Correlation of HER1/EGFR expression and degree of radiosensitizing effect of the HER1/EGFR-tyrosine kinase inhibitor erlotinib

Jae-Chul Kim*, M Aktar Ali, Animesh Nandi, Partha Mukhopadhyay, Hak Choy, Carolyn Cao and Debabrata Saha*

Department of Radiation Oncology, Division of Molecular Radiation Biology, University of Texas Southwestern Medical Center
2201 Inwood Road, Dallas, TX 75390-9187, USA

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Epidermal growth factor receptor (HER1/EGFR)-mediated signal transduction pathways are important in cellular response to ionizing radiation. High HER1/EGFR expression on cancer cells may contribute to radioresistance. In this pre-clinical study, we evaluated the radiosensitizing effect of erlotinib, a small molecule HER1/EGFR inhibitor in three human cancer cell lines with different HER1/EGFR expression — A431 (very high expression), H157 (moderate expression) and H460 (low expression). Our results demonstrated that A431 was the most radioreistant, while H460 was the most radiosensitive. However, A431 cells were the most sensitive to erlotinib (IC50 = 300 nM) and H460 cells the most resistant (IC50 = 8 μM). H157 had intermediate sensitivity to radiation and erlotinib (IC50 = 3 μM). With 300 nM erlotinib, the radiation dose enhancement ratios (DER) were 1.40, 1.17 and 1.04 in A431, H157 and H460, respectively. Treatment with erlotinib for 24 hr at 300 nM increased G1 arrest by 18.6, 2.0 and 4.8% in A431, H157 and H460, respectively. Erlotinib-induced apoptosis was augmented by radiation in A431 cells only. In conclusion, high HER1/EGFR expression may result in a high degree of radiosensitization with erlotinib combined with radiation. The extent of erlotinib-induced radiosensitization was proportional to HER1/EGFR expression, as well as autophosphorylation of the human epidermal growth factor receptor (HER1/EGFR).

Keywords: Epidermal growth factor (EGF), erlotinib, HER1/EGFR inhibitor, HER1/EGFR expression, radiosensitization, autophosphorylation, lung cancer, cell lines A431, H157 and H460, flow cytometric analysis, cell cycle analysis, apoptosis, tyrosine kinase activity

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Cell growth is mediated by a complex network of signaling pathways responsive to external influences, such as growth factors, as well as internal controls and checks. Epidermal growth factor (EGF) was one of the first growth factors to be described, and was shown to be mitogenic, with the effect mediated by its binding to the cell-surface receptor known as EGF receptor (HER1/EGFR or erbB1). HER1/EGFR and other EGF family of receptors HER2/neu (erbB2), HER3 (erbB3) and HER4 (erbB4) have a common molecular structure, consisting of an amino-terminal extracellular domain, a single trans-membrane anchoring region and a carboxyl-terminal intracellular domain having tyrosine kinase (TK) activity. Many molecules collectively known as the EGF-like growth factors have been identified that can bind and activate the EGF family of receptors. Receptor activation initiates signaling through a variety of down-stream pathways, including those involving PI3K/Akt, Ras/Raf/mitogen-activated protein kinase (MAPK) and STAT3, which are important for a variety of cellular processes involved in promoting tumour growth including proliferation, survival, angiogenesis, invasion and metastasis.

HER1/EGFR is expressed on the surface of normal cells, excluding hematopoietic cells and cells of
epidermal origin\textsuperscript{4,6}. HER signaling pathway plays an important role in the normal regulation of cell growth, proliferation and migration. Alterations in expression or the activity of HER family members may cause a severe imbalance in cell growth leading to proliferation, migration and survival of cancer cells. HER1/EGFR expression is up-regulated in a variety of human solid tumours including squamous cell carcinoma of the head and neck (SCCHN), glioma, colon, pancreatic, non-small cell lung, breast, renal, ovarian and bladder carcinomas\textsuperscript{7}. Most SCCHN (80 to 100\%\textsuperscript{7,8} and human pancreatic cancers\textsuperscript{9} (90\%) express high level of HER1/EGFR. Similarly, human colon carcinomas (about 70\%), 84\% of non-small cell lung cancers (NSCLCs) and squamous carcinomas (84\%) frequently overexpress HER1/EGFR\textsuperscript{7,10,11}. The over-expression also occurs in adenoc (65\%) and large-cell carcinomas (65\%), but is rare in small-cell carcinomas\textsuperscript{11,12}. The increased expression of HER1/EGFR may be due to a variety of mechanisms including amplification of the HER1/EGFR gene, mutations of HER1/EGFR mRNA transcription or translation and HER1/EGFR mutations, resulting in a constitutively active tyrosine kinase. Several strategies have been used to target HER1/EGFR, such as i) monoclonal antibodies that compete with activating ligands for binding with the extracellular domain, ii) small molecules that inhibit the intracellular TK activity, iii) immunotoxin conjugates that directed by anti-HER1/EGFR monoclonal antibodies delivering toxins to the surface of the HER1/EGFR-expressing tumour cells, iv) antisense oligonucleotides to reduce the level of HER1/EGFR expression, and v) targeting the downstream effectors of the HER1/EGFR signaling cascade\textsuperscript{13-16}. In transformed cells, HER1/EGFR signaling pathways may play an important role in response to ionizing radiation. The tumour radiosensitivity is shown to be related to HER1/EGFR overexpression\textsuperscript{17,18}. A positive correlation was found between HER1/EGFR expression and the resistance to radiation therapy in mouse adenocarcinoma\textsuperscript{19,20}. In addition, a higher rate of failure in local tumour control and a poor overall survival after radiotherapy was reported in patients with advanced head and neck cancers, with significantly elevated expression of HER1/EGFR\textsuperscript{7}. Therefore, modifying the receptor expression or mutating HER1/EGFR function could influence not only tumour growth and progression, but also the response to radiation therapy. Thus, the HER1/EGFR pathway is an attractive target in the fight against various human cancer types\textsuperscript{22}. In a preclinical study, it was shown that HER1/EGFR inhibitors could enhance the radiosensitivity and promote radiation-induced apoptosis\textsuperscript{25}. The mechanism by which HER1/EGFR inhibitors may enhance radiosensitivity is not yet known. Preliminary findings suggest their capacity to inhibit cellular proliferation, DNA damage repair and tumour angiogenesis may play a role\textsuperscript{24,26}. In the present study, an attempt has been made to develop a cancer therapeutic strategy using HER1/EGFR inhibitor erlotinib, in combination with radiation. Erlotinib is a small molecule designed to inhibit the tyrosine kinase activity of the HER1 signaling pathway, which plays a key role in the development of non-small cell lung cancer (NSCLC). We analyzed the effect of the combined treatment of erlotinib and radiation in three cell lines (A431, H157 and H460), expressing different levels of HER1/EGFR. In this study, colony-forming ability was measured to assess the modulation of sensitivity. Cell-cycle redistribution and per cent apoptosis were measured by flow cytometric analysis. Materials and Methods

Materials

Erlotinib was obtained from OSI Pharmaceuticals, Inc., Melville, NY. Cell culture medium RPMI 1640 and DMEM, fetal bovine serum, penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen (formerly GIBCO BRL, Gaithersburg, MD). Dimethyl sulfoxide (DMSO), crystal violet, phosphatase inhibitors, EGTA, SDS, NP-40 and Trizma base were obtained from Sigma-Aldrich (Saint Louis, MO). RNase was purchased from Stratagene (La Jolla, CA) and 7-aminoactinomycin-D (7-AAD) from Molecular Probes (Eugene, OR). Phospho-tyrosine antibodies (monoclonal, PY-69) were purchased from BD Biosciences (San Diego, CA) and EGFR polyclonal antibodies from Santa Cruz Bio-technology. Enhanced chemiluminescence (ECL) system and Hyperfilm were obtained from GE Healthcare (formerly Amersham Pharmacia Biotech, Piscataway, NJ).

Cell culture

Three different human cancer cell lines A431 (squamous vulvar cancer cell line), H157 (squamous lung cancer cell line) and H460 (a large-cell variant of
In vitro clonogenic assay

Mid-log phase cells from 75 cm² flasks were trypsinized and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL). They were diluted serially to appropriate concentrations and plated out, in triplicate into 25 cm² flasks in 5 ml medium (RPMI 1640 for H460 and H157 and DMEM for A431). First, all three cell lines were exposed to radiation alone. Cells were irradiated using [137Cs] irradiator (J. L. Shepherd & Associates, Glendale, CA) at room temperature at the dose rate of 1.8 Gy/min. Different doses of single γ-irradiation were given 24 hr after the cells were plated. Next, all cell lines were treated with erlotinib alone to assess their sensitivity. Fresh stock solutions of erlotinib were prepared before each experiment by dissolving in 100% DMSO to give a 5 mM concentration. Stock solutions were diluted with medium to yield appropriate final concentrations. The final DMSO concentration in the culture medium was 0.1% or less in all experiments. Sham treatment with 0.1% or less in all experiments. Sham treatment with

After 10 days and then fixed for 15 min with methanol: acetic acid (3:1) and stained for 15 min with 0.05% crystal violet in methanol. After staining, colonies were counted. The surviving fraction was calculated as (mean colony counts)/(cells inoculated) × (plating efficiency), where plating efficiency was defined as (mean colony counts)/(cells inoculated for non-irradiated controls). Surviving fractions for radiation plus drug were normalized by dividing by the surviving fraction with drug alone. The radiation dose enhancement ratio (DER) was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus drug (normalized for drug toxicity) for a surviving fraction of 0.25. Error bars were calculated as ± SE by pooling the results of three independent experiments.

Cell-cycle analysis

Cell cycle analysis was performed using a modified Krishan technique. Briefly, 4 × 10⁵ cells were plated into 25-cm² flasks for each data point. After 24 hr incubation at 37°C, cells were treated with 300 nM (IC₅₀ for A431 cells) erlotinib for 24 hr. Cells were trypsinized, rinsed once (by gently aspirating the old media and washing once with PBS), resuspended in PBS, and fixed in ice-cold ethanol (70%) for 20 min. Fixed cells were rinsed again with PBS and resuspended in 50 μg/ml propidium iodide (Sigma) with 40 KU/ml of DNase-free RNase. Thereafter, they were analyzed using a Beckton Dickinson FACScan flow cytometer and the percentage of cells in each cell-cycle phase was calculated. Data from at least 1 × 10⁴ cells were collected and analyzed using ModFit softwares (Becton Dickinson). Cell aggregates were identified and removed from the analysis by gating. Experiments were repeated three times, and mean ± SE values were calculated.

Measurement of apoptosis

Per cent apoptosis was measured by using 7-AAD with flow cytometry. The 4 × 10⁵ cells were plated into 25-cm² flasks for each data point and after 24 hr, erlotinib was added at 300 nM (IC₅₀ for A431 cells). Cells were irradiated (5 Gy), 2 hr later rinsed off (by gently aspirating the old media and washing once with PBS) and fresh medium was added. At 48 hr, cells were trypsinized (keeping all floating cells), counted for each sample and 1 × 10⁶ cells were centrifuged (1000 rpm, 5 min, 4°C) and resuspended in 200 μl of PBS + 25 μg/ml of 7-AAD. After incubating for 15 min at room temperature, 3 ml of PBS was added to each tube and the cells were centrifuged (1000 rpm, 5 min, 4°C) again, resuspended in 200 μl PBS + 1% paraformaldehyde and analyzed using FACScan. Cells with intermediate
levels of 7-AAD staining (“7-AAD dim”) were considered apoptotic.

**Detection of HER1/EGFR and phospho-HER1/EGFR**

The cell lines were cultured for 24 hr after plating, and after removing the culture medium, the cells were rinsed with cold 1× PBS. Cells were lyzed using a lysis buffer (containing a mild detergent and protease and phosphatase inhibitors: 50 mM HEPES, pH 7.5; 1% Triton X-100; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 2 mM sodium orthovanadate; 1.5 mM MgCl₂; and 1 mM EGTA), sonicated briefly and incubated on ice for 30 min. Lysates were clarified by centrifugation at 12000 rpm for 5 min.

Immunoprecipitation studies were carried out to detect HER1/EGFR autophosphorylation. Cell lysates (1000 μg of protein) were incubated with 5 μg polyclonal HER1/EGFR antibodies and incubated for 4 hr at 4°C. Protein G agarose beads (30 μl) were added to the reaction mixture and incubated overnight at 4°C. Beads were centrifuged at 4000 rpm for 5 min and washed four times with 1× PBS containing 0.1% NP-40 and 0.1% SDS + phosphatase inhibitors. After the final wash, sample-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.5% 2-mercaptoethanol, 10% glycerol) was added and boiled at 100°C for 5 min. Samples were centrifuged at 12000 rpm for 5 min and the supernatants subjected to 7% SDS-PAGE. Proteins were transferred on to the nitrocellulose membrane (at 30 V overnight) and immunoblotted with anti-phosphotyrosine monoclonal antibody PY69. Membranes were developed using the ECL system and exposed to Hyperfilm.

**Results**

**Level of HER1/EGFR expression and phosphorylation**

To investigate the correlation between HER1/EGFR expression and radiosensitization by erlotinib, we assessed endogenous HER1/EGFR expression and the level of HER1/EGFR autophosphorylation in two NSCLC cell lines (H157 and H460) and the high HER1/EGFR-expressing A431 cells. Fig. 1 shows that HER1/EGFR expression was extremely high in A431 cells, moderate in H157 cells and very low in H460 cells. The level of HER1/EGFR autophosphorylation was also consistent with the level of endogenous HER1/EGFR expression (Fig. 1). We detected an extremely high level of HER1/EGFR autophosphorylation in A431 cells, a moderate level in H157 cells, and a very low level in H460 cells. In another experiment (data not shown), we observed EGF-mediated HER1/EGFR phosphorylation in all three cell lines. These data show that the level of HER1/EGFR phosphorylation was clearly proportional to the level of endogenous HER1/EGFR expression.

**Sensitivity to erlotinib**

The sensitivity of the three selected cell lines to erlotinib (50 nM-5 μM) was assessed in clonogenic assay. Fig. 2 shows the cytotoxicity curves for serial doses of erlotinib. The mean inhibitory concentration representing 50% survival (IC₅₀) of erlotinib was 300 nM, 3 μM and 8 μM for A431, H157 and H460, respectively. We used 300 nM of erlotinib for the subsequent radiosensitization studies using these three cell lines.

**Radioresistance**

To determine the radioresistance of the three cell lines, a clonogenic assay was performed using graded doses of γ-radiation and the results are shown in Fig. 3. A431 was the most resistant, and H460 the most sensitive to radiation. Surviving fraction analysis at 2 Gy showed 74.2 and 66.4% survival of A431 and
H460 cells, respectively. Significant differences in survival were observed at 4 Gy, where 31.7% of A431 cells survived, compared with only 9.2% of H460 cells, when exposed to a similar dose of radiation, whereas, at 6 Gy, 8.7% of A431 and less than 1% of H460 cells survived. The surviving fractions of H157 cells were 61%, 23% and 5.8% at 2, 4 and 6 Gy, respectively.

Radiosensitization
To determine the radiosensitizing effect of erlotinib, cells were exposed to graded doses of γ-radiation and/or 300 nM concentration of erlotinib (IC50 dose of erlotinib for A431 cells; Fig. 2). The results are presented in Fig. 4. At a surviving fraction of 0.25, DERs of 1.40, 1.17, and 1.04 were observed for A431, H157 and H460, respectively. A431, having the highest level of HER1/EGFR expression displayed the greatest sensitivity to the radiosensitizing effect of erlotinib when combined with radiation.

Cell-cycle analysis
To address the mechanism of radioenhancement by erlotinib, cell-cycle analysis was performed in the cell lines and the results are presented in Fig. 5. In A431 cells, treatment with erlotinib alone (300 nM) for 24 hr significantly increased the percentage of cells in G0-G1 phase from 42.3 to 60.9% (p<0.05) and decreased the proportion in S phase (45.4 to 33.0%, p<0.05). However, in H157 or H460, no significant increase was observed in the percentage of cells in G0-G1 phase or decrease in S-phase cells.

Apoptosis
Erlotinib-induced apoptosis was augmented by radiation (5 Gy) only in A431 cells (Fig. 6). In A431 cells, treatment with erlotinib at 300 nM resulted in 9.4% apoptosis whereas in H157 and H470 cells, it was 5.4 and 3.8% respectively. When these cells were exposed to radiation (5 Gy) alone, the percentage of apoptotic cells was 13, 17.6 and 17.1% for A431, H157 and H460 cells, respectively. Interestingly, a more than 2 fold increase (27%) in apoptotic cells was observed when erlotinib was combined with a single dose of radiation (5 Gy) in A431 cells. However, the combined treatment of erlotinhib and radiation at the specified doses did not show any additional effect on apoptosis in either H157 (14.5%) or H460 (17.9%) cells (Fig. 5).

Discussion
The HER1/EGFR is a trans-membrane receptor, which plays a pivotal role in the development of
cancer and tumour progression. HER1/EGFR-dependent signaling is involved in cell proliferation, apoptosis, angiogenesis and metastasis. Therefore, HER1/EGFR represents an attractive target in cancer treatment. Several drugs that target HER1/EGFR are in phase II and III stages as single agents, or in combination with other anticancer modalities. The most advanced are: (i) cetuximab (IMC-C225,
Erbitux™, ImClone Systems Inc., New York, NY), a chimeric human-mouse monoclonal IgG1 antibody which blocks ligand binding and functional activation of the HER1/EGFR; and (ii) erlotinib and (iii) gefitinib (ZD1839, Iressa®, AstraZeneca, Wilmington, PA), both small molecules and selective inhibitors of HER1/EGFR-TK activity, which prevent autophosphorylation and activation.

In this study, erlotinib was used in combination with radiation. In phase I and II studies, erlotinib as a single agent has shown a good safety profile and encouraging antitumor activity in patients with various tumor types. A number of phase III studies in several tumor types including NSCLC and pancreatic cancer are complete or ongoing. In summary, erlotinib is a novel inhibitor of HER1/EGFR-TK activity with promising efficacy in initial studies and is currently undergoing extensive clinical evaluation as an anticancer drug.

Previous pre-clinical studies reported the radiosensitizing effect of cetuximab. Gefitinib also increased the antitumor effect of radiation in lung, colon and SCCHN models. In a recent study, erlotinib has also shown the radiosensitizing effect in human carcinoma cells expressing HER1/EGFR. In this study, we reported a correlation between HER1/EGFR expression and the degree of radiosensitizing effect of erlotinib. A431 that expresses a very high level of HER1/EGFR was the most radioresistant among three cell lines tested, while H460, expressing the least HER1/EGFR was the most radiosensitive. A431 was most sensitive to erlotinib (IC50 = 300 nM), while H460 was the most resistant to erlotinib (IC50 = 8 µM). H157 had intermediate sensitivity to both radiation and erlotinib (IC50 = 3 µM) (Table 1). Our results are consistent with the earlier findings, where the IC50 values of 0.1 µM and 24 µM were reported for gefitinib in A431 and H460, respectively. In the present study, erlotinib-induced apoptosis was also augmented by radiation in A431 cells only.

The mutational status of phosphatase and tension homolog (PTEN) and p53 in these three cell lines is also important, since these molecules are the major contributors to the growth of tumours and their interactions with HER1/EGFR inhibitors. HER1/EGFR-overexpressing cancer cells with mutant PTEN are less sensitive to gefitinib. Our observations with A431 containing wild-type PTEN and overexpressing HER1/EGFR demonstrated a significant responsiveness to erlotinib alone and combined with radiation. However, very low HER1/EGFR-expressing H460 with wild-type PTEN did not respond to either single or combined therapy. Moreover, H157, having a moderate level of HER1/EGFR expression and mutant PTEN also showed a low level of response to similar treatment. In a recent study, it was reported that the activity of gefitinib did not depend on the mutational status of the p53, but on HER1/EGFR signaling. The cell lines used in the present study have different p53 mutational status. A431 has mutated p53, H157 has nonsense mutation and expressed a truncated p53, while H460 contains wild-type p53. Our results demonstrated that the responsiveness of these cell lines to erlotinib mainly depends on the level of HER1/EGFR expression.

Radiation induces G2M arrest. In this study, we found that HER1/EGFR inhibition by erlotinib induced G1 cell-cycle arrest. The growth inhibitory effect of erlotinib combined with radiation might be due to the perturbation of the cell cycle with a decrease of cell numbers in S phase and accumulation in both the G0-G1 and G2M phases. The HER1/EGFR inhibitors such as gefitinib also initiate cell-cycle arrest in the G1 phase. The radioenhancement effect by the combined treatment of gefitinib and radiation in oral squamous carcinoma cells is also reported. In murine ovarian carcinoma cells, it was found that anti-EGF monoclonal antibody C225 not only reduced the EGF receptor phosphorylation, but also down-regulated the levels of EGF receptors and reversed the radioresistance due to the over-expression of EGFR.

### Table 1—Combined effect of erlotinib and radiation on differentially expressed EGFR in lung carcinoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR level</th>
<th>Surviving fraction at 6 Gy (%)</th>
<th>IC50 Erlotinib</th>
<th>DER</th>
<th>%G1 Arrest</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>++++</td>
<td>8.6</td>
<td>300 nM</td>
<td>1.40</td>
<td>18.6</td>
</tr>
<tr>
<td>H157</td>
<td>++</td>
<td>5.8</td>
<td>3 µM</td>
<td>1.17</td>
<td>2.0</td>
</tr>
<tr>
<td>H460</td>
<td>+</td>
<td>0.85</td>
<td>8 µM</td>
<td>1.04</td>
<td>4.8</td>
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In conclusion, we demonstrated that cancer cells with elevated HER1/EGFR expression might contribute to a higher degree of radiosensitization by erlotinib, when combined with radiation. The extent of erlotinib-induced radiosensitization depends on the level of HER1/EGFR expression and autophosphorylation. More sensitization was observed in the cells with higher expression level of EGFR. Therefore, determination of cell types and measurement of their HER1/EGFR expression and concomitant level of autophosphorylation might be helpful to predict an outcome in a cancer treatment.

References: