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 EGFR has been proved useful in enhancing the effect of radiotherapy, but the advantages of new-generation EGFR tyrosine kinase inhibitors (TKI) in radiosensitization are not well known. We used two human bladder cancer cells with wild-type EGFR 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, the 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 vitro. 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 TKI better inhibits the growth of bladder cancer cells both in vitro and in vivo. The absence of radiosensitization by EGFR inhibition alone and the greater radiosensitizing effect of EGFR inhibitor in HER2 knocked down cells suggest 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. Mol Cancer Ther; 14(3): 810–20. ©2015 AACR.

Introduction
One strategy to enhance the effect of cancer radiotherapy is by using signaling pathways activated by irradiation. Exposure of cells to ionizing radiation activates multiple signaling pathways such as PI3K-Akt (1, 2) and 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 (RTK) such as 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 patients with head and neck cancer (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 (TKI) 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.

Because EGFR heterodimerizes with other members of the ErbB family, a broader inhibition of the receptors may enhance anti-tumor activity (10). EGFR inhibitors with intrinsic activity or combined with agents against HER2 (11, 12), HER3 (13), or insulin-like growth factor-1 receptor (14) have been reported to enhance radiosensitivity. Afatinib (BIBW2992) is an anilinoquinazoline 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 in vitro and in vivo radiosensitizing activity of afatinib in a murine bladder cancer model (17).

It remains unclear whether new generation of 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

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study, we used in vitro and 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 ATCC/Bioresource Collection and Research Center (BCRC) in 2011. The cells were authenticated in BCRC by short-tandem repeat–PCR profiling. They were cultured in DMEM supplemented with 10% FBS. Another human bladder carcinoma cell line, NTUB1, was established from human bladder cancer tissue and kindly provided by Dr. Hong-Jeng Yu (Department of Urology, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan; ref. 18). It was not authenticated. NTUB1 cells were cultured in RPMI-1640 medium with 10% FBS. 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. For in vitro experiments, afatinib and erlotinib stock solutions were prepared in 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 (w/v) and 0.4% Tween 80 (v/v) in sterile water] for oral administration to ICR nude mice (BioWest) for daily oral administration to ICR nude mice (BioWest) for 10 days. The distance from the radiation source to the body of the mouse or the thigh tumor of nude mice was set at 100 cm.

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.). 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 flank or the thigh tumor of nude mice was set at 100 cm.

RTK signaling antibody array

The PathScan RTK Signaling Antibody Array Kit from Cell Signaling Technology contained 39 antibodies against phosphorylated forms of RTKs or key signaling proteins. T24 cells were first treated with 100 nmol/L afatinib or erlotinib for 30 minutes, and then with 10 Gy of radiation. After 24 hours of incubation, the cells were processed for RTK array analysis according to the manufacturer’s instructions. The membrane was developed with LumiGLO and Peroxide reagent (Cell Signaling Technology), 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 × 105/well) were cultured in 6-well plates, treated with different doses of radiation following a 1-hour pretreatment with afatinib or erlotinib on day 1, retreated with the drugs on days 2 and 3 using the same concentrations, incubated for 7 days, and stained with 0.5% crystal violet (Sigma-Aldrich) in 10% methanol for 30 minutes 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 three independent experiments.

Cell-cycle analysis

Cell-cycle stages were analyzed using a BD FACSCan Flow Cytometer (Becton Dickinson). In brief, T24 or NTUB1 bladder cancer cells were pretreated for 30 minutes with vehicle, 200 nmol/L afatinib, or 200 nmol/L erlotinib, irradiated (2.5 Gy), incubated 24 hours, fixed in 70% ethanol, and stained with a solution containing 50 μg/mL propidium iodide and 0.1 mg/mL RNase (both from Sigma-Aldrich) in the dark for 30 minutes. 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) 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 (%–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, electrophoresed 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 μg tissue. The homogenate was then centrifuged (13,000 × g) for 10 minutes, 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). The antibodies used were EGFR, HER2, phosphor-Akt (Ser473), caspase-3, cleaved caspase-3, PARP, cleaved PARP (Cell Signaling Technology), phospho-EGFR (pY1086), phospho-HER2 (pY1139; Epitomics), and β-actin (Santa Cruz Biotechnology).

Chemical cross-linking

Samples for the cross-linking analysis were obtained at 60 minutes after various treatments. The cells were washed with PBS containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM EDTA, and 1 mM PMSF and then with 1% Triton X-100. Cellular extracts were mixed with 1% formaldehyde and incubated for 15 minutes. The reaction was stopped with a 5× SDS-PAGE sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.1% Bromophenol blue. Fifty micrograms of nuclear extract were fractionated on a 10% SDS–polyacrylamide gel, and the separated proteins were transferred to PVDF membranes and visualized by enhanced chemiluminescence (Roche Diagnostics). The antibodies used were EGFR, HER2, phosphor-Akt (Ser473), caspase-3, cleaved caspase-3, PARP, cleaved PARP (Cell Signaling Technology), phospho-EGFR (pY1086), phospho-HER2 (pY1139; Epitomics), and β-actin (Santa Cruz Biotechnology).
three times and incubated for 60 minutes at room temperature with 5 mmol/L Suberic acid bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt (Sigma-Aldrich) in PBS, and the reaction was terminated using 20 mmol/L Tris-HCl, pH 8.5 for 15 minutes 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 RNAi 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% to 90% confluent for transfection. A mixture of HER2 shRNA plasmid or scramble plasmid (Santa Cruz Biotechnology; cat. no. sc-108083), blank DMEM, and lipofectamine 2000 transfection reagent (Invitrogen) 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 to 7 hours 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 analysis.

Mouse xenograft models
Male athymic ICR nude mice (6- to 8-weeks-old; BioLASCO) were housed in sterilized cages under 12 hours light/dark cycles and given ad libitum access to food and water. NTUB1 human bladder cancer cells (2 × 10⁶ cells/site) were suspended in Matrigel (BD Biosciences) and injected into the right hind leg of mice. As the tumors became established (mean starting tumor volume = 101 mm³), mice were randomized into six groups (n = 6) to receive the following treatments: (i) methylcellulose/Tween 80 vehicle; (ii) oral afatinib (daily 10 mg/kg of body weight) on days 1 to 7; (iii) oral erlotinib (daily 50 mg/kg of body weight) on days 1 to 7; (iv) vehicle plus 10 Gy of radiotherapy on day 4; (v) oral erlotinib (daily 50 mg/kg of body weight) on days 1 to 7 plus 10 Gy of radiotherapy on day 4; (vi) oral erlotinib (daily 50 mg/kg of body weight) on days 1 to 7 plus 10 Gy of radiotherapy on day 4. Mice were sacrificed when tumor volume reached 1,500 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 PET/CT scans with [18F]-2-fluoro-2-deoxy-D-glucose (FDG) were performed on days 0 and 14. The mice were i.v. 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 a P value of <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 RTKs 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 B demonstrates that phosphorylation of EGFR, HER2, and downstream signals were enhanced after irradiation of these bladder cancer cells. Treatment with 100 nmol/L afatinib 30 minutes before irradiation significantly suppressed the level of phosphorylated EGFR, HER2, Akt (Thr308 and Ser473),
MAPK, and S6 ribosomal proteins. In contrast, 100 nmol/L erlotinib pretreatment followed by irradiation suppressed only levels of phospho-EGFR and phospho-MAPK. These results showed that afatinib (a TKI with activity against EGFR and HER2) inhibits radiation-activated signaling pathways and is a more efficient suppressor than erlotinib (a TKI 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 3 consecutive days and irradiation on the first day demonstrated that afatinib dose dependently decreased the clonogenic survival of both cells (Fig. 2A and B), whereas erlotinib had no such effect, even at 1,500 nmol/L (Fig. 2C and D). 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 Fig. 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 nmol/L afatinib 30 minutes before irradiation (compared with irradiation alone or irradiation plus 200 nmol/L 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 biologic significance of combined treatments in enhancing apoptosis, we performed fluorescence microscopic analysis of annexin V binding of both T24 and NTUB1 cells (Fig. 3C and D). The results showed that the percentage of...
annexin V–binding cells increased dramatically after radiotherapy. The combined treatment of radiotherapy and afatinib, not the radiotherapy 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 Supplementary Fig. S1. These data suggested that this EGFR/HER2 dual inhibitor (in contrast with 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

Because 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 phosphor-ylated histone H2AX (γ-H2AX), which forms foci at DNA double-strand breaks (DSB) and recruits DSB repair proteins. As shown in Fig. 4A–D, 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 minutes after irradiation. Adding 1 hour of 100 nmol/L afatinib pretreatment (but not 100 nmol/L 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, a 7-day oral treatment with afatinib in combination with radiotherapy...
on day 4 suppressed the growth of tumor xenografts to a greater extent than radiotherapy alone or erlotinib–radiotherapy 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 mm³ in the sham treatment, erlotinib alone, afatinib alone, radiotherapy alone, erlotinib–radiotherapy combination, or afatinib–radiotherapy combination groups.

We then used 18F-FDG-micro-PET/CT to assess the effect of combining irradiation with dual blockade of EGFR and HER2. 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 mm³. B, representative images of animal PET/CT scans 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–radiotherapy combination are the least viable. C, Western blot analysis shows that the radiotherapy–afatinib combination (compared with the radiotherapy–erlotinib combination) effectively suppresses radiation-activated EGFR, HER2, and Akt signals and enhances cleaved PARP expression.

Figure 5. Comparison of the in vivo radiosensitizing effect of afatinib with that of erlotinib. A, a 7-day oral treatment of nude mice with afatinib in combination with radiotherapy on day 4 suppresses the growth of tumor xenografts to a greater extent than radiotherapy alone or the erlotinib–radiotherapy combination. The results are expressed as mean ± SEM values from 6 mice in each group.
tyrosine kinases on glucose metabolism 1 week after the completion of drug treatment (day 14). The day-14 image [as compared with the pretreatment (day-0) image] showed that the metabolic tumor volume and level of glucose metabolism, representative of tumor viability, decreased after the afatinib–radiotherapy combination (Fig. 5B). In contrast, the metabolic tumor volume and level of glucose metabolism increased in mice treated with erlotinib–radiotherapy, whereas metabolic tumor volume increased even more in mice treated with sham, radiotherapy, 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, radiotherapy induced the activation of these proteins, and only the radiotherapy–afatinib combination treatment effectively suppressed all these signals. Similarly, radiotherapy combined with afatinib (but not radiotherapy 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 in vitro and in vivo superiority of the EGFR/HER2 dual TKI (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 RNAi. The expression of HER2 in T24 and NTUB1 bladder cancer cells decreased dramatically after the transfection with this plasmid (Fig. 6A and Supplementary Fig. S2A). 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 Supplementary Fig. S2B), but not on T24 and NTUB1 cells transfected with control vector (Fig. 6C and Supplementary Fig. S2C). Meanwhile, pretreatment with afatinib in control vector–transfected T24 and NTUB1 cells retained the radiosensitizing effect (Fig. 6D and Supplementary Fig. S2D).

The number of γ-H2AX foci in T24 and NTUB1 cells transfected with HER2 shRNA was higher after treatment with the erlotinib–radiotherapy combination than with radiation alone (Fig. 6E and F and Supplementary Fig. S2E and S2F), indicating that by inhibiting HER2 gene expression, bladder cancer cells treated with EGFR TKI 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 Supplementary Fig. S3A and S3B, 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 Supplementary Fig. S4, 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 patients with bladder cancer 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 this study, we tested T24 and NTUB1 cells with low baseline EGFR expression (21), a lack of EGFR activating mutations, and low probability of benefitting 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 downregulated HER2 expression as compared with 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: (i) an immediate early phase involving DNA damage repair; (ii) a later phase of suppressed DNA damage-induced apoptosis, and (iii) 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 resestizes resistant cell lines to radiation (25). Toulany and colleagues (26) reported that radiation, but not EGF, enhances EGFR/HER2 heterodimerization and activates the downstream Akt signaling pathway. 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 C failed to support this idea because clonogenic survival after radiation alone is basically the same in HER2 downregulated and control (vector treated) T24 cells. Interestingly, afatinib also overcomes resistance to cetuximab (an anti-EGFR monoclonal antibody) in T24 cells, probably through down-regulation of 611-CIF (a C-terminal fragment of HER2; ref. 31). Because 611-CIF is robustly expressed in T24 cells and perhaps...
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 RNAi. 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 compared with the 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 γ-H2AX foci per cell is greater in the erlotinib plus radiation group, indicating more radiation-induced DNA damage. F, representative images of γ-H2AX foci.

**Significant when compared with RT alone, \( P < 0.05 \)
afatinib could affect its expression, the role of 611-CTF in afatinib-enhanced radiosensitivity deserves further investigation.

On the basis of 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 radiosensitivity 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 with 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). 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 deletion plus erlotinib is not as good as the radiosensitizing effect of afatinib alone suggests that other factors may be involved, such as a higher afatinib radiosensitizing effect of afatinib alone suggests that other factors may be involved, such as a higher afatinib radiosensitizing effect of HER2 depletion plus erlotinib is not as good as the increased DNA damage by radiation plus afatinib is not confirmed.

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 in vitro and in vivo growth of bladder cancer cells. The radiosensitizing effect of an EGFR inhibitor was much higher in HER2 knockdown 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Y.-C. Tsai, P.-Y. Ho, Y.-S. Pu, J.C.-H. Cheng.
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-C. Tsai, P.-Y. Ho, T.-F. Tuan, A.-L. Cheng, Y.-S. Pu, J.C.-H. Cheng.

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