Epidermal Growth Factor Receptor Tyrosine Kinase Inhibition Represses Cyclin D1 in Aerodigestive Tract Cancers

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ABSTRACT

Purpose: **Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are active in cancer therapy. Mechanisms engaged during these clinical responses need to be determined. We reported previously that epidermal growth factor stimulation markedly increased cyclin D1 protein expression in human bronchial epithelial (HBE) cells, and this was opposed by chemoprevention with all-*trans*-retinoic acid. The current study sought to determine whether the EGFR TKI erlotinib repressed cyclin D1 protein expression in immortalized HBE cells, lung cancer cell lines, and clinical aerodigestive tract cancers.**

Experimental Design: **The BEAS-2B immortalized HBE cell line was exposed to varying concentrations of erlotinib, and effects on proliferation, cell cycle distribution, G_1 cyclin expression, and cyclin D1 reporter activity were measured. Non–small-cell lung cancer cell lines were also evaluated for changes in proliferation and cyclin protein expression after erlotinib treatments. A proof of principle clinical trial was conducted. During this study, patients underwent a 9-day course of erlotinib treatment. Pretreatment and posttreatment tumor biopsies were obtained, and changes in candidate biomarkers were determined by immunostaining. Plasma pharmacokinetics and tumor tissue erlotinib concentrations were measured.**

Results: **Erlotinib, at clinically achievable dosages, repressed BEAS-2B cell growth, triggered G_1 arrest, and preferentially reduced cyclin D1 protein expression and transcriptional activation. Erlotinib also preferentially repressed proliferation and cyclin D1 protein expression in responsive, but not resistant, non–small-cell lung cancer cell lines. This occurred in the presence of wild-type EGFR sequence at exons 18, 19, and 21. Five patients were enrolled onto an erlotinib proof of principle clinical trial, and four cases were evaluable. Pharmacokinetic studies established therapeutic erlotinib plasma levels in all patients, but tissue levels exceeding 2 μmol/L were detected in only two cases. Notably, these cases had pathological evidence of response (necrosis) in posttreatment biopsies as compared with pretreatment biopsies. In these cases, marked repression of cyclin D1 and the proliferation marker Ki-67 was detected by immunohistochemical assays. Cases without pathological response to erlotinib did not exhibit changes in cyclin D1 or Ki-67 immunohistochemical expression and had much lower erlotinib tissue levels than did responding cases.**

Conclusions: **Taken together, these *in vitro* and *in vivo* findings provide direct evidence for repression of cyclin D1 protein as a surrogate marker of response in aerodigestive tract cancers to erlotinib treatment. These findings also provide a rationale for combining an EGFR TKI with an agent that would cooperatively repress cyclin D1 expression in clinical trials for aerodigestive tract cancer therapy or chemoprevention.**

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the United States (1). Improved therapeutic and preventive strategies are needed to combat this major public health problem. We have reported the frequent overexpression of the epidermal growth factor receptor (EGFR) and its ligand, transforming growth factor α, in non–small cell lung cancer (NSCLC) as well as in bronchial preneoplasia (2–4). This EGFR autocrine loop is important in regulating epithelial carcinogenesis, as reviewed previously (5–8). EGFR tyrosine kinase inhibitors (TKIs) and anti-EGFR antibodies are in clinical use (5, 6, 8–10). EGFR TKIs are active in aerodigestive tract cancer therapy (6, 8–11).

Using the retinoid all-*trans*-retinoic acid (RA) as a pharmacological tool, we uncovered mechanisms engaged during *in vitro* blockade of carcinogenic transformation using BEAS-2B immortalized human bronchial epithelial (HBE) cells. Exposing these cells to tobacco carcinogens caused cellular transforma-

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tion to occur. However, RA treatment opposed this carcinogenic transformation (12, 13). By comparing transformed and RA-treated HBE cells, the EGFR and cyclin D1 proteins were identified as repressed in this chemoprevention model (12–14).

To evaluate the functional role of EGFR signaling in this model, transformed and RA-treated HBE cells were independently treated with epidermal growth factor (EGF; ref. 14). EGF treatment prominently induced the phosphorylated (activated) form of EGFR in transformed cells. In contrast, this activation was repressed in RA-treated HBE cells (14). Cyclin D1 protein was also markedly increased by EGF treatment. This EGF effect was antagonized by treatment with the chemopreventive agent RA (14). These *in vitro* findings highlighted EGFR-mediated induction of cyclin D1 as an attractive target for lung cancer therapy or chemoprevention. Consistent with this view, immunostaining of human bronchial preneoplastic and malignant lung lesions confirmed that cyclin D1 and EGFR were frequently overexpressed early during lung carcinogenesis *in vivo* (2–4, 15, 16).

EGF affects cyclin D1 protein expression through several pathways. Among these pathways are activation of STAT3, activation of mitogen-activated protein kinase (MAPK), and direct induction of cyclin D1 transcription by binding and activation of the cyclin D1 promoter (17–23). Cyclin D1 induction that occurs as a consequence of activation of these different pathways implicates a critical role for this cell cycle regulator in mediating EGFR responses.

This study sought to determine whether EGFR inhibition by the EGFR TKI erlotinib repressed cyclin D1 expression in erlotinib-sensitive immortalized HBE cells and NSCLC cell lines. The mechanistic basis for cyclin D1 repression was then examined by assessing erlotinib effects on second messenger pathways as well as cyclin D1 transcriptional activation. A proof of principle clinical trial was also conducted with erlotinib to perform pharmacodynamic and plasma and tissue pharmacokinetic studies. Findings that will be presented here indicate that cyclin D1 is a surrogate marker of *in vitro* and *in vivo* response to this EGFR TKI. A critical role for intratumoral erlotinib levels was also found in these observed clinical responses.

MATERIALS AND METHODS

Cell Culture. BEAS-2B cells were derived from normal HBE cells by immortalization with SV40, as described previously (24). These cells were passaged in LHC-9 media and cultured in a humidified incubator at 37°C with 5% CO_2 (12, 13, 25). Cells were passaged every 3 to 5 days. EGF deprivation was achieved using EGF-depleted LHC-9 media (14). Cells were maintained without EGF for 72 hours before repletion with media containing EGF at a concentration of 50 ng/mL. Erlotinib (OSI Pharmaceuticals, Boulder, CO) was dissolved in the vehicle, dimethyl sulfoxide (DMSO). H226, H358, H441, and A549 cell lines were each passaged as recommended by American Type Culture Collection (Manassas, VA). H226, H358, and H441 cells were each cultured in RPMI 1640 media containing 10% fetal bovine serum. A549 cells were cultured in Ham's modification of F-12 media containing 10% fetal bovine serum. Cells were typically passaged every 5 to 7 days.

Proliferation and Cell Cycle Assays. Cellular proliferation was measured using the CellTiter-Glo assay (Promega, Madison, WI). Assays were plated in sextuplicate in 96-well plates at a density of 3,000 cells per well. Basal luminescence activity and an ATP standard curve were determined. Cells were then treated with either erlotinib at various clinically achievable dosages or vehicle (DMSO) for 72 hours, with media replaced at 48 hours. Luminescence activity and an ATP standard curve were determined each day that the assay was performed. Growth was calculated by subtracting the basal ATP content from ATP content measured after erlotinib or vehicle treatment, respectively. Growth rates were normalized to rates in vehicle-treated cells as a control. Cell cycle analyses were independently performed on BEAS-2B HBE cells after 6 and 24 hours of treatment with erlotinib (1 μmol/L) or vehicle using propidium iodide staining, as described previously (25). This dosage of erlotinib has previously been shown to abrogate the mitogenic effects of EGF but not fibroblast growth factor, insulin-like growth factor 1, or platelet-derived growth factor (26).

Immunoblot Assays. BEAS-2B HBE cells were deprived of EGF for 72 hours, and EGF was restored to these cultures as described above in the presence of either erlotinib (1 μmol/L) or vehicle. Cells were harvested at several time points using radioimmunoprecipitation assay buffer containing phosphatase inhibitors. Protein concentrations were determined using the Bradford assay, and equal amounts of total protein were subjected to SDS-PAGE size fractionation before transfer to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Antibodies were purchased that recognized cyclin D1 (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-

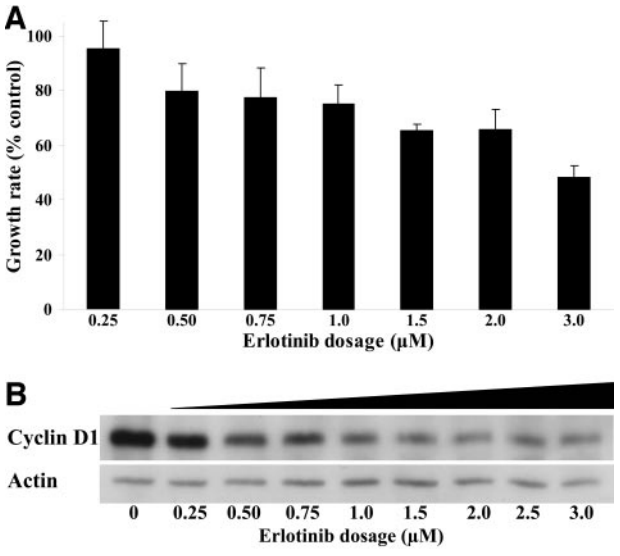

Fig. 1 Dose-dependent effects of erlotinib on BEAS-2B growth and cyclin D1 protein expression. *A*. Erlotinib, at clinically achievable dosages, suppresses BEAS-2B cell growth in a dose-dependent manner. A representative experiment is depicted from three independent replicate experiments. *Bars*, SD. *B*. Immunoblot analyses performed over a similar dosage range as described in *A* revealed dose-dependent repression of cyclin D1 protein. A prominent decline in cyclin D1 immunoblot expression occurred at doses as low as 500 nmol/L.

EGFR (PY-20; MP Biomedicals, Irvine, CA), phospho-Akt (9271; Cell Signaling, Beverly, MA), phospho-MAPK (9101; Cell Signaling), or actin (C-11; Santa Cruz Biotechnology). Appropriate secondary antibodies were used (Amersham Life Sciences, Arlington Heights, IL) along with detection by chemiluminescence (ECL Plus; Amersham Life Sciences). Immunoblot analyses were also performed after erlotinib treatments to detect cyclin E (HE-12; Santa Cruz Biotechnology), cyclin D2 (C-17; Santa Cruz Biotechnology), cyclin D3 (C-16; Santa Cruz Biotechnology), and p27 (C-19; Santa Cruz Biotechnology), respectively.

Luciferase Assay. BEAS-2B cells were transfected with equal amounts of a cyclin D1 reporter construct containing the firefly luciferase gene (14) and a *Renilla* luciferase reporter plasmid (pRL-TK vector; Promega) as a control for transfection efficiency. Transfections were performed for 24 hours using Effectene reagent (Qiagen, Valencia, CA) as per the manufacturer's protocol. Cells were then deprived of EGF for 24 hours. Culture media were then replaced with EGF-depleted or EGF-supplemented media containing erlotinib (3 μmol/L) or vehicle as a control. After 6 hours, firefly luciferase and *Renilla* luciferase activities were each recorded using the Dual Luciferase Assay Reporter System (Promega). Normalized luciferase activity was determined by dividing firefly luciferase activity by *Renilla* luciferase activity.

EGFR Genomic DNA Sequencing. Genomic DNA was harvested individually from H226, H358, H441, and A549 cell lines using DNAzol (Molecular Research Center, Cincinnati, OH). Genomic DNA from clinical tumors was extracted from paraffin-embedded tissues using the DNEasy Tissue Kit (Qiagen) according to the manufacturer's protocol. Polymerase chain reaction (PCR) assays were performed using established techniques to amplify EGFR exons 18, 19, and 21 (27, 28). PCR products were then purified using QIAquick (Qiagen) and sequenced using previously described techniques (27, 28). PCR products from clinical tumors were also subjected to clonal sequence analysis. The products were ligated into TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Competent cells (Invitrogen) were transformed with the cloned vectors and selected according to the manufacturer's protocol. Individual colonies were expanded, and plasmid DNA was extracted from each using the Wizard Plus Miniprep system (Promega). Five clones of exons 18, 19, and 21 were independently sequenced. If a previously described EGFR mutation was identified (27, 28), 10 additional clones were sequenced to estimate mutation frequency.

Proof of Principle Trial. Patients with advanced aerodigestive tract cancers were enrolled onto an institutional review board-approved trial conducted at Dartmouth-Hitchcock Medical Center. After obtaining informed consent, patients underwent a pretreatment tumor biopsy. Patients were then given erlotinib at an oral dosage of 150 mg daily for 9 days. On days 1 and 8, detailed plasma pharmacokinetics were measured. On day 9, a posttreatment tumor biopsy was performed between 60 and 90 minutes after administration of the erlotinib dose, which correlates closely with the observed time to peak plasma erlotinib concentrations. These tumor tissues were analyzed for erlotinib concentrations using a validated liquid chromatographic method with tandem mass spectroscopic detection as performed by MDS Pharma Services (St. Laurent, Quebec, Canada).

Immunohistochemistry. Expression of candidate biomarkers of erlotinib response was determined by comparing immunohistochemical assay results in pretreatment *versus* posttreatment biopsies. Biopsy specimens were fixed in cold acetone, cleared in methyl benzoate, and embedded in paraffin. The immunohistochemical markers examined were EGFR, phospho-EGFR, cyclin D1, p27, and Ki-67. Antibodies used to detect these species were EGFR (clone EGFR.113; Novocastra Laboratories, Newcastle, United Kingdom), phospho-EGFR (4404; Cell Signaling), cyclin D1 (CP236A; Biocare Medical, Walnut Creek, CA), Ki-67 (clone MIB1; Dako, Glostrup, Denmark),

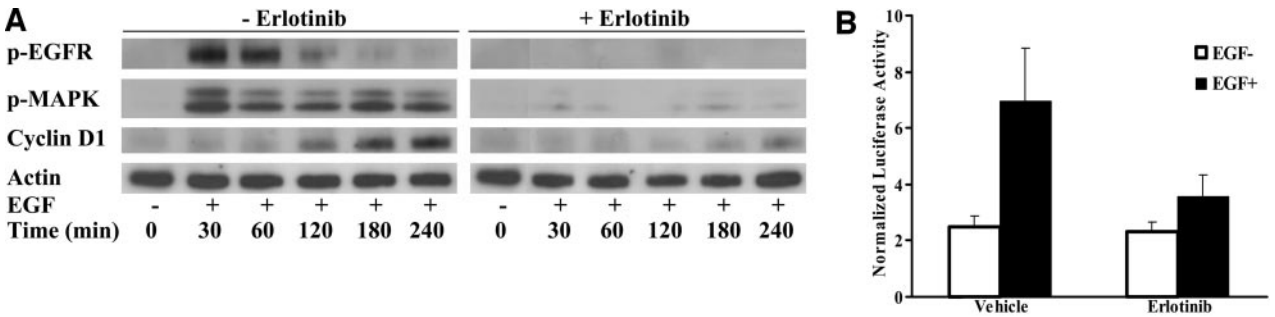

Fig. 2 Erlotinib effects on EGF-mediated changes in signal transduction and cyclin D1 expression. *A*. Kinetics of the expression of the activated form of EGFR (phospho-EGFR, *p-EGFR*), phospho-MAPK (*p-MAPK*), and cyclin D1 protein expression after EGF stimulation are shown. This study reveals inhibition by erlotinib (1 μmol/L) of EGF-mediated augmentation of phospho-EGFR, phospho-MAPK (migrating as the expected p44/42 doublet), and cyclin D1 expression. In the *top two panels*, phospho-EGFR and phospho-MAPK are shown as increased by EGF treatment as compared with untreated cells ($t = 0$) as early as 30 minutes after treatment. This effect was markedly repressed in erlotinib-treated cells, with only a minimal phospho-EGFR signal detected at 2 hours, even with prolonged chemiluminescence (data not shown). EGF-mediated induction of cyclin D1 protein is evident in vehicle-treated cells within 2 hours of treatment, and this is subsequent to the induction of phospho-EGFR and phospho-MAPK. These responses were inhibited in erlotinib-treated BEAS-2B HBE cells. Immunoblot analysis of actin served as a loading control. *B*. Cyclin D1 reporter plasmid luciferase assays were performed in BEAS-2B HBE cells after EGF treatment or no treatment in the presence or absence of erlotinib. The EGF-mediated transcriptional activation of the cyclin D1 reporter plasmid was repressed by erlotinib treatment at doses of 3 μmol/L and 1 μmol/L (data not shown). *Bars*, SD.

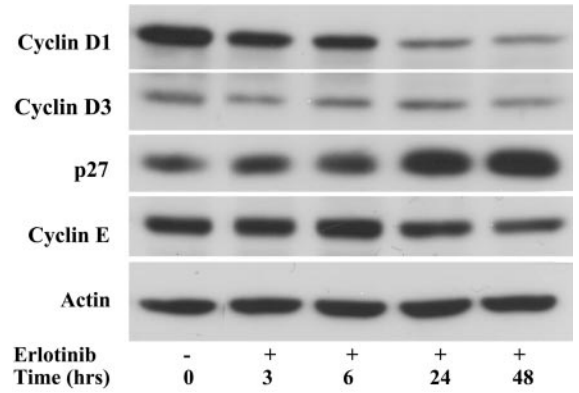

Fig. 3 The kinetics of changes in immunoblot expression of key G_1 cell cycle regulators in asynchronously growing BEAS-2B HBE cells cultured in complete media are displayed before and after erlotinib (1 μmol/L) treatment. The prominent repression of cyclin D1 immunoblot expression was observed in BEAS-2B cells growing asynchronously in EGF-containing media. A minor increase in p27 expression was observed without appreciable changes in cyclin D2 or cyclin E expression (data not shown). No such changes were observed in cells treated with vehicle alone (data not shown).

and p27 (C-19; Santa Cruz Biotechnology). Positive controls used for each were EGFR (tonsil mucosa), phospho-EGFR (adrenal cortical carcinoma), cyclin D1 (mucosal lymphoid tissue and mantle cell lymphoma), Ki-67 (colonic mucosa crypt cells), and p27 (spleen). The cyclin D1 antibody used for immunohistochemical analyses was evaluated for specificity by immunoblot analysis of BEAS-2B total cellular protein. Staining was categorized based on the intensity and location of staining as well as the percentage of tumor cells staining positive. Immunostaining results were scored by a pathologist (V. A. M.) who was blinded to the pharmacokinetic and clinical data. Scoring was as follows: 0, no tumor cells staining positive; 1+, 0% to 50% of tumor cells staining positive; 2+, 50% to 75% of tumor cells staining positive; and 3+, 75% to 100% of tumor cells staining positive. Patterns of expression of each biomarker in posttreatment as compared with pretreatment biopsies were then categorized as repressed or not repressed. Statistical correlation between biomarker repression and pathological response was determined using the χ^2 method with NCSS statistical software (Kaysville, UT). Significance was defined as a $P < 0.05$.

RESULTS

Erlotinib treatment at clinically relevant dosages led to dose-dependent reduction of BEAS-2B cell growth as shown in Fig. 1*A*. This was accompanied by induced G_1 arrest (data not shown). Immunoblot analyses in Fig. 1*B* revealed that erlotinib repressed cyclin D1 expression in a dose-dependent manner over a similar dosage range. These findings implicated repression of cyclin D1 protein and induced G_1 cell cycle arrest as important in HBE cellular response to this EGFR TKI.

To explore this effect further after EGF deprivation, BEAS-2B cells were treated with EGF in the presence of 1 μmol/L erlotinib or vehicle (DMSO). Cells were harvested before and at 30, 60, 120, 180, and 240 minutes after these treatments. Immunoblot analyses revealed marked induction of phospho-EGFR and phospho-MAPK in the vehicle-treated cells, but not in the erlotinib-treated cells, as shown in Fig. 2*A*. Phospho-Akt expression was similarly increased in both vehicle- and erlotinib-treated HBE cells (data not shown). Cyclin D1 immunoblot expression was augmented within 2 hours of EGF treatment in vehicle-treated HBE cells. This induction was markedly inhibited in erlotinib-treated cells. This inhibition was mediated at least in part through transcriptional repression of cyclin D1 based on the cyclin D1 reporter assay depicted in Fig. 2*B*. No change in p27 immunoblot expression was detected in vehicle- or erlotinib-treated cells (data not shown).

To compare changes in cell cycle-regulatory proteins in asynchronously growing cells, BEAS-2B HBE cells were cultured in complete media containing EGF. These cells were then independently treated with 1 μmol/L erlotinib or vehicle (DMSO), and changes in key cell cycle-regulatory proteins were compared by immunoblot analyses. As shown in Fig. 3, analyses performed on lysates from a representative experiment revealed a rapid decline in cyclin D1 protein expression within 6 hours of

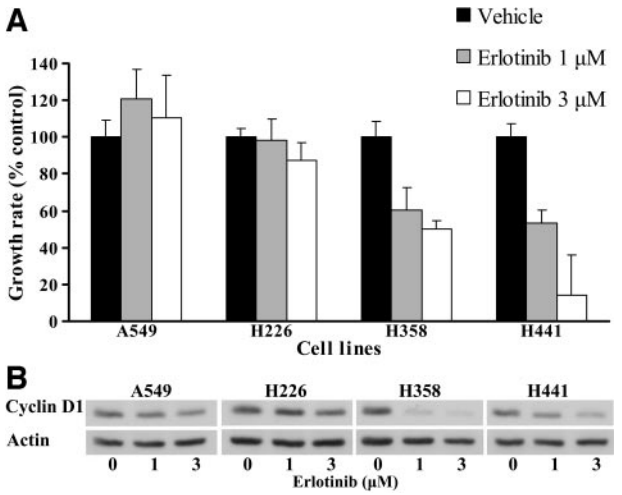

Fig. 4 Effects of erlotinib on lung cancer cell line growth and cyclin D1 protein expression. *A*. At clinically achievable dosages, erlotinib suppressed H358 and H441 cell growth, whereas this inhibition was not observed for A549 cells and was blunted in H226 cells. Similar patterns of sensitivity were seen in three independent assays. *Bars*, SD from a representative assay. *B*. Immunoblot assays performed with the same erlotinib dosages revealed preferential repression of cyclin D1 protein with minimal repression of cyclin D2 and cyclin D3 proteins (data not shown).

Table 1 Patient characteristics ($N = 4$)

Age (y)	57.5
Median (range) (y)	53–62
Male	4
ECOG performance status 1	4
Histopathology	
Squamous cell cancer (lung)	2
Adenocarcinoma (lung)	1
Squamous cell cancer (larynx)	1

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

Table 2 Erlotinib pharmacokinetic and pharmacodynamic effects

Case	Pathologic response	Pretreatment cyclin D1	Posttreatment cyclin D1	Peak plasma erlotinib concentration (μmol/L)	Tumor tissue erlotinib concentration (μmol/L)
1	No	2+	2+	6.5	0.3
2	No	2+	2+	3.9	1.7
3	Yes	3+	1+	9.7	4.1
4	Yes	2+	1+	3.0	4.8
Median concentration				5.2	2.9

erlotinib treatment. At 24 hours, a modest decline in cyclin D3 and no appreciable change in cyclin D2 expression (data not shown) occurred, whereas a minor increase in p27 expression was detected.

Changes in cyclin D1 immunoblot expression were also examined after erlotinib treatments of lung cancer cell lines. A549, H226, H358, and H441 lung cancer cell lines were treated with dosages of erlotinib that are clinically achievable. As shown in Fig. 4*A*, erlotinib markedly suppressed H358 and H441 cell growth but did not appreciably affect A549 or H226 cell growth. Immunoblot analyses demonstrated prominent repression of cyclin D1 in erlotinib-sensitive but not erlotinib-resistant cells at the same dosages used to assess cellular proliferation, as shown in Fig. 4*B*. Minor repression of cyclin D3 and no change in cyclin D2 protein expression were observed in erlotinib-sensitive cells (data not shown). Repression of phospho-MAPK occurred in one erlotinib-sensitive and one erlotinib-resistant cell line, whereas

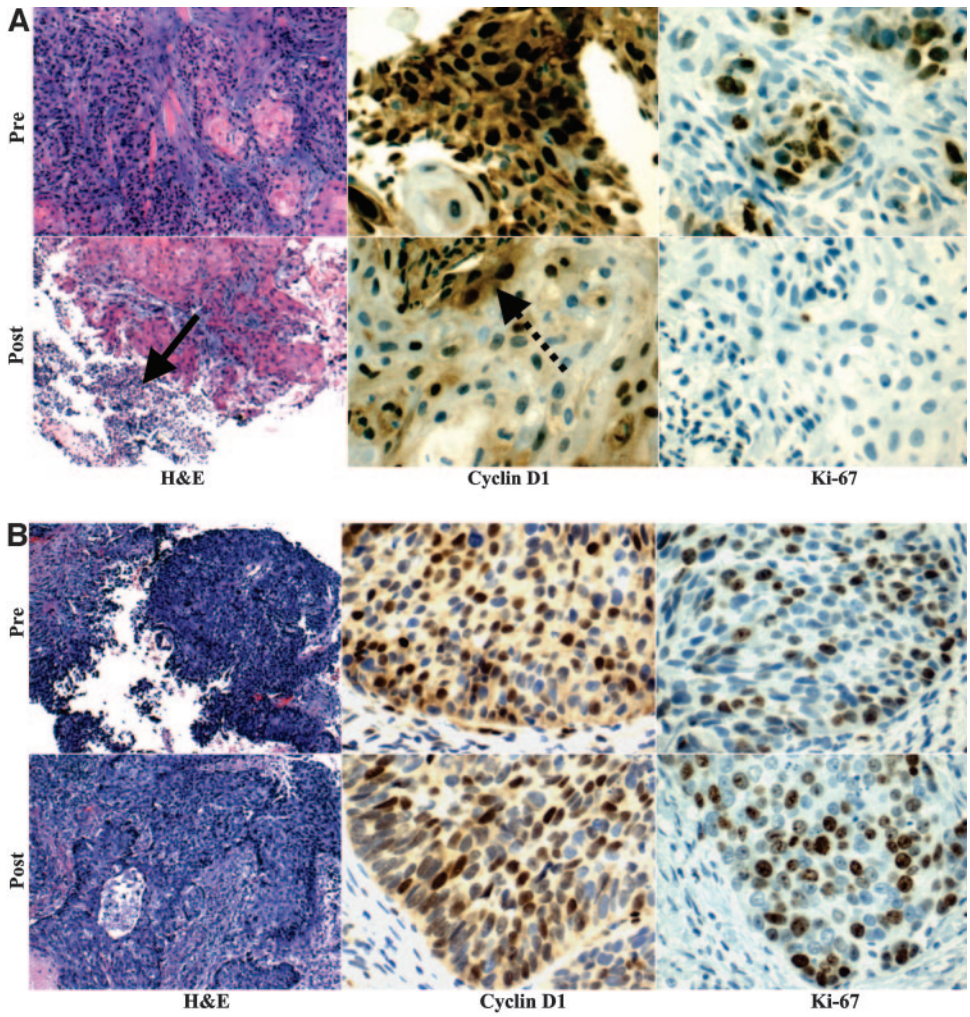

Fig. 5 Cyclin D1 and Ki-67 immunohistochemical expression in cases of erlotinib-sensitive or -resistant aerodigestive tract cancers. *A*. These species were both repressed in a representative responding case. This was observed both in nonnecrotic nests of tumor cells and in necrotic regions. The *black arrow* indicates a region of tumor necrosis, as shown by hematoxylin and eosin staining. The *dashed black arrow* depicts cyclin D1 immunostaining within cancer cells. *B*. This representative nonresponding case did not exhibit histopathological evidence of necrosis or an appreciable reduction in cyclin D1 or Ki-67 immunohistochemical expression.

Table 3 Nonclonal EGFR genomic DNA sequence analyses

	Exon 18	Exon 19	Exon 21
Cell lines			
A549	wt	wt	wt
H226	wt	wt	wt
H358	wt	wt	wt
H441	wt	wt	wt
Case			
3	wt	wt	wt
4	wt	wt	wt

Abbreviation: wt, wild-type.

phospho-Akt was not repressed in any of these lung cancer cell lines (data not shown). EGFR genomic DNA sequence analyses revealed that each examined cell line was wild-type at exons 18, 19, and 21.

To directly evaluate potential biomarkers of clinical response, patients with aerodigestive tract cancers, who were candidates for subsequent tumor biopsies, were enrolled onto the described proof of principle trial. The clinical characteristics of these patients appear in Table 1. Pharmacokinetic analyses confirmed therapeutic plasma concentrations in all patients, whereas tumor tissue concentrations above 2 μmol/L were noted in only two cases (cases 3 and 4), with lower concentrations noted in two other cases (cases 1 and 2; Table 2). Notably, the two cases with higher erlotinib tumor tissue concentrations had evidence of histopathological responses and changes in biomarker expression, but the cases with the lower concentrations did not. The histopathology of responding cases was as follows: case 3, lung adenocarcinoma; and case 4, laryngeal squamous cell carcinoma.

Of four evaluable patients, two had histopathological evidence of erlotinib response. This was based on the appearance of tumor necrosis in posttreatment biopsies as compared with pretreatment biopsies, as depicted in Fig. 5. Both patients having pathological response reported symptomatic improvement during the 9-day course of treatment. Nonclonal and clonal sequencing of exons 18, 19, and 21 revealed that the responding cases were predominantly wild-type at the regions reported to predict response (Table 3). One of the previously reported EGFR mutations was identified at a low frequency in case 3 (Table 4). The nonclonal and clonal sequence data for this case appear in Fig. 6.

Candidate biomarkers of erlotinib response were assessed by performing immunohistochemical assays in pretreatment as compared with posttreatment biopsies, as displayed in Table 2. EGFR expression declined in one responding and one nonresponding case, whereas p27 was not detected immunohistochemically in these examined cases (data not shown). In responding cases, cyclin D1 and Ki-67 immunostaining decreased markedly in posttreatment biopsies as compared with pretreatment biopsies, as shown in Fig. 5. In contrast, in nonresponding cases, an appreciable change in cyclin D1 or Ki-67 immunostaining was undetected. The cyclin D1 antibody used for these immunohistochemical analyses was confirmed to recognize a single predominant immunoreactive cyclin D1 species by immunoblot analysis of BEAS-2B total cellular protein (data not shown). The correlation between pathological response and cyclin D1 repression was significant ($P = 0.046$).

Table 4 Clonal EGFR genomic DNA sequence analyses

	EGFR mutation							
Case	G719S	Del-1a	Del-1b	Del-2	Del-3	Del-4	Del-5	L858R
3	1/15	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

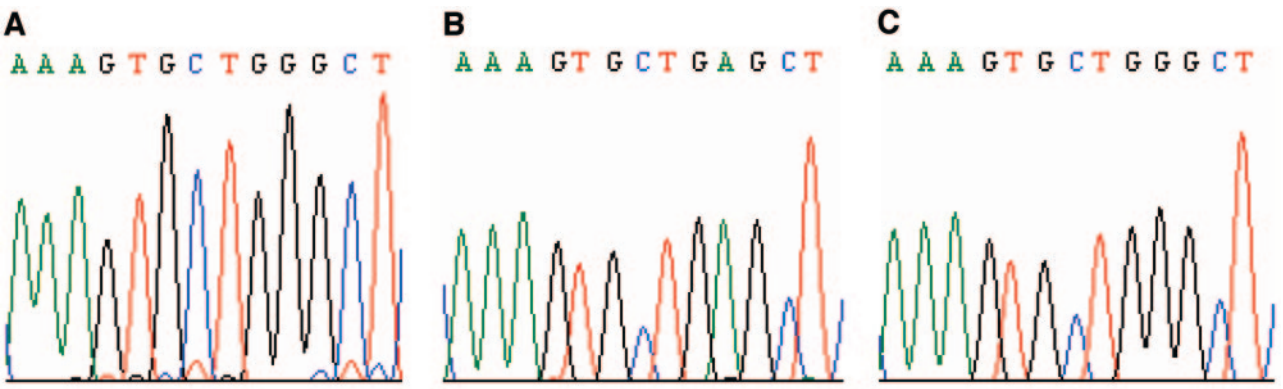

Fig. 6 EGFR genomic DNA sequence analyses from a responding case (case 3). *A*. Nonclonal DNA sequence analysis reveals wild-type EGFR sequence at the region of exon 18 that is known to harbor a mutation reported to predict enhanced clinical response to an EGFR TKI. *B*. Clonal sequence analysis reveals a rare mutation of exon 18 in a single clone of 15 independently sequenced clones. The remaining clones were found to contain wild-type EGFR exon 18 genomic DNA sequence at this region. *C*. A representative wild-type clonal sequence of this EGFR genomic DNA region is displayed.

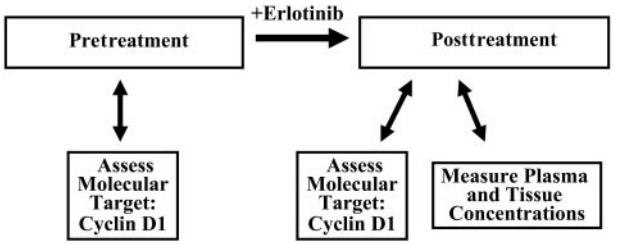

Fig. 7 Proof of principle clinical trial design. This figure displays the strategy taken in the described erlotinib clinical trial. This trial used pretreatment and posttreatment tumor biopsies to uncover pharmacodynamic and pharmacokinetic relationships that existed for this EGFR TKI.

DISCUSSION

EGFR TKIs are active in the treatment of aerodigestive tract cancers (8–11). Several clinical characteristics and molecular genetic features have been reported that may predict response to this class of agents including the development of rash or the presence of EGFR mutations (27–30). Identifying mechanisms of response to EGFR inhibition is important for progress in this field (31–33). We reported in this study that erlotinib suppressed growth of immortalized HBE cells and some NSCLC cell lines in a dose-dependent manner and at clinically relevant dosages. Erlotinib also inhibited the expected EGFR-mediated induction of cyclin D1 expression and transcriptional activation that are evident after EGF stimulation. Cyclin D1 was preferentially repressed by erlotinib treatment of immortalized HBE cells and erlotinib-sensitive NSCLC cell lines, whereas erlotinib-resistant NSCLC cell lines did not exhibit this effect. This association was not observed in prior studies of other cancer cell lines, suggesting that this may be cell context dependent (34). Notably, all examined cell lines evaluated in this study had wild-type EGFR DNA sequence at the regions recently reported to predict clinical response to gefitinib (Iressa; ref. 27, 28).

These *in vitro* findings implicated cyclin D1 repression as a predictor of therapeutic response to erlotinib. These findings were extended to the clinic by conducting a proof of principle clinical trial in aerodigestive tract cancers. Responding cases exhibited repression of cyclin D1 protein, whereas no appreciable change in cyclin D1 expression was observed in nonresponding cases. This highlighted cyclin D1 repression as a marker of clinical erlotinib response. Although a small number of cases were assessed in this clinical trial, finding that responding cases repressed cyclin D1 expression, whereas non-responding cases did not, has translated these *in vitro* observations to the *in vivo* setting.

Determining molecular therapeutic mechanisms responsible for cyclin D1 repression might uncover additional species that could be targeted to augment clinical activity. Perhaps currently available therapeutic agents would cooperatively affect cyclin D1 expression as part of a targeted combination regimen. In this regard, we reported that certain classical and nonclassical retinoids trigger proteasomal degradation of cyclin D1 (35). Perhaps a regimen that combined an EGFR TKI with a classical or nonclassical retinoid would cooperatively reduce cyclin D1 expression and thereby augment therapeutic or chemopreventive activity in aerodigestive tract cancers (36, 37).

This study established a relationship between tumor tissue (but not plasma) erlotinib concentrations and histopathological responses. Tissue erlotinib concentrations exceeding 2 μmol/L were found in responding cases, whereas substantially lower levels were observed in nonresponding cases. These findings indicated that a threshold relationship might exist between tumor tissue erlotinib concentrations and the pharmacodynamic effects of this EGFR TKI on cyclin D1 expression. If so, dose-escalation strategies could be an attractive approach to consider. Toxicities from this approach may limit clinical feasibility.

The results from this erlotinib proof of principle clinical trial confirmed and extended *in vitro* results. Cyclin D1 and Ki-67 were highlighted as biomarkers of erlotinib response *in vivo*. Confirmatory trials are indicated in this and other clinical settings in which EGFR TKIs have activity. Elevated tissue erlotinib concentrations were also found to affect pathological responses and changes in biomarker expression. Such relationships may only become apparent through the conduct of trials that incorporate biomarker assessment and tumor tissue drug measurements, as depicted in Fig. 7. This proof of principle approach is attractive to consider for future investigations of other targeted agents.

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