phase and an increase in cells in sub-G1 phase. This implies that Akt may be critical in modulating the cell cycle distribution and promoting apoptosis. Akt can also directly phosphorylate the DNA damage checkpoint kinase Chk1 on serine 280 and block checkpoint function (34, 35). However, whether Chk1 is correlated with increased DNA damage by radiation plus afatinib is not confirmed.

This study also has limitations. The finding that the radiosensitizing effect of HER2 depletion plus erlotinib is not as good as the radiosensitizing effect of afatinib alone suggests that other factors may be involved, such as a higher affinity to EGFR (15) and inhibition of HER3 (36) or HER4 (37) by afatinib. In addition, since chemotherapeutic agents like cisplatin are widely used to enhance radiosensitivity in bladder cancer therapy (38, 39), whether EGFR/HER dual inhibition can further improve the outcome of chemoradiation deserves more studies. Finally, although we clearly demonstrated the in vivo benefit of afatinib to enhance radiosensitivity, the response of a subcutaneous tumor graft may differ from that of the orthotopic bladder tumor model (21).
In summary, the radiosensitizing effects of different generations of clinically useful EGFR TKIs were compared for the first time. We show the inadequacy of EGFR inhibition alone and the advantage of concomitant blockade of radiation-activated EGFR and HER2 signaling to inhibit the \textit{in vitro} and \textit{in vivo} growth of bladder cancer cells. The radiosensitizing effect of an EGFR inhibitor was much higher in HER2 knocked-down than wild-type cells, therefore HER2 may play a synergistic role with EGFR in determining radiosensitivity. The regained radiosensitizing activity of erlotinib implies that with proper HER2 inhibition, EGFR tyrosine kinase is still a potential target to enhance radiotherapy effect in these seemingly unresponsive bladder cancer cells.
References


6. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle


11. Sambade MJ, Camp JT, Kimple RJ, Sartor CI, Shields JM. Mechanism of lapatinib-mediated radiosensitization of breast cancer cells is primarily by inhibition of the Raf>MEK>ERK mitogen-activated protein kinase cascade and radiosensitization of lapatinib-resistant cells restored by direct inhibition of MEK. Radiotherapy and oncology :
journal of the European Society for Therapeutic Radiology and Oncology.

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Figure legends

**Figure 1.** The effect of combining radiation with EGFR tyrosine kinase inhibitors on radiation-sensitive signals. (A) Treatment of T24 cells with irradiation stimulates phosphorylation of EGFR, HER2, and downstream signals as detected by the PathScan® RTK Signaling Antibody Array Kit 24 hours after treatment. Pretreatment with 100 nM afatinib suppresses the phosphorylation of EGFR, HER2, Akt, MAPK, and S6 proteins. In contrast, pretreatment with 100 nM erlotinib only inhibits the phosphorylation of EGFR and MAPK. (B) The original results of PathScan® RTK Signaling Antibody Array.

**Figure 2.** Clonogenic survival analysis shows a difference in the radiosensitizing effect of TKIs that inhibit either EGFR/HER2 or EGFR alone. Afatinib, an EGFR/HER2 dual inhibitor, has a significant radiosensitizing effect and causes a dose-dependent decrease in clonogenic cell survival 7 days after initial treatment in both cell lines (A and B). Erlotinib, an EGFR TKI, fails to radiosensitize either cell line even at high-dose (C and D). The results are expressed as mean ± SD values from three independent experiments. (*, p < 0.05 when comparing with control group.)

**Figure 3.** Tyrosine kinase blockade of both EGFR and HER2 by afatinib, not blockade of EGFR alone, promotes radiation-induced apoptosis. (A) Propidium iodide staining and flow
cytometry analysis 24 hours after the treatment in T24 cells reveals that irradiation significantly increases the proportion of cells in sub-G1 phase, and pretreatment with afatinib, not erlotinib, further increases that proportion. Irradiation also increases the proportion of cells in G2-M phase, and pretreatment with afatinib, not erlotinib, abolishes this effect. The results are expressed as mean ± SD values from three independent experiments. (B) The effects on NTUB1 cells are similar. (C) Fluorescence microscopic analysis of annexin V binding in T24 cells showed that percentage of annexin V-binding cells increased dramatically after RT, and the combined treatment of RT and afatinib further increased annexin V-positive cells. The results are expressed as mean ± SD values from three independent experiments. (D) Analysis of annexin V binding in NTUB1 cells. (E) Western blots show that irradiation increases the expression of the apoptosis markers (cleaved forms of caspase 3 and PARP) which are further enhanced by afatinib pretreatment.

**Figure 4.** The EGFR/HER2 dual inhibitor afatinib, not the EGFR inhibitor erlotinib, promotes radiation-induced DNA damage. (A) The average number of γ-H2AX foci per cell increases 30 min after irradiation and is further enhanced by pretreatment with afatinib (but not with erlotinib) in T24 cells. (B) The representative images after different treatments in T24 cells. (C and D) The effects in NTUB1 cells are similar. The results are expressed as mean ± SD values in a minimum of 150 cells per treatment group.
Figure 5. Comparison of the *in vivo* radiosensitizing effect of afatinib with that of erlotinib.

(A) Seven-day oral treatment of nude mice with afatinib in combination with RT on day 4 suppresses the growth of tumor xenografts to a greater extent than RT alone or the erlotinib-RT combination. The results are expressed as mean ± SEM values from 6 mice in each group. Intersection of the dashed line with each curve indicates the number of days required to reach a tumor volume of 500 mm$^2$. (B) Representative images of animal positron emission tomography/computed tomography (PET/CT) before treatment (day 0) and 2 weeks after initial treatment (day 14). Arrows indicate the viable right thigh tumors. The standard uptake value (SUV) and the viable volume of tumor are shown above the image. Tumors in mice receiving the afatinib-RT combination are the least viable. (C) Western blot analysis shows that the RT-afatinib combination (compared to the RT-erlotinib combination) effectively suppresses radiation-activated EGFR, HER2, and Akt signals and enhances cleaved PARP expression.

Figure 6. The radiosensitizing effect of erlotinib in HER2 knocked-down T24 cells emphasizes the synergism between EGFR and HER2 in determining radiosensitivity. (A) The expression of HER2 in T24 cells decreases dramatically after the transfection with the lentiviral vector plasmid for HER2 RNA interference. (B and C) Clonogenic survival analysis
shows the radiosensitizing effect of erlotinib in HER2 knocked-down cells but not in control vector-treated cells. (*, \( p < 0.05 \) when comparing with control group.) (D) Afatinib still has a radiosensitizing effect in control vector-treated T24 cells. (E) In T24 cells transfected with HER2 shRNA, the number of \( \gamma \)-H2AX foci per cell is greater in the erlotinib plus radiation group, indicating more radiation-induced DNA damage. (F) Representative images of \( \gamma \)-H2AX foci.
Fig. 1

A

Expression ratio (%)

- EGFR
- HER2
- Akt (Thr308)
- Akt (Ser473)
- MAPK (ERK1/2)
- S6 Ribosomal Protein
- Reference spots

B

- EGFR
- HER2
- Akt (Thr308)
- Akt (Ser473)
- MAPK (ERK1/2)

Control/DMSO
RT
RT+Afatinib
RT+Erlotinib
Fig. 2

A. T24 cells

B. NTUB1 cells

C. T24 cells

D. NTUB1 cells
Fig. 3

A

B

C

D

E

**Annexin V positive cell (%)**

*: Significant when comparing with control group, \( p<0.05 \)

**: Significant when comparing with RT only group, \( p<0.05 \)

T24 cells

NTUB1 cells

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*Significant when comparing with RT alone, p<0.05
**Significant when comparing with RT alone, p<0.05**
Molecular Cancer Therapeutics

Synergistic Blockade of EGFR and HER2 by New Generation EGFR Tyrosine Kinase Inhibitor Enhances Radiation Effect in Bladder Cancer Cells

Yu-Chieh Tsai, Pei-Yin Ho, Kai-Yuan Tzen, et al.

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