Epigallocatechin gallate sensitizes CAL-27 human oral squamous cell carcinoma cells to the anti-metastatic effects of gefitinib (Iressa) via synergistic suppression of epidermal growth factor receptor and matrix metalloproteinase-2

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Received June 6, 2012; Accepted July 30, 2012

DOI: 10.3892/or.2012.1991

Abstract. Human head and neck squamous cell carcinoma (HNSCC) is a major cause of cancer-related death during the last decade due to its related metastasis and poor treatment outcomes. Gefitinib (Iressa), a tyrosine kinase inhibitor has been reported to reduce the metastatic abilities of oral cancer. Previous studies have shown that epigallocatechin gallate (EGCG), a green tea polyphenol, possesses cancer chemopreventive and anticancer activity. However, the mechanisms involved in the suppression of invasion and metastasis of human oral cancer cells following co-incubation with gefitinib and EGCG remain poorly understood. In the present study, we attempted to investigate the synergistic effects of a combined treatment of gefitinib and EGCG in CAL-27 cells *in vitro* and to elucidate the underlying molecular mechanisms associated with the supression of cell migration and invasion. In the present study, we found that the individual treatments or the combined treatment of gefitinib and EGCG synergistically inhibited the invasion and migration of CAL-27 cells using Transwell invasion and wound-healing scratch assays, respectively. Similarly, gefitinib in combination with EGCG synergistically attenuated enzymatic activity and the protein expression of MMP-2 in CAL-27 cells. Furthermore, individual or combined treatment with EGCG and gefitinib suppressed the protein expression of p-EGFR and the phosphorylated protein levels of ERK, JNK, p38 and AKT and displayed inhibitory effects on metastatic ability of CAL-27 cells. Combined effects of EGCG and gefitinib-altered anti-metastatic actions for related gene expression were observed using DNA microarray analysis. Importantly, EGCG sensitizes CAL-27 cells to gefitinib-suppressed phosphorylation of epidermal growth factor receptor (EGFR *in vitro*. Taken together, our results suggest that the synergistic suppression of the metastatic ability of CAL-27 cells after EGCG and gefitinib individual or combined treatment are mediated through mitogen-activated protein kinase (MAPK) signaling. Our novel findings provide potential insights into the mechanism involved with synergistic responses of gefitinib and EGCG against the progression of oral cancer.

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Key words: epigallocatechin gallate, gefitinib, synergistic effect, migration, invasion, head and neck squamous cell carcinoma, matrix metalloproteinase-2

Introduction

In Taiwan, head and neck squamous cell carcinoma (HNSCC) is a highly prevalent malignancy and is associated with the habit and common risk factor of betel nut chewing (1-3). Clinical therapies for HNSCC patients consist of multiple-modality treatment with surgery, radiation and multi-drug chemotherapy (4,5). Systemic and nodal metastases are the major causes of mortality associated with HNSCC patients (6,7). Metastasis involves the matrix metalloproteinases (MMPs), a group of proteolytic enzymes, which contribute in the degradation of the basement membrane and extracellular matrix (ECM) (8-10). The matrix metalloproteinase-2 (MMP-2) is intensely involved in the invasion and metastasis of HNSCC. Thus, inhibition of metastasis or downregulation of MMP-2 expression are important goals for successful therapy (11,12).

Epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase (RTK) family. EGFR is expressed

in a number of cell types, including epithelial and mesenchymal cells (13,14). It has been reported that EGFR is highly expressed in 80-100% of HNSCC patients, and increased expression of EGFR is often associated with a poor prognosis in HNSCC (15,16). The EGFR signaling pathways contribute to the regulation of cancer cell proliferation, angiogenesis, adhesion, migration, invasion and anti-apoptosis. In addition, EGFR signaling is triggered by the binding of epidermal growth factor (EGF), resulting in the dimerization of EGFR molecules (17,18). Autophosphorylation of the EGFR through the tyrosine kinase domains leads to the stalling of downstream signals such as mitogen-activated protein kinases (MAPKs) (ERK, JNK and p38), serine/threonine kinase AKT and protein kinase C (PKC) pathways (19,20). MAPKs are associated with the expression of the components mediated in MMP promoter induced through AP-1, and its association with c-fos and c-Jun (20). A number of studies have suggested that the MAPKs play a central role in regulating the activities of MMPs (19,20). Several anti-metastatic agents that target EGFR or their downstream signal have been studied in HNSCC (14-16). Clinical studies involving HNSCC treatments have shown that the combination of cetuximab (Erbitux) with other drugs is an EGFR inhibitor (21-23). Cetuximab-radiation or cetuximab-cisplatin combinations exhibited significant improvement in adverse effects and a significant increase in survival compared with radiation alone. However, a number of HNSCC patients eventually manifest acquired resistance to cetuximab or cisplatin (24).

Gefitinib (Iressa), an EGFR inhibitor, has shown obvious *in vitro* and *in vivo* anticancer activity through reduced EGFR expression in cancer cell lines, including prostate, breast, ovarian, colon and HNSCC (25,26). Preclinical therapy evidence suggests that gefitinib may enhance anticancer activity compared to a variety of cytotoxic drugs including platinum derivatives, taxanes, doxorubicin or topotecan (27). Gefitinib was found to competitively inhibit the autophosphorylation of the catalytic domain of the EGFR (19,20). To investigate the potential of gefitinib and the enhancement of its effects in combination with other chemotherapeutic agents or natural products, we evaluated the combination treatment of gefitinib and epigallocatechin gallate (EGCG). It has been reported that EGCG has a number of biological functions including induction of cell apoptosis, cell cycle arrest, cell growth inhibition, anti-angiogenesis and suppression of metastasis (28-33). EGCG is the most abundant and most active phenolic constituent of green tea (34). EGCG, has also been extensively studied in regards to its anticancer activity in a number of cancer cell lines and in animal tumor models (28-33). Several studies have also demonstrated that EGCG inhibits the activation of the receptor tyrosine kinases, such as EGFR, insulin-like growth factor-1 receptor (IGF-1R), vascular endothelial growth factor receptor (VEGFR), and their downstream effectors such as AKT and MAPKs (35,36). Chen *et al* has demonstrated that EGCG inhibits cell invasion of SCC-9 oral cancer cells through the downregulation of MMPs and u-PA expressions (37). Thus, EGCG may be useful as an effector for the prevention of cancer metastasis. In the current study, we aimed to ascertain whether that combined treatment with EGCG and gefitinib synergistically inhibits cancer cell invasion and migration by targeting EGFR signaling pathways. As evidenced by our results, EGCG/gefitinib combination treatment modulates anti-metastatic effects in an HNSCC culture system *in vitro*.

Materials and methods

Materials and reagents. EGCG, dimethyl sulfoxide (DMSO) and anti-actin were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Gefitinib was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were purchased from Gibco/Life Technologies (Carlsbad, CA, USA). The primary antibodies were obtained as follows: antibodies for MMP-2, TIMP-2, p-ERK, p-JNK, p-p38, p-AKT and AKT were obtained from EMD Millipore Corp. (Billerica, MA, USA); antibodies for p-EGFR, EGFR, ERK, JNK, p38, PKCα and horseradish peroxidase (HRP)-linked goat anti-mouse IgG, goat anti-rabbit IgG, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. The HNSCC CAL-27 cell line was kindly provided by Dr Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan). Cells were cultured in 75 cm^2 tissue culture flasks (TPP, Techno Plastic Products AG., Trasadingen, Switzerland) with DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and grown at 37˚C in a humidified 5% CO_2 atmosphere and detached by 0.25% Trypsin/0.02% EDTA (38-40).

Transwell invasion assay. The invasive ability of CAL-27 cells was evaluated using the Boyden chamber assay with Matrigel matrix-coated filters as previously described (41,42). Cells ($1x10^4$ cells/0.4 ml) were seeded in the upper chamber of the Transwell inserts (8 μm pore size, EMD Millipore, Temecula, CA, USA) pre-coated with Matrigel (BD Biosciences, Bedford, MA, USA) and exposed to DMSO (0.5%, as a control; CTL), EGCG (25 μM), gefitinib (10 μM) or the combination of gefitinib (10 μM) and different concentrations (25, 50 and 100 μM) of EGCG. DMEM containing 10% FBS was placed in the lower chamber and cells for each treatment were incubated for 48 h at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. Then, the non-invasive cells in the upper chamber were removed with a cotton swab, and the invaded cells were fixed with 4% formaldehyde for 15 min and stained with 2% crystal violet in 2% ethanol for 15 min after being washed with PBS. The number of cells that penetrated the membrane was counted and images were captured under a light microscope at a magnification of x200, as previously described (41,42). Each experiment was repeated 3 times.

Wound-healing scratch assay. Approximately $2x10^5$ CAL-27 cells/well were cultured in 12-well plates after cell monolayers were attached overnight to 80% confluency by scratching with a 200-μl pipette tip and then incubated in the presence or absence of EGCG (25 μM), gefitinib (10 μM) or the combination of gefitinib (10 μM) and different concentrations (25, 50 and 100 μM) of EGCG for 48 h. Cells that migrated into the wound region were determined and images were captured

using a phase-contrast microscope (x100) as previously described (9,41). Five randomly chosen fields were analyzed for each well and each experiment was performed in triplicate.

Gelatin zymography analysis. CAL-27 cells ($5x10^5$/well) in 12-well plates were incubated in a serum-free medium with either 25 μM EGCG alone, 10 μM gefitinib alone or in combination with gefitinib (10 μM) and EGCG at 25, 50 and 100 μM. After a 48-h incubation, conditioned medium was collected to perform a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin (Sigma-Aldrich Corp.). After electrophoresis, the gel was washed twice with 2.5% Triton X-100 in dH_2O twice for a total of 60 min at 25˚C, then were incubated in substrate buffer (pH 7.6, 50 mM Tris, 10 mM $CaCl_2$, 50 mM and 0.05% Brij-35) at 37˚C for 24 h. After incubation, the gel was stained with 0.3% Coomassie brilliant blue R250 (Bio-Rad Laboratories, Hercules, CA, USA) in 50% methanol and 10% acetic acid for 20 min and de-staining was subsequently performed with 10% acetic acid and 30% methanol to visualize MMP-2 activity as previously described (43,44). Bands of gelatinolytic activity were assessed using NIH ImageJ software. The results were performed in 3 independent experiments.

Western blot analysis. The CAL-27 cells ($1x10^7$/flask) were placed in a 75T flask and exposed to 25 μM EGCG, 10 μM gefitinib or the combination of gefitinib (10 μM) and EGCG at 25, 50 and 100 μM for indicated time intervals. Cells were harvested and resuspended in lysis buffer (PRO-PREP™ protein extraction solution; iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea) as previously described (45-47). After being centrifuged at 13,000 x g for 10 min at 4˚C, the whole-cell protein extracts were collected and quantitated using a Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as the standard. The protein lysates were determined by 10-12% SDS-PAGE, and then electro-transferred onto a nitrocellulose membrane using an iBlot™ Dry Blotting System (Invitrogen/Life Technologies) before being blocked with PBS containing 0.2% Tween-20 and 5% non-fat powdered milk for 1 h. The membrane was incubated first with antibodies overnight and bound antibodies were detected using horseradish peroxidase-conjugated secondary antibody, followed by Immobilon Western Chemiluminescent HRP substrate (Millipore) and X-ray film (GE Healthcare, Piscataway, NJ, USA). The protein abundance was quantified and NIH ImageJ software was used to determine the band intensity from immunoblotting analysis (47,48).

RNA purification. CAL-27 cells at a density of $1x10^7$ cells were placed in a 75T flask and incubated without and in combination with gefitinib (10 μM) and EGCG (100 μM) for 24 h. Cells were scraped and collected by centrifugation, and total RNA was subsequently isolated using an Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) after being harvested as previously described (49,50). RNA quantity and purity were assessed at 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (Labtech International Ltd., East Sussex, UK).

Microarray analysis. After RNA extractions, 300 ng of each sample was amplified and labeled using the GeneChip WT Sense Target Labeling and Control Reagents (Affymetrix, Inc. Santa Clara, CA, USA) for expression analysis. Thereafter, hybridization was performed against the Affymetrix GeneChip Human Gene 1.0 ST array (Affymetrix, Inc.). The arrays were hybridized for 17 h at 45˚C and 60 rpm. Arrays were subsequently washed (Affymetrix Fluidics Station 450; Affymetrix, Inc.) and stained with streptavidin-phycoerythrin (GeneChip Hybridization, Wash, and Stain kit; Affymetrix, Inc.), and were scanned on an Affymetrix GeneChip® Scanner 3000 (Affymetrix, Inc.). Resulting data were analyzed using Expression Console software (Affymetrix, Inc.) with default RMA parameters. Genes regulated by EGCG and gefitinib with a 1.2-fold change in expression were identified. Moreover, bioinformatics analysis for these candidate genes was determined utilizing MetaCore (GeneGo, Inc., St. Joseph, MI, USA) as previously described (51,52).

Statistical analysis. All data represent the means ± SD from 3 independent experiments. The differences were evaluated using the Student's t-test and were considered statistically significant at $P<0.001$.

Results

Combined or individual treatment with EGCG and gefitinib inhibits invasive behavior of CAL-27 cells. The invasion assay revealed that CAL-27 cells invaded the Matrigel-coated filters from the upper to the lower chamber in the absence and presence of EGCG, gefitinib or the combination of both compounds. Our study indicated that individual treatment with EGCG (25 μM) or gefitinib (10 μM) alone suppressed the cell invasive ability of CAL-27 cells at 48 h by approximately 31 and 33%, respectively (Fig. 1). We next investigated the combined effect of gefitinib (10 μM) and EGCG (25-100 μM). The combination of EGCG and gefitinib exhibited a synergistic inhibition (at least by 2.12-fold) of the invasive ability of CAL-27 cells (Fig. 1).

Combined or individual treatment with EGCG and gefitinib suppresses migratory ability of CAL-27 cells. To measure the effect of cell migration we used the wound-healing scratch assay. The ability of cells to migrate to a wounded area in a monolayer was observed. Individual treatment of EGCG (25 μM) and gefitinib (10 μM) had a significant inhibitory effect on cell migration of 22 and 34%, respectively, in CAL-27 cells (Fig. 2). We also observed that the combination treatment of gefitinib (10 μM) and EGCG (25-100 μM) for 48 h dramatically inhibited the migration of CAL-27 cells into the wounded area; these synergistic effects were increased by at least 1.56-fold when compared with the effects of EGCG or gefitinib treatment alone (Fig. 2).

Gefitinib in combination with EGCG synergistically attenuates the enzymatic MMP-2 activity in CAL-27 cells. We aimed to explore whether gefitinib, EGCG or the combined treatment of both compounds influence MMP-2 activity in the conditioned medium of CAL-27 cells. Results from the gelatin zymographic analysis (Fig. 3) demonstrated that co-incubation of gefitinib and EGCG (25, 50 and 100 μM) for 48 h synergistically enhanced the suppressive effect on the activities of

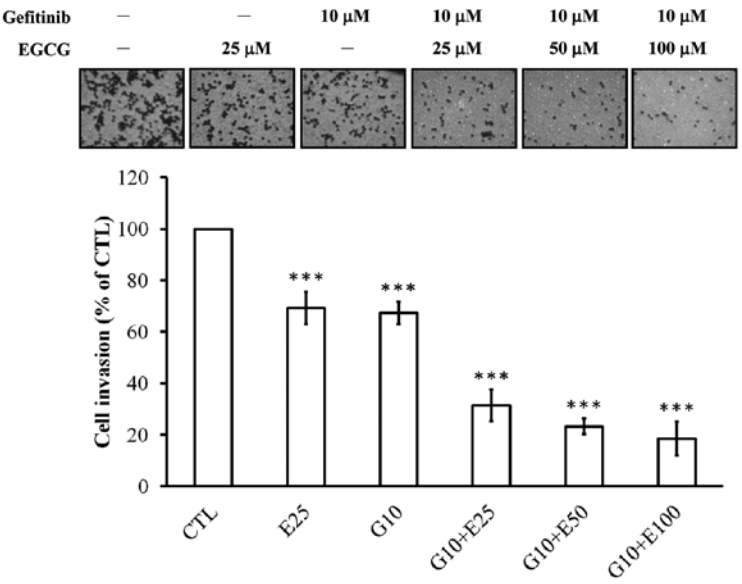

Figure 1. Effects of the combined or individual treatment of EGCG and gefitinib on CAL-27 cell invasion. Cells ($1x10^4$/0.4 ml) were seeded into the upper chamber of Transwell inserts coated with Matrigel and exposed to DMSO (0.5%, as the control; CTL), EGCG (25 μM) (E25), gefitinib (10 μM) (G10) or a combination of gefitinib (10 μM) and various concentrations of EGCG (25, 50 and 100 μM) (G10+E25, G10+E50, G10+E100, respectively) for 48 h. The invasive ability of CAL-27 cells was determined as described in Materials and methods. The number of cells that penetrated the membrane was counted and images were captured under a light microscope at x200 magnification. Data represent the means ± SD of 3 experiments. ***P<0.001 is considered statistically significant when compared with the untreated control. Each experiment was repeated 2 times with similar results.

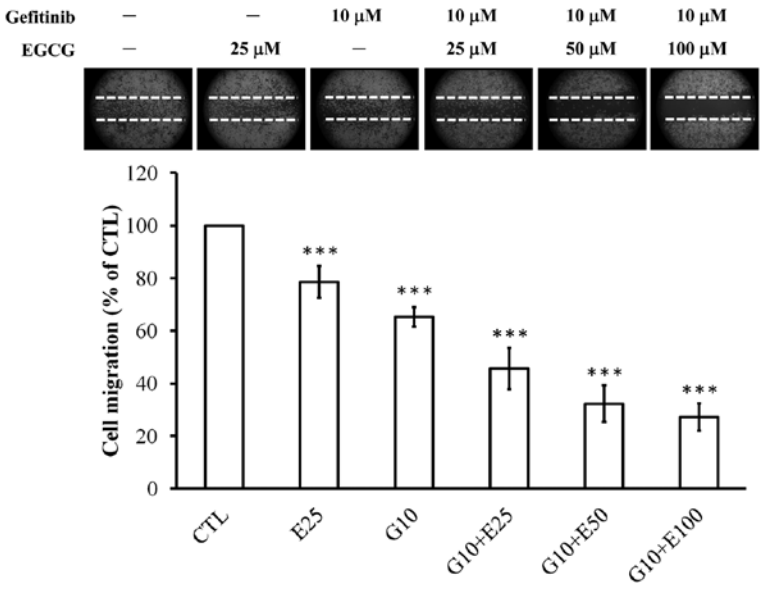

Figure 2. Individual and combined effects of EGCG and gefitinib on CAL-27 cell migration. Cells, after scratching with a 200-μl pipette tip, were incubated in the absence (CTL) or presence of EGCG (25 μM) (E25), gefitinib (10 μM) (G10) or the combination of gefitinib (10 μM) and different concentrations of EGCG (25, 50 and 100 μM) (G10+E25, G10+E50, G10+E100, respectively) for 48 h as described in Materials and methods. Cells which migrated into the wounded region were determined and images were captured using a phase-contrast microscope (x100). Values represent the means ± SD of 3 independent experiments. ***P<0.001 is considered statistically significant when compared with the untreated control. Each experiment was performed in triplicate with similar results.

MMP-2 in CAL-27 cells, resulting in an additive inhibition by at least 1.16-fold in comparison to EGCG or gefitinib individually treated samples. However, EGCG or gefitinib individually inhibited the enzymatic MMP-2 activity by 57 and 68%, respectively, in CAL-27 cells.

Combined or individual exposure to EGCG and gefitinib alters the protein expression associated with the metastatic ability of CAL-27 cells. To investigate the expression levels of the proteins associated with the inhibitory effects on the migration and invasion of CAL-27 cells by the individual and combined effects of EGCG and gefitinib, western blot analysis was applied and results are presented in Fig. 4. Individual or combined treatment with gefitinib and EGCG (25-100 μM) for 48 h synergistically decreased the protein expression of MMP-2, but significantly increased the protein levels of

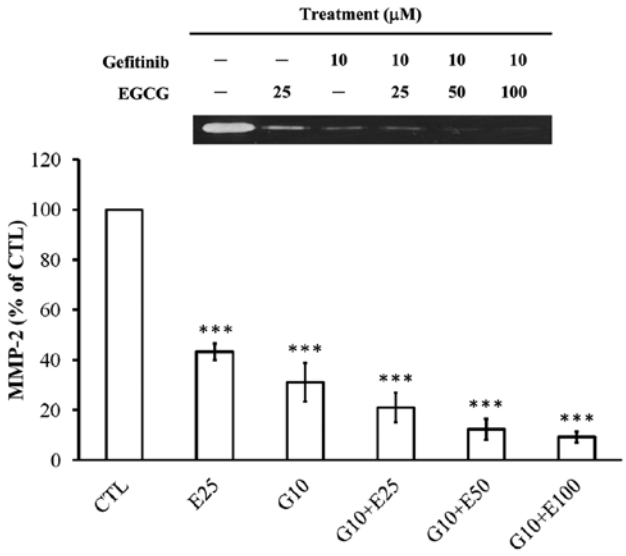

Figure 3. Effects of gefitinib in combination with EGCG on the MMP-2 activity in CAL-27 cells. Cells were incubated with either 25 μM EGCG or 10 μM gefitinib alone or in combination with gefitinib (10 μM) (G10) and various concentration of EGCG (25, 50 and 100 μM) (G10+E25, G10+E50, G10+E100, respectively) for 48 h. Gelatin zymography of MMP-2 and densitometric analysis were carried out as described in Materials and methods. Values are the means ± SD, (n=3). ***P<0.001 indicates a statistically significant difference between the treated groups and the untreated control.

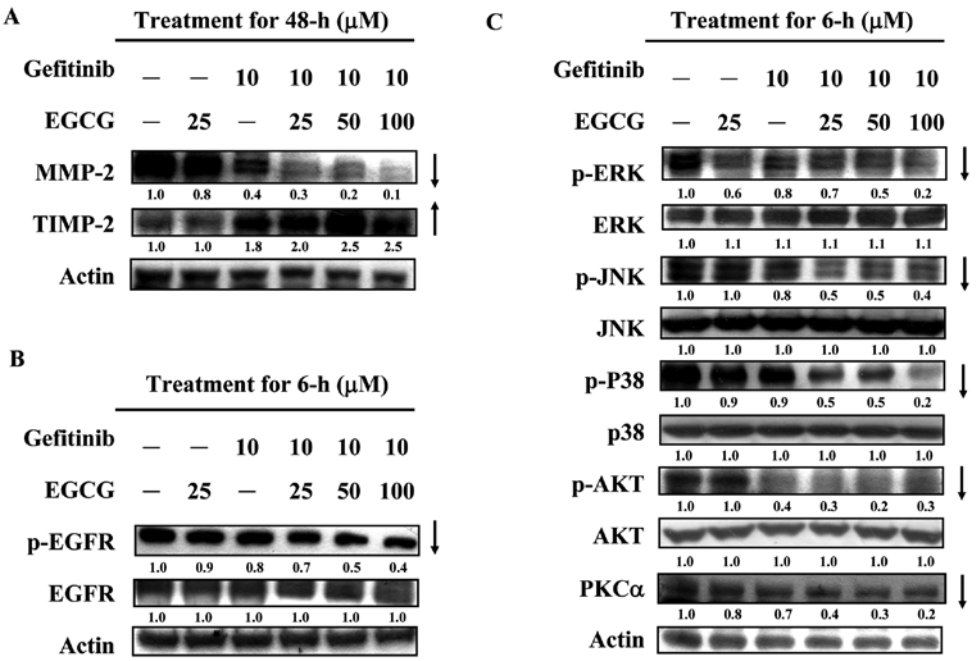

Figure 4. Individual and combined effects of gefitinib and EGCG on the expression levels of protein associated with metastatic ability of CAL-27 cells. Cells ($1x10^7$/flask) were placed in a 75T flask and exposed to 25 μM EGCG, 10 μM gefitinib or the combination of gefitinib (10 μM) and EGCG at 25, 50 and 10 μM for indicated time intervals. After incubation, the total proteins were collected, and the proteins levels of (A) MMP-2 and TIMP-2; (B) EGFR and p-EGFR; (C) p-ERK, ERK, p-JNK, JNK, p-p38, p-38, p-AKT, AKT and PKCα were subjected to western blotting as described in Materials and methods. Actin protein level was used as the internal control for equivalent loading. The results are representative of 3 separate experiments.

TIMP-2 in CAL-27 cells (Fig. 4A). We further explored the effect of EGCG and gefitinib on upstream signaling pathways in CAL-27 cells. Data in Fig. 4B revealed that p-EGFR protein expression was suppressed in the CAL-27 cells after being treated or co-incubated with gefitinib and EGCG (25, 50 and 100 μM) for 6 h. However, there was no significant difference in EGFR levels among CAL-27 cells treated with a combination of gefitinib and EGCG, either agent alone or the control. Previous studies have reported that the involvement of MAPKs may be essential for the expression of MMPs and it is involved in cell invasion and migration during tumor metastasis (10,53,54). Our results indicated that gefitinib combined with EGCG synergistically suppressed the phosphorylated protein expression of ERK, JNK and p38 but no impact on the protein levels of ERK, JNK and p38 in the CAL-27 cells was observed compared with the untreated control (Fig. 4C).

Table I. Genes with more than a 4-fold change in mRNA level in CAL-27 cells after 24 h treatment with gefitinib (10 μM) and EGCG (100 μM) as identified by DNA microarray.

Input IDs	Gene name	Gene symbol	Description	Fold-change
7916609	c-Jun	JUN	Transcription factor AP-1	5.91
7938154	ILK	ILK	Integrin-linked protein kinase	5.04
7930074	NF-κB	NFKB2	NF-κB	4.62
8149638	DOK2	DOK2	Docking protein 2	4.54
8062377	c-Src	SRC	Proto-oncogene tyrosine-protein kinase Src	4.45
7945436	H-Ras	HRAS	GTPase HRas	4.37
8131406	Rac1	RAC1	Ras-related C3 botulinum toxin substrate 1	4.33
8085374	c-Raf-1	RAF1	RAF proto-oncogene serine/threonine-protein kinase	4.33
8018364	GRB2	GRB2	Growth factor receptor-bound protein 2	4.32
8101002	Betacellulin	BTC	Probetacellulin	4.31
8089801	GSK3 β	GSK3B	Glycogen synthase kinase-3 β	4.27
8115831	MKP-1	DUSP1	Dual specificity protein phosphatase 1	4.26
8005029	MEK4	MAP2K4	Dual specificity mitogen-activated protein kinase kinase 4	4.17
8172345	Elk-1	ELK1	ETS domain-containing protein Elk-1	4.15
8062623	PLC-γ 1	PLCG1	PLC-γ 1	4.07
8082911	NCK1	NCK1	Cytoplasmic protein NCK1	-4.04
8006906	ErbB2	ERBB2	Receptor tyrosine-protein kinase erbB-2	-4.06
8096845	EGF	EGF	Pro-epidermal growth factor	-4.13
8051670	SOS	SOS1	SOS	-4.16
8095728	Epiregulin	EREG	Proepiregulin	-4.23
8153223	FAK1	PTK2	Focal adhesion kinase 1	-4.23
7927389	JNK1	MAPK8	Mitogen-activated protein kinase 8	-4.28
8032761	MEK2	MAP2K2	Dual specificity mitogen-activated protein kinase kinase 2	-4.29
8150076	MKP-2	DUSP4	Dual specificity protein phosphatase 4	-4.32
8132860	EGFR	EGFR	Epidermal growth factor receptor	-4.43
8015607	STAT3	STAT3	Signal transducer and activator of transcription 3	-4.50
8074791	ERK1/2	MAPK1	ERK1/2	-4.51
7920600	Shc	SHC1	SHC-transforming protein 1	-4.71
8148317	c-Myc	MYC	Myc proto-oncogene protein	-4.87
8154178	JAK2	JAK2	Tyrosine-protein kinase JAK2	-4.96
7925531	AKT	AKT3	AKT (PKB)	-4.98
7981494	AKT	AKT1	AKT (PKB)	-5.06
8009301	PKC-α	PRKCA	Protein kinase C α type	-5.29
7978644	I-κB	NFKBIA	I-κB	-5.34
8057744	STAT1	STAT1	Signal transducer and activator of transcription 1-α/β	-5.38
7916747	JAK1	JAK1	Tyrosine-protein kinase JAK1	-5.48
7995681	MMP-2	MMP2	72 kDa type IV collagenase	-5.65
8091009	PI3K cat class IA	PIK3CB	PI3K cat class IA	-5.76
8116402	JNK2	MAPK9	Mitogen-activated protein kinase 9	-6.10
8106784	p120GAP	RASA1	Ras GTPase-activating protein 1	-10.33
8095736	Amphiregulin	AREG	Amphiregulin	-25.41

We also revealed that the activation of phospho-AKT (Ser473) and PKCα protein levels was downregulated in CAL-27 cells after exposure to gefitinib, EGCG or co-incubation with both compounds for a 6-h exposure (Fig. 4C).

DNA microarray analysis for combined effects of EGCG and gefitinib-altered anti-metastatic actions in CAL-27 cells. To examine the gene expression profile in the combined EGCG and gefitinib-treated CAL-27 cells, DNA microarray analysis

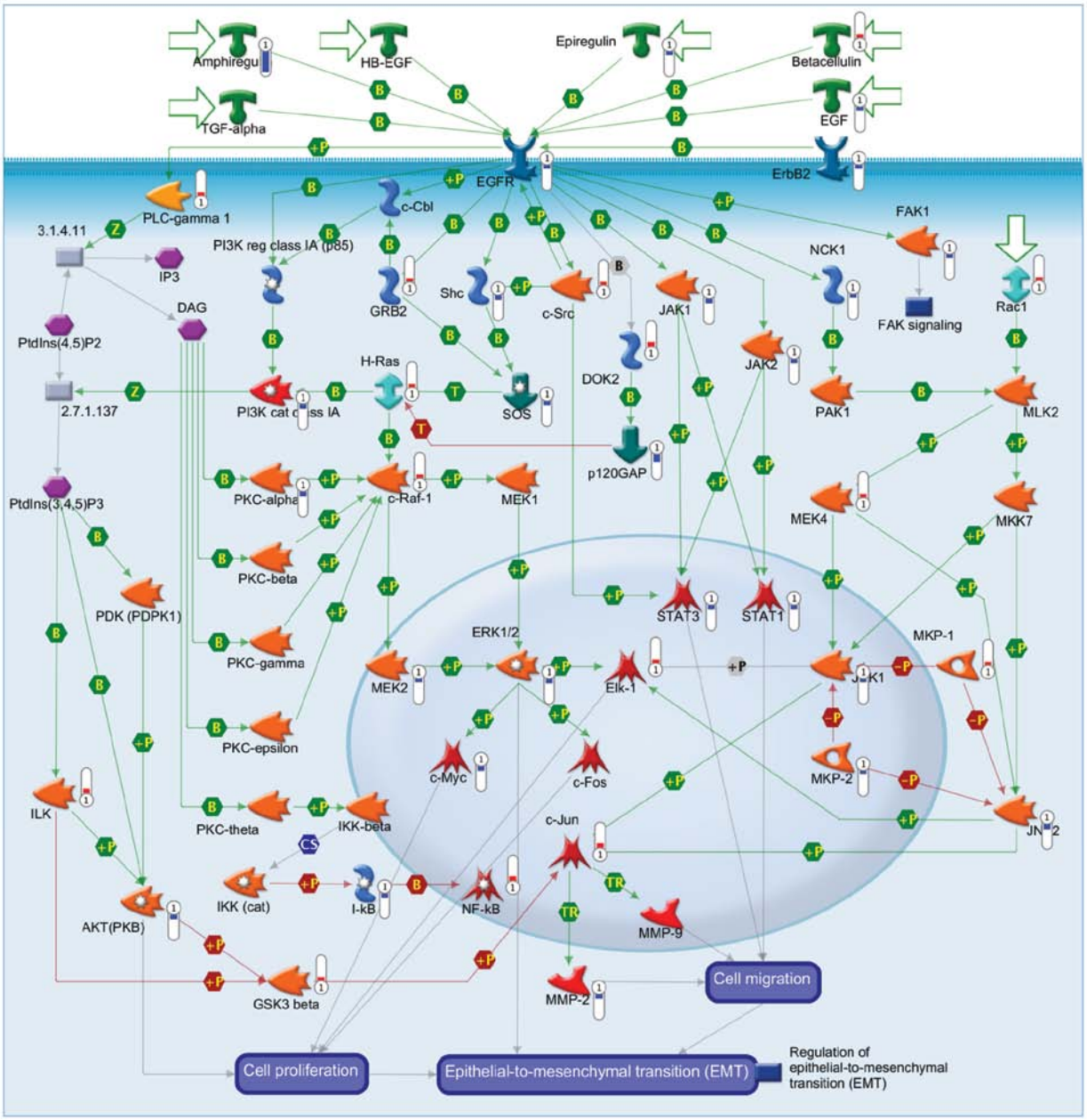

Figure 5. Bioinformatics analysis demonstrated the molecular canonical pathways from MetaCore GeneGo analysis in human oral cancer CAL 27 cells. (Red, upregulation; blue, downregulation).

was performed after treatment for 24 h. Our data demonstrated that 41 genes (15 genes, upregulated; 26 genes, downregulated) were expressed using microarray analysis. As shown in Table I, we found that the levels of c-Jun, ILK, NF-κB, DOK2, c-Src, H-Ras, Rac1, c-Raf-1, GRB2, betacellulin, GSK3 β, MKP-1, MEK4, Elk-1, PLC-γ 1 were upregulated in the CAL-27 cells treated with the combination of EGCG and gefitinib. On the other hand, expression of NCK1, ErbB2, EGF, SOS, epiregulin, FAK1, JNK1, MEK2, MKP-2, EGFR, STAT3, ERK1/2, Shc, c-Myc, JAK2, AKT, AKT, PKC-α, I-κB, STAT1, JAK1, MMP-2, PI3K cat class IA, JNK2, p120GAP and amphiregulin were downregulated in treated CAL-27 cells (Table I). The schematic diagram shown in Fig. 5 was observed for the top scorers by the number of network pathways from the GeneGo analysis program.

Discussion

EGFR is a major target of TRKs in tumor therapy, especially in HNSCC (15,16). Grandis *et al* suggested that EGFR was overexpressed in ~90% of HNSCC tumors and overexpression of EGFR was significantly associated with poor prognosis (56). Targeting EGFR is a strategy in antitumor metastasis in preclinical HNSCC models. In Asia, 7% HNSCC patients carry the EGFR mutation, and the EGFR mutation in HNSCC is associated with altered therapeutic responses to EGFR inhibitors (56,57). Therefore, the study of the resistance to EGFR inhibition and combinational strategies is required. Previous studies have shown that enhancement or synergistic antitumor effects in both *in vitro* and *in vivo* models of HNSCC were found when EGCG was combined with erlotinib (58). In this

study, we demonstrated that a combined treatment of EGCG and gefitinib synergistically inhibited invasion (Fig. 1) and migration (Fig. 2) in CAL-27 cells. In addition, gefitinib in combination with EGCG synergistically attenuated enzymatic activity and protein level of MMP-2 (Fig. 3). Importantly, EGCG may enhance gefitinib-suppressed phosphorylation of EGFR in CAL-27 cells *in vitro*. With the results presented in this study, the combination of EGCG and gefitinib may be considered a useful strategy to pursue in clinical trials.

Our earlier study discovered that EGCG induced apoptosis through death-receptor, mitochondrial and ER stress pathways in human adrenal tumor NCI-H295 cells (59). We also found that EGCG-provoked apoptotic death in TSGH-8301 cells was mediated through targeting AKT and HSP27 and modulating p-BAD, activating the intrinsic apoptotic cascade pathway (60). The previous study reported that EGCG and EGFR inhibitors induce apoptosis in a number of cancer types, including HNSCC (58). Many studies have also provided related evidence that EGCG has the potential to reverse the process of carcinogenesis in HNSCC patients and targeted multiple signaling pathways (such as EGFR, IGF-1R, VEGFR, MAPKs, AKT and PKC pathways) resulting in the inhibition of cell metastasis (35,36). Our results showed that combined exposure to EGCG and gefitinib suppressed the protein expression of p-EGFR and inhibited the phosphorylated protein levels of ERK, JNK, p38 and AKT associated with metastatic actions on CAL-27 cells (Fig. 4). The combined effects of EGCG and gefitinib altered the anti-metastatic responses of related gene expression as observed using DNA microarray analysis. Our results from the DNA microarray analysis demonstrated that the mRNA levels of *ErbB2*, *SOS*, *FAK1*, *JNK1*, *MEK2*, *MKP-2*, *EGFR*, *STAT3*, *ERK1/2*, *JAK2*, *AKT*, *AKT*, *PKC-α*, *JAK1*, *MMP-2*, *PI3K cat class IA*, *JNK2* were downregulated in treated CAL-27 cells (Table I). Our results suggest that EGCG may enhance gefitinib-suppressed phosphorylation of EGFR in HNSCC CAL-27 cells *in vitro*. EGCG may be developed as a new class of chemopreventive or chemo-therapeutic agent for HNSCC.

In conclusion, EGCG exhibited a synergistic anti-metastatic activity when combined with gefitinib. In addition to targeting the common EGFR downstream signaling pathways, our study suggested novel mechanisms by which the combination of gefitinib and EGCG results in the depletion of EGFR and ultimately decreases both total and activated EGFR levels. Our results provide a promising regimen for future chemoprevention and treatment of HNSCC.

Acknowledgements

We thank the grant-in-aid DMR-101-023 from the China Medical University Hospital. This study was also supported by the grant NSC-101-2313-B-039-008 from the National Science Council, Republic of China (Taiwan).

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