Deregulated Cyclin D1 Expression Is Associated with Decreased Efficacy of the Selective Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Gefitinib in Head and Neck Squamous Cell Carcinoma Cell Lines

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ABSTRACT

Purpose: Despite promising initial results, recent Phase III trials of the selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (“Iressa”; AstraZeneca, Wilmington, Delaware) in advanced head and neck squamous cell carcinoma (HNSCC) have been equivocal. Cyclin D1, an EGFR target gene, is frequently overexpressed in HNSCC, has been implicated in its pathogenesis, and is strongly associated with poor prognosis in this disease. Therefore, we examined the relationship between deregulated cyclin D1 expression and sensitivity to gefitinib to determine whether this frequently occurring oncogenic change affected the cellular response to gefitinib.

Experimental Design: A panel of six EGFR-overexpressing HNSCC cell lines was used to correlate CCND1 gene copy number, cyclin D1 expression, and response to gefitinib. The effect of constitutive overexpression of cyclin D1 was assessed by establishing stably transfected clonal SCC-9 cell lines.

Results: Three of six cell lines displayed cyclin D1 amplification and/or overexpression, and these cell lines were resistant to gefitinib. SCC 9 clones overexpressing cyclin D1 continued to proliferate and maintained their S-phase fraction when treated with gefitinib, whereas empty vector control clones and the parental SCC 9 cells were profoundly inhibited and displayed marked reductions in S-phase. The resistance of cyclin D1-overexpressing clones and cyclin D1-amplified cell lines was associated with maintenance of cyclin D1 expression after gefitinib treatment.

Conclusions: These data suggest that deregulated cyclin D1 overexpression may be associated with resistance of HNSCC to EGFR inhibitors. Therefore, the role of cyclin D1 as a marker of therapeutic response and its utility as a prognostic marker in HNSCC warrant additional analysis.

INTRODUCTION

Head and neck cancers represent the sixth most common malignancy worldwide. The vast majority (>90%) of these cancers are squamous cell carcinomas. Despite advances in the treatment of head and neck squamous cell carcinomas (HNSCC), the 5-year survival rate has remained <50% for the past four decades, and the overall mortality rate is either stable or on the increase in Western countries (1).

The epidermal growth factor receptor (EGFR) has been implicated in cancer development and progression through overexpression (in the absence of gene amplification) in 36 to 100% of HNSCC and this is commonly associated with the activation of one of its ligands, transforming growth factor α, in an autocrine loop (reviewed in refs. 2, 3–6). These events occur early in the pathogenesis of HNSCC (4) and are associated with reduced relapse-free survival and poor overall survival in many studies of HNSCC patients (6, 7). Alterations in the function of EGFR and other members of the tyrosine kinase receptor family have been linked with oncogenic transformation, autonomous cell growth, invasion, angiogenic potential, and development of distant metastases in a variety of cancers (8–10).

Advances in the understanding of the molecular biology of various cancers have resulted in the development of novel targeted therapeutics. Among these are therapies directed at the EGFR, which may have particular utility in HNSCC because of the frequent and early overexpression of EGFR in this disease. Potential therapeutic approaches include the use of monoclonal antibodies that compete with the binding of activating ligands to the extracellular domain of the receptor, through to newer modalities such as immunotoxins and antisense oligonucleotides. HNSCC patients have participated in clinical trials with most of the available classes of inhibitors and have seen some promising results (8, 11, 12). Gefitinib (“Iressa”; AstraZeneca, Wilming-
ton, DE) is an orally active, low molecular weight (M, 447), synthetic quinazoline derivative that acts as a selective reversible inhibitor of EGFR tyrosine kinase activity (13). It is also active against other tyrosine kinases such as HER2, but only when they are coexpressed with EGFR (14, 15). In one Phase II clinical trial, 52 patients with advanced or metastatic HNSCC were treated with gefitinib and showed an 8% complete response, 12% showed a partial response, and 35% of patients had stable disease (16). Ongoing Phase III trials with gefitinib as a monotherapy in similar stage HNSCC patients have shown antineoplastic activity, although the results of these trials are still premature (17) compared with the more established role of gefitinib in non–small-cell lung carcinoma (18).

The underlying mechanism for the differential sensitivity of cancer cells to EGFR inhibitors is yet to be elucidated. The level of EGFR expression alone is not sufficient to predict the response of cell lines to EGFR inhibitors in vitro (19, 20). EGFR mutations that correlate with sensitivity to gefitinib have recently been documented in small cell lung cancer (21, 22), but similar mutations were not found in any of the seven HNSCC cell lines examined (22). The cell cycle regulatory proteins cyclin D1 and p27KIP1 are targets for EGFR signaling (23–25) and are commonly deregulated in various cancers (26). Cyclin D1 is well characterized as a marker of poor prognosis in HNSCC (27–35) and has been correlated with poor histological differentiation of HNSCC and local invasion (34–36). Cyclin D1 associates with the cyclin-dependent kinases (CDK) CDK4 and CDK6, activating them in mid-late G1 phase, and thereby controlling progression through G1 phase (37). In addition, cyclin D1-CDK4/CDK6 complexes sequester the CDK inhibitors p27KIP1 and p21WAF1/CIP1 (38). Because this titrates p21WAF1/CIP1 away from cyclin E-CDK2, another cyclin-CDK complex controlling progression through the G1-S-phase transition, the balance between cyclin D1 and p27KIP1 levels can profoundly affect the rate of cell cycle progression. EGFR inhibition induces arrest in G1 phase, and this is associated with decreased cyclin D1 expression and increased p27KIP1 expression (23, 38). Investigation of the role of these molecules in the sensitivity of HNSCC to gefitinib has largely focused on the role of p27KIP1 and has shown that sensitivity is reduced in cells expressing antisense p27KIP1 constructs (39). However, the central roles of cyclin D1 and EGFR in the pathogenesis of HNSCC make cyclin D1 an attractive downstream molecule of the EGFR pathway to explore for a potential association with clinical susceptibility to EGFR inhibitors. We hypothesized that EGFR-overexpressing HNSCC cell lines with coexisting amplification of the cyclin D1 gene (CCND1) might show increased resistance to gefitinib, and we have used six EGFR-overexpressing HNSCC cell lines and overexpression of cyclin D1 in SCC 9 cells to test this idea.

**MATERIALS AND METHODS**

**Cell Culture and Conditions.** Established human squamous cell carcinoma lines, FaDu, Detroit 562, SCC 9, SCC 15, SCC 25, and CAL 27 were obtained from American Type Culture Collection (Rockville, MD) and cultured in a humidified atmosphere of 5% CO2 and 95% air at 37°C. SCC 9, SCC 15, and SCC 25 cells were grown in 1:1 mixture of DMEM and Ham’s F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mmol/L L-glutamine, 15 mmol/L HEPES, and 0.5 mmol/L sodium pyruvate (DMEM-F12) supplemented with 10% FCS, 400 ng/mL hydrocortisone, and 80 μg/mL Gentamicin. Detroit 562 and FaDu were grown in EMEM supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 10% FCS, and 80 μg/mL Gentamicin. CAL 27 cells were grown in DMEM containing 10% FCS, 2 mmol/L L-glutamine, 0.5 mmol/L sodium pyruvate, and 80 μg/mL Gentamicin. All of the cell lines were derived from primary SCC of the tongue except for the FaDu cell line, which was derived from a primary SCC from the hypopharynx, and Detroit 562, which is derived from a metastatic pharyngeal SCC. The control cell lines MDA-MB-134 (American Type Culture Collection) and MCF-7 human breast cancer cells (Michigan Cancer Foundation, Detroit, MI) were cultured in RPMI 1640 supplemented with 5% FCS, insulin (10 μg/mL), and gentamicin (10 μg/mL) at 37°C.

**DNA Purification and Southern Blot Analysis.** For Southern blot analysis, genomic DNA was prepared from the cell lines with the Qiagen DNAeasy kit (Qiagen Pty Ltd.; Clifton Hills, Victoria, Australia) according to the manufacturer’s instructions. The cyclin D1 probe was a 1.3-kb HindIII restriction fragment encompassing the coding region. Fifty nanograms of this DNA fragment were labeled by random priming with [α-32P]dCTP (110 Bq/μmol/L; Amersham, Castle Hill, Australia) and hybridized at a final probe concentration of 1 to 2 ng/mL. The progesterone receptor (PR) probe used as a control was hPR1, a 1.2-kb human PR fragment encoding most of the DNA binding domain and part of the A/B domain.

Hybridization was carried out for 16 hours at 65°C in 0.5 mol/L sodium phosphate (pH 6.9), 7% SDS, 0.5% Blotto instant skim milk, 0.2% sodium azide, and 1 mmol/L EDTA. Filters were washed to a final stringency of 0.05 × SSC [20 × SSC: 3 mol/L sodium chloride, 0.3 mol/L sodium citrate (pH 7.0)] + 0.1% SDS for 30 minutes at 65°C. Autoradiography was done at -70°C with 2 intensifying screens (DuPont, Wilmington, DE). Images were also captured by PhosphorImager (Molecular Dynamics 445 SI, Molecular Dynamics, Sunnyvale, CA).

**Proliferation Assays.** Cells (500 to 2,000) were plated in 96-well microtiter plates (six replicates per plating density) according to predetermined growth characteristics and optimal plating densities for the respective cell lines. Inoculates were incubated overnight at 37°C to allow cell attachment before addition of gefitinib at t = 0. Control cells were treated with an equivalent concentration of DMSO (vehicle). Relative cell numbers were estimated daily for 5 days, with the Cell Titer 96 assay (Promega, Madison, WI) in accordance with the manufacturer’s instructions. The IC50 was defined as the drug concentration required to reduce the cell number to 50% of the control.

**Clonogenic Assays.** These were established under conditions similar to the proliferation assays. Cells (6,000 to 12,000) were plated overnight in 6-well plates before addition of gefitinib or DMSO vehicle. Plated cells were observed twice weekly, and the medium was replaced weekly with fresh medium containing either gefitinib (at 2 μmol/L or 10 μmol/L concentration) or DMSO. At the conclusion of the experiment, the cells were fixed and stained with a Diff Quick Stain (Lab
Aids, Narrabeen, New South Wales, Australia) in accordance with the manufacturer’s guidelines.

**Expression of Cyclin D1.** The entire open reading frame of cyclin D1 was amplified by PCR with PFU polymerase (Promega) with the pLib-D1 vector as template (40) and then cloned into the Gateway pDONR201 vector (Invitrogen, Rockville, MD). After confirmation by sequencing, recombination into the destination vector pcDNA-Dest 47 (Invitrogen) produced a mammalian construct for expression.

SCC 9 cells were transfected for 24 hours with pcDNA-Dest 47–cyclin D1 and FuGENE (Roche) in a ratio of 1 μg to 3 μL. Control SCC 9 cells were transfected with the empty pcDNA-Dest 47 vector with the same protocol. The cells were selected for 21 days in 400 μg/mL Geneticin (Invitrogen), and clones isolated and expanded in the presence of 400 μg/mL Geneticin.

**Cell Lysis.** Cells were lysed as follows: cell monolayers were washed twice with ice-cold PBS, and then scraped into ice-cold “normal” lysis buffer [0.5% deoxycholate, 150 mmol/L NaCl, 1% sodium PP, 50 mmol/L Tris (pH 8.0), 0.1% SDS, 10% glycerol, 5 mmol/L EDTA, 20 mmol/L NaF, 10 μg/mL aprotinin, 10 μg/mL Leupeptin, 1 mmol/L phenyl methyl sulfonyl fluoride, and 200 μmol/L sodium orthovanadate] or Sherr lysis buffer [50 mmol/L HEPES (pH 7.5), 1 mmol/L dithiothreitol, 150 mmol/L NaCl, 10% (v/v) glycerol, 0.1% Tween 20, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 10 μg/mL aprotinin, 10 μg/mL Leupeptin, 1 mmol/L phenyl methyl sulfonyl fluoride, 0.1 mmol/L sodium orthovanadate, and 1 mmol/L NaF]. Alternatively, cell monolayers were trypsinized at the selected time points, and an aliquot was resuspended in DMEM-F12 10% FCS then stained for later flow cytometric DNA analysis by addition of ethidium bromide (50 μg/mL) and Triton X-100 (0.2%), with the remainder resuspended in lysis buffer. Cell suspensions in lysis buffer were incubated for 5 minutes on ice, and the cellular debris was cleared by centrifugation (13,000 rpm, 5 minutes, 4°C). The cleared lysates were stored at −80°C. After normalization of protein concentration, SDS sample buffer was added, and the lysates were heated to 95°C for 5 minutes, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated (2 hours at room temperature or overnight at 4°C) with primary antibodies directed against the following: cyclin D1 (DCS6; Novacastra Laboratories, Newcastle-upon-Tyne, United Kingdom); p27KIP1 (K25020) and p21WAF1/CIP1 (C24420) from Transduction Laboratories (Lexington, KY); phospho-Rb (Ser-780) from Cell Signaling Technology (Beverly, MA); and β-actin antibody (Sigma, St. Louis, MO). After incubation (1 hour at room temperature) with horse-radish peroxidase-conjugated antimonium or antirabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), proteins were visualized with the enhanced chemiluminescence detection system (Amersham).

**Flow Cytometry.** Flow cytometric analysis was done on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) with CELLQuest 2.0 software (Becton Dickinson Immunocytometry Systems). The proportions of cells in G1, S, and G2/M phases of the cell cycle were calculated from the resulting DNA histograms with ModFit LT analysis software (Verity Software House, Inc., Topsham, ME).

**Image and Data Analysis.** Images captured by Phospho-Imager (Molecular Dynamics 445 SI; Molecular Dynamics) or densitometer scanning (Molecular Dynamics PDSI) of X-ray film were quantitated with IP Lab Gel H analysis program (Signal Analytics, Vienna, VA). Quantification of protein levels by this method was linear over the range of intensities measured. All statistical analysis was done with Statview 4.5 Software (Abacus Concepts, Berkeley, CA).

**RESULTS**

**Cyclin D1 Overexpression and CCND1 Gene Amplification in HNSCC Cell Lines.** We hypothesized that cyclin D1 overexpression, as a consequence of gene amplification, may be sustained despite inhibition of EGFR signaling. To test this premise, we characterized the response to gefitinib of six EGFR-overexpressing HNSCC cell lines, FaDu, Detroit 562, SCC 9, SCC 15, SCC 25, and CAL 27, after first determining their cyclin D1 protein expression levels and gene copy number compared with MCF-7 and MDA-MB-134, two well-characterized breast cancer cell lines with normal and amplified CCND1 (41). Western blots showed an ∼5-fold variation in cyclin D1 protein expression within the HNSCC cell lines (Fig. 1A). The lowest expression of cyclin D1 in the HNSCC cell lines was observed in SCC 9, SCC 15, and CAL 27 (relative expression 1.0 to 1.6), whereas SCC 25 and Detroit 562 had intermediate expression (relative expression 2.5 to 3.5), and FaDu expressed the highest levels of cyclin D1 (relative expression 5.3). Thus, we classified SCC 25, Detroit 562, and FaDu as overexpressing cyclin D1 relative to the other three HNSCC cell lines. Both breast cancer cell lines expressed high levels of cyclin D1 compared with the HNSCC cell lines (relative expression 4.8 to 6.9; Fig. 1A), likely reflecting tissue-specific control of basal expression of this gene. Because of the differences in protein expression levels and evidence for CCND1 amplification in HNSCC, we went on to examine gene copy number by Southern blot of HNSCC cell lines compared with MCF-7 and MDA-MB-134 (Fig. 1B). CCND1 is located at 11q13; therefore, to control for chromosome copy number, the same filters were reprobed with PR, because the PR gene is also located at chromosome 11 (11q22), and PR amplification has not been previously reported in HNSCC.

SCC 9, SCC 15, and CAL 27 had CCND1 gene copy numbers of 0.99 to 1.55 relative to the MCF-7 cell line and were not considered to be amplified (Fig. 1B). Similarly, SCC 25 cells, which overexpressed cyclin D1 but with a gene copy number of 2.0, were not considered amplified. The FaDu and Detroit 562 cell lines had increased CCND1 copy numbers of 24.3 and 3.3, respectively, after taking chromosome 11 number into account, and thus displayed CCND1 amplification (Fig. 1B). These results are consistent with published data for these cell lines (42). The large increase in CCND1 copy number in the FaDu cell line was similar to that of MDA-MB-134, and these cell lines both expressed high levels of cyclin D1, although the relative increase in protein expression was smaller than the increase in gene copy number. The two HNSCC cell lines with the highest cyclin D1 expression (FaDu and Detroit 562) displayed CCND1 amplification, whereas those with the lowest
cyclin D1 expression (SCC9, SCC15, and CAL 27) had no evidence for amplification at this locus.

**EGFR-Overexpressing HNSCC Cell Lines That Overexpress Cyclin D1 Are Less Sensitive to Gefitinib.** Because **CCND1** amplification and cyclin D1 protein overexpression were common in HNSCC cell lines, we examined whether the sensitivity of the six HNSCC cell lines to gefitinib was related to cyclin D1 overexpression. We treated cell lines that either did (FaDu, Detroit 562, and SCC 25) or did not (SCC 9, SCC 15, and CAL 27) overexpress cyclin D1, with 2 μmol/L gefitinib, 10 μmol/L gefitinib, or DMSO (vehicle). After treatment, colony formation was assessed, and relative cell numbers were measured with colorimetric assays (Fig. 2). The cell lines that overexpressed cyclin D1 formed numerous colonies in the presence of 2 μmol/L or 10 μmol/L gefitinib, although these were smaller than for vehicle-treated cells (Fig. 2A). In contrast, the remaining cell lines formed very few colonies in the presence of gefitinib (Fig. 2A). Similarly, the cell lines that overexpressed cyclin D1 continued to proliferate when treated with up to 10 μmol/L gefitinib, but cell numbers remained static or decreased after gefitinib treatment in the cell lines that did not overexpress cyclin D1 (Fig. 2B). Thus, the clonogenic assays and growth curves both indicated that the cell lines overexpressing cyclin D1 were resistant to gefitinib.

DNA analysis by flow cytometry indicated that 10 μmol/L gefitinib induced arrest in G1 phase, with a resulting mean decrease in S phase in all of the cell lines (Table 1). After gefitinib treatment, the relative S-phase fraction varied from 0.65 in Detroit 562 to 0.25 in SCC 9. As a group, the three cell lines overexpressing cyclin D1 (FaDu, Detroit 562, and SCC 25) displayed a significantly smaller reduction in S-phase fraction than the remaining three cell lines (P = 0.020).

In more detailed experiments, we examined the response of the panel of six cell lines to a range of concentrations of gefitinib. Data from replicate experiments with colorimetric assays to measure relative cell number were pooled (Fig. 3) and used to estimate the concentration at which the relative cell number was reduced to 50% relative to control cells, after 5 days of treatment (IC50). The IC50 ranged from 0.4 μmol/L for SCC 9 to 14.4 μmol/L for FaDu (Table 1). Previous studies with a range of cancer cell lines have determined that resistant cell lines have an IC50 of 3 to 16 μmol/L and sensitive cell lines have an IC50 of 0.07 to 1.4 μmol/L (19). Consequently, we classified FaDu, Detroit 562, and SCC 25 as resistant, and SCC 9, SCC 15, and CAL 27 as sensitive to gefitinib. Interestingly, the most resistant cell line, FaDu, displayed the highest level of **CCND1** amplification and overexpression. Collectively, the data in Figs. 2 and 3 and Table 1 suggest that resistance to gefitinib is associated with cyclin D1 overexpression. These data prompted us to additionally investigate the possibility that gefitinib resistance might result from the deregulation of cyclin D1 expression.

**Effects of Cyclin D1 Overexpression in SCC 9 Cells.** To additionally explore the role of deregulated cyclin D1 expression in sensitivity to gefitinib, we constitutively overexpressed cyclin D1 in the SCC 9 cell line. This cell line is representative of the majority of HNSCC, displaying overexpression of EGFR and c-erb2 as well as mutation of p53. There is no evidence of amplification of the cyclin D1, D2, and D3 genes or deletion or inactivation of p16INK4A, a CDK inhibitor specifically targeting cyclin D-dependent kinases. We confirmed the lack of **CCND1** amplification (Fig. 1B) and noted low cyclin D1 protein expression compared with other EGFR-overexpressing HNSCC cell lines (Fig. 1A).

SCC 9 cells were transfected with a vector expressing cyclin D1 or the corresponding empty vector, and clonal cell lines were selected. The SCC 9 cells tolerated transfection with cyclin D1 or the corresponding empty vector, and clonal cell lines were selected. The SCC 9 cells tolerated transfection with either vector and were used for all of the additional experiments, and these gave consistent results. The levels of cyclin D1 protein expression achieved, as measured by densitometry, were at least 3-fold greater in the two cyclin D1-
Fig. 2  Sensitivity of HNSCC cell lines to gefitinib (ZD1839) treatment. A. Clonogenic assays were done in 6-well plates treated with DMSO (vehicle control), 2 μmol/L gefitinib, or 10 μmol/L gefitinib. The medium was replaced weekly with fresh medium containing either DMSO or gefitinib. Colonies were observed twice weekly and then fixed and stained after 14 days (SCC 25, SCC 15, Cal 27, and SCC 9) or when the cells had reached confluence (FaDu and Detroit). Clonogenic assays were done in duplicate. Inserts are ×10 magnifications of the adjacent well. B. Exponentially growing cells were treated with DMSO [vehicle control, (■), 2 μmol/L gefitinib (□), or 10 μmol/L gefitinib (○)], and relative cell number was estimated daily for 5 days with the Cell Titer 96 assay system. The data points are the means of 6 replicates per experiment, and the experiment was repeated at least twice.
overexpressing clones compared with the parental cell line and two empty vector clones (Fig. 4A). Because p27\textsuperscript{KIP1} and p21\textsuperscript{WAF1/CIP1} have been implicated in gefitinib-induced growth arrest, expression of these proteins was also examined; this revealed increased relative expression of p21\textsuperscript{WAF1/CIP1} in the cyclin D1-overexpressing clones but no consistent relationship between p27\textsuperscript{KIP1} expression and cyclin D1 expression (Fig. 4A).

As expected, during exponential growth cyclin D1-overexpressing clones had an increased mean percentage of cells in S phase, when compared with empty vector clones and the parental cell line (Fig. 4B). There was no difference between the mean %S-phase of the two overexpressing clones (\(P = 0.30\)) or between the empty vector clone and the parental cell line SCC 9 (\(P = 0.78\)). There was, however, a significant difference between the mean %S-phase of the cyclin D1-overexpressing clones and both an empty vector clone (clone D1.F5, \(P = 0.0004\); D1.F7, \(P = 0.002\)) and the parental cell line (clone D1.F5, \(P = 0.0001\); D1.F7, \(P = 0.0006\)).

**Overexpression of Cyclin D1 Impairs gefitinib-Induced G\textsubscript{1} Arrest.** To assess the effect of cyclin D1 overexpression on the sensitivity to gefitinib, we examined the concentration-dependence of gefitinib-mediated growth inhibition in the overexpressing clones compared with the empty vector-transfected cells (Fig. 5). It is apparent from the growth curves that there was ongoing proliferation after treatment of the cyclin D1-overexpressing clones with up to 5 \(\mu\)M gefitinib (Fig. 5A), although the rate of proliferation was slightly reduced compared with vehicle-treated control cells. In contrast, empty vector clones, like the parental SCC 9 cells (see Fig. 2A), were markedly inhibited by gefitinib (Fig. 5A). The results of the clonogenic assays were similar, with visible colonies in the wells treated with 2 \(\mu\)M gefitinib in the cyclin D1-overexpressing clones but not in the empty vector clone (Fig. 5B).

More detailed examination of the concentration-dependence of the response to gefitinib (Fig. 6) revealed that the IC\textsubscript{50} of two empty vector clones was 0.24 \(\mu\)M and 0.44 \(\mu\)M, similar to the IC\textsubscript{50} for the parental SCC 9 cells (0.42 \(\mu\)M). The IC\textsubscript{50} of two cyclin D1-overexpressing clones was significantly higher, at 2.55 \(\mu\)M and 1.25 \(\mu\)M. At each concentration of gefitinib there was a significantly higher relative overexpressing clones compared with the parental cell line and two empty vector clones (Fig. 4A). Because p27\textsuperscript{KIP1} and p21\textsuperscript{WAF1/CIP1} have been implicated in gefitinib-induced growth arrest, expression of these proteins was also examined; this revealed increased relative expression of p21\textsuperscript{WAF1/CIP1} in the cyclin D1-overexpressing clones but no consistent relationship between p27\textsuperscript{KIP1} expression and cyclin D1 expression (Fig. 4A).

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number of viable cells in the overexpressing clones than in the empty vector clones.

We also measured the cell cycle phase distribution of the clones after EGFR inhibition with gefitinib. After 24 hours of treatment with 1 μmol/L or 10 μmol/L gefitinib (Fig. 7), it was apparent that a greater proportion of empty vector and parental cells were arrested in G1 phase after gefitinib treatment, compared with the cyclin D1-overexpressing clones. In the SCC 9 parental cell line and empty vector-transfected clonal SCC 9 cells (EV.E10) after treatment with 10 μmol/L gefitinib (Fig. 7), the proportion of cells in S phase was significantly reduced and that in G1 phase increased relative to vehicle-treated control cells (Fig. 7). In comparison, the relative S-phase fraction was reduced ~20% in both of the cyclin D1 overexpressing clones after 1 μmol/L of treatment with gefitinib and ~30% after treatment with 10 μmol/L gefitinib. The continued proliferation of the cyclin D1-overexpressing clones at these concentrations of gefitinib is thus associated with maintenance of a substantial proportion of cells in S phase. Taken together, the 2- to 6-fold increase in IC50 combined with the continued exponential growth and high S-phase fraction of cyclin D1-overexpressing clones at concentrations of gefitinib that resulted in growth arrest or cell death in parental cells and empty vector clones is consistent with a role for cyclin D1 overexpression in resistance to gefitinib treatment.

To better appreciate the molecular basis of the G1 arrest, we studied the expression of the molecules involved in the G1-S transition.
transition after gefitinib treatment of the cyclin D1-overexpressing clones. Initial examination of whether cyclin D1 overexpression was maintained on gefitinib treatment of the cyclin D1-overexpressing clones revealed that cyclin D1 protein expression was substantially reduced in the empty vector clones but maintained in the cyclin D1-overexpressing clones after 24 hours treatment (Fig. 8). To determine whether this cyclin D1 overexpression led to increased CDK4 activity, we used an antibody recognizing phosphorylation at serine 780, a residue of retinoblastoma protein (pRb) specifically phosphorylated by cyclin D1-CDK4. This revealed that after gefitinib administration, pRb serine 780 phosphorylation was sustained in the overexpressing clones. Because both the CDK inhibitors p27KIP1 and p21WAF1/CIP1 have been implicated in the antiproliferative effect induced by gefitinib in HNSCC (39), we also examined their expression. None of the cell lines examined displayed increased p21WAF1/CIP1 expression after gefitinib treatment. However, treatment of the empty vector-transfected cells with 2 μmol/L gefitinib for 24 hours resulted in an up-regulation of p27KIP1 protein that was not apparent in either of the cyclin D1-overexpressing clones (Fig. 8).

These data suggested that failure to down-regulate cyclin D1 and up-regulate p27KIP1 was a central component of the resistance of the cyclin D1-overexpressing clones. To determine whether this was also the case in the gefitinib-resistant cell lines displaying cyclin D1 overexpression and amplification at the CCND1 locus, we examined cyclin D1 and p27KIP1 expression after gefitinib treatment in the resistant FaDu and Detroit 562 cell lines, compared with the gefitinib-sensitive CAL 27 and SCC 9 cell lines. The expression of p27KIP1 was increased after gefitinib treatment of all of the cell lines, although the increase was greater in the sensitive cell lines (Fig. 9). However, after gefitinib treatment, the cyclin D1 levels were reduced by ~50% in both CAL 27 and SCC 9 (Fig. 9). Thus, in both the cyclin D1-overexpressing cell lines and cyclin D1-transfected SCC 9 cells, resistance to gefitinib was associated with maintenance of cyclin D1 levels.

DISCUSSION

Clinical studies have shown amplification of the cyclin D1 gene (CCND1) in up to 58% of HNSCC and overexpression of cyclin D1 in up to 68% of HNSCC; overexpression in the absence of gene amplification occurs in 4 to 55% of cases (27–34, 43). Both cyclin D1 overexpression as measured by immunohistochemistry and gene amplification measured by differential PCR or FISH are consistent and strong markers of poor prognosis. Comparative studies have either shown no prognostic advantage of protein expression over gene amplification or have favored cyclin D1 gene amplification as a better marker of poor prognosis (33, 34, 44–46). The consequences of cyclin D1 overexpression for response to therapy are not well understood and may differ between cell types. We hypothesized that cyclin D1 overexpression may affect the efficacy of therapies that reduce cyclin D1 expression, including those targeted at the EGFR. We have tested this hypothesis using six EGFR-overexpressing HNSCC cell lines and SCC 9 tongue cancer cells constitutively overexpressing cyclin D1.

CCND1 gene amplification was present in two of six EGFR-overexpressing HNSCC cell lines displaying differential sensitivity to gefitinib. The FaDu cell line showed ~24-fold CCND1 gene amplification, associated with high cyclin D1 protein expression and resistance to gefitinib treatment (IC50 14.4 μmol/L). Similarly, the resistant Detroit 562 cell line (IC50 12 μmol/L) has evidence of increased gene copy number and overexpression of cyclin D1 protein. In contrast, the sensitive SCC 9, CAL 27, and SCC15 cell lines (IC50 0.4 to 1.4 μmol/L) had no evidence of increased gene copy number and relatively low levels of cyclin D1 protein expression. The SCC 25 cell line had an IC50 of 9 μmol/L, similar to the other two resistant cell lines. However, at concentrations of 2 to 5 μmol/L gefitinib, the response of SCC 25 was intermediate between the sensitive cell lines, which were inhibited by ~80%, and the other resistant cell lines, which were inhibited by <20% (Fig. 3A). This may reflect the modest degree of cyclin D1 overexpression and lack of CCND1 amplification of this cell line. Consistent with our hypothesis, overall there seemed to be a relationship between the degree of cyclin D1 overexpression and resistance to gefitinib, with the most resistant cell lines displaying CCND1 amplification.

The SCC 9 cyclin D1 overexpression model showed that deregulated expression of cyclin D1 could dampen the cellular effects of the EGFR inhibitor gefitinib. Cyclin D1 transfection resulted in an increased number of cells in S phase in this already transformed and fast-growing malignant cell line. The cyclin D1-overexpressing clones were more resistant to gefitinib, resulting in a higher IC50, ongoing exponential growth after treatment with up to 5 μmol/L gefitinib, and a significantly reduced, although not totally ablated, G1 arrest. Colony formation was impaired after treatment with 2 μmol/L of gefitinib. The highest concentrations of gefitinib tested (5 to 10 μmol/L)
caused modest growth arrest in cyclin D1-overexpressing cells but substantially impaired colony formation. In addition, in empty vector and parental cell lines, cell numbers decreased after treatment with $10^{-5}$M gefitinib. This suggests that gefitinib is cytotoxic rather than cytostatic at these concentrations, consistent with evidence that epidermal growth factor can act as a survival signal, and consequently that inhibition of EGFR signaling may trigger apoptosis (47).

In contrast with our observation of resistance to gefitinib after cyclin D1 overexpression in SCC 9 cells, ectopic expression of cyclin D1 alone was not sufficient to prevent G1 arrest induced by epidermal growth factor deprivation in MCF10A breast cells, and overexpression of cyclin D1 still resulted in a G1 arrest after EGFR inhibition with a monoclonal antibody (MAb) 225 [ref. 38]. Interestingly, however, the levels of cyclin D1 were rapidly reduced after EGFR inhibition in all but two clones in this model. In these two clones, at 16 hours, the MAb
225-treated cells had more cyclin D1 bound to p27KIP1 than control cells, and it was concluded that the remaining unbound cyclin D1 protein was at a level inadequate to maintain cell cycle progression. In contrast, in both SCC 9 cells expressing ectopic cyclin D1 and two HNSCC cell lines with amplification of the CCND1 gene, gefitinib insensitivity was associated with failure to appreciably decrease cyclin D1 expression after treatment. These data are consistent with the conclusion that maintenance of cyclin D1 levels is critical to the resistance these cell lines display to gefitinib and confirm the central role of cyclin D1 down-regulation in the response to inhibition of EGFR signaling. No clinical studies have addressed whether cyclin D1 expression is maintained in CCND1-amplified cancers after EGFR inhibition, and this is an issue warranting additional investigation.

The CDK inhibitors p27KIP1 and p21WAF1/CIP1 are members of the CIP/KIP family, which interact with and inhibit the cyclin-CDK complexes essential for G1 to S-phase progression (37). Both p21WAF1/CIP1 and p27KIP1 have been implicated in the growth arrest after disruption of EGFR kinase activity in EGFR- and HER2-overexpressing cells (23, 25, 39). Di Gennaro et al. (39) showed that in HNSCC-derived cell lines treated with gefitinib, there was an increase in p27KIP1 and p21WAF1/CIP1 protein expression. The resultant inhibition of cyclin E- and cyclin A-CDK2 complexes resulted in G1 phase arrest, growth inhibition, and apoptosis. In this study, p21WAF1/CIP1 expression was unchanged in all of the clones and parental cell lines after gefitinib treatment. We observed an increase in p27KIP1 expression as a response to gefitinib treatment in the SCC 9 parental cell line and the empty vector clones, which was not apparent in the cyclin D1-transfected cells. However, p27KIP1 expression was increased after gefitinib treatment of the resistant cell lines FaDu and Detroit 562, and thus there was no clear relationship between p27KIP1 induction and sensitivity to gefitinib.

In summary, we have provided several lines of evidence in support of the idea that deregulation of cyclin D1 confers resistance to the EGFR-targeted therapy with gefitinib: the relationship between CCND1 amplification, cyclin D1 overexpression, and insensitivity to gefitinib; the ability of constitutive overexpression of cyclin D1 to confer resistance to gefitinib; and the correlation between the ability of gefitinib to down-regulate cyclin D1 and sensitivity to growth inhibition by this compound. CCND1 amplification is a frequent finding in HNSCC and thus warrants additional investigation as a potential marker of resistance. Identifying cyclin D1 amplification and targeting it directly with antisense cyclin D1 gene therapy (42, 48) or indirectly through CDK inhibitors (e.g., flavopiridol) together with EGFR inhibitors may be a useful therapeutic strategy in a significant subset of HNSCC patients.

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