

Thyroid Cancer Cell Resistance to gefitinib Depends on the Constitutive Oncogenic Activation of the ERK Pathway

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Context: Poorly differentiated thyroid carcinomas are refractory to common anticancer therapies, and novel inhibitors are being tested in these deadly malignancies. The epidermal growth factor receptor (EGFR) tyrosine kinase represents an attractive target for treatment because it is up-regulated in thyroid cancer and plays a role in cancer progression. However, EGFR inhibitors have provided poor results in thyroid carcinomas.

Objective: We evaluated the possible mechanism underlying the resistance of thyroid cancer cells to EGFR inhibitors.

Design: We tested the effect of the EGFR tyrosine kinase inhibitor gefitinib in a panel of thyroid cancer cell lines.

Results: We found that in most of the cell lines, although gefitinib inhibited EGFR phosphorylation, it was poorly effective in reducing cell viability. gefitinib, however, was able to inhibit epidermal growth factor-induced cell migration and matrix invasion. In most thyroid cancer cell lines, gefitinib significantly inhibited Akt phosphorylation by inhibiting EGFR activation, but it had limited or no effect on ERK phosphorylation. The poor cell response to gefitinib was associated with genetic alterations, leading to constitutive activation of the ERK pathway, including BRAF^(V600E) and HRAS^(G12A/Q61R) mutations and RET/PTC1 rearrangement. When BRAF^(V600E)-positive thyroid cancer cells were incubated with the specific BRAF inhibitor PLX4032, sensitivity to gefitinib was restored. Similar results were obtained with rat sarcoma and RET/papillary thyroid cancer inhibitors.

Conclusions: These results indicate that thyroid cancer resistance to gefitinib is due to the constitutive activation of the mitogenic pathway by either signals downstream of EGFR or other tyrosine kinase receptors. This resistance can be overcome by the combined use of selective inhibitors. (*J Clin Endocrinol Metab* 98: 2502–2512, 2013)

Epidermal growth factor (EGF) belongs to a family of related peptides (EGF-like growth factors), including TGF α , amphiregulin, heparin binding-EGF, epiregulin, heregulins, neuregulins, and β -cellulin (1–3). Receptors for EGF-like growth factors include the following: the

EGF receptor (EGFR or ErbB1/HER1), ErbB-2/Neu/HER2, ErbB-3/HER3, and ErbB-4/HER4 (4).

The EGFR is overexpressed in most human carcinomas (5, 6). The up-regulation of the EGF system has made this pathway an attractive target for anticancer therapies (7,

8). Two classes of EGFR antagonists have been developed: monoclonal antibodies and small-molecule tyrosine kinase inhibitors (9). Cetuximab is a monoclonal antibody against the extracellular domain of the EGFR, inhibiting EGF binding (9), whereas gefitinib and erlotinib are 2 small-molecule tyrosine kinase inhibitors that block EGFR autophosphorylation (10).

Thyroid cancer is the most common endocrine malignancy and accounts for approximately 1% of total cancers (11). Although most well-differentiated thyroid cancers (papillary and follicular) can be successfully treated by surgery, radioiodine and TSH-suppressive T₄ therapy (12), no efficacious options are available for poorly differentiated or undifferentiated (anaplastic) thyroid carcinomas, which display important chemoresistance (13). Therefore, novel drugs and targeted therapies may represent an alternative option for these patients.

EGFR is overexpressed in thyroid cancer cells and tissues (14–18), and its expression is associated with a negative prognosis (5, 14) and a poorly differentiated phenotype (19). Studies of thyroid cancer cells indicate that EGF promotes cell proliferation, an invasive phenotype, and epithelial-to-mesenchymal transition (20–23). Moreover, EGF is able to enhance vascular endothelial growth factor receptor secretion in cells lacking TSH receptors (24). In thyroid cancer cells, the activation of EGFR by its ligand is accompanied by the activation of both the ERK and Akt pathways (25). Accordingly, anti-EGF and anti-TGF- α antibodies inhibit thyroid cancer cell proliferation in vitro (26–28), indicating that EGFR is a potential target for the inhibition of thyroid tumor growth in patients with poorly differentiated thyroid carcinomas.

Gefitinib is the first EGFR-targeting drug showing promising results in various solid tumors in preclinical and clinical studies (9, 29, 30). In thyroid cancer, gefitinib decreased tumor cell growth in vitro and in thyroid cancer xenografts that overexpress EGFR (17). Moreover, gefitinib potentiates the effect of ionizing radiation on thyroid cancer cells, suggesting that combined treatment could achieve a similar growth inhibition effect with a reduced radiation dose and toxicity (31). Despite these promising results, thyroid cancer cell sensitivity to EGFR inhibition has been reported to be variable and unpredictable (32).

The present study was aimed at identifying predictive factors for the response of thyroid cancer to gefitinib. We evaluated the effect of gefitinib in a panel of thyroid cancer cell lines and studied the possible signaling pathways involved in resistance to the drug. In particular, we studied the effect of gefitinib on the EGFR signaling pathway in different thyroid cancer cells and evaluated the combined effect of gefitinib with classical chemotherapeutic agents

or inhibitors of the most common thyroid cancer oncogenes: BRAF^(V600E), renin-angiotensin system (RAS), and RET/papillary thyroid carcinoma (PTC).

Materials and Methods

Cells and materials

Papillary thyroid cancer cell TPC-1 lines (RET/PTC positive) and BC-PAP (BRAF^{V600E} positive) were provided by Drs A. Fusco and M. Santoro (Naples, Italy). Anaplastic thyroid cancer cells SW1736 (BRAF^{V600E} positive), Hth-74, and C-643 (RAS^{G12A/Q61R} positive) were provided by Dr N. E. Heldin (Uppsala, Sweden), whereas the FF-1 (BRAF^{V600E} positive) cell line was established in our laboratory. Papillary 8505-C (BRAF^{V600E} positive) and follicular FTC-133 (phosphatase and tensin homolog deleted from chromosome 10 negative) thyroid cancer cells were purchased from European Animal Cell Culture (Salisbury, United Kingdom). The follicular cancer cell line WRO was provided by A. Fusco (Naples, Italy). Normal thyrocytes in primary culture (pooled from 10 different samples) were established from surgical human specimens as previously reported (33). RAS and BRAF mutations in thyroid cancer cell lines were confirmed by PCR amplification and sequencing, as previously reported (34). These cell lines were grown in complete RPMI 1640 (Sigma, St Louis, Missouri), with the exception of FTC-133 cells, which were grown in complete DMEM F-12. The human esophagus carcinoma cells A431 (Dr D. Weir, Boston, Massachusetts) were grown in complete MEM.

The EGFR tyrosine kinase inhibitor gefitinib (Iressa, ZD1839), the BRAF inhibitor PLX4032, the multikinase inhibitor BAY73–4506 (regorafenib), and the farnesyltransferase inhibitor tipifarnib were purchased from Selleck (Houston, Texas).

Cell viability

Cell proliferation was evaluated by the methylthiazolyl tetrazolium test (MTT; Amersham Biosciences), which measures the ability of viable mitochondria to reduce soluble tetrazolium salts to insoluble formazan. MTT was measured by dissolving formazan in dimethylsulfoxide and reading the absorbance at 405 nm. Cells (1.5×10^3) were seeded in 96-well plates. After 24 hours, increasing concentrations of gefitinib, PLX4032, regorafenib, and tipifarnib, either alone or in combination, were added to the incubation medium for 48 hours in dose-response experiments. Cells were also treated with gefitinib alone or in combination with chemotherapeutic drugs (2 μ M doxorubicin, 5 μ g/mL cisplatin, or 5 μ g/mL Taxol).

Fluorescence-activated cell sorting analysis

The effect of gefitinib was evaluated by fluorescence-activated cell sorting (FACS) analysis. The cell cycle distribution and apoptosis of thyroid cancer cells were evaluated after 48 hours of treatment with 1 μ M gefitinib. For cell cycle analysis, cells were fixed and permeabilized with absolute ethanol and incubated with propidium iodide and ribonuclease (Sigma-Aldrich) for 1 hour at 4°C. Cells were then subjected to FACS analysis to determine DNA content (FL2). Apoptosis was measured by staining intact cells with fluorescein isothiocyanate-annexin and propidium iodide (BD Biosciences, Franklin Lakes, New Jersey)

according to the manufacturer's protocol. The cells were then evaluated by FACS analysis (fluorescein isothiocyanate, 1/4 FL1, propidium iodide 1/4 FL2).

Invasion assay

For inhibition studies, cells were preincubated with 1 μ M gefitinib for 12 hours and maintained with this compound for the duration of the experiment. Approximately 10^5 cells resuspended in 200 μ L medium were placed on 6.5-mm-diameter polycarbonate filters (8 μ m pore size; Corning Costar Corp, Cambridge, Massachusetts) coated on the lower and upper side with 1.2 mg/mL of Matrigel (BD Biosciences Labware, Bedford, Massachusetts). A total of 1 μ g/mL EGF in 1 mL of medium was added to the lower compartment. The plates were incubated at 37°C with 5% CO₂ for 48 hours. At the end of the incubation, the cells and the Matrigel on the upper side of the filter were removed with a cotton swab. Cells that had migrated to the lower side of the filter were stained with 0.1% crystal violet in 20% methanol for 20 minutes. After 3 washes with water and complete drying, the crystal violet was solubilized by filter immersion in 10% acetic acid. The solubilized crystal violet concentration was measured as absorbance at 590 nm.

Immunoblot analysis

Cells were incubated with or without 1 μ M gefitinib for 2 hours and lysed with radioimmunoprecipitation assay buffer containing 0.1% sodium dodecyl sulfate and protease inhibitor cocktail (Roche Biochemical Inc, Basel, Switzerland). Samples were resuspended in loading buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Amersham, Little Chalfont, United Kingdom). The membranes were blocked with 5% milk-Tris-buffered saline and 0.1% Tween 20 and immunoblotted with primary antibody (1 μ g/mL). Appropriate horseradish peroxidase-conjugated secondary antibodies were added at 1:2000 (Amersham Biosciences), and proteins were visualized by enhanced chemiluminescence (Amersham). Densitometric analysis was performed by ImageJ software (National Institutes of Health, Bethesda, Maryland).

Immunoprecipitation analysis

Cells were incubated with gefitinib as above and lysed in cold radioimmunoprecipitation assay buffer. Cell lysates were incubated at 4°C for 2 hours with 4 μ g of the anti-EGFR antibody coated with protein G-sepharose. Immunoprecipitates were subjected to SDS-PAGE and proteins transferred to nitrocellulose membranes, immunoblotted with antiphosphotyrosine 4G10 monoclonal antibody, and detected by enhanced chemiluminescence. Membranes were stripped and reprobed with an anti-EGFR rabbit polyclonal antibody.

The following antibodies were used for Western blotting: polyclonal anti-phosphorylated (p) EGFR and anti-EGFR antibodies (Santa Cruz Biotechnology, Santa Cruz, California); polyclonal anti-pERK, anti-ERK, anti-pAKT, and anti-AKT antibodies (Cell Signaling Technology Inc, Danvers, Massachusetts); and a monoclonal antiphosphotyrosine antibody (4G10) from Upstate Biotechnology Inc (Lake Placid, New York).

Statistical analysis

The results were compared by a 2-way ANOVA. Significance was determined with a *t* test (*, $P < .05$; **, $P < .01$; ***, $P < .001$).

Statistical analysis was carried out with Microsoft Excel software (Redmond, Washington).

Results

Gefitinib inhibits both basal and EGF-stimulated EGFR phosphorylation in thyroid cancer cells

We preliminarily tested the effect of increasing doses of EGF on viability of cell lines belonging to different thyroid cancer histotypes by MTT assay (Figure 1A). The indicated doses of EGF stimulated proliferation of thyroid cancer cells at variable extent (Figure 1A). The effect was stronger in A431 cells, overexpressing EGFR (1, 8) and used as a positive control. In contrast, EGF was without effect in normal thyrocytes (Figure 1A).

To explore the role of EGFR activation in thyroid cancer cell biology, we tested the effect the tyrosine kinase inhibitor gefitinib on EGFR phosphorylation in the same panel of thyroid cancer cell lines. A431 cells were used as a positive control. Cells were exposed to 1 μ M gefitinib for 2 hours. This concentration is physiologically relevant because the plasma levels of the drug obtained in humans with the current orally administered doses are 0.5–1.0 μ M or more (35). Western blot analysis with anti-EGFR and anti-pEGFR antibodies indicated that thyroid cancer cells expressed the EGFR protein to various extents (Figure 1B). Among the thyroid cancer cells tested, the highest EGFR protein content was observed in SW-1736 cells, and the lowest was observed in 8505-C cells. Anti-pEGFR Western blotting showed a variable degree of basal EGFR phosphorylation in thyroid cancer cells (Figure 1B). In all thyroid cancer cells and A431 cells, gefitinib markedly inhibited basal EGFR phosphorylation (Figure 1B). It was interesting to note that normal thyrocytes displayed a low level of EGFR phosphorylation (Figure 1A). We then studied the effect of gefitinib on ligand-stimulated EGFR phosphorylation. TPC-1 (RET/PTC positive), BC-PAP and SW-1736 (BRAF^(V600E) positive), and WRO and Hth74 (BRAF^(V600E) negative) cells were treated with 1 μ g/mL EGF for 5 minutes in either the presence or the absence of 1 μ M gefitinib. Cell lysates were immunoprecipitated with an anti-EGFR antibody and then blotted with an antiphosphotyrosine antibody as indicated in the Materials and Methods (Figure 1C). These results indicate that, at variance with normal thyrocytes, EGF variably stimulated thyroid cancer cell proliferation, and the mitogenic effect of EGF did not correlate with the level of expression/phosphorylation of EGFR. Moreover, gefitinib markedly inhibited both basal EGFR and ligand-stimulated EGFR phosphorylation (Figure 1C, lower panels).

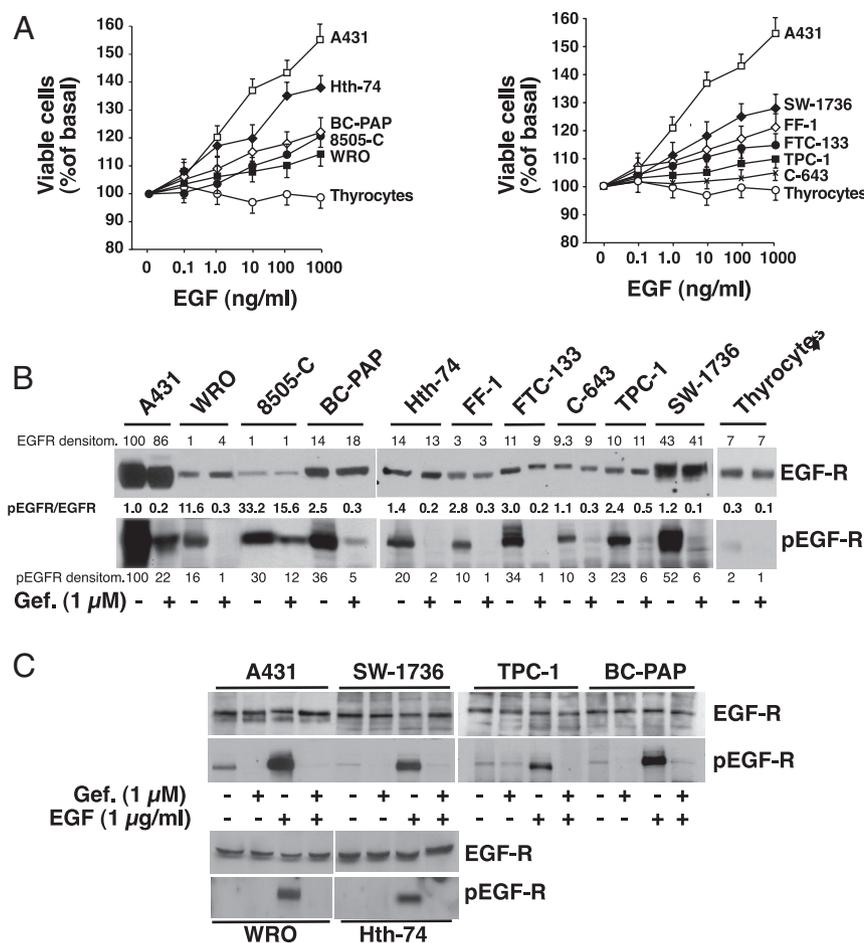


Figure 1. Gefitinib inhibits both basal and EGF-stimulated EGFR phosphorylation in thyroid cancer cells. **A**, The indicated cell lines were seeded in 96-well plates and incubated with increasing doses of EGF (0.1, 1.0, 10.0, 100.0, and 1000.0 ng/ml) for 48 hours. Cell viability was measured by the MTT assay. Each point represents the mean \pm SE of 3 separate experiments performed in quadruplicate. **B**, Cell monolayers were incubated in the presence or absence of 1 μ M gefitinib for 2 hours. Lysates were then subjected to Western blot analysis with an anti-pEGFR antibody, as described in Materials and Methods. A431 cells (overexpressing EGFR) were used as a positive control. Filters were reblotted with an anti-EGFR antibody for protein normalization. A representative experiment is shown and results of densitometric analysis are also indicated. Top, EGFR densitometry (first lane is expressed as 100); bottom, pEGFR densitometry (first lane is expressed as 100); middle (bold), pEGFR to EGFR ratio. **C**, Cell monolayers were incubated with 1 μ g/ml EGF for 5 minutes in the presence or absence of 1 μ M gefitinib. Cell lysates were first subjected to immunoprecipitation with an anti-EGFR antibody and then Western blotted with an anti-PY antibody, as described in Materials and Methods. Filters were reblotted with an anti-EGFR antibody. PY, phospho tyrosine.

Gefitinib has little effect on thyroid cancer cell viability and apoptosis

We then tested the effect of gefitinib on thyroid cancer cell viability compared with A431 cells. At 10 μ M, gefitinib consistently reduced (approximately 40%) the number of viable WRO and Hth-74 cells, which was close to the 60% inhibition observed in A431 cells (Figure 2A, right panels). In the other thyroid cancer cell lines, gefitinib's effect was variable but always less than 40%, even at the highest concentration tested (Figure 2A, right panels). Parallel anti-pEGFR Western blots with increasing doses of gefitinib indicated that 1 μ M gefitinib was able to inhibit EGFR phosphorylation in all cell lines tested (Fig-

ure 2A, left panel). These results indicate that, with the exception of WRO and Hth-74 cells, most thyroid cancer cells are refractory to the effect of gefitinib on cell viability.

To better characterize the effect of gefitinib on thyroid cancer cell viability, we evaluated the cell cycle distribution and apoptosis by FACS analysis (Figure 2B). Cells were incubated for 48 hours in the presence or absence of 1 μ M gefitinib and then subjected to FACS analysis. Propidium iodide staining and FACS analysis indicated that gefitinib induced significant G1 arrest in A431, WRO, and Hth-74 cells but not in FTC-133, C643, and SW-1736 cells (Figure 2B). Annexin staining and FACS analysis indicated that gefitinib significantly increased apoptosis in A431, WRO, and Hth-74 cells but not in FTC-133, C643, and SW-1736 cells (Figure 2B). These results indicate that despite the marked inhibition of EGFR phosphorylation, gefitinib reduces cell viability, cell cycle progression, and apoptosis only in some thyroid cancer cells and has only a minor effect in most of the tested cell lines. These observations suggest that, despite the inhibition of EGFR by gefitinib, most thyroid cancer cells are resistant to the effect of gefitinib on cell viability.

Gefitinib enhances sensitivity to chemotherapeutic drugs in only some thyroid cancer cells

It has been reported that gefitinib may also improve the chemosensitivity of cancer cells via several mechanisms, including down-regulation of anti-gen-presenting cell transporter (36). Given the poor effect of gefitinib alone on thyroid cancer cell viability, we investigated whether gefitinib could increase the cytotoxic effects of chemotherapeutic drugs. Cells were treated with 2 μ M doxorubicin, 5 μ g/ml cisplatin, or 5 μ M Taxol in the presence or absence of 1 μ M gefitinib as indicated in Materials and Methods. The MTT assay revealed that gefitinib potentiated the effect of all 3 chemotherapeutic drugs in A431, Hth74, and WRO cells; was minimally effective in FF-1, SW-1736, and BC-PAP cells; and had no

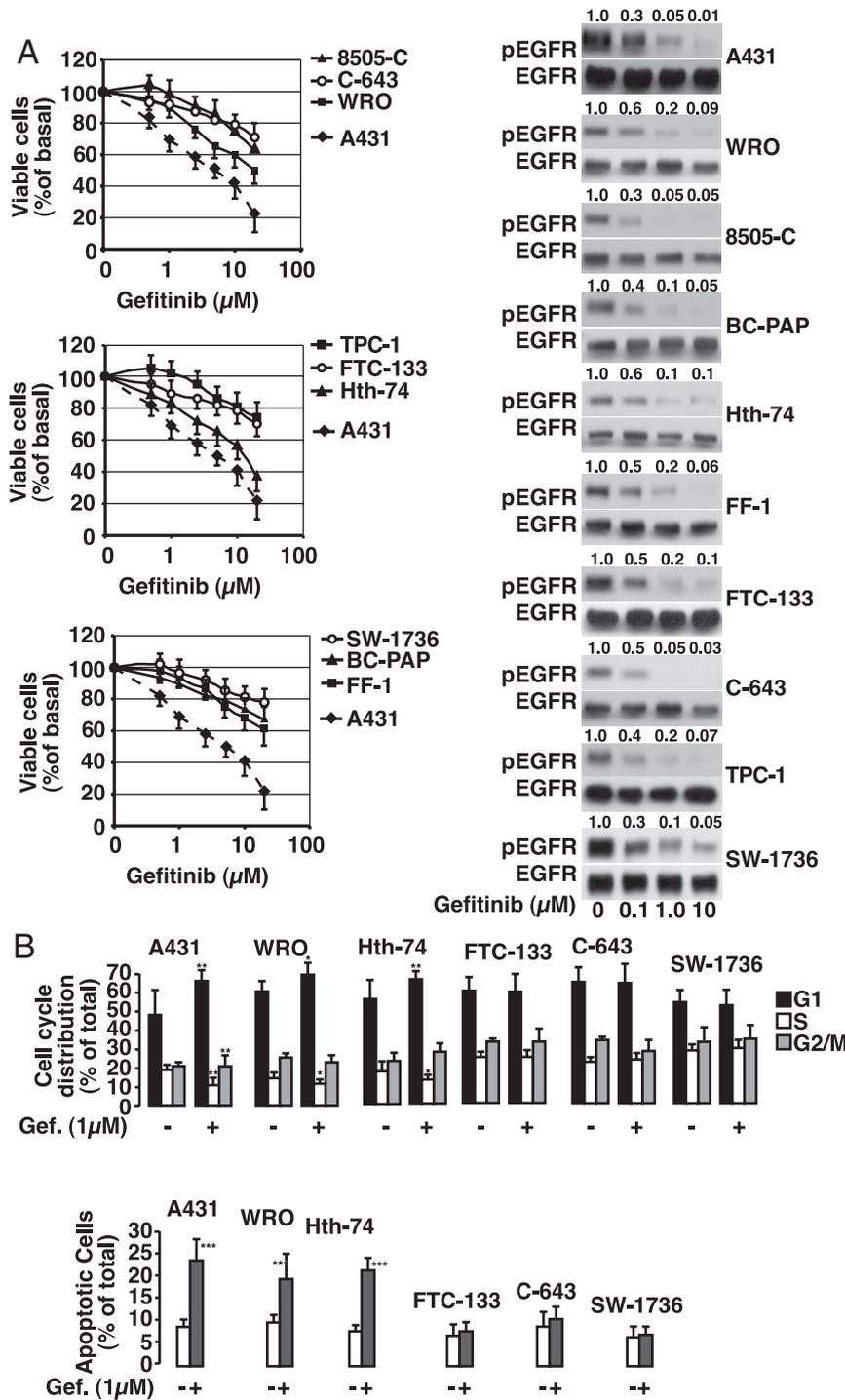


Figure 2. Effects of gefitinib on thyroid cancer cell viability, the cell cycle, and apoptosis. A, Right, The indicated cell lines were seeded in 96-well plates and incubated with increasing doses of gefitinib (0.5, 1.0, 2.0, 5.0, 10, and 20 μM) for 48 hours. Cell viability was measured by the MTT assay. Each point represents the mean ± SE of 3 separate experiments performed in quadruplicate. Left, Cell monolayers were incubated in the presence or absence of 0.1, 1.0, or 10 μM gefitinib for 2 hours. Lysates were then subjected to Western blot analysis with an anti-pEGFR antibody, as shown above. A representative experiment is shown and on top of panels are indicated results of densitometric analysis (first lane densitometry expressed as 1.0). B, The indicated cell lines were incubated with 1 μM gefitinib for 48 hours. Upper panel, propidium iodide staining and FACS analysis of cell cycle distribution by DNA content. Columns indicate the percentage of total cells in each cell cycle phase and represent the mean ± SE of 3 separate experiments. Statistical comparisons with untreated cells are shown for each cycle phase. Lower panel, Annexin staining and FACS analysis of apoptotic cells. Columns indicate the percentage of total cells that are apoptotic and represent the mean ± SE of 3 separate experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

effect in FTC-133, 8505, TPC-1, and C-643 cells (Table 1). These results indicate that gefitinib enhanced the cytotoxic effect of doxorubicin, cisplatin, and Taxol in the same thyroid cancer cell lines in which it also reduced viability when used alone. In the other thyroid cancer cell lines (with the partial exception of FF-1 cells), gefitinib had only a minor or no effect. Collectively, these results confirm that gefitinib is effective mainly in the WRO and Hth-74 thyroid cancer cells.

Gefitinib inhibits EGF-induced cell invasiveness

Evidence indicates that EGFR activation is involved in invasiveness and the epithelial-mesenchymal transition of cancer cells (37). We therefore investigated whether gefitinib was able to inhibit EGF-induced thyroid cancer cell invasiveness in a reconstituted basal membrane system (Matrigel; BD Biosciences Labware). To this end, TPC-1, 8505-C, and BC-PAP cells were selected because these lines were poorly sensitive to gefitinib in terms of cell viability. Gefitinib-sensitive A431, Hth-74, and WRO cells were used as controls (Figure 3B). Cells were allowed to migrate through modified Boyden chambers in the presence or absence of EGF, as described in Materials and Methods. Gefitinib had minimal or no effect on basal (unstimulated) cell migration in all cells tested (Figure 3). EGF significantly enhanced migration through Matrigel (BD Biosciences Labware) in all cells tested, with a more prominent effect in A431, WRO, and Hth-74 cells (Figure 3). Incubation with gefitinib (1 μM) significantly reduced EGF-stimulated cell invasiveness in all cell lines. This effect, however, was quantitatively variable, with less effect in TPC-1 cells, which are poorly responsive to EGF (Figure 3). These results indicate that independent of the effect on cell proliferation, the inhibition of EGFR ac-

Table 1. Effect of Gefitinib on the Cytotoxic Effect of Chemotherapeutics

	Untreated	Doxorubicin, 2 μ M	Cisplatin, 5 μ g/mL	Taxol, 5 μ g/mL
A431				
Basal	100 \pm 3.3	9.6 \pm 19.1	96.4 \pm 4.5	25.9 \pm 11.7
Gefitinib, 1 μ M	44.1 \pm 16.0 ^a	5.7 \pm 5.9 ^b	24.2 \pm 4.3 ^a	10.2 \pm 9.1 ^c
WRO				
Basal	100 \pm 9.5	12.4 \pm 4.8	84.8 \pm 11.2	58.7 \pm 12.0
Gefitinib, 1 μ M	46.6 \pm 11.5 ^a	3.5 \pm 4.5 ^c	19.6 \pm 8.4 ^a	15.3 \pm 5.3 ^a
Hth-74				
Basal	100 \pm 6.4	47.9 \pm 9.1	19.9 \pm 6.9	55.2 \pm 5.5
Gefitinib, 1 μ M	45.1 \pm 10.1 ^a	13.5 \pm 5.3 ^c	6.3 \pm 4.6 ^c	16.5 \pm 9.1 ^c
FTC-133				
Basal	100 \pm 3.5	39.3 \pm 6.3	28.9 \pm 8.3	51.3 \pm 4.7
Gefitinib, 1 μ M	97.1 \pm 2.9	41.9 \pm 4.5	35.5 \pm 13.1	51.67 \pm 6.6
FF-1				
Basal	100 \pm 8.7	13.4 \pm 7.4	26.3 \pm 10.8	52.4 \pm 6.9
Gefitinib, 1 μ M	79.3 \pm 8.1 ^c	16.3 \pm 9.7	14.2 \pm 5.4 ^c	44.49 \pm 9.5 ^b
SW-1736				
Basal	100 \pm 9.4	36.5 \pm 5.3	56.7 \pm 9.2	39.0 \pm 6.3
Gefitinib, 1 μ M	87.9 \pm 10.2 ^b	34.6 \pm 4.6	48.8 \pm 14.5 ^b	32.6 \pm 7.1 ^b
8505-C				
Basal	100 \pm 3.5	28.8 \pm 9.6	78.7 \pm 4.7	27.4 \pm 7.8
Gefitinib, 1 μ M	106.1 \pm 4.6	22.9 \pm 6.0	82.8 \pm 5.8	33.9 \pm 2.2
TPC-1				
Basal	100 \pm 4.5	13.8 \pm 13.3	42.7 \pm 9.9	34.3 \pm 11.1
Gefitinib, 1 μ M	86.2 \pm 17.4	12.7 \pm 5.3	37.2 \pm 14.2	35.7 \pm 9.6
C-643				
Basal	100 \pm 4.7	102.1 \pm 4.5	49.5 \pm 13.7	51.0 \pm 9.1
Gefitinib, 1 μ M	107.2 \pm 3.1	102.5 \pm 6.3	51.9 \pm 12.8	55.8 \pm 10.3
BC-PAP				
Basal	100 \pm 4.9	32.0 \pm 7.8	50.4 \pm 7.4	30.3 \pm 5.2
Gefitinib, 1 μ M	90.6 \pm 6.2	29.7 \pm 8.1	46.3 \pm 5.7 ^b	36.2 \pm 19.1

Table shows the effect of gefitinib, alone or in combination with chemotherapy drugs, on cell viability. The indicated cell lines were seeded in 96-well plates and grown in 10% fetal bovine serum. Then cells were incubated in the presence or the absence of 1 μ M gefitinib alone or in combination with 2 μ M doxorubicin, 5 μ M cisplatin, or 5 μ M Taxol for 48 hours. Cell viability was then measured by the MTT assay. The data indicate the mean \pm SE of 3 separate experiments performed in quadruplicate and are expressed as a percentage of the control.

^a $P < .001$.

^b $P < .05$.

^c $P < .01$.

tivation by gefitinib reduces the effect of EGF on the invasiveness and migration of most thyroid cancer cells.

Gefitinib inhibits EGFR downstream signaling molecules to variable degrees in different thyroid cancer cells

To better understand why most thyroid cancer cells are not responsive to gefitinib in terms of viability and apoptosis despite the marked inhibition of EGFR phosphorylation, we studied the intracellular pathways downstream of EGFR phosphorylation. We evaluated the phosphorylation of both ERK1/2 (p42 and p44 MAPKs) and protein kinase B/Akt, 2 major downstream targets of activated EGFR. Cell lines were exposed to 1 μ M gefitinib, and anti-pAkt or anti-pERK Western blotting was performed. Cell treatment with gefitinib completely inhibited ERK1/2 phosphorylation in A431 cells and inhibited ERK phosphorylation in WRO, Hth-74, and FTC-133 cells. No

significant effect was observed in the other thyroid cancer cell lines (Figure 4) despite the inhibition of EGFR phosphorylation. No change in the total ERK protein expression level was observed (Figure 4). It is interesting to note that gefitinib inhibited ERK phosphorylation in thyroid cancer cells expressing wild-type BRAF (WRO, Hth-74, and FTC-133) (38, 39) but not in cell lines harboring the BRAF^(V600E) mutation (8505-C, SW-1736, BC-PAP, and FF-1) (38, 39). Gefitinib also showed no effect in 2 cell lines expressing wild-type BRAF but harboring either the HRAS^{G12A/Q61R} mutation or the RET/TPC-1 rearrangement (C-643 and TPC-1 cells, respectively).

Gefitinib at 1 μ M significantly reduced Akt-stimulated phosphorylation in all thyroid cancer cell lines with the exception of C-643 cells [which harbor HRAS^{G12A/Q61R}, which may directly activate the phosphatidylinositol 3-kinase (PI3K) pathway] (40) and FTC-133 cells (which are

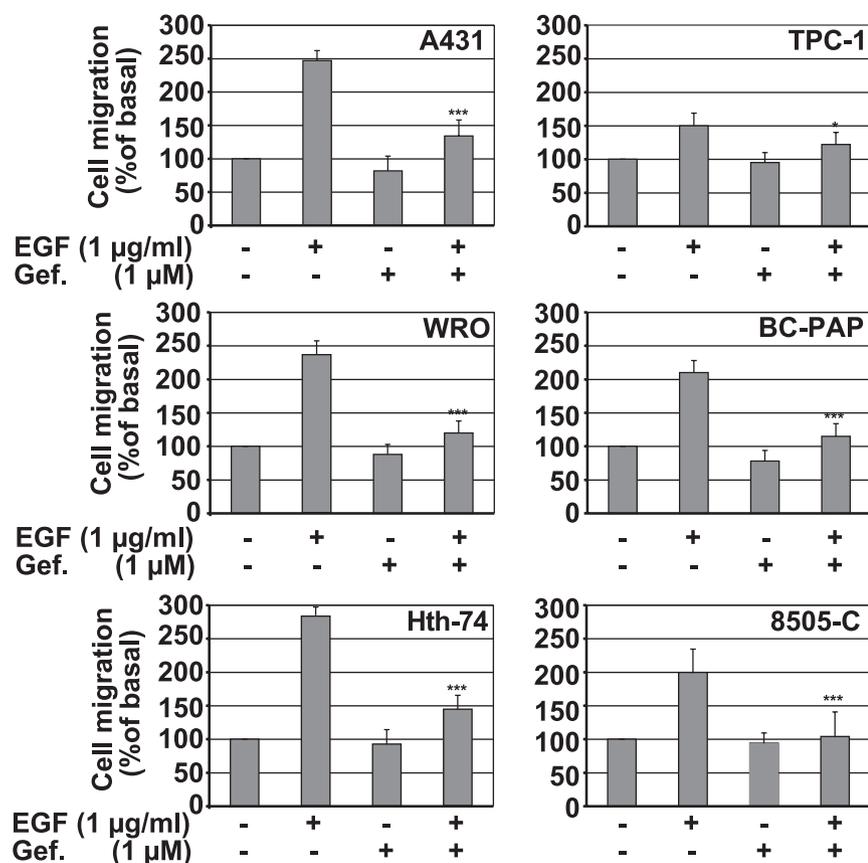


Figure 3. Effect of gefitinib (Gef.) on EGF-stimulated Matrigel invasion (BD Biosciences Labware). The indicated cells were incubated in the presence or absence of 1 μ M gefitinib, collected, and allowed to migrate through Matrigel-coated transwells for 48 hours in the presence or the absence of 1 μ M gefitinib, 1 μ g/mL EGF, or both as indicated in Materials and Methods. Migrated cells were stained with crystal violet, and the absorbance was measured at 590 nm. Columns indicate the percentage of basal condition and are the mean \pm SE of 3 separate experiments performed in triplicate. *, $P < .05$, **, $P < .01$, ***, $P < .001$ for the effect of gefitinib in EGF-stimulated cells.

phosphatase and tensin homolog deleted from chromosome 10 deficient and therefore have PI3K pathway up-regulation) (41) (Figure 4). No significant change was observed in Akt protein levels (Figure 4). Akt phosphorylation was fully inhibited in A431 cells (Figure 4).

Taken together, these results indicate that in thyroid cancer cells, EGFR inhibition by gefitinib may not necessarily imply the inhibition of the downstream ERK pathway. This discrepancy may be explained by the oncogene-driven activation of downstream signaling molecules that may stimulate the signaling pathway independent of EGFR activation, promoting cell growth.

Inhibition of BRAF increases sensitivity to gefitinib in thyroid cancer cells harboring the BRAF^(V600E) mutation

Based on the hypothesis that constitutively activated pathways downstream of EGFR make gefitinib poorly effective (42), we first explored the role of BRAF^(V600E), the most common genetic alteration in thyroid cancer. To this

end, thyroid cancer cell lines carrying either wild-type BRAF (WRO and Hth-74) or the BRAF^(V600E) mutant (BC-PAP and SW-1736) were studied. Cells were incubated with increasing doses of gefitinib along with increasing doses of the selective BRAF inhibitor PLX4032 (39, 43). Although PLX4032 did not change the effect of gefitinib in cell lines harboring wild-type BRAF (WRO and Hth-74), it caused an additive, dose-dependent inhibition of cell viability in thyroid cancer cell lines harboring BRAF^(V600E) (BC-PAP and SW-1736) (Figure 5A). Anti-pERK Western blotting indicated that the combination of the 2 drugs also caused ERK phosphorylation inhibition in cells that were not responsive to gefitinib (Figure 5A, bottom panels). These results indicate that BRAF^(V600E), by activating the ERK pathway independently of EGFR, may play a major role in determining gefitinib resistance in thyroid cancer cells harboring this mutation.

Inhibition of RAS or RET/PTC increases sensitivity to gefitinib in thyroid cancer cells harboring a RAS mutation or RET/PTC rearrangement

We then explored whether, similar to BRAF^(V600E), other molecular abnormalities leading to the constitutive activation of the RAS pathway may also be responsible for thyroid cancer cell resistance to gefitinib. C-643 cells harboring the HRASG12A/Q61R mutation and TPC-1 cells harboring a RET/TPC1 rearrangement were incubated with the RAS inhibitor tipifarnib (44) and the RET inhibitor regorafenib (45), respectively. Tipifarnib significantly enhanced the effect of increasing doses of gefitinib on cell viability in C-643 cells harboring the HRASG12A/Q61R mutation (Figure 5B) but did not change the effect of gefitinib in BC-PAP cells harboring wild-type RAS. In C-643 cells, anti-pERK Western blotting indicated that combined treatment with tipifarnib and gefitinib almost completely inhibited the phosphorylation of ERK (Figure 5B). Similarly, regorafenib significantly enhanced the effect of gefitinib on cell viability in TPC-1 cells harboring the RET/PTC-1 rearrangement (Figure 5C) but showed no effect in FTC-133 cells harboring wild-type RET/PTC-1 and RAS.

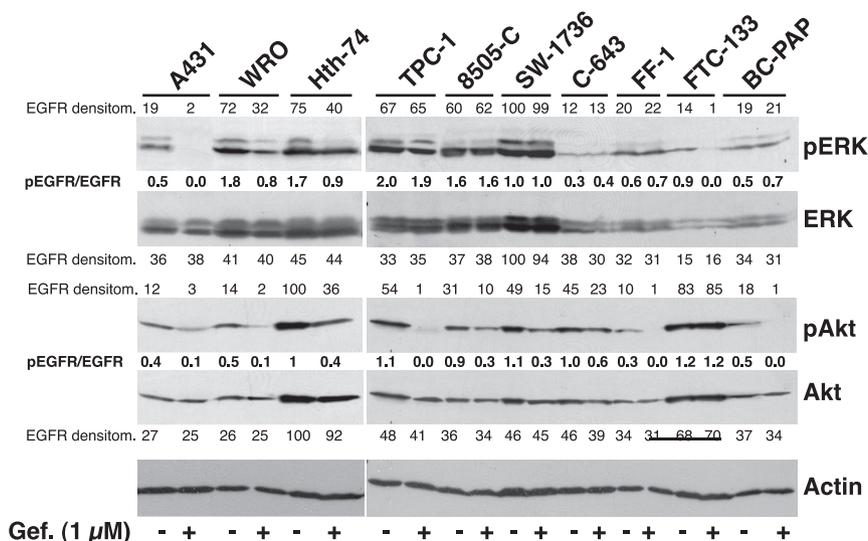


Figure 4. Effects of gefitinib on the major EGFR downstream signaling pathways in thyroid cancer cells. The indicated cell lines were incubated with $1 \mu\text{M}$ gefitinib for 2 hours. Lysates were then blotted with either anti-pERK or anti-pAkt antibodies. Filters were reblotted with anti-ERK antibody, anti-Akt antibody, or an anti- β -actin antibody for protein normalization. A representative experiment is shown and results of densitometric analysis are also indicated. Top, pERK and pAkt densitometry (higher signal is expressed as 100); bottom, pERK and pAkt densitometry (higher signal is expressed as 100); middle (bold), pERK/ERK and pAkt to Akt ratio.

Anti-pERK Western blotting indicated that combined treatment with regorafenib and gefitinib nearly completely abolished ERK phosphorylation in TPC-1 cells (Figure 5C). These results indicate that the constitutive activation of the RAS pathway caused by BRAF or RAS mutations or RET/PTC rearrangement makes thyroid cancer cells resistant to the gefitinib inhibition of EGFR.

Discussion

In accordance with previous reports, the present study indicates that all thyroid cancer cells express active EGFR (17), promoting cancer cell proliferation and invasiveness, and may be effectively blocked by the selective EGFR inhibitor gefitinib.

However, despite the marked inhibition of EGFR phosphorylation, gefitinib significantly affected cell viability and apoptosis in only a few cell lines (WRO and HTh-74). This limited effect of gefitinib on thyroid cancer cell proliferation paralleled its poor inhibitory effect on the activation of the ERK pathway. In contrast, gefitinib inhibited Akt activation in most thyroid cancer cell lines. Overall, gefitinib was not effective in thyroid cancer cell lines harboring mutations of molecules that cause the constitutive activation of the ERK pathway, downstream of EGFR or independent of EGFR activation (42). Therefore, 8505-C, SW-1736, BC-PAP, and FF-1 cells, which harbor BRAF^(V600E), C-643 cells, which harbor HRAS^{G12A/Q61R} (46), and TPC-1 cells, which harbor RET/PTC-1 (47), do

not respond to gefitinib because the marked inhibition of EGFR phosphorylation does not inhibit the ERK pathway. Gefitinib therefore is effective only in WRO and Hth-74 cells, in which activation of the mitogenic ERK pathway depends only on EGFR activation.

It was also interesting to note that EGFR activation is not a mechanism of chemoresistance in most thyroid cancer cell lines with the exception of WRO and Hth-74, in which gefitinib enhanced the cytotoxic effect of chemotherapy compounds, such as doxorubicin and cisplatin. This observation is in accordance with previous data obtained in other cancer cell types indicating that inhibition of the EGFR pathway results in impaired DNA repair capacity after cell exposure to DNA-damaging agents (48, 49) and increased expression of

antigen-presenting cell transporter (36). Gefitinib, however, was able to inhibit invasiveness in most thyroid cancer cells independently of the presence of mutations activating downstream EGFR signaling on the ERK pathway. This effect of gefitinib therefore occurs via an ERK-independent pathway, most likely by inhibiting the EGFR postreceptor PI3K/Akt pathway, in accordance with the hypothesis that this pathway is important in mediating EGF-induced cancer cell migration (50).

The poor effect of gefitinib on the proliferation and apoptosis of most thyroid cancer cells is due therefore to the EGFR-independent overactivation of the ERK pathway, regardless of the cause. When the specific oncogenic mechanism of each cell line was addressed by the selective inhibition of BRAF^(V600E) by PLX4032 and mutated RAS or rearranged RET/PTC (by tipifarnib and regorafenib, respectively), the effect of gefitinib on those thyroid cancer cells was restored. Moreover, experiments with siRNA for BRAF indicated that depletion of BRAF in BRAF^(V600E) positive cells restores the effect of gefitinib, thereby confirming the specific effect of PLX4032 (see Supplemental Table 1B, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). These observations are in line with previous data indicating that BRAF and KRAS mutations are a frequent mechanism of resistance to EGFR-targeting therapies (51–53). Because both BRAF and RAS belong to the RAS/RAF/MEK/ERK pathway, when either BRAF or RAS is constitutively ac-

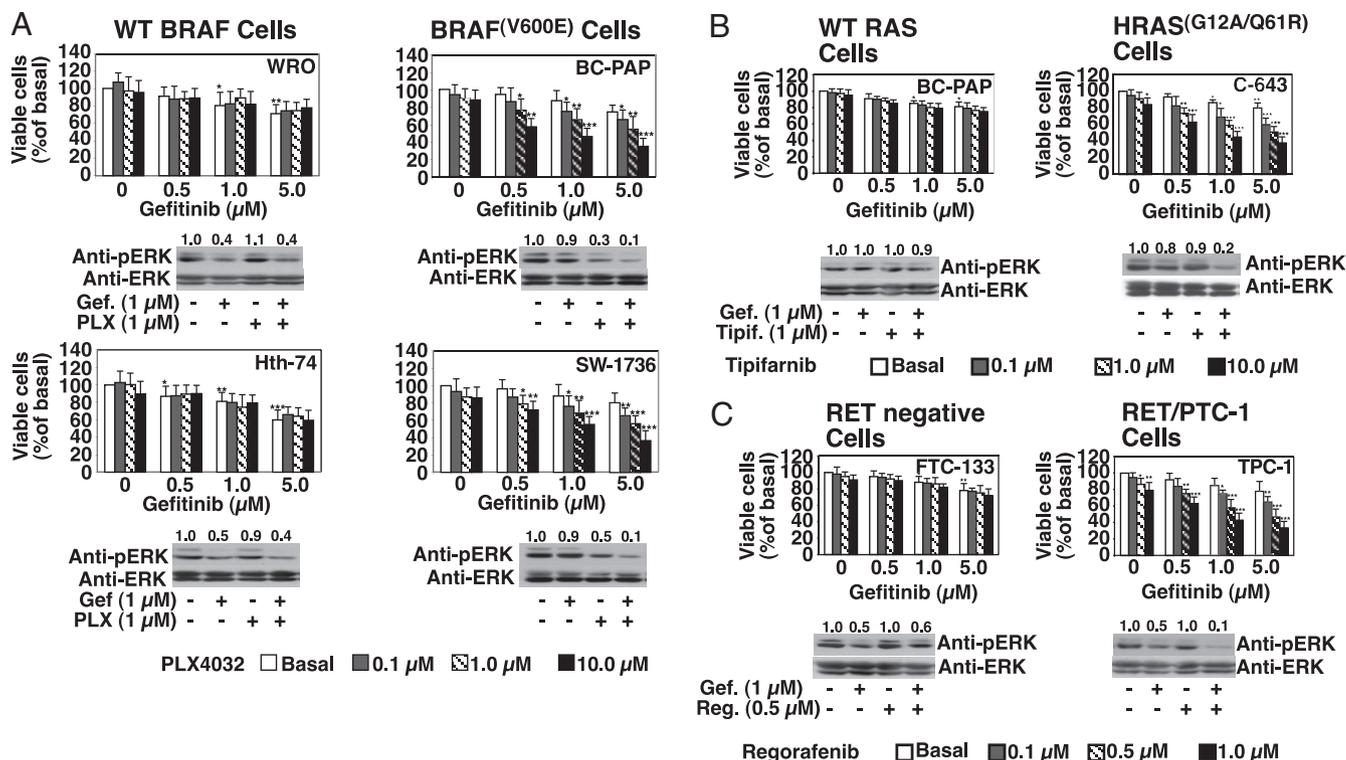


Figure 5. Effect of oncogene inhibition on thyroid cancer cell response to gefitinib. A, Effect of BRAF inhibition on thyroid cancer cell response to gefitinib. The indicated cell lines were seeded in 96-well plates and incubated with increasing doses of gefitinib (0.5, 1.0, and 5.0 μM), PLX4032 (0.1, 1.0, and 10 μM), or both for 48 hours. Cell viability was measured by the MTT assay. B, Effect of the inhibition of mutated RAS on thyroid cancer cell response to gefitinib. C-643 (RAS mutated thyroid cancer cells) and BC-PAP (wild type RAS thyroid cancer cells) were seeded in 96-well plates and incubated with increasing doses of gefitinib (Gef.; 0.5, 1.0, and 5.0 μM), the RAS inhibitor tipifarnib (0.1, 1.0, and 10 μM) or both for 48 hours. Cell viability was then measured by the MTT assay. C, Effect of the inhibition of rearranged RET/PTC-1 on thyroid cancer cell response to gefitinib. TPC-1 thyroid cancer cells harboring the RET/PTC1 rearrangement and FTC-133 thyroid cancer cells expressing wild-type RET/PTC1 were seeded in 96-well plates and incubated with increasing doses of gefitinib (0.5, 1.0, and 5.0 μM), the RET inhibitor regorafenib (0.1, 0.5, and 1.0 μM), or both for 48 hours. Cell viability was measured by the MTT assay. Each column represents the mean \pm SE of 3 separate experiments performed in quadruplicate. Statistical comparison is made with untreated cells. * $P < .05$; ** $P < .01$; *** $P < .001$. Representative anti-pERK Western blots are shown to visualize the effect of the inhibitors on ERK phosphorylation. On top of blots are indicated the results of densitometric analysis (first lane densitometry is expressed as 1).

tivated, the inhibition of EGFR by gefitinib has no effect on cell proliferation.

The RET/PTC rearrangement similarly causes constitutive activation of ERK and gefitinib resistance. Experiments performed with the ERK inhibitor PD98059 confirm that ERK activation is responsible for the lack of effect of gefitinib in BRAF(V600E)-positive cells because ERK inhibition results in a decrease of cell viability at an extent similar to that obtained in BRAF-depleted cells (see Supplemental Table 1A). Although these results were obtained in vitro, they may have potential clinical applications in targeted therapies for poorly differentiated thyroid carcinomas, because these malignancies often harbor BRAF(V600E) and RAS mutations. Clinical trials with gefitinib, performed in small series of patients with this type of cancer, have provided poor results (54). It is reasonable to speculate that most of the cancers treated harbored either BRAF or RAS mutations and therefore were poorly responsive to gefitinib. Hence, genotyping for BRAF and RAS can be useful to select patients and combined thera-

pies. For instance, combined treatment with EGFR and BRAF inhibitors has provided good results in colon carcinoma cells, in which EGFR up-regulation is responsible for the poor response to the BRAF inhibitor PLX4032 (55). Moreover, a multiple-target tyrosine kinase inhibitor, such as vandetanib, which inhibits 3 receptors (EGFR, vascular endothelial growth factor receptor, and RET), has recently been documented to improve the clinical outcome of patients with locally advanced or metastatic thyroid cancer (56).

In conclusion, although EGFR is highly expressed in thyroid cancer cells, this does not imply an EGFR-dependent oncogene addiction that may be easily targeted by effective EGFR inhibitors. Resistance to gefitinib is present in all thyroid cancer cells in which an EGFR-independent mechanism for ERK pathway activation is present. Even in the presence of constitutive activation of the ERK pathway, gefitinib is still able to inhibit the PI3K pathway and, via this mechanism, reduce thyroid cancer cell invasiveness. Further in vivo investigations are required to val-

idate these in vitro results and confirm that a multitarget anticancer therapy may be tailored according to the specific molecular abnormalities of each cancer to override the malignant cell resistance to one inhibitor, even though the inhibitor is effective for its target.

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References

- Watanabe T, Shintani A, Nakata M, et al. Recombinant human β -cellulin. Molecular structure, biological activities, and receptor interaction. *J Biol Chem*. 1994;269:9966–9973.
- Toyoda H, Komurasaki T, Ikeda Y, Yoshimoto M, Morimoto S. Molecular cloning of mouse epiregulin, a novel epidermal growth factor-related protein, expressed in the early stage of development. *FEBS Lett*. 1995;377:403–407.
- Wells A. EGF receptor. *Int J Biochem Cell Biol*. 1999;31:637–643.
- Yarden Y. The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *Eur J Cancer*. 2001;37(suppl 4):S3–S8.
- Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37(suppl 4):S9–S15.
- Baselga J. Targeting the epidermal growth factor receptor with tyrosine kinase inhibitors: small molecules, big hopes. *J Clin Oncol*. 2002;20:2217–2219.
- Cohen S. The stimulation of epidermal proliferation by a specific protein (EGF). *Dev Biol*. 1965;12:394–407.
- Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res*. 1984;44:1002–1007.
- Ciardello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res*. 2001;7:2958–2970.
- Grunwald V, Hidalgo M. Developing inhibitors of the epidermal growth factor receptor for cancer treatment. *J Natl Cancer Inst*. 2003;95:851–867.
- Wartofsky L. Increasing world incidence of thyroid cancer: increased detection or higher radiation exposure? *Hormones (Athens)*. 9:103–108.
- Mallick UK. The revised American Thyroid Association management guidelines for patients with differentiated thyroid cancer: an evidence-based risk-adapted approach. *Clin Oncol (R Coll Radiol)*. 2009;22:472–474.
- Patel KN, Shaha AR. Poorly differentiated and anaplastic thyroid cancer. *Cancer Control*. 2006;13:119–128.
- Akslen LA, Myking AO, Salvesen H, Varhaug JE. Prognostic impact of EGF-receptor in papillary thyroid carcinoma. *Br J Cancer*. 1993; 68:808–812.
- Lee DH, Lee GK, Kong SY, et al. Epidermal growth factor receptor status in anaplastic thyroid carcinoma. *J Clin Pathol*. 2007;60:881–884.
- Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol*. 1995;19:183–232.
- Schiff BA, McMurphy AB, Jasser SA, et al. Epidermal growth factor receptor (EGFR) is overexpressed in anaplastic thyroid cancer, and the EGFR inhibitor gefitinib inhibits the growth of anaplastic thyroid cancer. *Clin Cancer Res*. 2004;10:8594–8602.
- van der Laan BF, Freeman JL, Asa SL. Expression of growth factors and growth factor receptors in normal and tumorous human thyroid tissues. *Thyroid*. 1995;5:67–73.
- Landriscina M, Pannone G, Piscazzi A, et al. Epidermal growth factor receptor 1 expression is upregulated in undifferentiated thyroid carcinomas in humans. *Thyroid*. 21:1227–1234.
- Hoelting T, Siperstein AE, Clark OH, Duh QY. Epidermal growth factor enhances proliferation, migration, and invasion of follicular and papillary thyroid cancer in vitro and in vivo. *J Clin Endocrinol Metab*. 1994;79:401–408.
- Rothhut B, Ghoneim C, Antonicelli F, Soula-Rothhut M. Epidermal growth factor stimulates matrix metalloproteinase-9 expression and invasion in human follicular thyroid carcinoma cells through focal adhesion kinase. *Biochimie*. 2007;89:613–624.
- Yeh MW, Rougier JP, Park JW, et al. Differentiated thyroid cancer cell invasion is regulated through epidermal growth factor receptor-dependent activation of matrix metalloproteinase (MMP)-2/gelatinase A. *Endocr Relat Cancer*. 2006;13:1173–1183.
- Zhang Z, Liu ZB, Ren WM, Ye XG, Zhang YY. The miR-200 family regulates the epithelial-mesenchymal transition induced by EGF/EGFR in anaplastic thyroid cancer cells. *Int J Mol Med*. 2012;30(4): 856–862.
- Hoffmann S, Hofbauer LC, Scharrenbach V, et al. Thyrotropin (TSH)-induced production of vascular endothelial growth factor in thyroid cancer cells in vitro: evaluation of TSH signal transduction and of angiogenesis-stimulating growth factors. *J Clin Endocrinol Metab*. 2004;89:6139–6145.
- Landriscina M, Piscazzi A, Fabiano A, et al. Targeting epidermal growth factor receptor 1 signaling in human thyroid-stimulating hormone-independent thyroid carcinoma FRO cells results in a more chemosensitive and less angiogenic phenotype. *Thyroid*. 2009; 19:629–637.
- Broecker M, Hammer J, Derwahl M. Excessive activation of tyrosine kinases leads to inhibition of proliferation in a thyroid carcinoma cell line. *Life Sci*. 1998;63:2373–2386.
- Grande M, Franzen A, Karlsson JO, Ericson LE, Heldin NE, Nilsson M. Transforming growth factor- β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. *J Cell Sci*. 2002;115:4227–4236.
- Holting T, Siperstein AE, Clark OH, Duh QY. Epidermal growth factor (EGF)- and transforming growth factor α -stimulated invasion and growth of follicular thyroid cancer cells can be blocked by antagonism to the EGF receptor and tyrosine kinase in vitro. *Eur J Endocrinol*. 1995;132:229–235.
- Baselga J. Why the epidermal growth factor receptor? The rationale for cancer therapy. *Oncologist* 2002;7(suppl 4):2–8.
- Siegel-Lakhai WS, Beijnen JH, Schellens JH. Current knowledge and future directions of the selective epidermal growth factor receptor inhibitors erlotinib (Tarceva) and gefitinib (Iressa). *Oncologist*. 2005;10:579–589.
- Lopez JP, Wang-Rodriguez J, Chang CY, et al. Gefitinib (Iressa) potentiates the effect of ionizing radiation in thyroid cancer cell lines. *Laryngoscope*. 2008;118:1372–1376.
- Landriscina M, Maddalena F, Fabiano A, Piscazzi A, La Macchia O, Cignarelli M. Erlotinib enhances the proapoptotic activity of cytotoxic agents and synergizes with paclitaxel in poorly-differentiated thyroid carcinoma cells. *Anticancer Res*. 2010;30:473–480.
- Malaguarnera R, Mandarino A, Mazzon E, et al. The p53-homologue p63 may promote thyroid cancer progression. *Endocr Relat Cancer*. 2005;12:953–971.

34. Frasca F, Nucera C, Pellegriti G, et al. BRAF(V600E) mutation and the biology of papillary thyroid cancer. *Endocr Relat Cancer*. 2008;15:191–205.
35. Cohen MH, Williams GA, Sridhara R, et al. United States Food and Drug Administration Drug Approval summary: gefitinib (ZD1839; Iressa) tablets. *Clin Cancer Res*. 2004;10:1212–1218.
36. Hoffmann K, Xiao Z, Franz C, et al. Involvement of the epidermal growth factor receptor in the modulation of multidrug resistance in human hepatocellular carcinoma cells in vitro. *Cancer Cell Int*. 2011;11:40.
37. Saloman DS, Bianco C, Ebert AD, et al. The EGF-CFC family: novel epidermal growth factor-related proteins in development and cancer. *Endocr Relat Cancer*. 2000;7:199–226.
38. Leboeuf R, Baumgartner JE, Benezra M, et al. BRAFV600E mutation is associated with preferential sensitivity to mitogen-activated protein kinase inhibition in thyroid cancer cell lines. *J Clin Endocrinol Metab*. 2008;93:2194–2201.
39. Salerno P, De Falco V, Tamburrino A, et al. Cytostatic activity of adenosine triphosphate-competitive kinase inhibitors in BRAF mutant thyroid carcinoma cells. *J Clin Endocrinol Metab*. 2010;95:450–455.
40. Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, et al. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature*. 1997;385:544–548.
41. Weng LP, Gimm O, Kum JB, et al. Transient ectopic expression of PTEN in thyroid cancer cell lines induces cell cycle arrest and cell type-dependent cell death. *Hum Mol Genet*. 2001;10:251–258.
42. Kruser TJ, Wheeler DL. Mechanisms of resistance to HER family targeting antibodies. *Exp Cell Res*. 2010;316(7):1083–1100.
43. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467(7315):596–599.
44. End DW, Smets G, Todd AV, et al. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 in vivo and in vitro. *Cancer Res*. 2001;61:131–137.
45. Wilhelm SM, Dumas J, Adnane L, et al. Regorafenib (BAY 73-4506): a new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. *Int J Cancer*. 2011;129:245–255.
46. Hou P, Liu D, Shan Y, et al. Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. *Clin Cancer Res*. 2007;13:1161–1170.
47. Carlomagno F, Vitagliano D, Guida T, et al. Efficient inhibition of RET/papillary thyroid carcinoma oncogenic kinases by 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). *J Clin Endocrinol Metab*. 2003;88:1897–1902.
48. Friedmann B, Caplin M, Hartley JA, Hochhauser D. Modulation of DNA repair in vitro after treatment with chemotherapeutic agents by the epidermal growth factor receptor inhibitor gefitinib (ZD1839). *Clin Cancer Res*. 2004;10:6476–6486.
49. Friedmann BJ, Caplin M, Savic B, et al. Interaction of the epidermal growth factor receptor and the DNA-dependent protein kinase pathway following gefitinib treatment. *Mol Cancer Ther*. 2006;5:209–218.
50. Shien T, Doihara H, Hara H, Takahashi H, et al. PLC and PI3K pathways are important in the inhibition of EGF-induced cell migration by gefitinib ('Iressa', ZD1839). *Breast Cancer*. 2004;11:367–373.
51. Amado RG, Wolf M, Peeters M, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008;26:1626–1634.
52. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol*. 2008;26:5705–5712.
53. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med*. 2008;359:1757–1765.
54. Pennell NA, Daniels GH, Haddad RI, et al. A phase II study of gefitinib in patients with advanced thyroid cancer. *Thyroid*. 2008;18:317–323.
55. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012;483:100–103.
56. Leboulleux S, Bastholt L, Krause T, et al. Vandetanib in locally advanced or metastatic differentiated thyroid cancer: a randomised, double-blind, phase 2 trial. *Lancet Oncol*. 2012;13:897–905.