Effects of Epigallocatechin-3-gallate on Growth, Epidermal Growth Factor Receptor Signaling Pathways, Gene Expression, and Chemosensitivity in Human Head and Neck Squamous Cell Carcinoma Cell Lines[1]

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ABSTRACT

The antitumor effects of the green tea compound epigallocatechin-3-gallate (EGCG) have not been studied in detail previously in head and neck squamous cell carcinoma (HNSCC) cells. Overexpression of the epidermal growth factor receptor (EGFR) occurs frequently in HNSCC, which is an adverse prognostic factor. Therefore, we examined in detail the molecular effects of EGCG on two human HNSCC cell lines, YCU-N861 and YCU-H891, focusing on the EGFR signaling pathway. The 70% lethal dose (IC_{70}) of EGCG for both cell lines was 10 μg/ml. Treatment with EGCG increased the proportion of cells in the G_1 phase of the cell cycle and induced apoptosis. In cells treated with EGCG, there was a decrease in the cyclin D1 protein, an increase in the $p21^{Cip1}$ and $p27^{Kip1}$ proteins, and a reduction in the hyperphosphorylated form of pRB, changes that may account for the arrest in G_1. EGCG also caused a decrease in the Bcl-2 and Bcl-X_L proteins, an increase in the Bax protein, and activation of caspase 9, suggesting that EGCG induces apoptosis via a mitochondrial pathway. Treatment with EGCG inhibited phosphorylation of the EGFR, signal transducer and activator of transcription3 (Stat3), and extracellular regulated kinase (ERK) proteins and also inhibited basal and transforming growth factor-α-stimulated c-*fos* and *cyclin D1* promoter activity. EGCG at 0.1 μg/ml (a concentration found in serum after oral administration) markedly enhanced the growth-inhibitory effects of 5-fluorouracil. Taken together, these findings provide insights into molecular mechanisms of growth inhibition by EGCG and suggest that this naturally occurring compound may be useful, when used alone or in combination with other agents, in the chemoprevention and/or treatment of HNSCC.

INTRODUCTION

Epidemiological and rodent carcinogenesis studies provide evidence that green tea has chemopreventive effects for a wide range of malignancies (1). Mechanistic studies indicates that EGCG, a major component of tea, and related compounds have various anticancer effects, including inhibition of carcinogen-induced mutagenesis (2), induction of G_1 arrest (3–5), induction of apoptosis (6–10), inhibition of growth factor-mediated proliferation (11–13), inhibition of transformation (11), inhibition of angiogenesis (14), and inhibition of telomerase activity (15). However, there have been very few studies on this subject related to HNSCC, and yet studies in this area might lead to new approaches in the prevention and treatment of this important group of human cancers.

It is well known that 80–100% of HNSCCs[3] overexpress the EGFR, and that these cells also produce the EGFR ligand TGF-α (16–19). This autocrine effect causes sustained activation of EGFR through autophosphorylation of a critical tyrosine residue (16). This, in turn, activates several downstream signaling pathways that enhance cell proliferation, tumor growth, and tumor progression (16, 20, 21). Indeed, HNSCCs that overexpress EGFR have a poor prognosis (19). ERK is an important signaling molecule that lies downstream of EGFR (22). Several studies indicate that Stat3 also lies downstream of the EGFR, and activation of Stat3, *in vitro* and *in vivo*, is strongly related to carcinogenesis and tumor progression in several types of human malignancies (23, 24), including HNSCCs (25). A major target of ERK and Stat3 is AP-1, a complex of transcription factors (26, 27) that play important roles in the growth of various types of cancer (28). Transcription of the cell cycle control protein cyclin D1 is regulated by both the ras-ERK (26) and Stat3 signaling pathways (23, 24). Furthermore, cyclin D1 overexpression occurs in several types of human malignancies (29), including HNSCC (30, 31), and overexpression of cyclin D1 in HNSCC is associated with a poor prognosis (32).

One of the most striking effects of EGCG is its ability to directly inhibit activation of the EGFR (12). Therefore, in the

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; EGCG, epigallocatechin-3-gallate; 5-FU, 5-fluorouracil; EGFR, epidermal growth factor receptor; Stat3, signal transducer and activator of transcription 3; ERK, extracellular regulated kinase; TGF, transforming growth factor; AP-1, activator protein-1; β-gal, β-galactosidase; IC_{70}, 70% lethal dose.

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present study we used two human HNSCC-derived cell lines, YCU-H891 and YCU-N861, to examine possible effects of EGCG on activation of EGFR, ERK, and Stat3 and on cyclin D1 expression. In parallel studies, we also examined the effects of EGCG on cell proliferation, cell cycle progression, and apoptosis. Because 5-FU is used frequently in the treatment of patients with HNSCC, we also examined the combined effects of EGCG and 5-FU on proliferation in these two cell lines.

MATERIALS AND METHODS

Chemicals and Drugs. EGCG and 5-FU were obtained from Sigma Chemical Co. (St. Louis, MO) and TGF-α from Life Technologies, Inc. (Grand Island, NY). EGCG was dissolved in water, and 5-FU was dissolved in DMSO.

Cell Lines and Cell Culture. The human head and neck cancer cell lines YCU-H891 (derived from a tumor of the hypopharynx) and YCU-N861 (from a tumor of the nasopharynx) were generously provided by Dr. M. Tsukuda (Yokohama City University, Yokohama, Japan). The YCU-H891 cells express high levels of the EGFR (33). Both cell lines were maintained in a 5% CO_2 atmosphere at 37°C in RPMI 1640 containing 10% fetal bovine serum (Life Technologies, Inc.).

Colony Formation Assays. The cytotoxic effects of EGCG and/or 5-FU were measured by colony formation assays. One thousand cells were seeded into 35- or 100-mm dishes. Twenty-four h later, the indicated concentrations of drugs were added. After incubation for 48 h, the drugs were washed out, and the cells were incubated for an additional 5 days (35-mm dishes) or 7 days (100-mm dishes). The colonies were then stained with Giemsa solution and counted, in triplicate assays. The relative surviving fraction, compared with cells treated with only the vehicle, was then calculated.

Cell Cycle Assays. Exponentially growing cells were treated with the indicated concentrations of EGCG and were harvested by trypsinization at the indicated time. The cells were then washed twice with PBS, fixed in 70% ethanol for 30 min at room temperature, washed again with PBS, treated with 0.1 μg/ml RNase, and stained with 25 μg/ml propidium iodide. Flow cytometric analyses were carried out on a FACScalibur instrument using the CELL Quest program (Becton Dickinson, San Diego, CA).

Protein Extraction and Western Blot Analysis. Control or treated cells were harvested at the indicated times. Total cellular protein was extracted using the M-Per Mammalian Protein Extraction Reagent (Pierce Corp., Rockford, IL) plus leupeptin (10 μg/ml), aprotinin (2 μg/ml), and phenylmethylsulfonyl fluoride (2.5 mM), and the amount of protein was quantified with the Coomassie Protein Assay Reagent kit (Pierce). The gel was loaded with 40–50 μg of protein/lane, and the proteins were subject to electrophoresis on this 7.5–12.5% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes. The following primary antibodies were then used for immunoblotting: p21 (H-164), cyclin D1 (M-20), pRB (SC-50), Bcl-2 (N-19), Bcl-X_L (H-62), Bax (N-20), and caspase 9 (H-38), all from Santa Cruz Biotechnology (Santa Cruz, CA). The p27 antibody (DCS-72) was from Oncogene (Cambridge, MA), and the actin antibody (20–33) was from Sigma Chemical Co. Protein bands were detected using the ECL-enhanced chemiluminescence system (Amersham International, Buckinghamshire, United Kingdom).

EGFR, Stat3, and ERK Phosphorylation Assays. Forty to 50% confluent cultures were incubated in RPMI 1640 minus serum for 24 h and then incubated in the presence or absence of 10 μg/ml of EGCG for an additional 24 h and then harvested. One half of these cultures were also stimulated with 50 ng/ml of TGF-α, for the indicated time, just prior to harvesting. Proteins were extracted, and Western blot analysis was performed as described above. For the detection of specific proteins, the following antibodies were used: phospho-EGFR (clone 74) and EGFR (clone 13) from Transduction Laboratory (Lexington, KY); phospho-Stat3 (B-7) and Stat3 (F-2) from Santa Cruz Biotechnology; phospho-ERK1/2 (9106S) from Cell Signaling (Beverly, MA); and ERK1/2 (15868) from Upstate Biotechnology (Lake Placid, NY). The phospho-EGFR antibody reacts only with the tyrosine autophosphorylated (activated form) of the EGFR.

***Cyclin D1* and c-*fos* Reporter Assays.** The *cyclin D1*-promoter luciferase reporter plasmid-1745CD1LUC was constructed and provided by Dr. R. Pestell (Albert Einstein Cancer Center, New York, NY) (34). The c-*fos* promoter-luciferase reporter plasmid p-FOS-wt-luc and the method we used for transient transfection luciferase reporter assays were described previously (22). Triplicate samples of 1×10^5 cells in 35-mm plates were transfected using lipofectin (Life Technologies, Inc., Rockville, MD). One μg of DNA of the indicated luciferase reporter plasmid and 10 ng of the control pCMV-β-gal plasmid were cotransfected in opti-MEM I medium (Life Technologies, Inc., Rockville, MD). After 16 h, the medium was changed to serum-free RPMI 1640 containing the indicated concentration of EGCG. For growth factor stimulation assays, 50 ng/ml of TGF-α were added 30 min after adding the EGCG. The cells were then incubated for 24 h, and luciferase activity was determined with the luciferase assay system (Promega Corp., Madison, WI). β-gal activities were determined using the β-gal enzyme assay system (Promega Corp., Madison, WI). Luciferase activities were then normalized with respect to β-gal activities.

Cell Proliferation Assays. Cell proliferation was determined using a PreMix WST-1 Cell Proliferation Assay System (Takara, Tokyo, Japan), according to the manufacture's instructions. Briefly, 3×10^3 cells were seeded in a 96-well plate. Twenty-four h later, the indicated concentrations of 5-FU and/or EGCG were added. After incubation for 48 h, the cells were washed twice with PBS and then incubated for 2 h in medium containing the 10% PreMix WST-1 reagent. Absorbance was then measured using a microplate reader at a wavelength of 450 nm. Cell proliferation was calculated by dividing the absorbance of the treated cells by that of the vehicle-treated control cells (taken as 100%). Each point represents the average of triplicate wells.

RESULTS

Effects of EGCG on Growth, Cell Cycle Progression, and Apoptosis. To assess the cytotoxic effects of EGCG on two human HNSCC cell lines, dose-response curves were generated using colony formation assays. We found that EGCG

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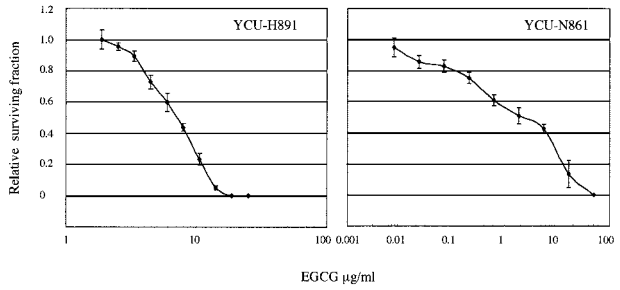

Fig. 1 Cytotoxic effect of EGCG on YCU-H891 and YCU-N861 cells. Cells were treated with the indicated concentration of EGCG, and the surviving fraction was determined by clonogenic assays. A survival fraction of 1.0 corresponds to the numbers of colonies obtained with control untreated cultures. Each *point* indicates the average of triplicate dishes; *bars*, SD.

showed strong cytotoxic effects on both the YCU-H891 and YCU-N861 cell lines (Fig. 1). Thus, the IC_{70} for both cell lines was ~10 μg/ml. The YCU-N 861 cells were, however, more sensitive to lower concentrations of EGCG than the YCU-H891 cells (Fig. 1). Thus, 1.0 μg/ml of EGCG caused ~50% inhibition of YCU-N861 cells but had no effect on YCU-H891 cells (Fig. 1).

To determine whether these cytotoxic effects are associated with specific changes in cell cycle progression or the induction of apoptosis, cell cycle analyses were carried out using DNA flow cytometry. Both the YCU-H891 and YCU-N861 cells were treated with 10 μg/ml of EGCG (the IC_{70} concentration). In addition, the YCU-H891 cells were treated with 20 μg/ml of EGCG, and the YCU-N861 cells were treated with 40 μg/ml of EGCG, which are the approximate IC_{100} concentrations for the respective cell lines. The cells were then harvested at 24-, 48-, and 72-h time points. With both cell lines, treatment with EGCG resulted in an increase, within 24 h, of cells in the G_1 phase, with both doses of EGCG (Fig. 2). However, the YCU-H891 cells showed a greater response to EGCG. Thus, within 48 h the fraction of cells in the G_1 phase increased by 17–20% with the YCU-H891 cells but by only 10% with the YCU-N861 cells (Fig. 2*B*). In addition, at the 72-h time point, there was a significant and dose-dependent increase in the sub-G_1 population with both cell types, indicative of the induction of apoptosis (Fig. 2). In contrast, no significant changes in cell cycle distribution or the sub-G_1 population were observed in the control untreated cells during this time course (Fig. 2*B*).

Effects of EGCG on the Expression of Cell Cycle and Apoptosis-related Proteins. In view of the above-described effects of EGCG on G_1 cell cycle arrest and induction of apoptosis, we examined the effects of EGCG on the levels of expression of G_1-related cell cycle control proteins and apoptosis-related proteins, using Western blot analyses (Fig. 3*A*). Both YCU-H891 and YCU-N861 cells were treated with 10 μg/ml of EGCG, and cells were then harvested at the 24- and 48-h time points. In the YCU-H891 cells, treatment with EGCG was associated with increased levels of the $p21^{Cip1}$ and $p27^{kip1}$ proteins and a decreased level of the cyclin D1 protein. In the YCU-N861 cells, the $p21^{Cip1}$ protein was not detected, either before or after treatment with EGCG, but there was a marked increase in the $p27^{kip1}$ protein, and at 48 h, there was a decrease in the level of the cyclin D1 protein. In both cell lines, there was a decrease in the phosphorylated form of the pRB protein. Presumably, the latter change resulted from the above-described changes in the levels of the $p21^{Cip1}$, $p27^{kip1}$, and cyclin D1 proteins. In YCU-H891 cells, the antiapoptotic Bcl-2 and Bcl-X_L proteins were slightly decreased after treatment with EGCG, whereas the proapoptotic Bax protein increased within 24 h of treatment with EGCG. In YCU-N861 cells, EGCG treatment decreased the levels of the Bcl-2 and Bcl-X_L proteins and increased the level of the Bax protein (Fig. 3*A*).

Effects of EGCG on Caspase 9 Activation. It was of interest to evaluate the effect of EGCG on caspase 9 activation, because the above-mentioned changes in the Bcl-2 family proteins could trigger the release of cytochrome *c* from mitochondria and thus activate caspase 9, which is the initial target of the cytochrome *c*/Apaf-1 complex (35–37). YCU-H891 and YCU-N861 cells were treated with 10 or 50 μg/ml of EGCG for 72 h. Cells were harvested at 36 or 72 h, and extracts were prepared and examined by Western blot analyses. Activation of caspase 9 was not seen with the 10 μg/ml dose with either cell line (data not shown). The activated M_r 37,000 caspase 9 protein fragment was detected after 36 h of treatment with the 50 μg/ml dose of EGCG (Fig. 3*B*).

Effects of EGCG on Signal Transduction. As described in the "Introduction," previous studies with EGCG in other cell types indicated that it can inhibit signaling pathways related to the activation of growth factor receptors (11–13). HNSCCs often display up-regulation of TGF-α/EGFR signal transduction pathways (16–19). Therefore, it was of interest to examine in our two cell lines the effects of EGCG on cellular levels and the phosphorylation status of the EGFR protein and also the Stat3 and ERK proteins, which lie downstream of the EGFR (22–25), by performing Western blot analyses with the respective non-phospho-specific and phospho-specific antibodies. YCU-H891 and YCU-N861 cells were grown in serum minus medium for 24 h to reduce exposure to external growth factors, and half of these cultures were then treated with 10 μg/ml of EGCG for 24 h. Half of both sets of cultures were stimulated with 50 ng/ml of TGF-α for 6 h (YCU-H891) or 18 h (YCU-N861), just before harvesting. Then, extracts were prepared and examined by Western blot analyses (Fig. 4).

In the presence or absence of TGF-α, the serum-starved YCU-H891 cells expressed rather high levels of the EGCG, ERK and Stat3 proteins. In addition, these serum-starved cells displayed rather high levels of the phosphorylated forms of these three proteins, and the levels and phosphorylation status of these proteins did not significantly increase after TGF-α stimulation (Fig. 4). Presumably, this reflects the fact that these cells overexpressed the EGFR (33) and also have a TGF-α/EGFR autocrine loop, because this is often observed in EGFR-overexpressing HNSCCs (18, 21). In the YCU-H891 cells, treatment with EGCG did not appreciably affect the levels of the EGFR, ERK, and Stat3 proteins, but it did markedly decrease the phosphorylation of these three proteins, both in the absence and presence of TGF-α (Fig. 4).

On the other hand, in the absence of TGF-α, the YCU-N861 cells did not display detectable levels of the EGFR or Stat3 proteins or the phosphorylated forms of these two proteins

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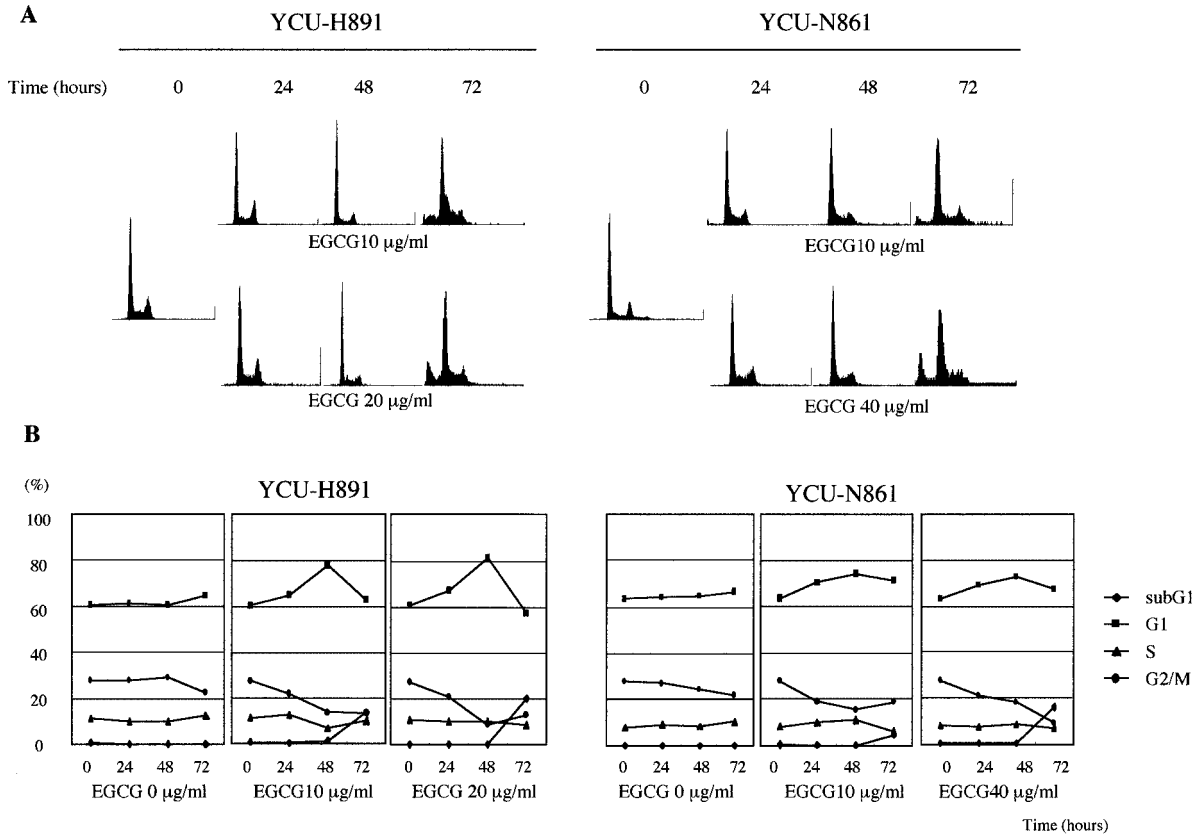

Fig. 2 Effects of EGCG on cell cycle progression and apoptosis. Exponentially growing cells were grown with the indicated concentrations of EGCG and then analyzed by DNA flow cytometry at the indicated time points (*A*). The distribution of cells in the sub-G_1 fraction (apoptosis) and in the G_1, S, and G_2-M phases of the cell cycle were calculated and plotted (*B*). An additional separate experiment gave similar results.

(Fig. 4). They did, however, display an appreciable level of the ERK protein and also the phosphorylated form of ERK. Stimulation of these cells with TGF-α induced increased expression of the EGFR and Stat3 proteins and also the phosphorylation of both these two proteins, as well as increased phosphorylation of the ERK protein. Treatment with EGCG in the absence of TGF-α led to a decrease in phosphorylation of the ERK protein. In the TGF-α-treated cells, EGCG markedly inhibited induction of the EGFR and Stat3 proteins caused by TGF-α and also markedly decreased the levels of the respective phosphoproteins (Fig. 4).

These results suggest that both the autocrine activation of EGFR signaling in YCU-H891 cells and the activation induced by exogenous TGF-α in YCU-N861 cells are markedly inhibited by EGCG, and as a consequence EGCG also inhibits signaling to the ERK and Stat3 proteins, in both cell lines.

Effects of EGCG on c-fos and Cyclin D1 Promoter Activity. There is evidence that both activated ERK and Stat3 enhance transcription of the c-*fos* and *cyclin D1* genes (24, 26, 27). Therefore, it was of interest to determine in our two cell lines the effects of EGCG on the transcription of these two genes, using c-*fos* promoter-luciferase and *cyclin D1* promoter-luciferase constructs in transient transfection reporter assays. The cells were transfected for 16 h with the respective reporter plasmid and then cultured for 24 h in serum minus medium, with or without the indicated concentrations of TGF-α and/or EGCG (Fig. 5). We found that in both the YCU-H891 and YCU-N861 cell lines, EGCG inhibited basal, and also TGF-α-stimulated, c-*fos* promoter and *cyclin D1* promoter activities. It is of interest that the absolute levels of luciferase activities were lower in the YCU-N861 cells than in the YCU-H891 cells (Fig. 5). This may reflect, in part, the difference between these two cell lines, with respect to the expression and phosphorylation of EGFR, ERK, and Stat3 shown in Fig. 4.

Effects of Combined Treatment with EGCG and 5-FU. Because 5-FU is one of the major chemotherapeutic agents used in the treatment of patients with HNSCC (38), it was of interest to determine whether EGCG can enhance the cytotoxic effects of 5-FU in our two cell lines. Because the plasma concentrations of EGCG after drinking several cups of tea, or receiving an oral dose of extracts containing EGCG, are in the range of 0.04–0.45 μg/ml (39, 40), we used a concentration of 0.1 μg/ml of EGCG in these assays. We tested 5-FU across a concentration range of 0.003–30 μg/ml. YCU-H891 cells were more resistant to 5-FU (18-fold) than YCU-N861 cells, when IC_{50}s were compared between these two cell lines (Fig. 6*A*). In both YCU-H891 and YCU-N861 cells, treatment with various concentrations of 5-FU plus 0.1 μg/ml of EGCG for 48 h markedly enhanced the inhibition of cell proliferation by 5-FU, when compared with treatment with 5-FU alone (Fig. 6*A*). The IC_{50} with YCU-H891 cells treated with 5-FU alone

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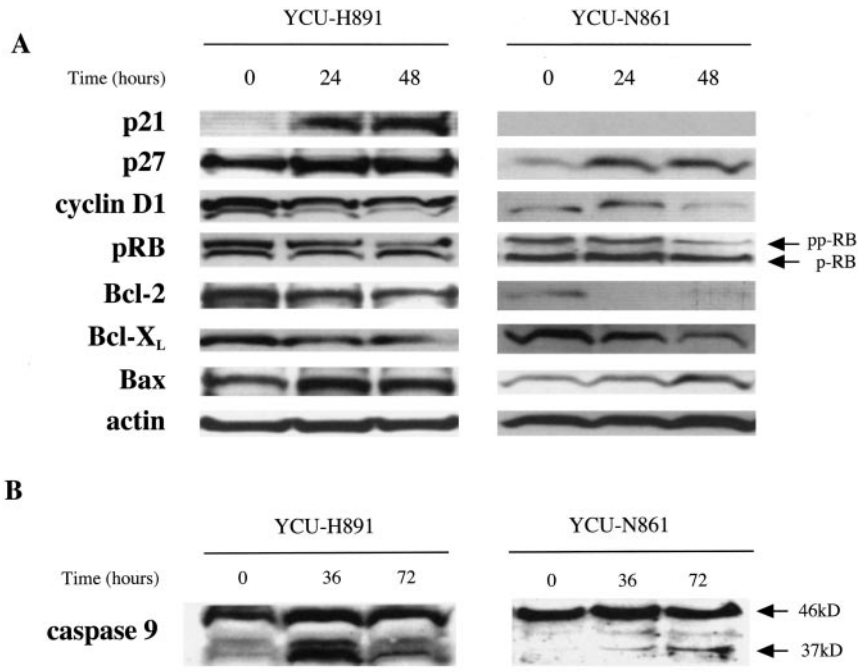

Fig. 3 Western blot analyses for the $p21^{Cip1}$, $p27^{Kip1}$, cyclin D1, pRB, Bcl-2, Bcl-X_L, and Bax proteins (*A*) and for activated caspase 9 protein (*B*) in extracts of YCU-H891 and YCU-N861 cells. Cells were treated with 10 μg/ml (*A*) or 50 μg/ml (*B*) of EGCG for the indicated times, and extracts were prepared. An antibody for actin was used as a loading control (*A*). The M_r 46,000 and M_r 36,000 caspase 9 protein band represents the inactivated and activated forms of caspase 9, respectively (*B*). *pp-RB*, hyperphosphorylated pRB.

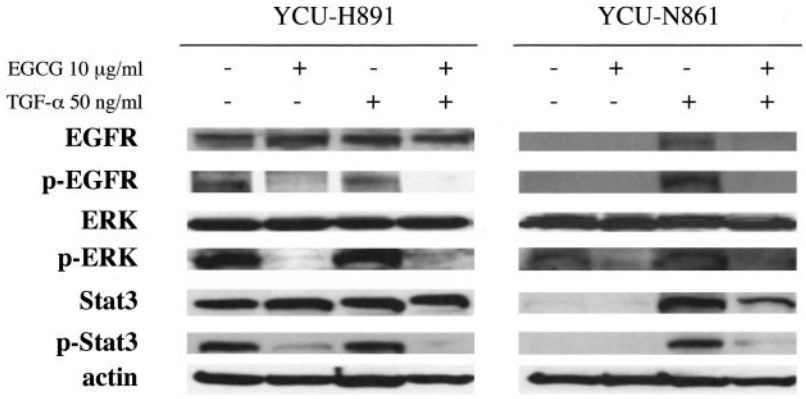

Fig. 4 Western blot analysis for the EGFR, phosphorylated EGFR (*p-EGFR*), ERK, phosphorylated ERK (*p-ERK*), and Stat3 and phosphorylated Stat 3 (*p-Stat 3*) proteins. Cells were cultured in serum-free medium for 24 h, incubated with or without 10 μg/ml of EGCG for an additional 24 h, and then harvested. For TGF-α stimulation, cells were treated with 50 ng/ml of TGF-α for 6 h (YCU-H891) or for 18 h (YCU-N861) before harvesting. An antibody for actin was used as a loading control.

was 4.5 μg/ml, whereas that of cells treated with 5-FU plus 0.1 μg/ml of EGCG was 0.1 μg/ml. The IC_{50} with YCU-N861 cells changed from 0.25 to 0.07 μg/ml with addition of 0.1 μg/ml of EGCG to 5-FU. Thus, 0.1 μg/ml of EGCG enhanced the growth-inhibitory effects of 5-FU 45-fold with YCU-H891 cells and 3.6-fold with YCU-N861 cells, respectively. As a result, both cell lines showed almost similar IC_{50}s when treated with 5-FU plus EGCG. Because in similar assays 0.1 μg/ml of EGCG alone did not show detectable inhibitory effects in either YCU-H891 or YCU-H861 cells (Fig. 6*B*), these results demonstrate that 0.1 μg/ml of EGCG synergistically enhances the inhibition of cell proliferation by 5-FU. To confirm that this combination actually induces cell death, clonogenic assays were also conducted. Fig. 7*A* illustrates the results obtained with the YCU-N861 cells by showing photographs of stained plates, and Fig. 7*B* quantitates the results obtained with both cell lines. Cells were treated with vehicle alone, EGCG alone, 5-FU alone, or EGCG plus 5-FU. Because the YCU-H cells were more resistant to 5-FU treatment than the YCU-N861 cells (Fig. 6*A*), we used 0.5 μg/ml of 5-FU for the YCU-H891 cells and 0.1 μg/ml for the YCU-N861 cells. For both cell types, we used 0.1 μg/ml of EGCG. With the YCU-N861 cells, treatment with 0.1 μg/ml EGCG alone slightly reduced the number of colonies, but it had no effect on YCU-H891 cells, when compared with the vehicle-treated control cultures (Fig. 7). Treatment with 5-FU alone inhibited colony formation ~40% with both cell lines (Fig. 7*B*). Treatment with 5-FU plus EGCG markedly inhibited colony formation in both cell lines (Fig. 7).

DISCUSSION

In the present study, we found that EGCG inhibited cell growth, caused G_1 arrest of the cell cycle, and also induced apoptosis in two cell lines originally derived from human HNSCC (Figs. 1 and 2). These results are consistent with

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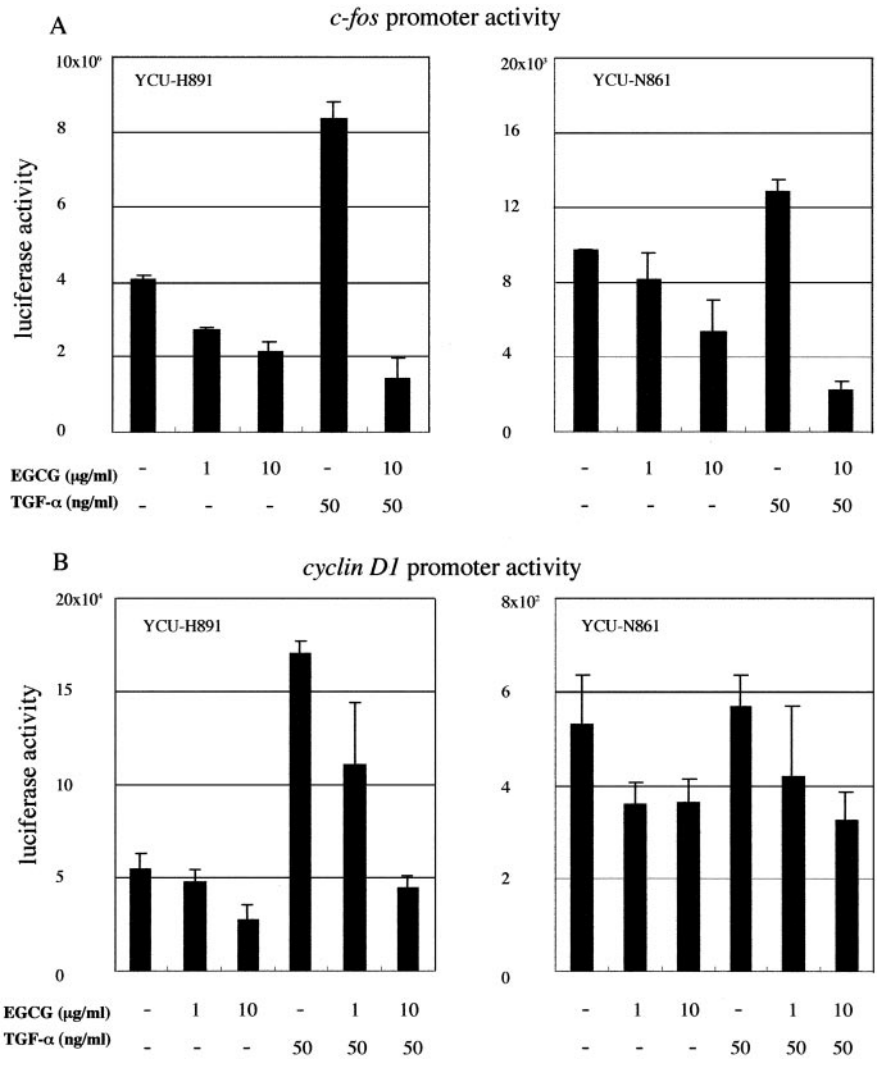

Fig. 5 Reporter assays for c-*fos* (*A*) and *cyclin D1* (*B*) in YCU-H891 (*left*) and YCU-N861 (*right*) cells. Cells were transfected in opti-MEM I medium with the c-fos promoter-luciferase or cyclin D1 promoter-luciferase reporter plasmids for 16 h and were then cultured in serum-free medium with the indicated concentrations of EGCG for 24 h. For the growth factor stimulation assays, TGF-α was added 30 min after EGCG. Luciferase activities were normalized to parallel assays for β-gal activities; *bars*, SD.

previous studies indicating that EGCG inhibited growth and induced apoptosis in human prostate, lung, colon, and gastric carcinoma and human leukemia cancer cell lines (6–9, 41). The EGCG concentrations used in the latter studies varied considerably, ranging from 10 to 200 μg/ml. The YCU-H891 cell line used in the present study was derived from a squamous cell carcinoma of the hypopharynx, and the YCU-N861 cell line was derived from a squamous cell carcinoma of the nasopharynx. Both cell lines showed similar responses to EGCG, with a 70% lethal dose of ~10 μg/ml (Fig. 1). Therefore, it appears that HNSCC cells are very sensitive to the inhibitory effects of EGCG, when compared with cells from other human malignancies.

We found that the cell death induced by EGCG in both of these two cell lines (Fig. 1) was associated with a decrease in the antiapoptotic Bcl-2 and Bcl-X_L proteins and an increase in the proapoptotic Bax protein and also activation of the caspase 9 protein (Fig. 3). Taken together, these results provide evidence that the apoptosis induced in these cells (Fig. 2) is mediated by a mitochondrial pathway (35–37). We also obtained evidence that EGCG treatment inhibited the activity of Stat3 (Fig. 4). Several studies (23, 42, 43) have demonstrated that Stat3 can up-regulate the expression of Bcl-X_L and Bcl-2. Therefore, inhibition of Stat3 by EGCG may be the cause for the decrease in the levels of the Bcl-X_L and Bcl-2 proteins, but the precise mechanism by which EGCG causes an increase in the level of the Bax protein remains to be determined.

As mentioned above, in the present study we also found that the inhibition of cell proliferation by EGCG in our two cell lines was associated with a G_1 arrest of the cell cycle (Fig. 2). Previous studies also demonstrated that EGCG causes a G_1 arrest in cell lines from various types of malignancies including HNSCCs (3–5, 7). In the present study, Western blot analyses indicated that EGCG treatment causes an increase in the levels of the cell cycle inhibitor proteins $p21^{Cip1}$ and $p27^{Kip1}$ and a decrease in the level of cyclin D1, an important G1 cyclin, and a decrease in the hyperphosphorylated form of the pRB protein. The latter change is probably a consequence of the changes in expression of the cyclin D1, $p21^{Cip1}$, and $p27^{Kip1}$ proteins and presumably explains the arrest in G_1 induced by EGCG. Similar

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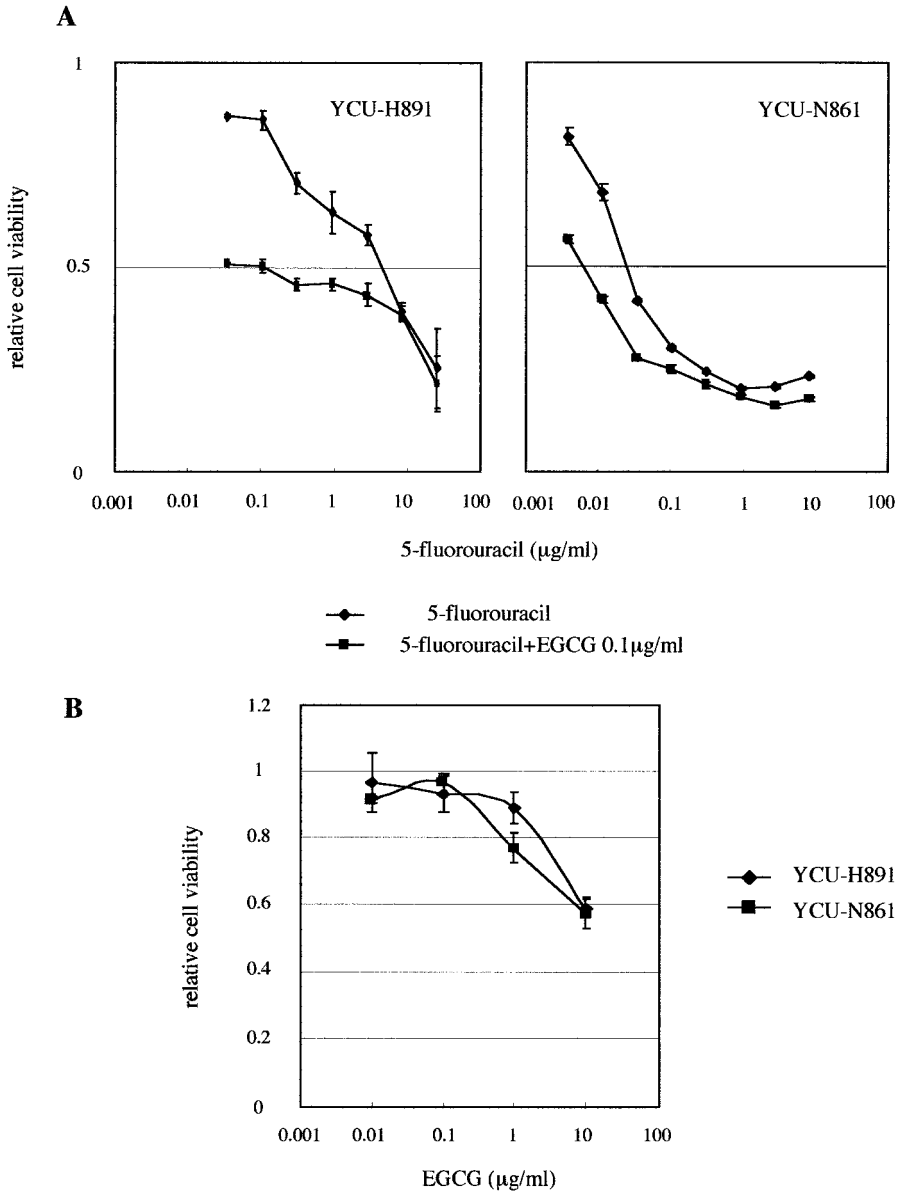

Fig. 6 Cytotoxic effects of the combination of EGCG and 5-FU (*A*) or of EGCG alone (*B*) on YCU-H861 and YCU-N861 cells, using cell proliferation assays. Cells were treated with the indicated concentrations of drugs for 48 h, and the extent of inhibition of cell proliferation was determined by proliferation assays (See "Materials and Methods"). A value of 1.0 corresponds to the vehicle-treated control cells; *bars*, SD.

findings were reported recently by other investigators. Thus, Liang *et al.* (4) found that EGCG inhibited the activation of cyclin-dependent kinases 2 and 4 through induction of the $p21^{Cip1}$ and $p27^{Kip1}$ proteins in breast cancer cells, and Liberto and Cobrinik (5) found that EGCG induced the $p21^{Cip1}$ protein and inhibited the cyclin D1-associated pRB kinase activity in a human breast epithelial cell line (MCF10A). Our *cyclin D1* promoter-luciferase reporter assays (Fig. 6) suggest that EGCG inhibits the expression of cyclin D1 at the level of transcription, but the mechanism by which EGCG increases the levels of the $p21^{Cip1}$ and $p27^{Kip1}$ proteins remains to be determined. The p53 status of the two cell lines used in this study is not known. However, in future studies it will be important to evaluate the possible role of the p53 protein in modulating some of the above-described effects of EGCG, because ~50% of HNSCCs carry mutations in the *p53* gene (32).

It is well known that increased EGFR expression is often an early event and also an adverse prognostic factor in patients with HNSCCs (16, 18, 19). Studies by Grandis and co-workers (25, 43–45) demonstrated that in HNSCC, Stat3 lies downstream of the TGF-α/EGFR signaling pathway, and Stat3 is strongly implicated in the growth of these carcinomas and in protecting them from apoptosis. Furthermore, *cyclin D1* is one of the genes that is activated by Stat3 (23, 24), although the precise mechanism by which the expression of cyclin D1 is enhanced by Stat3 remains to be determined. As discussed in the "Introduction," increased expression of cyclin D1 is seen in many types of human cancer including HNSCCs (29–31), and expression of an antisense cyclin D1 sequence in human cancer cells can inhibit their growth and tumorigenicity and also enhance their responsiveness to several chemotherapeutic agents (46–49).

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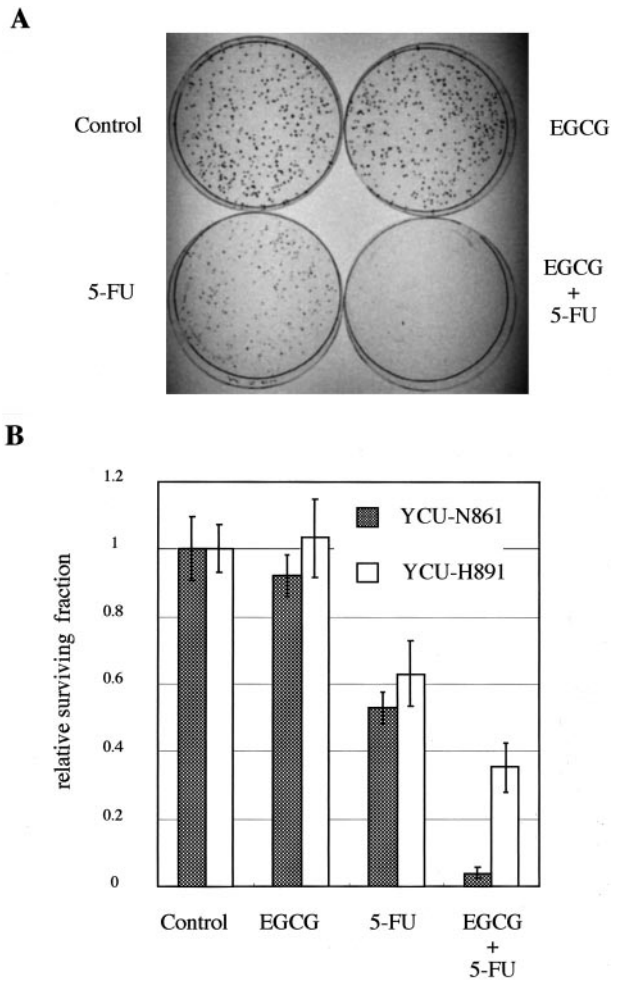

Fig. 7 Cytotoxic effects of EGCG plus 5-FU using colony formation assays with YCU-N861 and YCU-H861 cells. One thousand cells were seeded and cultured for 24 h in 100-mm dishes. The cells were then treated with DMSO alone, with 0.1 μg/ml of EGCG alone, with 5-FU alone, or with 5-FU plus 0.1 μg/ml of EGCG. With YCU-N861 cells, the concentration of 5-FU was 0.1 μg/ml, and with YCU-H891 cells, the concentration of 5-FU was 0.5 μg/ml. The drugs were washed out 48 h later, and the cells were cultured for an additional 7 days. The colonies were then stained with Giemsa solution (*A* is a photograph of the results obtained with YCU-N861 cells), and the numbers of colonies were counted and plotted as a fraction of the vehicle-treated cultures (*B*). Each *column* indicates the average of triplicate dishes; *bars*, SD. A relative survival of 1.0 corresponds to the control cells; *bars*, SD.

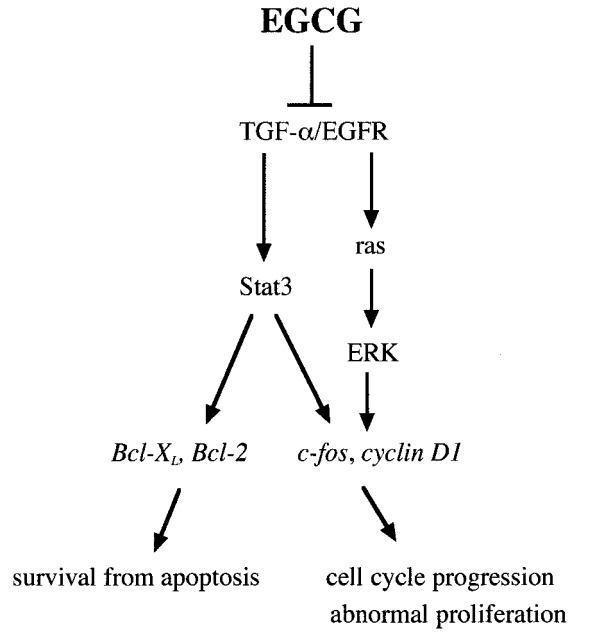

Fig. 8 A hypothetical mechanism by which EGCG inhibits growth and induces apoptosis in HNSCCs and thereby synergizes with 5-FU. The EGFR is activated by TGF-α, in an autocrine manner, thus leading to activation of the ras-ERK and Stat3 pathways. The c-*fos, cyclin D1, Bcl-X_L,* and *Bcl-2* genes are targets of these signal transduction pathways; therefore, tumor growth is enhanced. EGCG inhibits these signal transduction pathways and thereby induces G_1 arrest and apoptosis and potentiates the cytotoxicity of 5-FU.

The present study provides the first evidence that EGCG inhibits Stat3 activation and cyclin D1 promoter activity in human cancer cell lines. Our study extends previous evidence that EGCG inhibits activation of growth factor receptors and ERK proteins and also inhibits AP-1 activity and *c-fos* promoter activity (11–13, 28, 50–52). A striking finding by Liang *et al.* (12) is that in A431 epidermal carcinoma cells, EGCG directly inhibits binding of EGF to EGFR and also directly inhibits EGFR kinase activity. It remains to be determined whether these effects in themselves explain the multiple effects of EGCG on signal transduction, gene expression, growth inhibition, and induction of apoptosis seen in the present study or whether EGCG has other direct molecular targets in HNSCC cells.

In the present study, we found that even a low concentration of EGCG (0.1 μg/ml) synergistically enhances the cytotoxic effects of 5-FU (Fig. 7). This concentration of EGCG corresponds to the plasma concentration of EGCG, after an individual drinks several cups of tea (39) or after oral administration of EGCG-containing compounds (40). The mechanism of this synergy is not known but may relate to the above-described effects of EGCG on signal transduction and gene expression. A hypothetical scheme is shown in Fig. 8. It is of interest that the ability of EGCG to enhance the cytotoxicity of 5-FU was greater in the YCU-H891 cells than in the YCU-N861 cells (Fig. 7*A*), because the former cells have higher EGFR activity (Fig. 4) and may, therefore, be more dependent on this pathway. We should also emphasize that these two cell lines differ in other important respects that may influence their response to EGCG. Thus, the YCU-N861 cell line was derived from a nasopharyngeal carcinoma, and these tumors often carry the EBV, which can alter their physiology (53). In future studies, it will also be of interest to determine whether EGCG increases the sensitivity of various types of HNSCC cell lines to 5-FU, other chemotherapy agents, and/or radiation.

Taken together, the present studies suggest that the addition of EGCG to current therapeutic regimens used to treat HNSCCs may increase the efficacy of these therapies. At the present time, clinical trials for various types of cancer use anti-EGFR antibodies or specific tyrosine kinase inhibitors in combination with

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radiation and certain chemotherapy agents (54). The rationale for the latter studies is that inhibition of the EGFR-related signal transduction pathway enhances the cytotoxic effects of radiation or various chemotherapy agents (54–57). Although the clinical efficacy of EGCG remains to be determined, EGCG may have some advantages over EGFR antibodies or selected tyrosine kinase inhibitors, because it is a relatively inexpensive natural product, and because it appears to be nontoxic, it might be possible to administer it for a relatively long period of time without adverse side effects (1, 58).

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Effects of Epigallocatechin-3-gallate on Growth, Epidermal Growth Factor Receptor Signaling Pathways, Gene Expression, and Chemosensitivity in Human Head and Neck Squamous Cell Carcinoma Cell Lines

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