Targeting epidermal growth factor receptor/human epidermal growth factor receptor 2 signalling pathway by a dual receptor tyrosine kinase inhibitor afatinib for radiosensitisation in murine bladder carcinoma

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Abstract  Given the promising control of bladder cancer achieved by combined chemotherapy/radiotherapy with selective transurethral resection, obstacles remain to the treatment of unresectable bladder cancer. The aim of this study was to determine whether targeting epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor 2 (HER2) can radiosensitise a murine bladder tumour (MBT-2) cell line. Cell survival, expression of signal proteins and cell cycle changes in MBT-2 cells treated in vitro and in vivo with afatinib, an irreversible EGFR/HER2 inhibitor, plus radiotherapy were investigated by colony formation assay, Western blot assay and flow cytometry, respectively. Ectopic xenografts were established by subcutaneous injection of MBT-2 cells in C3H/HeN mice. Mice were randomised into 4 groups to receive afatinib (10 mg/kg/day on day 1–7) and/or radiotherapy (15 Gy on day 4). Positron emission tomography (PET) on day 8 was used to evaluate the early treatment response. Afatinib (200–1000 nM) increased cell killing by radiation (0–10 Gy). Pretreatment of irradiated cells with afatinib inhibited radiation-activated HER2 and EGFR phosphorylation. As compared to either treatment alone, the combination increased the level of the cleavage form of poly (ADP-ribose) polymerase, the expression of phospho-γH2AX and the percentage of cells in subG1 phase (indicating enhanced induction of apoptosis),

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and decreased tumour metabolism and inhibited tumour growth by 64%. Afatinib has therapeutic value as a radiosensitiser of murine bladder cancer cells. The synergism between afatinib and radiation likely enhances DNA damage, leading to increased cell apoptosis.

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1. Introduction

Bladder cancer is one of the most common cancers of the urinary tract in the world,1 and is the fourth most common cause of cancer death in males older than 80 years in the United States (US).2 Given the radiosensitivity of bladder cancer, bladder-sparing treatment is an alternative choice for patients who have localised disease but are medically unfit for radical surgery, or who are eligible for bladder-preserving protocol by transurethral tumour resection and chemoradiotherapy.3 Although the local control rate is good, the survival rate after combined modality treatment is only about 10% less than that after standard radical cystectomy.4 Moreover, cisplatinum has been shown to improve the effect of radiation on bladder cancer,5 but its toxicities hinder its use in patients with old age and impaired renal function.

A reasonable way to enhance the outcome of radiotherapy is by concomitantly using agents that inhibit radiation-activated signalling pathways.6 It was shown that cetuximab, an epidermal growth factor receptor (EGFR)-specific monoclonal antibody, significantly increased local–regional control and overall survival in head and neck cancer patients treated with radiotherapy.3 Gefitinib, an EGFR specific tyrosine kinase inhibitor, also enhances in vitro and in vivo growth inhibition of bladder cancer by ionising radiation. However, the effect of combination therapy was modest and only for the duration of gefitinib administration.8

One strategy to increase radiosensitisation in bladder cancer is by simultaneous blockade of the key radiation-activated signalling molecules, EGFR and other Erb-B family receptor tyrosine kinases, such as human epidermal growth factor receptor 2 (HER2).9 It is also noteworthy that both EGFR and HER2 are frequently overexpressed and associated with poor prognosis in bladder cancer.10,11

Afatinib, also known as BIBW2992, is an anilinoquinazoline designed to irreversibly bind EGFR and HER2 and suppresses the kinase activity of wild-type and activated EGFR and HER2 mutants.12,13 The activity of irradiation combined with an EGFR/HER2 dual inhibitor, like afatinib, has never been reported in bladder cancer. In this study, we assessed the antitumour effects of afatinib combined with ionising radiation in in vitro and in vivo models of murine bladder cancer (MBT-2) cell line. Our findings show that afatinib synergised with radiation to inhibit MBT-2 cell survival, as well as the growth of ectopic xenografts. Furthermore, we demonstrate the suppression of radiation-activated EGFR and HER2 by afatinib leading to enhanced DNA damage and apoptosis.

2. Materials and methods

2.1. Murine bladder tumour cell line

The murine (C3H/HeN) bladder tumour cell line, MBT-2, was obtained from the Japanese Collection of Research Bioresources (Okayama, Japan). Cells were cultured in RPMI-1640 supplemented with 10% foetal bovine serum and 50 U/ml penicillin/streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

2.2. Reagents

Afatinib was purchased from Selleck Chemicals (Houston, TX, USA). For in vitro studies, stock solutions of afatinib were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium containing 10% foetal bovine serum. For in vivo studies, afatinib was suspended in a vehicle (0.5% methylcellulose [wt/vol] and 0.4% Tween 80 [vol/vol] in sterile water) for oral administration to C3H/HeN mice bearing xenograft tumours.

2.3. Irradiation of cells

MBT-2 cells in culture flasks were irradiated with different doses of radiation, using a 6-MV photon linear accelerator. The distance from the radiation source to the bottom of the flask was set at 100 cm.

2.4. Colony formation assay

Cells (500/well) were seeded in six-well plates and treated with different doses of radiation following 30-min pretreatment with various doses of afatinib (200–1000 nM) or DMSO vehicle. Cells were then cultured for an additional 7 days, after which the number of colonies (clusters of more than 50 cells) was counted in each well using an inverted phase-contrast microscope at 100X magnification and photographed. The effect on colony number was analysed using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

2.5. Western blot analysis

Aliquots of cell lysates containing 90 µg of protein were separated by SDS–PAGE (6–15% polyacrylamide)
and then transferred onto polyvinylidene difluoride membranes and immunoblotted with various antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by enhanced chemiluminescence (ECL, Boehringer Mannheim, Mannheim, Germany). Antibodies to the phospho-HER2 and phospho-EGFR were obtained from Epitomics, Inc. (Burlingame, CA, USA), EGFR and H2AX from GeneTex, Inc. (Irvine, CA, USA), poly(ADP-ribose) polymerase (PARP) and cleaved PARP from Cell Signaling Technology (Danvers, MA, USA), beta-actin from Santa Cruz Biotechnology (Santa Cruz, CA) and histone variant H2AX, phospho-H2AX (Ser139) and clone JBW301 from Millipore Corporation (Billerica, MA, USA).

2.6. Cell cycle phase analysis

The distribution of cells among the phases of the cell cycle was determined by quantifying the cellular content of propidium iodide-stained DNA. Cells (10^6/ml) were treated as indicated, harvested by centrifugation, stained with propidium iodide (PBS containing 0.5% Tween 20, 15 µg/ml propidium iodide and 5 µg/ml DNase-free RNase), and analysed using a Becton Dickinson FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.7. γH2AX immunofluorescence microscopy

Cells were plated on polylysine-coated coverslips, allowed to attach overnight and exposed to ionising irradiation of 2.5 Gy either alone or combined with 100 nM afatinib. After treatment, cells were incubated for 30 min, washed three times with ice-cold phosphate-buffered saline (PBS), fixed in 4% formaldehyde/PBS for 30 min, permeabilised in 0.5% Triton X-100 in PBS for 1 h, blocked in 5% bovine serum albumin for 1 h at room temperature, incubated with the antibody (fluorescein isothiocyanate [FITC] conjugated anti-phospho-Histone γH2AX [Ser139; 1:1500; Millipore, Billerica, MA, USA]) for 2 h at room temperature in the dark, washed with PBS and mounted in Vectashield mounting medium containing diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). γ-H2AX foci were examined using a Zeiss Axio Imager A1 fluorescence microscope. In each sample, the number of γ-H2AX foci per nucleus was counted by focicounter under high power field and the average of 150 nuclei was calculated. The averaged number of γ-H2AX foci per nucleus represents the amount of double strand breaks.

2.8. In vivo studies

Female C3H/HeN mice (6 weeks of age) were obtained from the National Laboratory Animal Center and used for ectopic (subcutaneous) xenograft implantation. Body weights were measured weekly. Mice from each group were sacrificed on day 8. The tumour was fixed in 10% neutral buffered formalin and processed for histopathological and immunohistochemical evaluations. Tumour volumes were measured with a set of calipers and calculated using a standard formula: width^2 × length/2. All experimental procedures using these mice were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee.

2.9. Ectopic tumour model

Ectopic tumours were established by subcutaneous injection of MBT-2 cells (2 × 10^6) into the right hind leg of mice. As the tumours became established (mean starting tumour volume = 162 mm^3), the mice were randomised into 4 groups to receive the following treatments: (1) methylcellulose/Tween 80 vehicle; (2) afatinib (10 mg/kg/day of body weight) on day 1–7; (3) methylcellulose/Tween 80 vehicle plus 15 Gy of radiotherapy on day 4; (4) afatinib plus radiotherapy. Small animal positron emission tomography/computed tomography (PET/CT) scans with [18F]-2-fluoro-2-deoxy-D-glucose (FDG) were performed on the 8th day of treatment. The mice were intravenously injected with 14 MBq (378 Ci) of FDG in saline via the tail vein.

2.10. Irradiation of mice

Mice were immobilised using a customised harness. With the body shielded, the thigh tumour was irradiated with a half-beam rectangular field. A 6-MV photon beam from a linear accelerator was used to irradiate the thigh tumour with 15 Gy on day 4.

2.11. Histological evaluation

After fixation, tumour tissues were embedded in paraffin blocks and sectioned (5 µm). Tumour cells were detected in representative stained sections. The expressions of phospho-EGFR (Cell Signaling Technology, Inc., Danvers, MA, USA) and phospho-HER2 (Abcam PLC, Cambridge, UK) were evaluated after immunohistochemical staining using specific antibodies.

2.12. Statistical analysis

The tumour volume data satisfied the assumptions of normality and homogeneity of variance for parametric analysis; thus, group means on day 21 for the ectopic tumour models were compared with a one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference method for multiple comparisons. Differences were considered significant at p < 0.05.
3. Results

3.1. Radiosensitisation of MBT-2 cells by afatinib

Clonogenic cell survival decreased dose-dependently either with irradiation (2.5–10 Gy) or afatinib treatment (200–1000 nM (Fig. 1A). To determine if the interaction between afatinib and radiation was synergistic, combination index (CI) values were calculated from the dose–response data. In MBT-2 cells treated with highest doses of irradiation and afatinib, CI values of < 1 were achieved and indicative of synergism (Fig. 1B).

3.2. Radiation activates EGFR/HER2 and Akt protein expressions in a time-dependent manner

It has been reported that receptor tyrosine kinases, such as Erb-B family proteins, are activated by irradiation.9 Besides, activation of the phosphatidylinositol-3-kinase (PI3 K)/Akt pathway is associated with radioresistance. We found in Western blotting assays that levels of both HER2 and EGFR proteins increased time-dependently, starting at 2 h and 6 h after irradiation in MBT-2 cells with 2.5 Gy and 10 Gy, respectively (Fig. 2). Similarly, the increased expression of phospho-Akt was induced in a time-dependent manner.

3.3. Afatinib inhibits radiation-induced EGFR/HER2 and Akt protein expressions in MBT-2 cells

Since radiation induces Erb-B family protein expression, we investigated whether the dual EGFR/HER2 inhibitor, afatinib, can suppress induced expression of these proteins. In MBT-2 cells that received irradiation (either alone or in combination with afatinib), the increased expression of HER2 and EGFR proteins as well as expression of activated phospho-Akt were inhibited by afatinib at 6 h (Fig. 3).

3.4. Afatinib combined with irradiation increases the apoptosis of MBT-2 cells

Our analysis of the cell cycle distribution of MBT-2 cells at 6 h after irradiation (10 Gy) with or without pre-treatment of afatinib (100 nM, 30 min) revealed that the combination significantly increased the sub-G1 population ($p < 0.05$), indicating apoptotic cell death (Fig. 4A). Radiation alone failed to cause a statistically significant increase in the sub-G1 population, but it did insignificantly increase the proportion of cells in G2/M phase arrest and insignificantly decrease the proportion of cells in S phase. Afatinib alone, however, did not cause any significant change in the cell cycle phases. Moreover, Western blot analysis of cleaved PARP revealed that pretreatment with afatinib strongly increases the expression of this apoptotic marker in response to irradiation (Fig. 4B).

3.5. Afatinib combined with irradiation increased DNA damage of MBT-2 cells

Fig. 5A shows the immunofluorescence staining of γ-H2AX, a marker of DNA double-strand breaks. While sham-irradiated cells exhibited a minimal number of γ-H2AX foci (0 ± 0.05/cell), radiation alone induced immediate increases in γ-H2AX foci (13.0 ± 0.28/cell). 

Fig. 1. Radiosensitisation of murine bladder tumour (MBT-2) cells by afatinib was evaluated using the clonogenic assay. (A) Quantitative results of the clonogenic assays after combination treatment with afatinib and irradiation. Cells were cultured at a density of 500 cells per well in six-well plates and pretreated with different doses of afatinib (200–1000 nM for 30 min and then irradiated with different doses (2.5–10 Gy). After 7 days, the cells were fixed, stained and photographed (100X). The images were used to count the number of colonies containing more than 50 cells in each well. The number of MBT-2 colonies at each dose level is expressed as a percentage of those in the corresponding control group. Lines, mean (n = 3); Bars, S.D. (B) Combination indices (CI) for each dose level of irradiation and afatinib were calculated and plotted as a function of the MBT-2 cell fraction affected (Fa). CI values < 1 indicate synergism.
that were evident at 30 min as a result of cellular DNA damage. In contrast, treatment with afatinib had no effect on c-H2AX foci (0 ± 0.03/cell). However, in cells pretreated with afatinib prior to irradiation, the number of c-H2AX foci was significantly increased over that observed after irradiation alone (20.0 ± 0.46/cell versus 13.0 ± 0.28/cell, p < 0.001). Western blot assay revealed dose-dependent changes in γ-H2AX levels in MBT-2 cells pre-treated with afatinib (50 or 100 nM for 30 min followed by irradiation (2.5 or 10 Gy; Fig. 5C).

3.6. The combination of afatinib and radiotherapy exhibits an enhanced ability to control the growth of ectopic MBT-2 xenograft tumours

Daily oral treatment of mice with afatinib (10 mg/kg for 7 days) in combination with radiotherapy on day 4 suppressed the growth of xenograft tumours to a greater extent than radiotherapy alone (Fig. 6A). Afatinib itself did not satisfactorily control growth. Treatment with afatinib enhanced radiation-induced suppression of MBT-2 tumour growth by 64%.

One day after the treatment (day 8), thigh tumours were imaged by micro-PET/CT with [18F-FDG. Tumour viability was decreased after combined afatinib and radiotherapy, when compared to either modality alone or sham treatment (Fig. 6B). The treatment with afatinib alone failed to reduce metabolic tumour volume, but radiotherapy to thigh tumours (15 Gy) by itself partially reduced tumour size. Importantly, co-treatment with afatinib at 10 mg/kg significantly improved this radiotherapeutic effect.

The expressions of HER2 and EGFR were assessed immunohistochemically in MBT-2 tumours harvested at 8 days after initiation of treatments. Radiotherapy itself increased the expressions of both HER2 and EGFR (Fig 6C). Moreover, combined treatment with radiotherapy and afatinib suppressed radiation-activated expression of HER2 and EGFR in tumour tissues.

Taken together, the results indicate that afatinib radiosensitises MBT-2 cells in vitro and in vivo, and suggests that radiosensitisation occurs through the inhibition of radiation activated HER2/EGFR expression secondary to the enhancement of DNA damage.

4. Discussion

We demonstrated in this study the in vitro and in vivo radiosensitising effect of afatinib (an irreversible...
EGFR/HER2 dual inhibitor) on MBT-2 (a murine bladder cancer cell). The synergism with irradiation was likely mediated through enhancement of radiation-induced DNA damage and apoptosis. We also showed that afatinib suppressed radiation-activated EGFR and HER2 signalling. To our knowledge, the present study is the first to report that the combined blockade of EGFR and other Erb-B family receptor tyrosine kinases enhances the radiosensitivity of bladder cancer cells.

EGFR specific tyrosine kinase inhibitors, such as gefitinib\textsuperscript{8,14,15} and erlotinib,\textsuperscript{16} have been reported to enhance the radiosensitivity of bladder cancer cells. Compared to the radiosensitising effect of gefitinib, that of afatinib in our study was superior, indicating the critical role of HER2 signalling pathway in determining the radiosensitivity of bladder cancer cells. Similarly, monoclonal antibody to HER2/neu receptor has been shown to modulate the repair of radiation-induced DNA damage and enhance the radiosensitivity of breast and oesophageal cancer cells overexpressing HER2.\textsuperscript{17,18} Furthermore, the Radiation Therapy Oncology Group trial on bladder preservation demonstrated a significant association of HER2 staining of bladder tumour tissue with complete response after tri-modality treatment.\textsuperscript{19}

Lapatinib, a reversible EGFR/HER2 dual inhibitor, has been shown to have a radiosensitising effect in certain breast cancer cells\textsuperscript{20,21} and patients.\textsuperscript{22,23} However, its effect was unsatisfactory in our pilot study (data not shown). Compared to lapatinib, afatinib (an irreversible inhibitor of EGFR and HER2) was the more potent radiosensitiser.\textsuperscript{24} One large randomised trial recently reported the clinical benefit of afatinib for patients with non-small-cell lung cancer unresponsive to EGFR inhibitors or chemotherapy.\textsuperscript{25} Furthermore, afatinib has been shown to enhance responses to an EGFR specific monoclonal antibody in bladder cancer cells resistant to this treatment.\textsuperscript{26}

Schütze et al. investigated radiosensitisation by afatinib of human squamous cell carcinoma cells only marginally affected by radiation.\textsuperscript{27} The design of the present study differed from their design in the in vivo dosing schedule of afatinib (10 mg/kg for 7 days in our study versus 20 mg/kg until reaching a tumour diameter of 15 mm in their study) and radiotherapy (15 Gy on day 4 in this study versus 20 Gy after the last afatinib dose in their study). Moreover, we measured not only the volume of the ectopic tumour but also its glucose metabolism by PET, as well as the corresponding expression of EGFR and HER2 immunohistochemically. We additionally showed that afatinib enhanced radiation-induced DNA damage and apoptosis. The molecular response to irradiation combined with afatinib might vary between different malignant cells\textsuperscript{28,29} and this variation might account for discrepancies between our study and the one by Schütze et al.

MBT-2 is a poorly differentiated urothelial carcinoma cell line derived from a carcinogen-induced bladder tumour in C3H/HeN mice. The tumour resembles its human counterpart both grossly and histologically.\textsuperscript{30} The advantage of the MBT-2 murine bladder cancer model in this study is the immune-competent system for the in vivo data with the integrated physiological...
response after irradiation\textsuperscript{31} and inhibition of Erb-B family receptor tyrosine kinases.\textsuperscript{32,33}

In this study, radiosensitisation by afatinib was mediated through enhanced irradiation-induced DNA damage as indicated by the increase in $\gamma$-H2AX foci. Gamma H2AX ($\gamma$H2AX) is a marker of DNA double-strand breaks.\textsuperscript{34} Increased numbers of $\gamma$-H2AX foci have been found in bronchial carcinoma and breast adenoma cells treated with a EGFR inhibitor for radiosensitisation,\textsuperscript{35} and this increase has been correlated with dead or dying cells.\textsuperscript{36} Double-strand breaks of DNA are the principal lesions responsible for cell killing by ionising radiation. The radiosensitivity and clonogenic survival of irradiated bladder cancer cell lines have been previously assessed by alkaline comet assay, a method to detect DNA strand break damage.\textsuperscript{37} Our findings of reduced clonogenic survival and significantly increased numbers of $\gamma$-H2AX foci after combined afatinib and irradiation imply that afatinib augments DNA damage. Given that DNA is the most important cellular target of ionising radiation, afatinib is an ideal radiosensitisier for bladder cancer.

Although we demonstrate that afatinib suppresses irradiation-activated EGFR and HER2 pathways, the
full mechanism of enhanced radiosensitisation of bladder cancer cells remains elusive. Sambade et al. reported that lapatinib mediates radiosensitisation in breast cancer cells primarily by inhibiting the Raf > MEK > ERK mitogen-activated protein kinase cascade. We showed that afatinib inhibits post-radiation Akt phosphorylation in response to EGFR and HER2 signalling. Nicolle et al. similarly found that growth and invasiveness inhibition by gefitinib in urothelial carcinoma cell lines involves strong phosphorylation of Akt/MAPK pathways in association with activation of EGFR. Further investigation is ongoing to identify the pathways through which DNA damage and apoptosis signals are transduced.

In conclusion, in this murine bladder cancer model we demonstrated for the first time the in vitro and in vivo radiosensitising activity of afatinib, an orally bioavailable EGFR/HER2 dual inhibitor. Afatinib likely mediates its effect on bladder cancer cells by suppressing radiation-activated EGFR and HER2 signals and thereby causing enhanced DNA damage and cell apoptosis. The greater potency of afatinib as a radiosensitiser compared with previously reported EGFR inhibitors underscores the importance of other Erb-B family receptor tyrosine kinases such as HER2, and indicates a new direction for future clinical trials in bladder cancer.

Conflict of interest statement

None declared.

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References


