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# Gefitinib ('Iressa', ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor, up-regulates $p27^{KIP1}$ and induces G1 arrest in oral squamous cell carcinoma cell lines

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**KEYWORDS**
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**Summary** High expression of epidermal growth factor receptor (EGFR) is frequently observed in many solid tumor types including oral squamous cell carcinomas (OSCC). Recently, the results of preclinical studies and early clinical trials targeting the EGFR have shown evidence of the activity. In this study, gefitinib ('Iressa', ZD1839), an EGFR-tyrosine kinase inhibitor, inhibited cell proliferation and upregulated $p27^{KIP1}$ in OSCC cells. Growth inhibition was observed in OSCC xenografts when mice were treated with gefitinib in vivo. A flow cytometric analysis demonstrated that treatment with gefitinib induced accumulation in G1 phase, accompanied by a decrease in the percentage of cells in S phase. Apoptosis was not seen in this study. Cell growth was inhibited by an increase of the cell cycle inhibitor $p27^{KIP1}$ and a decrease of its ubiquitin ligase subunit Skp2.


## Introduction

Epidermal growth factor receptor (EGFR) is an important receptor involved in signaling pathways implicated in the proliferation and survival of cancer cells. EGFR is often highly expressed in human tumors, including oral squamous cell carcinomas (OSCC), and high expression of this receptor frequently accompanies development and growth of malignant tumors.[1–3] There is increasing evidence that high expression of EGFR is correlated with advanced tumor stage and metastasis, and poor clinical outcome of common human cancers such as breast, cervix, lung, and head and neck carcinoma.[1–3] Previous experimental and clinical work supports the view that the EGFR is a relevant target for cancer therapy. Two therapeutic approaches that appear most promising and are currently being used to inhibit the EGFR in clinical studies are monoclonal antibodies and small molecule inhibitors of EGFR tyrosine kinase enzymatic activity. A biological approach, using anti-EGFR

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## Nomenclature

| | |
|---|---|
| CDK | cyclin-dependent kinase; |
| CDKI | cyclin-dependent kinase inhibitor; |
| DMEM | Dulbecco's modified Eagle's medium; |
| EGFR | epidermal growth factor receptor; |
| mAb | monoclonal antibody; |
| OSCC | oral squamous-cell carcinoma; |
| PBS | phosphate-buffered saline; |
| PCNA | proliferating cell nuclear antigen; |
| SD | standard deviation; |
| SDS | sodium dodecyl sulfate; |
| TKI | tyrosine kinase inhibitor |

monoclonal antibody 225 (C225) to alter tumor proliferation, offers potential for improving tumor control.[4–6]

Among various quinazoline-derived agents that have been tested as anticancer agents in vitro and in preclinical models, the EGFR-TKI (EGFR tyrosine kinase inhibitor) gefitinib ('Iressa', ZD1839) shows antiproliferative activity in various human cancer cell types in vitro[7] and clinical trials in cancer patients are underway. Gefitinib demonstrated clinically significant antitumor activity and symptom relief in two Phase II trials of gefitinib monotherapy in patients with advanced non-small-cell lung cancer (IDEAL 1 & 2),[8,9] and has shown activity against head and neck cancer.[10]

EGFR signaling and cell cycle have been independently evaluated as targets for therapy, but the link between them has recently been identified.[11–13] Some reports showed that the blockade of EGFR kinase activity led to cell cycle arrest in the G1 phase.[14,15] Cell cycle progression is regulated by interactions between cyclins and cyclin-dependent kinases (CDKs).[16,17] In particular, the transition from G1 phase to S phase is known to be regulated by a family of negative cell cycle regulators, the CDK inhibitors (CDKIs). The mechanisms for cell cycle arrest in G1 phase with C225 were explored by examining the cyclins, CDKs, and their inhibitors in cultures of several carcinoma cells.[5,15] They suggested that cell cycle arrest in G1 phase was mediated by inhibition of CDK2 activity that was not accompanied by any changes in cyclin or kinase levels but could be accounted for by a rise in levels of the CDK inhibitor $p27^{KIP1}$. However, few previous studies have examined the mechanisms of gefitinib-induced growth arrest in OSCC cell lines.

The aim of this study was to examine the effect and mechanisms of gefitinib in human OSCC cell lines.

## Materials and methods

### Chemicals

Gefitinib was provided by AstraZeneca (Macclesfield, UK).

### Cell culture

OSCC cell lines (HSC2, HSC3) were grown in Ham/F12: Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with 10% fetal bovine serum, 24 g/ml adenine, 0.4 g/ml hydrocortisone and 50 units/ml penicillin and streptomycin. The cell lines HSC2 and HSC3 were provided by Japanese Collection Research Bioresources (Tokyo, Japan).

### Cell proliferation assays

The antiproliferative effect of gefitinib on the in vitro growth profile of each OSCC cell line was examined. The cells were treated with 1 μM gefitinib, then harvested by trypsinization and counted with a hemocytometer.

### Flow cytometric analysis

Cells treated with 1 μM gefitinib were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and then fixed in 95% ethanol and stored at 4 °C for up to 7 days prior to DNA analysis. After the removal of ethanol by centrifugation, the cells were washed with PBS. Cells were stained with solution containing 500 units/ml RNase (Sigma R-5000) at 37 °C for 15 min, and then 50 g/ml propidium iodide in 1.12% sodium citrate at room temperature for 15 min. Stained nuclei were analyzed for DNA-PI fluorescence using a Coulter EPICS® XL Flow Cytometer (Coulter Corp., Miami, FL, USA). Resulting DNA distributions were analyzed by the computer program Multicycle (Phoenix Flow Systems, San Diego, CA, USA), which was used for the populations of cells in apoptosis and in the G0/G1, S, and G2-M phases of the cell cycle.

### Western blot analysis

Primary antibodies against $p27^{KIP1}$ were obtained from BD Transduction Laboratories (Lexington, KY, USA), and antibodies against actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against EGFR, phospho-EGFR, mitogen-activated protein kinase (MAPK) kinase (MEK1/2), phospho-MEK1/2, p38 MAPK and phospho-MAPK were obtained from Cell Signaling Technology (USA), and antibody against SKP2 was obtained

from Zymed Laboratories, Inc. (San Francisco, CA, USA). For analysis of signal transduction pathways following treatment with 1 μM gefitinib, OSCC cells and xenograft tissues were lysed in a buffer: 1xPBS, 1% Nonidet-P 40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaF, 10 mg/ml leupeptin (Sigma Corp., St Louis, MO, USA), 0.1 mg/ml trypsin inhibitor (Sigma), 0.1 mg/ml aprotinin (Sigma) and 50 mg/ml PMSF (Sigma). Following sonication and incubation on ice for 30 min, supernatant was collected. Protein concentrations were determined with a DC protein assay kit (Bio Rad Laboratories, Hercules, CA, USA). The lysates were centrifuged and proteins (25 μg) subjected to electrophoresis on Tris—glycine SDS polyacrylamide gels. After blotting onto polyvinylidene fluoride membrane (BioRad Laboratories, Hercules, CA, USA), nonspecific binding sites were blocked using milk and incubations with primary antibodies carried out overnight at 4 °C in Tris-buffered saline. After being washed, proteins were detected by enhanced chemiluminescence (Amersham Corporation, Arlington Heights, IL, USA) according to manufacturer's instructions. Equal loading of extracts was confirmed using actin expression.

### Assay of tumor growth in athymic nude mice

Athymic BALB/c nude mice (4—5-week-old males) were obtained from Charles River Laboratories (Shizuoka, Japan). The care and treatment of experimental animals complied with institutional guidelines. Mice were injected s.c. with $1\times10^6$ OSCC cells into the dorsal flank. For each cancer cell line, after 3 weeks (when tumors were established with a mean volume of 100 $mm^3$), 10 mice/group were treated with i.p. gefitinib (100 mg/kg on days 1—5 each week for 3 weeks). Animals were assigned to one of two treatment groups: control or gefitinib. Tumor volume was determined by direct measurement with calipers and calculated by the formula: $\pi/6\times$(large diameter)$\times$(small diameter)$^2$.

### Immunohistochemical analysis of cell proliferation

Cell proliferation was assessed in histological sections of OSCC xenografts. Briefly, excised tumor specimens were fixed in 10% neutral-buffered formalin. After embedding in paraffin, 5 μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, slides were incubated at 4 °C overnight with 1:100 dilution of primary antibody. Immunostaining was performed with the Envision system (DAKO, Japan) in accordance with the manufacturer's instructions. Peroxidase activity was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxidase. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. The proliferating cell nuclear antigen (PCNA) index (percentage of PCNA-positive cells) was determined. Cells were examined at more than five areas in a magnification of $\times$200 and more than 2000 different tumor cells were analyzed for each specimen.

### Statistical analysis

All calculations were performed using the statistical computer program Statview 5.1 (Avacus, NC). Tumor volume, quantification of PCNA, and apoptotic cells were compared by unpaired Student's *t* test. The significance level was set at $P<0.05$ for each analysis.

## Results

### Gefitinib inhibition of OSCC proliferation in vitro

The antiproliferative effect of gefitinib was observed on two cell lines in a dose-dependent manner. Treatment with gefitinib in several doses

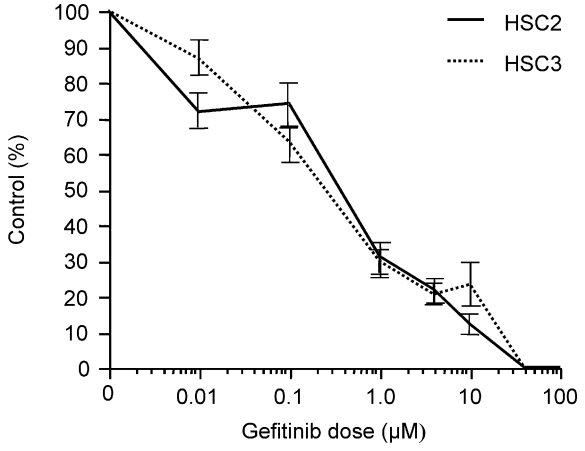


**Figure 1** Gefitinib inhibits proliferation of OSCC cells in vitro. Known numbers of single cells were plated into culture dishes in DMEM/F12 containing 10% FBS and treated with gefitinib in several doses for 48 h. Cells were then harvested by trypsinization and counted by a hemocytometer with trypan blue dye. Data points, mean of triplicate samples.

for 48 h led to a dose-dependent increase in the antiproliferative effects of gefitinib (Fig. 1). Growth curve profiles were evaluated in each OSCC cell line following the addition of 1 μM gefitinib directly to the culture medium. These growth inhibition profiles over a 72 h exposure time period are shown in Fig. 2. Exposure to gefitinib inhibited cellular proliferation of OSCC cell lines in a time-dependent manner.

## Flow cytometric analysis

Cell cycle profiles of asynchronously growing OSCC cells treated with gefitinib were analyzed. In both HSC2 and HSC3 cells, treatment with gefitinib alone led to an increase in G0-G1 phase. For example, the percentage of G0-G1 cells increased from 60.8% to 91.8% after treatment with gefitinib in HSC2 after 24 h (Fig. 3). The increase in the G1

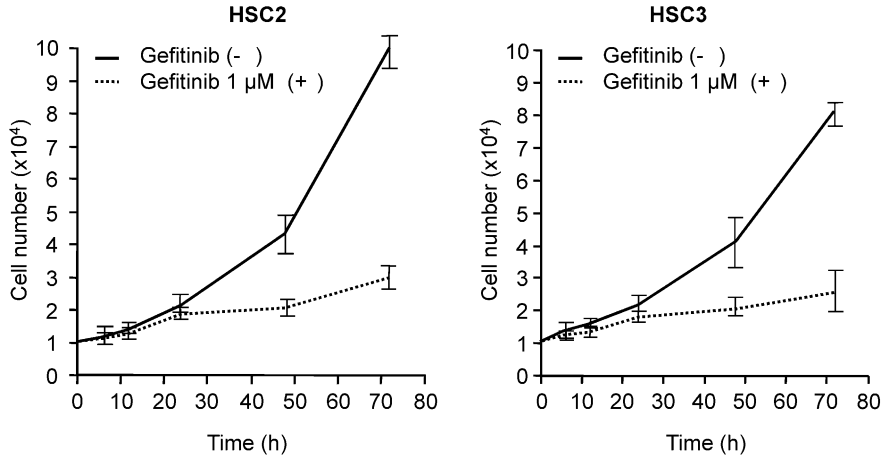


**Figure 2** In 1 μM, growth curve profiles were evaluated in each OSCC cell line following the addition of gefitinib directly to the culture medium over 72 h exposure time period. Cells were harvested by trypsinization and counted by a hemocytometer with trypan blue dye exclusion at the indicated time intervals. Data points, mean of triplicate samples.

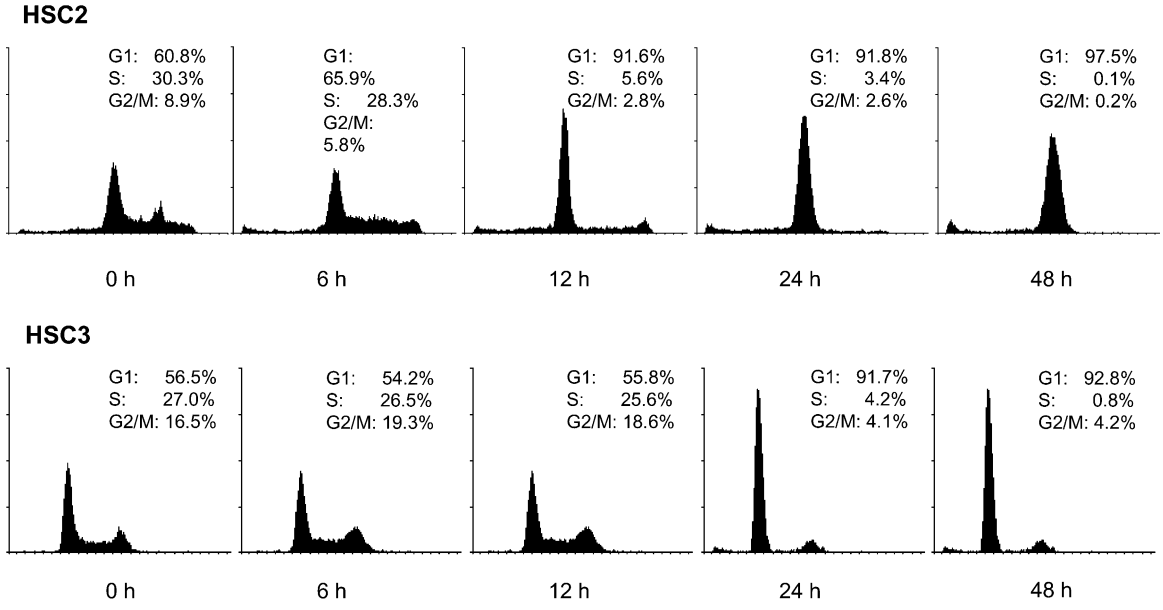


**Figure 3** Exponentially growing OSCC cell lines were treated with gefitinib for 48 h, and harvested cells were fixed and stained with propidium iodide and analyzed for DNA content by flow cytometric analysis. Gefitinib induces accumulation of cells in G1 with accompanying decrease in cells within the S phase. Columns, mean of triplicate samples.

population was accompanied by a decrease in cells in the S phase, whereas the G2-M population was not changed substantially.

## Cell cycle arrest associated with up-regulation of p27$^{KIP1}$ and down-regulation of Skp2

Transition from the G1 phase into the S phase is governed by the activation of CDKs. The expression of CDKs and CDKIs was compared. Immunoblot analysis of CDKs and CDKIs showed that treatment with 1 μM gefitinib increased the level of p27$^{KIP1}$ whereas CDKs (CDK2, CDK4, CDK6) and CDKI (p21$^{CIP1/WAF1}$, p16$^{INK4A}$) proteins did not change significantly (data not shown). Further, we found an inverse correlation between p27$^{KIP1}$ and Skp2 expression after gefitinib exposure. Thus, p27$^{KIP1}$ protein levels increased with decreasing of Skp2 expression in these cells (Fig. 4).

## Effect on downstream signaling of EGFR

This study evaluated whether the EGFR blockade affects the expression and/or the activation of intracellular proteins that are involved in downstream signaling of EGFR. As shown in Fig. 5, gefitinib (1 μM) for 24 h induced a significant suppression of EGFR autophosphorylation in HSC2 and HSC3 cells. Gefitinib completely inhibited MEK1/2 and p38 MAPK autophosphorylation (Fig. 5).

## Inhibition of OSCC tumor growth in athymic nude mice

Gefitinib inhibits in vivo tumor growth of OSCC xenografts. Two human SCC cell lines (HSC2, HSC3)

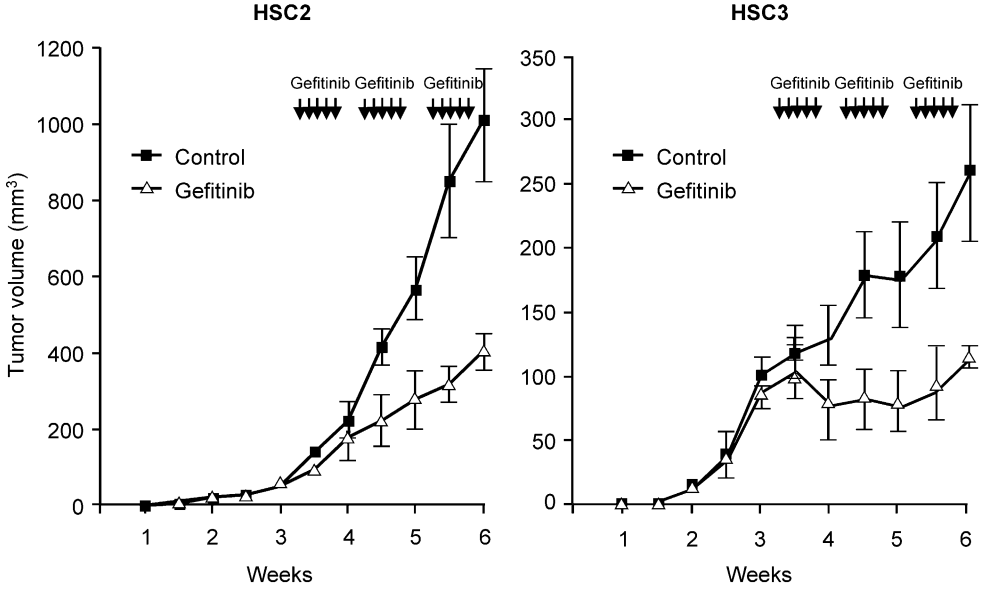


**Figure 4** p27$^{KIP1}$ and Skp2 protein content following treatment with gefitinib. Exponentially growing OSCC cells were treated or not treated with gefitinib for 48 h. These cells were harvested, lysed in RIPA buffer and tested in immunoblot procedures p27$^{KIP1}$ and Skp2.

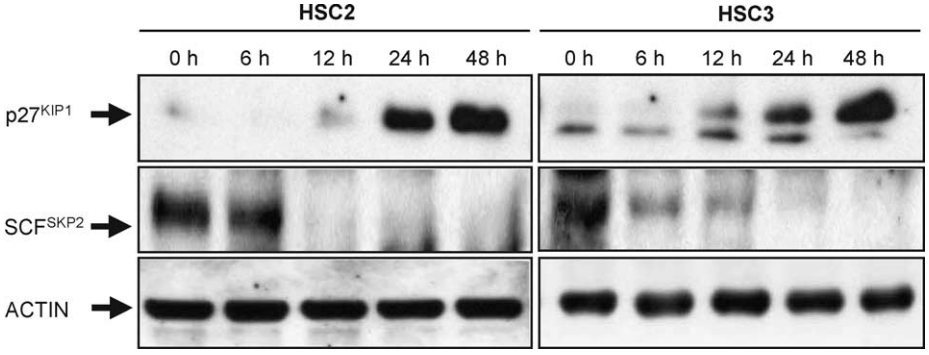


**Figure 5** Effects of gefitinib on EGFR downstream signaling pathway. OSCC cells (HSC2 and HSC3) were treated with 1 μM gefitinib or a combination of the two treatments: lane 1, control untreated cells; lane 2, 1 μM gefitinib.

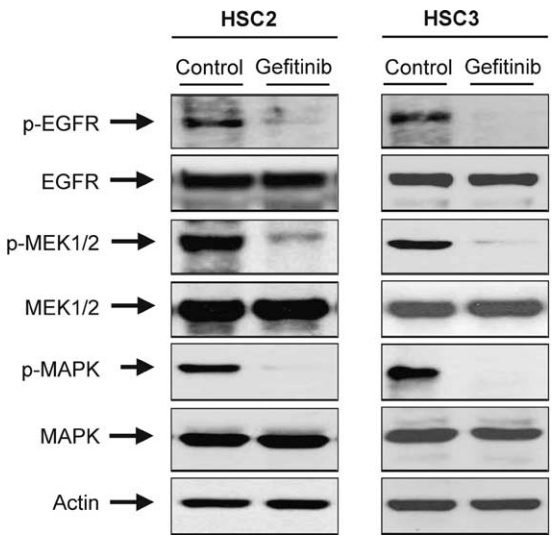


**Figure 6** Antitumor activity of gefitinib in well-established OSCC xenografts. $1\times10^6$ HSC2 or HSC3 cells were injected s.c. into the flank of athymic mice as described in Materials and methods Section. After 3 weeks (tumor mean size of 100 $mm^3$), mice were treated with i.p. gefitinib (100 mg/kg on days 1—5 each week for 3 weeks). Values represent mean tumor size±S.E. (*n*=10/

were inoculated s.c. into male athymic mice and allowed to grow until they had achieved a mean volume of 100 $mm^3$. Gefitinib was then administered via i.p. injection on days 1—5 for 3 weeks at a dose of 100 mg/kg. Treatment with gefitinib produced inhibition of tumor growth in both HSC2 and HSC3 xenografts (Fig. 6).

## Evaluation of cell proliferation and $p27^{KIP1}$, Skp2 expression

Cell proliferation, apoptosis and $p27^{KIP1}$, Skp2 expression were detected in histological sections of OSCC xenografts. Immunohistochemical staining with PCNA demonstrated the number of proliferating cells to be higher in the control group (HSC2, 36.2±8.1%; HSC3, 41.3±9.1%) than in the groups receiving treatment with gefitinib (HSC2, 19.2±7.8%; HSC3, 18.3±6.6%, Fig. 6). Inverse correlation of Skp2 overexpression was detected (Figs. 7 and 8). Control specimens showed negative immunostaining for $p27^{KIP1}$ and positive for Skp2. The specimens receiving gefitinib treatment showed negative immunostaining for Skp2 and positive for $p27^{KIP1}$. This correlation between $p27^{KIP1}$ and Skp2 was confirmed with Western blot analysis.

## Discussion

The cellular and biochemical mechanisms of growth arrest that follow interruption of EGFR signaling by gefitinib in high EGFR-expressing human OSCC cell lines were examined. Gefitinib is a potent antitumor agent that has been developed to target EGFR tyrosine kinase. In Phase II trials, gefitinib has shown the activity against head and neck cancer,[10] and Phase III clinical trials are planned. In this study, we have shown the antiproliferative effect of combined treatment with gefitinib in OSCC cell lines (HSC2 and HSC3). HSC2 and HSC3 were established from the mouth floor and tongue, and p53 gene mutations of these cells have been reported previously.[18,19] The number of EGFRs was shown to be high in HSC2 cells ($49.4\times10^5$/cell) and moderate in HSC3 cells ($18.5\times10^5$/cell).[20] A generally cytostatic growth inhibiting activity of gefitinib has been demonstrated in a wide range of human cancer cell lines that express functional EGFR.[7,21]

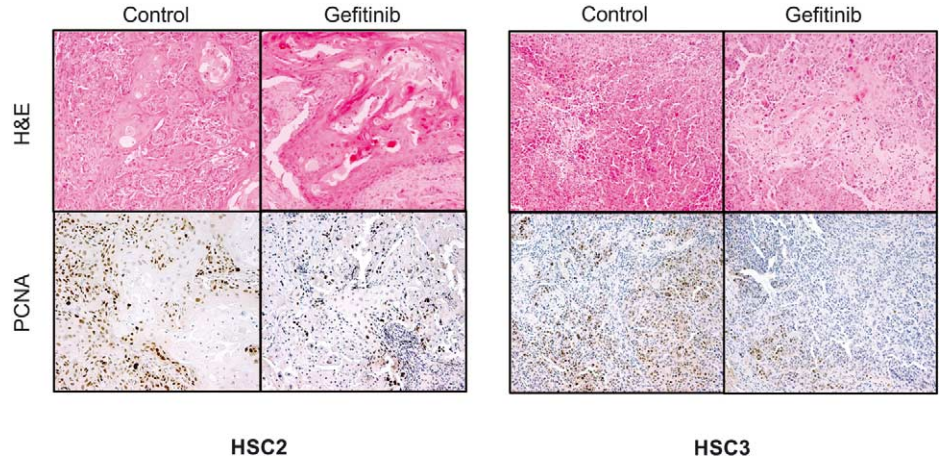


**Figure 7** Cell proliferation of HSC2 and HSC3 tumors. Mice bearing OSCC tumor xenografts were treated as described in Materials and methods Section. PCNA nuclear staining in OSCC control tumor and tumor treated with gefitinib, ×200.

Gefitinib, like C 225, has been shown to induce G1 arrest in human head and neck squamous cell carcinoma cell lines, via a dose- and time-dependent upregulation of p27$^{KIP1}$ cyclin-dependent kinase inhibitor.[22] Although regulation of p27$^{KIP1}$ gene expression by mitogenic pathways has been described,[23] post-translational modifications have been reported as the main mechanism of p27$^{KIP1}$ expression regulation, mainly through modulation of ubiquitin-dependent protein degradation rate.[24,25] In this study, it was observed that the addition of gefitinib to cultures produced marked up-regulation of p27$^{KIP1}$ protein. To clarify the association of ubiquitin-dependent protein degradation and p27$^{KIP1}$ up-regulation, this study investigated whether or not gefitinib might regulate the levels of expression of Skp2. The expression of Skp2 by Western blotting was assayed and gefitinib was found to decrease the levels of Skp2 in vitro and in vivo. These results showed that gefitinib increased the expression levels of p27$^{KIP1}$ and this may be regulated by the ubiquitin pathway with Skp2.

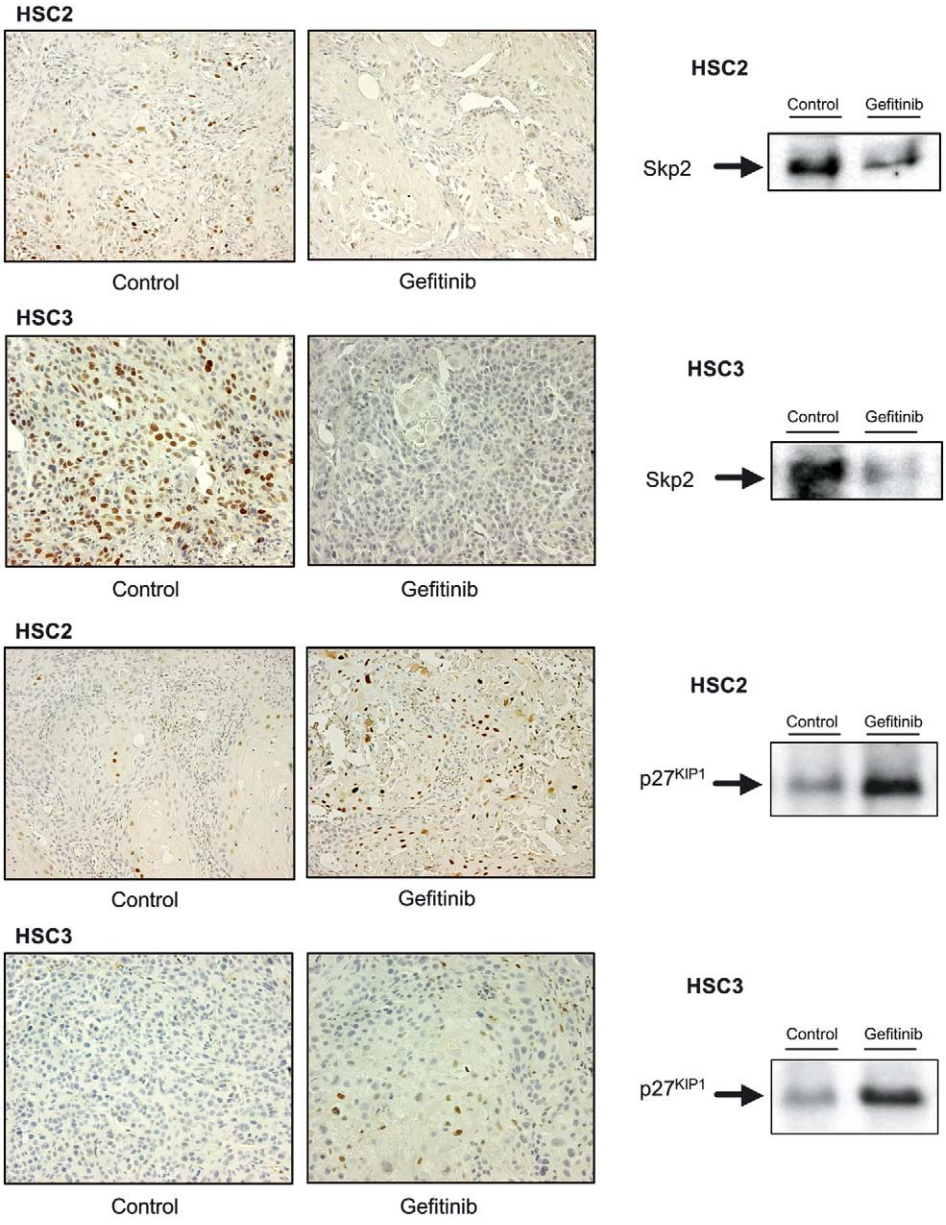


**Figure 8** p27$^{KIP1}$ and Skp2 expression in HSC2 and HSC3 xenografts. Mice bearing OSCC tumor xenografts were treated as described in Materials and methods Section. p27$^{KIP1}$ and Skp2 expression were evaluated by immunohistochmistry and western blotting. Control specimens showed negative immunostaining for p27$^{KIP1}$ and positive for Skp2. The specimens treated with gefitinib showed negative immunostaining for Skp2 and positive for p27$^{KIP1}$. This correlation between p27$^{KIP1}$ and Skp2 was confirmed with western blot analysis.

In addition, drug treatment reduced the activation status of MEK1/2 and p38 MAPK. A major signaling route of EGFR is Ras—Raf—MAPK pathway.[26] Activation of Ras initiates a multistep phosphorylation cascade that leads to the activation of MAPKs.[27] MAPK pathways stimulate cell cycle progression via induction of immediate early genes such as c-fos and through effects on cell cycle regulators such as cyclin D and $p27^{KIP1}$.[28–30] Elevated levels of MAPK activation in tumor tissues compared with their corresponding non-neoplastic tissues have been reported in several human tumors.[31,32] Therefore, the reduction in steady-state levels of MEK1/2 and p38 MAPK activity observed in tumors as a result of EGFR inhibition may be instrumental in the reduction in growth rate seen in these OSCC cells.

In conclusion, EGFR-blocking by gefitinib inhibited the growth of OSCC cell lines in vitro and in vivo. This inhibition was induced by arresting cell cycle progression in the G1 phase which is attributable to $p27^{KIP1}$ up-regulation with down-regulation of Skp2 in OSCC. Thus, gefitinib may be useful in this clinical setting, and results from further clinical trials are awaited.

## References


1. Hu G, Liu W, Mendelsohn J, et al. Expression of epidermal growth factor receptor and human papillomavirus E6/E7proteins in cervical carcinoma cells. *J Natl Cancer Inst* 1997;**89**:1271—1276.
2. Iihara K, Shiozaki H, Tahara H, et al. Prognostic significance of transforming growth factor-alpha in human esophageal carcinoma. Implication for the autocrine proliferation. *Cancer* 1993;**71**:2902—2909.
3. Grandis JR, Melhem MF, Gooding WE, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998;**90**:824—832.
4. Huang S-M, Bock JB, Harari PM. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* 1999;**59**:1935—1940.
5. Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. *J Biol Chem* 1994;**269**:27595—27602.
6. Prewett M, Rockwell P, Rockwell RF, et al. The biologic effects of C225, a chimeric monoclonal antibody to the EGFR, on human prostate carcinoma. *J Immunother Emphasis Tumor Immunol* 1996;**19**:419—427.
7. Ciardiello F, Caputo R, Bianco R, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000;**6**:2053—2063.
8. Nishiwaki Y, Vansteenkiste J, Kudoh S, et al. A multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (the IDEAL 1 trial). *J Clin Oncol* 2003;**21**:2237—2241.
9. Kris M, Natale RB, Herbst R, et al. A phase II trial of ZD1839 ('Iressa') in advanced non-small cell lung cancer (NSCLC) patients who had failed platinum- and docetaxel-based regimens (IDEAL 2). *Proc Am Soc Clin Oncol* 2002;**21**:292 [abstra 1166].
10. Cohen EE, Rosen F, Dekker A, et al. Phase II study of ZD1839 (Iressa) in recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). *Proc Am Soc Clin Oncol* 2002;**21**:225 [abstra 899].
11. Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH. Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1983;**1**:511—529.
12. Chou JL, Fan Z, DeBlasio T, Koff A, Rosen N, Mendelsohn J. Constitutive overexpression of cyclin D1 in human breast epithelial cells does not prevent G1 arrest induced by deprivation of epidermal growth factor. *Breast Cancer Res Treat* 1999;**55**:267—283.
13. Markowitz SD, Molkentin K, Gerbic C, Jackson J, Stellato T, Willson JK. Growth stimulation by coexpression of transforming growth factor-alpha and epidermal growth factor-receptor in normal and adenomatous human colon epithelium. *J Clin Invest* 1990;**86**:356—362.
14. Wu X, Fan Z, Masui H, Rosen N, Mendelsohn J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995;**95**:1897—1905.
15. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H, Mendelsohn J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G1 arrest in prostatic cancer cell line DU145. *Cancer Res* 1996;**56**:3666—3669.
16. Norbury C, Nurse P. Animal cell cycles and their control. *Annu Rev Biochem* 1992;**61**:441—470.
17. Marx J. How cells cycle toward cancer. *Science* 1994;**263**:319—321.
18. Momose F, Araida T, Negishi A, Ichijo H, Shioda S, Sasaki S. Variant sublines with different metastatic potentials selected in nude mice from human oral squamous cell carcinomas. *J Oral Pathol Med* 1989;**18**:391—395.
19. Jia LQ, Osada M, Ishioka C, et al. Screening the p53 status of human cell lines using a yeast functional assay. *Mol Carcinog* 1997;**19**:243—253.
20. Kiyota A, Shintani S, Mihara M, et al. Anti-epidermal growth factor receptor monoclonal antibody 225 upregulates $p27^{KIP1}$ and $p15^{INK4B}$ and induces G1 arrest in oral squamous carcinoma cell lines. *Oncology* 2002;**63**:92—98.
21. Chan KC, Knox F, Gandhi A, Slamon DJ, Potten CS, Bundred NJ. Blockade of growth factor receptors in ductal carcinoma in situ inhibits epithelial proliferation. *Br J Surg* 2001;**88**:412—418.
22. Di Gennaro E, Barbarino M, Bruzzese F, et al. Critical role of both p27KIP1and p21CIP1/WAF1 in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. *J Cell Physiol* 2003;**195**:139—150.
23. Choudhury GG. Akt serine threonine kinase regulates platelet-derived growth factor-induced DNA synthesis in glomerular mesangial cells: regulation of c-fos AND p27(kip1) gene expression. *J Biol Chem* 2001;**276**:35636—35643.
24. Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakidis TR, Roberts JM. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. *Nature* 2001;**413**:323—327.
25. Montagnoli A, Fiore F, Eytan E, et al. Ubiquitination of p27

is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev* 1999;**13**:1181—1189.
26. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998;**74**:49—139.
27. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* 1996;**271**:20608—20616.
28. Cheng M, Sexl V, Sherr CJ, Roussel MF. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci USA* 1998;**95**:1091—1096.
29. Kawada M, Yamagoe S, Murakami Y, Suzuki K, Mizuno S, Uehara Y. Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway. *Oncogene* 1997;**15**:629—637.
30. Loda M, Capodieci P, Mishra R, et al. Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. *Am J Pathol* 1996;**149**:1553—1564.
31. Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 1999;**18**:813—822.
32. Mandell JW, Hussaini IM, Zecevic M, Weber MJ, VandenBerg SR. In situ visualization of intratumor growth factor signaling: immunohistochemical localization of activated ERK/MAP kinase in glial neoplasms. *Am J Pathol* 1998;**153**:1411—1423.