Effect of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibition on Epithelial Proliferation in Normal and Premalignant Breast

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ABSTRACT

The factors controlling epithelial proliferation in ductal carcinoma in situ (DCIS) are unclear. Antiestrogens are effective in the prevention of the majority of estrogen receptor-positive, but not estrogen receptor (ER)-negative breast cancers, which suggests that other factor(s) are promoting proliferation in ER-negative DCIS. Mutated or overexpressed tyrosine kinases are frequently associated with tumor development. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is involved with mitogenesis and is expressed in ER-negative DCIS. We hypothesized that EGFR is central in driving proliferation in ER-negative/EGFR-positive DCIS. The purpose of this study was to establish whether the EGFR tyrosine kinase inhibitor (EGFR-TKI), ZD1839 (Iressa), can reduce epithelial proliferation and increase apoptosis in EGFR-positive DCIS.

Breast tissue from 16 women undergoing surgery for DCIS were implanted into 16–32 immunosuppressed mice/experiment (8 xenografts/mouse). Treatment commenced 2 weeks after implantation and consisted of once daily oral gavage with ZD1839 at doses ranging from 10 to 200 mg/kg for 14–28 days; appropriate controls were present. Xenografts were removed on days 14, 21, 28, and 42 after implantation and then assessed for proliferation (LI) by Ki67 immunostaining and apoptosis index (AI) by morphology. All P values reported are two-sided.

Overall, a 56% reduction in epithelial proliferation was seen with Iressa in EGFR-positive DCIS. EGFR-TKI inhibition compared with vehicle controls resulted in a fall in Geometric Mean Labeling Index (LI) after 14 days (day 28) of treatment both in ER-negative/EGFR-positive DCIS [6.5% interquartile range (IQR) 3.8–11.1] versus 13.9% (IQR, 12.0–16.3%); F(1,3) = 103; P = 0.002] and ER-positive/EGFR-positive DCIS [4.6% (IQR, 3.9–5.2%) versus 11.7% (IQR, 9.2–15.1%); F(1,2) = 32.3; P = 0.03]. EGFR-TKI inhibition had similar effects on the "at risk" normal breast epithelium adjacent to DCIS in the treated epithelium LI day 28 [ZD1839 2.2% (IQR, 1.7–3.3%) compared with control 3.8% (IQR, 2.4–5.4%); F(1,14) = 29.2; P = 0.00009] and in addition increased epithelial apoptotic index at day 21 [ZD1839 0.38 (0.23–0.83) compared with control 0.19 (0.1–0.25); F(1,6) = 12.2; P = 0.013].

The effect on epithelial proliferation was still significant after 28 days of treatment [for both DCIS (F1.29) = 24; P = 0.039 and normal breast F(1,6) = 47.3; P = 0.0005]. EGFR-TKI inhibition with ZD1839 offers a novel approach to the treatment of EGFR-positive DCIS, regardless of ER status, and provides a potential new chemopreventative approach in patients at high risk of breast cancer.

INTRODUCTION

Breast cancer is a major public health concern with ~180,000 cases/year in the United States (1). Prevention of ER-positive breast cancer has been reported by the use of the antiestrogens, tamoxifen, or raloxifene (2, 3). However, in both of these studies, development of tumors that were ER negative were not prevented, and a third of tumors that developed were ER positive, suggesting that antiestrogens do not fully inhibit carcinogenesis (2, 3). DCIS is a premalignant disease that accounts for 30% of mammographically detected breast cancer (4), and when untreated, progresses to invasive breast cancer in 25–30% of patients. Traditionally, most patients were treated by mastectomy, but this represents overtreatment for the majority of women who were not destined to develop breast cancer. However, after breast-conserving surgery, up to 40% of women with DCIS develop a recurrent lesion within 15 years, and of these, half will be invasive breast cancer and therefore potentially incurable (5).

Although radiotherapy reduces recurrences after breast-conserving surgery for DCIS, the use of antiestrogens as adjuvant therapy to prevent recurrence and progression of ER-negative DCIS after breast-conserving surgery is controversial (6, 7). In an in vivo model of human DCIS, we have reported previously that the pure antiestrogen Faslodex increased apoptosis in ER-positive DCIS but had no effect on epithelial proliferation or apoptosis in ER-negative DCIS, suggesting that there are other factors promoting proliferation in hormone-independent (ER-negative) DCIS (8, 9). High-grade DCIS expresses members of the type 1 tyrosine kinase family of receptors, including EGFR and c-erbB-2 (10, 11). Activation of the EGFR results in homodimerization or heterodimerization of EGFR with itself or c-erbB-2, c-erbB-3, or c-erbB-4 (12). Dimerization results in tyrosine kinase autophosphorylation activating the mitogen-activated protein kinase signaling pathway to induce cell proliferation and/or cell survival (13, 14). Heterodimerization of EGFR with c-erbB-2 can stimulate transformed growth of mammary epithelial cells through interaction with c-src protein kinase and EGFR/c-erbB-2 (15, 16), and heterodimers are critical for the initiation of tumor formation in transgenic mice models (17). Studies of invasive cancers treated with monoclonal antibodies to EGFR have shown reductions in malignant epithelial proliferation and increased apoptosis in preclinical studies (18).

ZD1839 (Iressa) is a p.o. active, selective EGFR-TKI that blocks signal transduction pathways implicated in proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth (19). In TK assays, EGFR-TKI occurs at IC50 doses of 0.023–0.079 μM. Inhibition of c-erbB-2 (IC50, 3 μm), and KDR (3.7–33 μm) also occurs but at doses 100-fold higher than EGFR inhibition. No other activity has been demonstrated against a range of other kinases (19). We hypothesized that ER-negative DCIS was likely to be using the EGFR signaling pathways for growth and tested this hypothesis by using ZD1839 on EGFR-positive DCIS xenografts using the in vivo model we have described previously (8, 9).

Phenotypically normal breast epithelium adjacent to DCIS expresses EGFR and frequently contains changes, such as loss of heterozygosity and mutation of the BRCA1 oncogene, and therefore represents breast epithelium with a high risk of malignant transformation (20). We, therefore, also studied the effect of Iressa on epi-

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2 The abbreviations used are: ER, estrogen receptor; DCIS, ductal carcinoma in situ; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; LI, labeling index; AI, apoptosis index; ZD1839, 4-(3-chloro-8-fluoranoimo)-7-methoxy-6-(3-morpholino)propoxy)quinazoline; MAP, mitogen-activated protein.
the epithelial proliferation (LI) and apoptosis (AI) in the normal breast adjacent to DCIS.

PATIENTS AND METHODS

Patients. Participants in this study were women who either attended the Nightingale Breast Screening Assessment Center or the Symptomatic Breast Clinic at the University Hospital of South Manchester, United Kingdom during the period November 1998 to January 2000. Women were included in the study if they had mammograms showing widespread microcalcification indicative of DCIS and either cytopathological or histopathological confirmation of the diagnosis (n = 16). All tissue specimens were obtained at therapeutic excision of DCIS and were subsequently reviewed by a Consultant Breast Pathologist. Approval to remove tissue from pathological samples in this study was granted by the South Manchester Medical Research Ethics Committee.

Animals. Intact, adult, female, athymic nude mice (BALB/c nu/nu), 8–10 weeks of age, were obtained from the breeding colony at the Paterson Institute for Cancer Research. They were housed under conventional conditions with a 12-h cycle of light and dark (lights off from 7:00 p.m. to 7:00 a.m.) in filter top cages. They were supplied ad libitum with irradiated feed and filtered water and irradiated bedding during breeding. Normal food, water, and bedding were used during the experiments. All care of the animals and surgical procedures were performed in accordance with Home Office Regulations and the United Kingdom Scientific Procedures (1986) Act. Halothane inhalational anesthesia (2–4% halothane on oxygen; Halovet Vaporiser, International Market Supplies, Congleton, United Kingdom) was used for all procedures.

Treatment of Tissue Samples. For preparation of tissue specimens for grafting to mice, 1–2 cm³ pieces of breast tissue, containing microcalcifications, were taken at the time of surgery from the main specimen and handled as described previously (6, 9). The tissue specimen was carefully dissected into 2 mm × 2 mm × 1 mm samples with a scalpel blade and, depending on the volume of tissue available, between 5 and 20 pieces were randomly selected from the Petri dish and not implanted into the mice but instead were used for day 0 histology. Half of these nonimplanted (day 0) grafts were immediately fixed in buffered formalin (4% formaldehyde) for 24 h, and the other half in Carnoy’s fixative for 1 h, and then all were stored in 70% alcohol. After at least 24 h, the fixed tissue samples were placed individually in tissue cassettes (Tissue Tek III; Bayer Diagnostics Ltd., Basingstoke, United Kingdom) and stored 70% alcohol until paraffin embedding. These samples, representing the DCIS excised from each patient, were labeled as the “day 0” specimens and were reserved for histological review, immunostaining, and apoptotic cell counts. The remaining xenografts were implanted into nude mice.

Implantation of Xenografts into Nude Mice. Breast tissue was implanted from 16 women as described previously (8, 9), and each patient’s sample was divided between 10 and 32 mice (depending on the volume of tissue available; median number, 16). Each mouse received tissue from 1 woman only, and transplantation was completed within 90 min of removal of tissue from the patient. Two small midline skin incisions were made across the dorsal skin through which eight tissue pieces were symmetrically placed (four on each side).

Retrieval of xenografts at the appropriate time points required reanesthetizing the mice and excising each graft using sharp dissection. The grafts were then processed for histology as described above for the day 0 specimens. For the 100–200 mg/kg ZD1839 experiments, grafts were removed on days 14, 21, and 28; at the lower doses, grafts were retrieved on days 14, 28, and 42. Two xenografts were removed at each interim time point and four xenografts at end time points from each mouse.

Treatment. Commencing on day 14 after removal of the first two xenografts, the mice were randomly allocated to be gavaged for 14–28 days with either ZD1839 (10–200 mg/kg) or vehicle. ZD1839, a p.o.-active, selective EGFR-TKI, was a kind gift of AstraZeneca Pharmaceuticals. The vehicle (control) was 0.5% polysorbate.

Histological Evaluation of Xenografts. All day 0 specimens and each xenograft were embedded into paraffin blocks. H&E-stained, 3-μm sections from each block were examined by a single experienced breast pathologist (W. F. K.) for the presence of DCIS or normal breast epithelium (8, 9); those containing DCIS or normal breast were assessed for apoptosis and Ki67 antigen immunogenicity (a marker of epithelial proliferation) as described previously (20).

Nuclear grade and the presence or absence of comedo-necrosis were also ascertained on all day O specimens containing DCIS (W. F. K.), and day 0 specimens were immunohistochemically stained for ER, c-erbB-2, and EGFR status.

Assessment of Apoptotic cell death. H&E-stained sections of DCIS samples were examined using light microscopy for morphological evidence of apoptosis. The criteria used to identify apoptotic cells have been described previously (9), and at least 1000 cells were counted at ×400 using a Zeiss microscope, and the number of cells displaying apoptotic morphology was expressed as a percentage of the total number counted (9). Immunohistochemical determination of ER and Ki67 nuclear antigen ER and Ki67 immunohistochemical methodology has been described previously (9).

The Ki67 LI was calculated from counting a minimum of 1000 epithelial cells, and the numbers of positively stained nuclei were expressed as a percentage of the total number counted. The ER status was determined also counting at least 1000 cells, and losses were considered ER positive if >5% of cells were positively stained for ER.

Immunohistochemical Determination of c-erbB-2, EGFR, and pErk1/ Erk2. Paraffin wax sections (3 μm thick) of tissue from each specimen were cut, mounted on 3-aminopropyltriethoxysilane-coated slides, dewaxed, and hydrated before immunohistochemical staining for the c-erbB-2, EGFR, and phosphorylated Erk1/Erk2. Antigen retrieval was achieved by a microwave method (650 W) for 30 min. Endogenous peroxidase activity was blocked by incubation in hydrogen peroxide [1.5% (v/v) for EGFR and c-erbB-2, 3% (v/v) for pErk1/Erk2] in PBS for 15 min.

For the c-erbB-2 and EGFR antigen, the slides were rinsed in TBS prior to using 10% normal rabbit serum to block nonspecific binding and then incubated with the primary antibody at a dilution of 1:20 for EGFR (mouse monoclonal antihuman, NCL-EGFR; Novocastra Labs, Newcastle upon Tyne, United Kingdom) and at a dilution of 1:40 for c-erbB-2 (mouse monoclonal, MAB4022; Chemicon, Harrow, United Kingdom) for 1 h at room temperature. A biotinylated rabbit antimouse immunoglobulin (E413; Dako, High Wycombe, United Kingdom) was applied as the secondary antibody (1:350 dilution, incubated for 30 min), and following this, a 30-min incubation at room temperature with the standard three-layered streptavidin-avidin-biotin peroxidase (K0377; Dako) for c-erbB-2 and EGFR.

For pErk1/Erk2 antigen detection, the sections were blocked with 20% normal human serum for 15 min prior to application of the polyclonal rabbit antihuman pErk1/Erk2 primary antibodies at a dilution of 1:20 (9101 L; New England Biolabs). Biogenex Multilink at a dilution of 1:100 (Euro/DPC, Llanberis, United Kingdom) was applied as the secondary antibody for 1 h. An avidin/biotin complex immunohistochemical kit procedure (Biogenex Concentrated Label, Euro/DPC) was used. The chromogen diaminobenzidine (Sigma Chemical Co., Poole, United Kingdom) was applied for 5 min, and then hematoxylin was applied as a counterstain for 5 min.

Negative (mouse IgG, X0931; Dako) and positive controls (sections of A431 cells for EGFR, and of DCIS tissue shown previously to be strongly positive for c-erbB-2 and pErk1/Erk2) were used. Staining for c-erbB-2 and EGFR were predominantly membranous, but there was also cytoplasmic and nuclear uptake. These were scored positive (scale 1–4) or negative in relation to the positive and negative control slides. Staining for pErk1/Erk2 was nuclear and cytoplasmic and scored as described previously (21).

All histological assessments were performed by investigators blinded to the treatment group. Reproducibility of counting was assessed by the same investigator resoring 10 slides stained with the Ki67 antibody and 10 H&E slides for apoptosis 6 months after initial estimation. The sets of results obtained were correlated by regression analysis (r = 0.95, P < 0.001).

Assessment of Activated EGFR. This method has been described previously (21). Sections were mounted, dewaxed, and rehydrated as described for EGFR. Antigen retrieval was achieved by a microwave method for 10 min. A mouse primary antibody to activated EGFR (Chemicon, Harrow, United Kingdom) was applied at a dilution of 1:50, followed by a secondary antibody detection system (En Vision DAKO) and a standard diaminobenzidine chromogen. Positivity for activated EGFR was scored by H scoring all slides without knowledge of the treatment given to the mouse. A positive control of activated EGFR in DU145 prostate cancer cells was used after growth of the cells on a coverslip and formalin fixation prior to assay (21).
Epithelial Proliferation in Normal Mouse Intestine. Removal, processing of intestinal tissue for Ki67 assessment, and scoring methodology have been described previously (22).

Statistical Methods. Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). The comparisons between Iressa and control samples were made “within-patient” using a repeated ANOVA model with patient and group (Iressa versus control) as factors. This statistical model effectively matched the Iressa and control readings from mice transplanted with tissue from the same woman. The residual sum of squares used in the Iressa versus control comparison is the appropriate “patient × drug” interaction term adjusting for multiple readings/patient. Data were restricted to those mice with tissue from patients with readings available from both “Iressa” and “control” mice. At each day, only one reading at most from each mouse was obtained, i.e., there were no multiple readings from the same mouse at the same time point. Hence, throughout the statistical analysis “mouse” is taken as the unit of analysis. All xenografts were removed in a standard way from each mouse so the effect of side or position in the mouse was the same in each experiment and mouse. Dose effects were adjusted for where necessary by including a co-variance term for dose in the statistical model. LI and AI values were log transformed to produce an adequate approximation to a normal distribution. Statistical analysis of proliferation in intestinal epithelium was by comparing medians at each epithelial cell position in the crypts (23).

RESULTS

Breast tissue from 16 women were used in this study (median age, 52 years; range, 34–88), of which 7 (43.8%) were identified through the United Kingdom Breast Screening Program with asymptomatic widespread mammographic microcalcification; the remainder (56.2%) presented with palpable breast lumps and had widespread mammographic microcalcification. Eight (50%) of these women were premenopausal, and the remainder were postmenopausal. All underwent mastectomy (9 with axillary node clearance). Seven women (43.8%) were diagnosed as having only DCIS, and 9 (56.2%) were diagnosed with invasive carcinoma and widespread surrounding DCIS.

Xenograft Implant and Retrieval: DCIS. Of the 16 breast tissue specimens found to contain DCIS at surgery, 13 (81.3%) produced day 0 specimens that contained DCIS, of which 11 were immunohistochemically EGFR positive and 2 were EGFR negative. Of these 11 EGFR-positive cases, 8 were ER-negative/c-erbB-2 positive (all high nuclear grade), 2 were ER-positive/c-erbB-2 positive (1 high grade, 1 intermediate grade), and 1 was ER-positive/c-erbB-2 negative (low grade). The 13 breast tissue specimens containing DCIS produced 273 day 0 samples, of which 44 (16.1%) contained foci of DCIS.

A total of 1904 xenografts were implanted, of which 1858 (97.5%) were successfully retrieved, and 329 (17.7%) contained DCIS. DCIS was retrieved at Days 14, 21, 28, and 42 xenografts in all of the 13 experiments.

Normal Breast. Histologically normal breast tissue was found in all 16 specimens removed from surgery for DCIS. The majority (97.6%) of the xenografts implanted were retrieved, of which 22.4% contained normal breast. The remainder contained stromal tissue with either ducts and no lobules or insufficient epithelium to score for Ki67 (≤500 cells). Normal breast was retrieved from all of the time points in 100% of all of the experiments. The normal breast in all 16 specimens expressed both ER-positive and EGFR-positive cells, and 2 showed some cells that were weakly c-erbB2 positive.

EGFR-positive DCIS. The geometric mean LI and AI in day 0 specimens was higher in the 8 cases of ER-negative/EGFR-positive DCIS than the 3 cases of ER-positive/EGFR-positive DCIS [15.4% (IQR, 13.9–16.1%) versus 8.2% (IQR, 6.5–11.2%); F(1,9) = 39.7; P < 0.001 and 1.29% (IQR, 1.0–1.6%) versus 0.80% (IQR, 0.6–1.0%); F(1,9) = 7.1; P = 0.062] (Fig. 1).

Mean LI in 11 EGFR-positive DCIS fell in the Iressa-treated group by 56% compared with the vehicle-treated group by day 28 [5.9% (IQR, 4.1–10.9%) versus 13.3% (IQR, 11.8–15.8%); F(1,6) = 101; P ≤ 0.001; see Table 1] and day 42 (P ≤ 0.05). Additionally, a rise in AI was seen at day 21 in ZD1839-treated DCIS [0.70% (IQR, 0.45–0.97) versus control 0.58% (0.30–0.77); F(1,2) = 14.1; P = 0.064], which was not dose dependent. These effects occurred on EGFR-positive DCIS proliferation whether the DCIS specimens were removed from a breast containing a co-occurring cancer or not.

Mean LI in ER-negative/EGFR-positive DCIS decreased in the Iressa-treated group compared with the vehicle-treated group by day 28 [6.5% (IQR, 3.8–11.1%) versus 13.9% (IQR, 12.0–16.3%); F(1,3) = 10.3; P = 0.002] and day 42 [11.0% (IQR, 10.8–11.8) versus 13.1% (IQR, 12.0–14.8%); F(1,3) = 31.3; P = 0.011] (Fig. 1a).

Similar changes in LI were seen in EGFR-TKI-treated versus vehicle-treated ER-positive/EGFR-positive DCIS (Fig. 1, a and b) with a fall in LI at day 28 [4.6% (IQR, 3.9–5.2%) versus 11.7% (IQR, 9.2–15.5%); F(1,2) = 32.3; P = 0.03]. There was no change in either LI or AI in the control groups at the different time points (see Fig. 1).

Increasing the dose of Iressa was associated with increasing inhibition of epithelial proliferation in both ER-negative/EGFR-positive and ER-positive/EGFR-positive DCIS but not in the normal breast epithelium (Fig. 2). There was a dose effect in DCIS [at day 28, F(1,6) = 132, P < 0.0001; but not at day 42, F(1,3) = 4.9, P = 0.11].

To explore whether there was any systematic bias introduced by using “mouse” as the unit of analysis in the 10.0% of mice in whom day 14 xenografts contained DCIS, the differences within each mouse between day 14 and day 28 readings were calculated. A comparison of these changes was then made between the control and Iressa mice using a similar ANOVA model as detailed previously. This analysis effectively uses 28% of the data set. Similar differences between control and Iressa were obtained [mean reduction control 0.2% versus Iressa 7.8%; F(1,3) = 14.3 P = 0.032].

Normal Breast. Normal breast xenografts had a decrease in geometric mean LI from days 0 to 14 [4.9% (IQR, 4.0–5.7%) versus 3.8% (IQR, 2.7–5.1%); F(1,15) = 29.7; P < 0.001], which then remained unchanged for the other time points in control-treated mice (Table 1). Mean LI decreased in the EGFR-TKI-treated group compared with controls at day 21 [F(1,6) = 6.4; P = 0.045], day 28 [2.2% (IQR, 1.7–3.3%) versus 4.1% (IQR, 2.4–5.4%); F(1,14) = 29.2; P = 0.00009], and day 42 [1.8% (IQR, 1.1–2.6%) versus 3.2% (IQR, 2.3–5.1%); F(1,6) = 47.3; P = 0.0005]. No effect of dose of ZD1839 on LI was seen at any time point.

In the normal breast, AI rose at day 21 in ZD1839-treated xenografts. A reduction in geometric mean AI was observed from day 0 to 14 [0.22% (IQR, 0.17–0.29%) versus 0.18% (IQR, 0.10–0.21%); F(1,15) = 6.8; P = 0.02]. At day 21, there was an increase in mean AI in Iressa-treated xenografts compared with controls [0.38% (IQR, 0.23–0.53%) versus 0.19% (IQR, 0.10 to 0.25%); F(1,6) = 12.2; P = 0.013]. By 14 days of EGFR-TKI treatment (day 28), the mean AI was not different from the controls (see Table 1).

EGFR-negative DCIS. There were 2 cases of EGFR-negative DCIS studied, and no significant changes in LI or AI were seen (data not shown).

Cell Turnover Indices. We have demonstrated previously a positive correlation of LI with AI in DCIS (20). Comparison of cell population using the geometric mean LI:AI ratio (as a ratio of cell turnover) revealed that Iressa treatment compared with controls inhibited cell turnover in both DCIS [6.1 (IQR, 4.6–9.8) versus control 16.4 (IQR, 9.4–27.3); P ≤ 0.0001] and normal breast at day 28 [8.1 (IQR, 6.2–23.5) versus 21.6 (IQR, 13.5–48.9), respectively; P = 0.0035].

Down-Regulation of Activated EGFR Expression and pErk1/2 in EGFR-TKI-treated DCIS and Normal Breast. Median DCIS H score for activated EGFR was 65 (50–80) and remained unaltered at
day 42 in the controls but fell by 36% to 52.5 (7.5–76.0) in ZD1839-treated xenografts (P = 0.038).

To determine whether the EGFR/TKI, immunohistochemical detection of pErK1/Erk2 was performed on day 0 and day 42 sections of DCIS and normal breast xenografts treated with Iressa or vehicle. Median day 0 DCIS and normal breast nuclear MAP kinase H score were 30 (IQR, 12–45) and 22 (IQR, 12–34). The nuclear H score of pErK1/Erk2 correlated with the Ki67 LI in DCIS (r = 0.59, P = 0.045) and in normal breast (r = 0.52, P = 0.020). In DCIS, the nuclear H score was decreased in the Iressa-treated group compared with controls after 28 days of treatment [8 (IQR, 5–18) versus 25 (IQR, 8–30); P = 0.017]. In normal breast, there was a similar difference [7 (IQR, 3–17.5) versus 18 (IQR, 8–32); P = 0.015].

**Minimal Toxicity from EGFR-TKI Therapy**. Toxicity was assessed by regular monitoring of the weight and general well-being of the mice. Median mouse weight (grams) at day 0 was 23.6 (IQR, 22.1–24.8) and at day 14 was 24.2 (IQR, 22.8–25.4). For mice treated with Iressa, there was no difference in median weight compared with controls [25.3 (IQR, 22.2–26.3) versus 25.6 (IQR, 21.0–26.2); P = 0.64] following 28 days (day 42) of treatment. To determine the effects of the EGFR-TKI on proliferation in a different organ system known to express EGFR, normal small and large intestine were removed at the end of the experiments, processed, and assessed for

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**Table 1** The overall effect of EGFR-TKI inhibition on epithelial proliferation (LI) and apoptosis (AI) on normal breast and DCIS epithelium

<table>
<thead>
<tr>
<th>Geometric mean</th>
<th>Day 0</th>
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<th>AI</th>
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<tr>
<td></td>
<td></td>
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*P < 0.001.

*a* P < 0.05.

*a* P < 0.01.
EGFR TYROSINE KINASE INHIBITION IN BREAST

proliferation as described previously. There was no difference in proliferation at the different crypt cell position measured in both the small intestine and colon in the group treated with 75 mg/kg Iressa compared with controls (Fig. 3).

DISCUSSION

Around 50% of screen-detected DCIS is ER negative (7–9), and 62% (8 of 13) of our xenograft DCIS experiments contained ER-negative DCIS. However, 3 of the 5 ER-positive DCIS specimens also expressed immunohistochemically detectable EGFR and responded to EGFR-TKI with a decrease in epithelial proliferation.

In invasive breast cancer, epithelial cells express either ER or EGFR (10), whereas in normal breast epithelium around 80–90% of cells express EGFR and 10–20% ER (11). Our small study suggests that expression of EGFR in DCIS is closer to that in normal breast with cells co-expressing both EGFR and ER and overall 88% of DCIS specimens expressing EGFR.

EGFR heterodimerizes with other type 1 tyrosine kinase receptors such as c-erbB2 oncoprotein (12–14) and signals intracellularly via the MAP kinase and RAS pathways (14, 13). ER-negative DCIS has a high proliferative rate (8, 20), which in pretreatment samples correlated with high expression of the activated MAP kinase signal transduction enzyme. Although ligand binding studies performed in the early 1990s suggested only 50% of ER-negative DCIS expressed EGFR, 11 of 13 (85%) of the DCIS we studied expressed EGFR, of which 10 had dual expression of the c-erbB2 oncoprotein on the same cell population. EGFR and other members of the type 1 tyrosine kinase receptor family are likely to have a more central role in controlling epithelial proliferation in DCIS and normal breast than considered previously.

We therefore hypothesized that the formation of EGFR/c-erbB-2 heterodimers or EGFR/EGFR homodimers were responsible for the high proliferation rate and MAP kinase expression, and we tested our theory by using an EGFR-TKI peptide, Iressa. On EGFR-TKI treatment, a prolonged fall in epithelial proliferation and a decrease in activated EGFR and MAP kinase expression was seen combined with an increase in apoptosis at an early stage, confirming our hypothesis. EGFR is known to produce a mitogenic signal and correlates with proliferation and tumor doubling in invasive breast cancer (24), and inhibition of this pathway led to a fall in Ki67 labeling index in DCIS xenografts.

ER-negative, high-grade DCIS is reported to be the most likely to relapse after wide local excision, and at relapse at least 50% had become invasive high-grade breast cancer with associated nodal or distant metastases (26, 38). Prevention of relapse and progression to invasive cancer require an agent that suppresses proliferation or increases apoptosis, and the EGFR-TKI described fulfills these criteria. The effects are seen in both ER-negative and ER-positive DCIS expressing the EGFR, indicating the potential value of this compound in mixed DCIS lesions, such as those found with breast screening mammography (29).

A similar suppression of normal breast epithelial proliferation combined with an increase in apoptosis indicates the potential of EGFR-TKIs as chemoprevention agents in women at increased risk of breast cancer (33). Primary culture of human mammary epithelial cells produces cells that respond to EGF in vitro by proliferation (27, 28), so that some inhibition of normal breast epithelial proliferation was expected in the absence of estrogen. The inherent sensitivity of normal breast to EGF and similar ligands is shown by the lack of any dose-response curve to the drug, whereas DCIS treated with ZD1839 showed a clear dose-response curve with a significant dose-drug interaction. Normal breast has low or no expression of the c-erbB-2 receptor (30, 31, 35–37), and its proliferation is inherently dependent on an intact EGFR pathway, whereas the overexpression of c-erbB-2 in DCIS may explain the reduced inhibition of epithelial proliferation seen with lower doses of ZD1839. Another explanation is that DCIS and invasive cancers are known to produce increased amounts of EGF and transforming growth factor-α ligands (compared with normal

Fig. 2. Effect of increasing dose of Iressa (from 10–200 mg/kg body weight) on epithelial proliferation (Ki67 LI) in EGFR-positive DCIS (F(1.6) = 132; P < 0.0001; a) and normal breast xenografts (b) after 14 days (day 28) of gavage (F(1.14) = 1.7; P = 0.21). In DCIS, a higher inhibition was obtained with higher doses of Iressa, whereas in normal breast, similar inhibition of proliferation was obtained at all doses. Numbers in parentheses represent the number of observations seen for that particular time point and for that group. Geometric mean values are shown as thick horizontal lines; boxes, IQR.

Fig. 3. Epithelial proliferation in small intestine. Normal intestine were removed from culled mice after 28 days of gavage with either ZD1839 or vehicle (day 42) and then assessed for epithelial proliferation by immunohistochemical staining for the Ki67 antigen. There was no difference in proliferation (median values) at the different crypt cell positions measured in both the small intestine and colon in the group treated with 75 mg/kg ZD1839 compared with controls.
breast), which will compete with ZD1839 for the EGFR (14, 18). It is also possible that at high doses of ZD1839, some inhibition of other type I growth factor receptors may occur (e.g., c-erbB-2; Ref. 39).

Robertson et al. (24) quantified EGFR and c-erbB-2 by ligand binding assay in invasive breast cancer and normal breast and found higher levels of EGFR binding in normal breast epithelium, although EGFR but not c-erbB-2 correlated with Ki67 labeling index and tumor doubling time in invasive cancers. In contrast, c-erbB-2 was overexpressed in tumors compared with normal breast. These findings, however, were on a small number of tumors and may potentially be explained by the presence of variant III EGFR, a mutant receptor that lacks the external binding domain and therefore does not bind ligand but is constitutively active (24). The EGFR-MAP kinase pathway is believed to be important in cell proliferation, survival, and differentiation (32, 40). EGFR can also signal via the phospholipase C and phosphatidylinositol 3-kinase as well (13). However, the significant positive correlation of pErk1/Erk2 with Ki67 labeling index in DCIS and normal breast suggests that MAP kinase signaling is important in mediating cell proliferation in breast epithelium. The decrease in phosphorylated EGFR coupled with a decrease in pErk1/Erk2 in the Iressa-treated group correlated with a fall in proliferation, and it is likely that the EGFR signals through this pathway, and MAP kinase changes will predict drug response.

Unexpectedly, an increase in apoptosis was seen in normal breast epithelium after 7 days of treatment. Although the insulin-like growth factor-1 is believed to be important in cell survival, recent work by Roudabush et al. (25) has indicated that insulin-like growth factor-1 receptor stimulation leads to secretion of heparin binding-EGF extracellularly, which promotes transactivation of the EGFR. Thus, the EGFR may be more important to cell survival in the normal breast than has been recognized previously (25). Because proliferation correlates with apoptosis in the normal breast, a reduction in proliferation would be expected to correlate with a reduction in apoptosis. After the initial increase in apoptosis after 7 days in the ZD1839-treated group, the fall in LI (proliferation) correlated with a fall in apoptosis by 14 days. However, the overall LI:AI ratio in the ZD1839-treated groups in both DCIS and normal breast was reduced, indicating a lower overall cell turnover with EGFR-TKI inhibition. Moreover, the overall fall in cell turnover indicates the potential chemopreventive effect of an EGFR-TKI on normal breast.

Despite the fact that the EGFR is expressed in many tissues, few signs of toxicity were seen at Iressa doses <100 mg/kg. General well-being, weight, blindness, and diarrhea were monitored. Redundancy for EGFR after chronic dosing with Iressa in the normal mice colon and small intestine may possibly explain the lack of effect on colon epithelial proliferation rate (40). In preclinical toxicology studies on normal patients and in Phase I and II clinical trials of Iressa, the only frequent side effects occur mainly at doses >600 mg/day and have been low-grade acne and mild diarrhea in 10% of patients, which both settled on stopping therapy (18, 34). However, the effect of chronic long-term dosing with Iressa and other inhibitors of EGFR signaling still remains to be determined.

Normal and premalignant breast epithelium contains a mixture of ER-positive and ER-negative cells (41). It is the ER-negative cells that proliferate in normal breast (41), and we have shown that EGFR-TKI therapy will reduce proliferation and increase apoptosis in the epithelium, and either alone or combined with antiestrogen therapy EGFR-TKI may represent a novel form of chemoprevention. These results suggest that antiestrogen therapy given concomitantly or alternately with an EGFR-TKI (given for 14 days with a 14-day drug holiday) will have minimal side effects while maximizing the effective anti-proliferative and apoptotic effects, and such combinations should be investigated in clinical trials. In particular, evidence that breast cancers that have become tamoxifen resistant use the EGFR signaling pathway suggests that the combination of tamoxifen and Iressa will be potent at chemopreventing cancer (42).

In conclusion, our results suggest a role for EGFR-TKI Iressa as adjuvant therapy for EGFR-positive DCIS and as a chemopreventative agent for high-risk breast cancer patients.

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