Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer

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Background: No proven targeted therapy is currently available for the treatment of triple-negative breast cancer (TNBC). Epidermal growth factor receptor (EGFR) is frequently overexpressed in TNBC. We studied the activity of EGFR antagonists alone, and in combination with chemotherapy, in TNBC cell lines.

Materials and methods: EGFR and phosphorylated EGFR were measured by enzyme-linked immunosorbent assay. Sensitivity to EGFR inhibitors alone and in combination with chemotherapy was assessed. Effects of gefitinib on EGFR signalling and cell cycle were also examined.

Results: EGFR was overexpressed in the TNBC compared with the human epidermal growth factor receptor 2 (HER-2)-positive cell lines. Phosphorylation of EGFR was detected in the TNBC cells in response to epidermal growth factor stimulation and was blocked by gefitinib treatment. However, the TNBC cell lines were less sensitive to EGFR inhibition than the HER-2-positive cell lines. Response to gefitinib was associated with reduced phosphorylation of both mitogen activated protein kinase (MAPK) and Akt and induction of G1 arrest. Gefitinib enhanced response to both carboplatin and docetaxel in the TNBC cells, and the triple combination of gefitinib, carboplatin and docetaxel was synergistic.

Conclusions: Although the TNBC cells are less sensitive to EGFR inhibition than the HER-2-positive cell lines, gefitinib enhanced response to chemotherapy. Gefitinib combined with carboplatin and docetaxel warrants further investigation in TNBC.

Key words: basal-like breast cancer, carboplatin, docetaxel, EGFR, gefitinib, triple-negative breast cancer

introduction

Reproducibly distinct clinicopathological subtypes of breast carcinoma, which have different outcomes, have been identified by molecular profiling [1]. These include luminal, HER-2 overexpressing, normal-like and basal-like breast cancers. Basal-like breast cancers are generally negative for expression of estrogen and progesterone receptors and HER-2 [triple-negative breast cancer (TNBC)] and express basal markers, such as cytokeratins 5/6, and epidermal growth factor receptor (EGFR) [2, 3]. TNBC is generally accepted as a clinical surrogate for basal-like breast cancer, although not all basal-like breast cancers are triple negative. The basal-like phenotype is associated with an early age of cancer onset, high chance of presentation with metastases and high proliferative index [4, 5]. In addition, BRCA-1-mutated tumours are associated with the basal-like phenotype [6–8].

At present, no targeted therapy has proven benefit in TNBC, although ‘drugable’ targets are being sought. EGFR mRNA is detected more frequently and at higher levels in basal-like breast cancers [1]. In a tissue microarray study, EGFR expression was observed in 54% of cases positive for basal cytokeratins. EGFR expression was also associated with poor survival, independent of nodal status and size [3].

EGFR inhibitors currently in clinical use include the small molecule tyrosine kinase inhibitors gefitinib and erlotinib and the mAb cetuximab, which are approved for treatment of a number of solid tumours [9, 10]. In this study, we have investigated EGFR inhibition as a therapeutic option in the treatment of TNBC.

materials and methods

cells and reagents

Three TNBC cell lines (BT20, HCC1937, MDA-MB-231) and two HER-2 overexpressing cell lines (BT474, SKBR3) were obtained from the American Tissue Culture Collection, Manassas, VA. Cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), at 37°C with 5% CO2. HCC1937 medium was supplemented with 1% sodium pyruvate (Sigma, Aldrich, Wicklow, Ireland). Gefitinib (Iressa®, Astrazeneca), cetuximab (Erbitux®, Merck), docetaxel (Taxotere®, Aventis), carboplatin (Paraplatin®, Bristol-Myers Squibb) and doxorubicin (Adriamycin®, Pharmacia & Upjohn) were purchased from the Pharmacy Department, St Vincent’s University Hospital, Dublin. Erlotinib was purchased from Sequoia Research Products (Pangbourne, UK). Western blotting was carried out using anti-MAPK, anti-phospho-MAPK, anti-Akt,
anti-phospho-Akt, anti-phospho-tyrosine (Cell Signalling Technology, Danvers, MA), anti-EGFR (Lab Vision, Fremont, CA), anti-α-tubulin (Sigma) antibodies, secondary anti-mouse antibody (Sigma) and anti-rabbit antibody (Pierce Biotechnology, Rockford, IL).

**protein extraction**

After 48-h gefitinib treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl RIPA buffer (Sigma) containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate. After 20-min incubation on ice, lysate was passed through a 21-gauge needle and centrifuged at 16 100·g for 5 min at 4°C. Protein quantification was carried out using the bicinchoninic acid assay (Pierce).

**enzyme-linked immunosorbent assay**

EGFR and phosphorylated epidermal growth factor receptor (P-EGFR) were used for the EGFR ELISA (R&D Systems, Minneapolis, MN). EGFR protein concentrations were estimated from a standard curve and expressed as pg EGFR per μg of total protein. For P-EGFR, TNBC cells were serum starved for 24 h and treated with serum-free medium (control), 10 ng/ml epidermal growth factor (EGF) for 10 min or 1 μM gefitinib for 15 min followed by 10 ng/ml EGF for 10 min. One microgram (BT20) or 5 μg (HCC1937, MDA-MB-231) protein was tested in the P-EGFR ELISA (R&D Systems). P-EGFR levels were expressed relative to control untreated cells.

**western blotting**

Twenty micrograms of protein in sample buffer was heated to 95°C for 5 min and proteins were separated using 7.5% or 10% polyacrylamide gels (Cambrex, East Rutherford, NJ). Protein was transferred to Hybond membrane. Protein bands were detected using anti-EGFR antibody (Labvision), and P-EGFR was detected using anti-phospho-tyrosine antibody (Cell Signaling). Using anti-EGFR antibody in 3% blocking solution for 1 h. Following three washes with PBS–0.1% Tween and one PBS wash, protein bands were detected using antibody in 1% blocking solution, three washes with PBS–0.1% Tween were carried out followed by incubation at room temperature with secondary antibody in 0.1% Tween and PBS–0.1% Tween and one PBS wash, protein bands were detected using Lumino! (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or ECL-Advance (Amersham Biosciences). Total EGFR was immunoprecipitated using anti-EGFR antibody (Labvision), and P-EGFR was detected using anti-phospho-tyrosine antibody (Cell Signalling).

**proliferation assays**

The 96-well plates were seeded with 3 × 10^4 cells per well. Following overnight incubation, drug was added at the appropriate concentrations and incubated for 5 days. Media were removed and cells washed once with PBS. One hundred microliters of acid phosphatase substrate [7.25 mM p-nitrophenyl-phosphate (Sigma) in sodium acetate buffer] was added to each well and incubated at 37°C for 1 h. Fifty microliters of 1 M NaOH was added to stop the reaction, and the absorbance was read at 405 nM with 620 nM as the reference wavelength.

**cell cycle assays**

The 24-well plates were seeded with 1 × 10^6 cells per well. Following overnight incubation, cells were treated with drugs for 48 h. An untreated control and a dimethyl sulfoxide control were also tested. Nonadherent cells were collected and combined with adherent cells after trypsinisation, then centrifugated at 500g for 5 min. Cells were washed in PBS, then resuspended in 20 μl PBS and fixed in 200 μl ice-cold 70% ethanol at 4°C overnight. Cell cycle assays were carried out using Cell Cycle Reagent (Guava Technologies, Hayward, CA) and analysed on the Guava EasyCyte. Data analysis was carried out using ModFit LT™ software (Verity, Software House, Topsham, ME).

**statistical analysis**

For fixed dose gefitinib combination assays, a two-tailed t-test was used to compare the effect of gefitinib combined with chemotherapy with the chemotherapy drug alone. For triple combination fixed ratio assays, combination indices (CIs) at the ED_{50} (effective dose of combination that inhibits 50% of growth) were determined using the Chou and Talalay equation [11], on CalcuSyn software (Biosoft, Cambridge, UK), CI < 1 implies synergy, CI = 1 implies additivity and CI > 1 implies antagonism. Percentages of cells in each phase of the cell cycle in drug-treated cells were compared with untreated cells using a two-tailed t-test. P < 0.05 was considered statistically significant.

**results**

**EGFR inhibition**

High levels of EGFR were detected in the TNBC cell lines (pg EGFR per μg total protein: BT20, 150.5 ± 11.7; HCC1937, 44.4 ± 8.1; MDA-MB-231, 72.1 ± 9.8) compared with the HER-2-positive cell lines, which overexpress HER-2 [12], but have low levels of EGFR (pg EGFR per μg total protein: BT474, 3.6 ± 1.1; SKBR3, 20.5 ± 2.5).

Despite high levels of EGFR expression, the TNBCs were less sensitive to gefitinib and erlotinib than the HER-2-positive cell lines (Table 1). Gefitinib was the most effective EGFR inhibitor tested, with HCC1937 the most sensitive of the TNBC cell lines [drug concentration which inhibits 50% of cell growth (IC_{50}): 8.4 ± 1.5 μM]. Cetuximab showed very little effect on proliferation in any of the cell lines tested. No significant difference in chemosensitivity, to the three

| **Table 1.** Response of the breast cancer cell lines to epidermal growth factor receptor inhibitors and chemotherapy drugs (IC_{50}: drug concentration which inhibits 50% of cell growth) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | BT20            | HCC1937         | MDA-MB-231      | BT474           | SKBR3           |
| Gefitinib, IC_{50} (μM) | 15.5 ± 1.4    | 8.4 ± 1.5      | 20.7 ± 1.1     | 0.25 ± 0.05     | 0.88 ± 0.31     |
| Erlotinib, IC_{50} (μM)  | 20.1 ± 2.7    | 26.2 ± 9.3     | 42.6 ± 3.1     | 3.1 ± 0.4       | 3.6 ± 0.4       |
| Cetuximab (% inhibition at 100 μg/ml) | 17.0 ± 2.0 | 19.7 ± 5.0 | 0              | 0               | 0               |
| Carboplatin, IC_{50} (μM) | 8.6 ± 4.8   | 11.6 ± 2.8     | 15.4 ± 3.3     | >20             | 9.7 ± 5.3       |
| Doxorubicin, IC_{50} (nM) | 34.7 ± 9.5  | 38.6 ± 1.6     | 59.6 ± 9.8     | 117.0 ± 40.0    | 27.0 ± 9.0      |
| Docetaxel, IC_{50} (nM)  | 2.4 ± 1.4     | 7.2 ± 2.5      | 3.0 ± 0.5      | 2.8 ± 0.3       | 1.2 ± 0.6       |

IC_{50} values were not achievable for cetuximab. Values displayed are for % inhibition achieved at 100 μg/ml.
chemotherapy drugs tested, was observed between the TNBC and the HER-2-positive cell lines (Table 1).

In BT20 and HCC1937 cells, addition of a fixed dose of gefitinib (approximate IC30 concentration for each cell line) enhanced the inhibitory effects of carboplatin and docetaxel, compared with the chemotherapy drugs alone (Figure 1A). Similar results were obtained for MDA-MB-231, but with a higher concentration of gefitinib, as this cell line is less sensitive to gefitinib (supplemental Figure 1, available online).

Treatment with the triple combination of gefitinib, carboplatin and docetaxel was also more effective in BT20 and HCC1937 cells than each agent alone (Figure 1B). CI values for the triple combination suggest a synergistic interaction in both cell lines (BT20, CI at ED50 = 0.5 ± 0.1; HCC1937, CI at ED50 = 0.8 ± 0.4), while the chemotherapy combination alone is slightly antagonistic (BT20, CI at ED50 = 1.2 ± 0.2; HCC1937, CI at ED50 = 1.3 ± 0.1).

effects of gefitinib on EGFR signalling and cell cycle arrest

P-EGFR was detected at high levels in BT20 cells and was reduced in response to gefitinib treatment. Low levels of

![Graphs showing proliferation assays of triple-negative breast cancer cells treated with carboplatin or docetaxel combined with gefitinib.](image)

Figure 1. (A) Proliferation assays of triple-negative breast cancer cells treated with carboplatin or docetaxel combined with a fixed dose of gefitinib. P values were calculated for chemotherapy drug alone versus gefitinib combined with chemotherapy, using a two-tailed t-test. (B) Proliferation assays with gefitinib (G), carboplatin (P) and docetaxel (D), alone and in combination, in BT20 and HCC1937 (drug ratios: G : P : D—10 000 : 10 000 : 1).
P-EGFR were detected in MDA-MB-231 and HCC1937 cells by western blot (Figure 2A). However, EGF treatment, following serum starvation, significantly increased phosphorylation of EGFR in all three TNBC cell lines and gefitinib treatment efficiently blocked EGF-induced phosphorylation of EGFR (Figure 2B).

Gefitinib decreased phosphorylation of MAPK and Akt in the sensitive HER-2-positive cell lines, BT474 and SKBR3. Gefitinib treatment also reduced phosphorylation of both MAPK and Akt in HCC1937, which is the most sensitive of the TNBC cell lines. Gefitinib decreased phosphorylation of MAPK in BT20 cells but no change in Akt phosphorylation was observed. No change in phosphorylation of MAPK was observed in MDA-MB-231 cells and consistent with previous results, phosphorylated Akt was not detected in MDA-MB-231 cells [13].

Response to EGFR inhibition is associated with induction of G1 cell cycle arrest [14, 15]. A significant increase in G1 was observed in BT474 and SKBR3 cells treated with 10 μM gefitinib for 48 h (Table 2). In contrast, no increase in G1 was observed in either BT20 or MDA-MB-231 cells treated with 10 μM gefitinib. A small increase in G1 was observed in the HCC1937 cells in response to 10 μM gefitinib but this increase did not achieve statistical significance. Treatment with

Figure 2. (A) Detection of EGFR, P-EGFR, MAPK, P-MAPK, Akt and P-Akt in triple-negative breast cancer and HER-2-positive cell lines treated with gefitinib for 48 h. (G: gefitinib; P-: phospho-; EGFR IP P-tyr: epidermal growth factor receptor immunoprecipitation, followed by phospho-tyrosine immunoblotting.). (B). Levels of phosphorylated epidermal growth factor receptor (P-EGFR) in epidermal growth factor-stimulated TNBC cells with and without gefitinib pretreatment, compared with untreated cells. P-EGFR levels were measured by enzyme-linked immunosorbent assay. (C) Analysis of the effects of 48 h of treatment with 10 μM G alone, 10 μM P and 1 nM D and the three drugs combined, on cell cycle progression in BT20 and HCC1937. P values were calculated for each treatment compared with untreated cells, using a two-tailed t-test.
sub-G0/G1 fraction was increased in HCC1937 cells treated with P-EGFR. In TNBC, EGF treatment induced positive cell lines, at least signalling for growth and this may explain their relative protein in TNBC, they may not be dependent on EGFR immunoblotting. This suggests that despite high levels of EGFR were only detected in BT20 cells. Very low levels of P-EGFR were detected in MDA-MB-231 cells. The BT-20 and HCC1937 cells are classified as basal-like or post-epithelial cell lines can also be divided into basal-like and post-epithelial EMT has been implicated in resistance to EGFR inhibition in basal-A while the MDA-MB-231 cells which show greatest sensitivity to gefitinib, also showed reduced phosphorylation of both MAPK and Akt. In BT20 cells, a decrease in phosphorylation of MAPK was observed with no change in phosphorylated Akt. No changes in either MAPK or Akt phosphorylation were observed in MDA-MB-231 cells. This suggests that response to EGFR inhibition requires efficient blockade of both MAPK and Akt signalling.

Although the TNBC cells are not inherently sensitive to EGFR inhibition, combined treatment with gefitinib and chemotherapy has a greater effect on proliferation than either gefitinib or the chemotherapy alone. Using CI, we have shown that the triple combination of gefitinib with carboplatin and docetaxel is synergistic in the TNBC cells. This synergy may be partly due to synergy between gefitinib and the individual chemotherapy drugs. However, the dual combination of carboplatin and docetaxel appears to be antagonistic while the addition of gefitinib results in synergy. Hoadley et al. [18] also showed that combinations of cetuximab or gefitinib with chemotherapy were synergistic in the basal-like breast cancer cell line SUM102.

Similar interactions between gefitinib and chemotherapy have been observed in the HER-2-positive SKBR3 and BT474 cells lines [25]. Pegram et al. [26] also showed that the triple combination of trastuzumab, carboplatin and docetaxel was highly synergistic in HER-2-positive breast cancer cells and this combination has shown efficacy in the treatment of early-stage HER-2-positive breast cancer [27]. Combined induction of G2/M and G1 arrest observed for treatment with gefitinib, carboplatin and docetaxel may, at least in part, explain the synergy observed with the triple combination in the TNBC cell lines. An increase in the sub-G0/ G1 fraction was also observed with the chemotherapy combination and the triple combination, suggesting an increase in apoptosis. Despite their relative resistance to gefitinib, our data show that combination of gefitinib with carboplatin and docetaxel improves response in the TNBC cell lines. Although the concentrations of gefitinib used in our in vitro assays are high, evidence from the BCIRG103 study suggests that a preferential distribution of gefitinib from blood into tumour tissue occurs in vivo [28]. Following treatment of breast cancer patients with oral gefitinib (250 mg/day for at least 14 days, n = 19), gefitinib concentrations in breast tumour tissue (mean = 16.7 μM) were 42 times higher than plasma concentrations.

Previous single-agent clinical trials in breast cancer with EGFR inhibitors, in general, have been disappointing. However, these have often been in heavily pretreated and unselected patient populations [29, 30]. Our results suggest that the triple combination of gefitinib with docetaxel and carboplatin is a rational combination that may provide additional benefit in TNBC patients and warrants further investigation.

discussion
Our data confirm the observation that EGFR is overexpressed in TNBC [2, 3, 16, 17]. The lack of a proven targeted therapy for TNBC, together with the availability of a number of approved EGFR inhibitors, provides a powerful rationale for the study of these agents, alone and in combination with chemotherapy in TNBC. While our data confirm the activity of the EGFR inhibitor gefitinib in TNBC cell lines, we found higher activity of this agent in HER-2-positive breast cancer cell lines, despite the fact that these cells had lower levels of EGFR. Cetuximab showed no inhibition of growth of the TNBC cell lines tested in this study. Lack of efficacy of cetuximab in breast cancer cells in vitro has previously been reported [18]. Hoadley et al. [18] identified a basal-like breast cancer cell line (SUM102) which displayed sensitivity to both cetuximab in breast cancer cells [23, 24]. Following treatment of breast cancer patients with gefitinib in early-stage HER-2-positive breast cancer [27]. Combined induction of G2/M and G1 arrest observed for treatment with gefitinib, carboplatin and docetaxel may, at least in part, explain the synergy observed with the triple combination in the TNBC cell lines. An increase in the sub-G0/ G1 fraction was also observed with the chemotherapy combination and the triple combination, suggesting an increase in apoptosis.

In the TNBC cell lines studied, significant levels of P-EGFR were only detected in BT20 cells. Very low levels of P-EGFR were detected in MDA-MB-231 and HCC1937 cells, by immunoblotting. This suggests that despite high levels of EGFR protein in TNBC cells, they may not be dependent on EGFR signalling for growth and this may explain their relative resistance to EGFR inhibition, compared with the HER-2-positive cell lines, at least in vitro. Although basal levels of P-EGFR in TNBC cells were low, EGF treatment induced significant phosphorylation of EGFR and gefitinib efficiently blocks the phosphorylation of EGFR in EGF-treated TNBC cells. Measurement of the phosphorylation status of EGFR in triple-negative tumours may help to clarify if TNBCs are dependent on EGFR signalling.

Phosphorylation of both MAPK and Akt were reduced in the gefitinib-sensitive, HER-2 overexpressing BT474 and SKBR3 cell lines in response to gefitinib. HCC1937 cells, which show the greatest sensitivity to gefitinib, also showed reduced phosphorylation of both MAPK and Akt. In BT20 cells, a decrease in phosphorylation of MAPK was observed with no change in phosphorylated Akt. No changes in either MAPK or Akt phosphorylation were observed in MDA-MB-231 cells. This suggests that response to EGFR inhibition requires efficient blockade of both MAPK and Akt signalling.

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Table 2. Cell cycle analysis of the percentage of cells in G1 phase in control and gefitinib-treated cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control (mean ± SD)</th>
<th>Gefitinib 10 μM (mean ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>51.2 ± 4.0</td>
<td>49.4 ± 3.4</td>
<td>0.453</td>
</tr>
<tr>
<td>HCC1937</td>
<td>57.7 ± 4.0</td>
<td>62.9 ± 4.0</td>
<td>0.082</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>44.0 ± 4.2</td>
<td>43.8 ± 2.1</td>
<td>0.897</td>
</tr>
<tr>
<td>BT474</td>
<td>54.3 ± 2.7</td>
<td>76.2 ± 2.5</td>
<td>0.003</td>
</tr>
<tr>
<td>SKBR3</td>
<td>49.2 ± 1.3</td>
<td>75.0 ± 3.6</td>
<td>0.003</td>
</tr>
</tbody>
</table>

P was calculated using a two-tailed t-test.
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references