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Activation of EGFR Promotes Squamous Carcinoma SCC10A Cell Migration and Invasion Via Inducing EMT-Like Phenotype Change and MMP-9-Mediated Degradation of E-Cadherin

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ABSTRACT

EGFR is a potent stimulator of invasion and metastasis in head and neck squamous cell carcinomas (HNSCC). However, the mechanism by which EGFR may stimulate tumor cell invasion and metastasis still need to be elucidated. In this study, we showed that activation of EGFR by EGF in HNSCC cell line SCC10A enhanced cell migration and invasion, and induced loss of epitheloid phenotype in parallel with downregulation of E-cadherin and upregulation of N-cadherin and vimentin, indicating that EGFR promoted SCC10A cell migration and invasion possibly by an epithelial to mesenchymal transition (EMT)-like phenotype change. Interestingly, activation of EGFR by EGF induced production of matrix metalloproteinase-9 (MMP-9) and soluble E-cadherin (sE-cad), and knockdown of MMP-9 by siRNA inhibited sE-cad production induced by EGF in SCC10A. Moreover, both MMP-9 knockdown and E-cadherin overexpression inhibited cell migration and invasion induced by EGF in SCC10A. The results indicate that EGFR activation promoted cell migration and invasion through inducing MMP-9-mediated degradation of E-cadherin into sE-cad. Pharmacologic inhibition of EGFR, MEK, and PI3K kinase activity in SCC10A reduced phosphorylated levels of ERK-1/2 and AKT, production of MMP-9 and sE-cad, cell migration and invasion, and expressional changes of EMT markers (E-cadherin and N-cadherin) induced by EGF, indicating that EGFR activation promotes cell migration and invasion via ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways. Taken together, the data suggest that EGFR activation promotes HNSCC SCC10A cell migration and invasion by inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin into sE-cad related to activation of ERK-1/2 and PI3K signaling pathways. J. Cell. Biochem. 112: 2508–2517, 2011.

KEY WORDS: EGFR; HNSCC; INVASION; MMP-9; E-CADHERIN

Head and neck squamous cell carcinoma (HNSCC) is an insidious, life threatening malignant epithelial neoplasm. It is the sixth most common cancer in the world [Schaaij-Visser et al., 2009], and includes epithelial malignancies of the oral cavity, pharynx, and larynx. Despite advances in our understanding and treatment of this disease, the 5-year survival rates have not improved significantly in the last 50 years [Swango, 1996; Grandis et al., 2004]. HNSCC is characterized by a highly invasive and

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metastatic malignancy [Nieuwenhuis et al., 2003; Schaaij-Visser et al., 2009; Kupferman et al., 2011]. Therefore, understanding of underlying mechanism that controls the HNSCC invasive and metastatic behavior is of pronounced importance.

Epidermal growth factor receptor (EGFR), a member of family of ErbB receptors, is a transmembrane glycoprotein consisting of an extracellular ligand binding domain, a transmembrane region, and an intracellular protein tyrosine kinase domain. The natural ligands EGF and TGF-α bind to the extracellular domain of EGFR, and activate the receptor and its downstream signal transduction pathways such as ERK-1/2, PI3K and Stat3 pathways, ultimately causing activation or modulation of various cellular processes. Over-expression of EGFR is common in the HNSCC [Thomas et al., 2003; Kalyankrishna and Grandis, 2006; Sheikh Ali et al., 2008]. The increased expression of the receptor is often associated with the increased amount of its ligands-EGF and TGF-α production in the HNSCC cells [Kalyankrishna and Grandis, 2006; Oc et al., 2000], which forms an autocrine stimulatory pathway in HNSCC. Moreover, overexpression of EGFR in primary tumors was associated with the tumor invasion, metastasis, recurrence, and poor survival in the patients with HNSCC [Mantha et al., 2003; Reuter et al., 2007; Valkova et al., 2010]. Recent data have proposed EGFR as a new target for anti-HNSCC therapy [Hoffmann et al., 1997; Le Tourneau et al., 2007; Chen et al., 2010; Fung and Grandis, 2010]. These studies suggest that EGFR plays a crucial role in the invasion and metastasis of HNSCC. However, the mechanism of EGFR-stimulated HNSCC cell invasion and migration still need to be elucidated.

Many different processes are involved in tumor cell invasion and metastasis such as an epithelial to mesenchymal transition (EMT), adhesion molecules downregulation, and matrix metalloproteinases (MMPs) upregulation in cancer cells. EMT frequently occurs during epithelial tumor progression to more aggressive metastatic tumors, and loss of epithelial protein marker E-cadherin and the concurrent upregulation of mesenchymal protein markers N-cadherin and vimentin are important cellular events observed during EMT [Bergers et al., 2000; Kang and Massague, 2004; Natalwala et al., 2008]. E-cadherin, an EMT marker, is a major cell–cell adhesion molecule in epithelial cells that functions as a tumor suppressor [Berx et al., 1998]. E-cadherin expression is frequently down-regulated in many different types of tumors including HNSCC [Eriksen et al., 2004; Marsit et al., 2008], and loss of its expression or function diminishes cell–cell contacts and contributes to tumor invasion and metastasis [Frixen et al., 1991; Vleminckx et al., 1991]. Matrix metalloproteinases (MMPs) are a family of metalloendopeptidases that cleave the protein components of the extracellular matrix (ECM) and endothelial cell basement membrane [Stamenkovic, 2003]. Most of the MMPs are synthesized as inactive latent enzymes and conversion to the active enzyme is generally mediated by activator systems [Malemud, 2006]. MMPs play a central role in tumor invasion and metastasis by the degradation of ECM and endothelial cell basement membrane. MMP-9 (gelatinase B), a member of MMPs family, has been found in large quantities in cancer tissues, and correlated with the processes of tumor invasion and metastasis in human cancers including HNSCC [Libra et al., 2009]. Furthermore, previous study reported that MMPs could degrade cell surface associated molecules [Stamenkovic, 2000], and MMP-9 could cleave E-cadherin ectodomain near plasma membrane into sE-cad [van Roy and Berx, 2008], which may be involved in MMPs-enhanced invasive and metastatic potentials of cancer cells.

In this study, we aimed to investigate the mechanism of EGFR-enhanced tumor migration and invasion in HNSCC cell line SCC10A. We found that EGFR activation promotes SCC10A cell migration and invasion by inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin into sE-cad related to activation of ERK-1/2 and PI3K signaling pathways in HNSCC SCC10A cells.

MATERIALS AND METHODS

MATERIALS

The following antibodies were used: E-cadherin and GAPDH (Santa Cruz Biotechnology), soluble E-cadherin and N-cadherin (Zymed), Vimentin (Epitomics), α-tubulin (Sigma), MMP-9, EGFR, p-EGFR, AKT, p-AKT, ERK-1/2, and p-ERK-1/2 (Cell Signaling Technology). Anti-mouse and anti-rabbit secondary antibodies, conjugated to horseradish peroxidase for Western blotting or conjugated to fluorescein isothiocyanate for immunofluorescent staining, were obtained from Vector Laboratories. MMP-9 siRNA and scrambled siRNA come from Santa Cruz Biotechnology. Pharmacological inhibitor of EGFR (AG1478) was from Calbiochem, pharmacological inhibitor of MEK-1/2 (U0126) was from Promega, and pharmacological inhibitor of PI3K (LY294002) was bought from Cell Signaling Technology.

CELL LINE AND CULTURE

Head and neck squamous cell carcinoma (HNSCC) cell line SCC10A was derived from the primary lesion of a larynx carcinoma, and has been extensively characterized for its in vitro and in vivo phenotypes [Momose et al., 1989; Jetten et al., 1990; Ballo et al., 1999]. Cells were normally maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

MIGRATION AND INVASION ASSAY

In vitro cell migration assay was performed in 24-well transwell chambers (Costar) as previously described by Chen et al. [2008]. Briefly, the upper and lower culture compartments of each well in the transwell chambers are separated by polycarbonate membranes (8 μm pore size). Briefly, 2×10^5 cells in 0.5 mL of serum-free DMEM medium were added on the upper compartment, and 0.75 mL of serum-free DMEM medium containing 0, 10, or 20 ng/mL EGF (Sigma) was placed into the lower compartment. Cells were incubated for 4 h at 37°C, fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Non-migrating cells retained on the upperside of the membrane were removed by wiping with a cotton swab. Cells that had migrated through the membrane and had reached the underside of the membrane were counted in 10 randomly chosen microscopic fields using a 20× objective. For in vitro cell invasion assays, the polycarbonate membranes were coated with Matrigel (1 mg/mL) (BD Biosciences) and allowed gel to dry at 37°C for 2 h. Next, 2×10^5 cells in 0.5 mL of DMEM were added on the upper compartment, and 0.75 mL of DMEM medium

containing 0, 10, or 20 ng/mL EGF was placed into the lower compartment. The cells were incubated for 24 h at 37°C, fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Non-invasive cells retained on the upperside of the membrane were removed by wiping with a cotton swab. Cells that had penetrated the Matrigel gel and had reached the underside of the membrane were counted in 10 randomly chosen microscopic fields using a 20× objective.

Time-lapse video microscopy was also performed to detect cell motility as previously described by us with slight modifications [Zhou and Kramer, 2005; Zuo et al., 2010]. Briefly, cells pretreated with 0 or 10 ng/mL EGF were seeded onto 6-well plates (Falcon), coated with Col-I (1 μg/mL), and maintained at 37°C and 5% CO_2. Plates were then examined in a Zeiss Axiovert inverted microscope and cell velocity was determined as previously described [Zhou and Kramer, 2005; Zuo et al., 2010].

CELL ADHESION ASSAY

Cell adhesion assay was performed as previously described with slight modifications [Lin et al., 2007]. Briefly, cells were cultured for 24 h in serum-free DMEM medium, and were pretreated with 5 μM AG14780 for 2 h, followed by incubation with 0, 10, or 20 ng/mL EGF for 8 h. Then cells were detached using 0.0125% trypsin (Promega) and 4 mM of EDTA solution in PBS. The cells were allowed to attach to 96-well culture plates (2×10^4/well) precoated with fibronectin (10 μg/mL; Sigma), laminin-1 (10 μg/mL; Sigma) or Col-I (10 μg/mL; Invitrogen) for 45 min, and then gently washed with PBS to remove non-adhering cells. The adherent cells were fixed using a 2% paraformaldehyde and stained with 0.1% crystal violet. A microplate reader was used to measure the bound dye (adherent cells) after eluting with 2% SDS at a wavelength of 560 nm. Three independent experiments were performed in triplicate.

IMMUNOFLUORESCENT STAINING

Cells were plated into chamber slides (Nalge Nunc International) for 24 h, treated with 0, 10, or 20 ng/mL EGF for 8 h, and then rinsed twice with 50 mM/L TBS (pH7.6). After fixation with 4% paraformaldehyde for 10 min and then permeabilization with 0.5% Triton X-100 in PBS for 10 min, cells were incubated with anti-E-cadherin monoclonal antibody for 1 h at RT, followed by incubation with fluorescein isothiocyanate (FITC) conjugated-goat anti-mouse antibodies for 1 h at RT. After washing in PBS, slides were mounted with Vectashield (Vector) and viewed using a Nikon fluorescence microscope or a laser scanning confocal microscope (Bio-Rad Laboratories).

REAL-TIME QUANTITATIVE PCR

Real-time quantitative PCR was performed to detect the mRNA expression of MMP-9 on an Applied Biosystem 7000 Sequence Detection System (Applied Biosystems). Briefly, cells cultured for 48 h in growth medium were serum starved 24 h, and then treated with or without 10 ng/mL EGF for 8 h before RNA extraction (Qiagen RNeasy MiniKit). Five micrograms of total RNA from each sample was reverse transcribed to cDNA using A3500 reverse transcription system (Promega). The following forward and reverse primers were used: MMP-9, 5′-ctctgctcctcctgttcgac-3′, 5′-ttgattttggagggatctcg-3′; and GAPDH, 5′-tggcagagatgcgtggaga-3′, 5′-ggcaagtcttccgag-tagtttt-3′. The PCR conditions used were as follows: 5-min denaturation at 95°C followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Amplification of the target gene was monitored as a function of increased SYBR green I fluorescence. An analysis threshold was set, and the cycle threshold (Ct) was computed for each sample. The comparative difference in gene expression was then determined.

WESTERN BLOTTING AND GELATINOLYTIC ZYMOGRAPHY

Cells were cultured in the complete DMEM medium for 24 h, then was washed with PBS and treated with 0, 10, or 20 ng/mL EGF for 8 h. Cells were extracted using lysis buffer [50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L $MgCl_2$, and complete protease inhibitor mixture (Roche Molecular Biochemicals)]; protein concentrations were measured with the bicinchoninic acid protein assay kit (Pierce) and processed for SDS-PAGE. After transferring onto nitrocellulose membranes (Millipore Corp.), proteins were probed with primary antibodies and secondary horseradish peroxidase-coupled antibodies. Blots were developed by chemiluminescence using the enhanced chemiluminescence system (Amersham Biosciences). Gelatinolytic zymography of MMP-9 activity in the conditioned medium was done in 7.5% (w/v) SDS-polyacrylamide gels containing 2.56 mg/mL gelatin under nonreducing conditions as previously described by us [Zuo et al., 2010].

TRANSIENT TRANSFECION

The cells were transfected with MMP-9 siRNA or control siRNA according to the siRNA transfection protocol provided by the manufacturer. Briefly, the day before transfection, SCC10A cells were plated into 6-well plates at the density of 10^5 cells/mL in DMEM medium containing 10% FBS. When the cells were 60–80% confluent, they were transfected with 10 nmol/L of MMP-9 siRNA or control siRNA after a preincubation for 20 min with siRNA transfection reagent in siRNA transfection medium (Santa Cruz Biotechnology). Four hours after the beginning of the transfection, the medium was replaced with in DMEM medium containing 10% FBS and continued to culture the cells for an additional 44 h. At the end of the transfection, MMP-9 expression level in the cells was determined by Western blot. In addition, E-cadherin expression vector pcDNA3.0-E-cadherin and control vector pcDNA3.0 [Yang et al., 2010], kindly provided by Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, were transfected into SCC10A cells with Lipofectamine 2000 reagent (Invitrogen). Fourty-eight hours after the beginning of the transfection, E-cadherin expression level in the cells was determined by Western blot.

STATISTICAL ANALYSIS

Student *t*-tests were used for statistical analysis of interval data with *P*-values less than 0.05 considered to be significant.

RESULTS

EGFR ACTIVATION PROMOTES SCC10A CELL MIGRATION AND INVASION

To investigate the role of EGFR in the migration and invasion of HNSCC, we took the approach of activating EGFR by EGF in the HNSCC cell line SCC10A, which normally expresses high levels of EGFR. Initially, we examined whether EGFR activation could potentiate cell migration by a transwell chamber. As shown in Figure 1A, the cells with EGF treatment migrated faster than the control cells. Next, cell motility was studied by time-lapse video microscopy. Compared with the control cells, the cells with EGF treatment significantly increased their velocity on Col-I substrate (Fig. 1B, left). Cell trace analysis depicting the movement of individual cells also revealed a significant increase in cell motility in the EGF-treated cells versus the control cells on Col-I substrate (Fig. 1B, right). We next analyzed whether EGF treatment could potentiate the cell invasive capability through Matrigel by a transwell chamber. As shown in Figure 1C, the cells with EGF treatment significantly increased their invasive ability as compared to the control cells. Furthermore, we detected SCC10A adhesive capacity on the different substrates (Ln-1, Fn, and Col-I)-coated culture plates, which is related to cell migration and invasion. The results showed that the EGF treatment significantly reduced cell adhesion to the substrates in dose dependent manner, and EGFR inhibitor AG1478 could inhibit the decreased cell adhesion caused by EGF treatment (Fig. 1D), indicating that EGFR activation decreased cell adhesion in HNSCC cells. These data suggest that the activation of EGFR by EGF promotes SCC10A cell migration and invasion.

EGFR ACTIVATION INDUCES CHANGE OF EMT-LIKE PHENOTYPE IN SCC10A CELLS

SCC10A cells without EGF treatment grown in culture at high densities showed classic epitheloid morphology, including growth in islands of closely apposed cells with cobblestone appearance. In contrast, the cells with the EGF treatment showed a scattered phenotype, losing their close cell–cell junctions and becoming spindle-shaped (Fig. 2A). These morphologic changes are reminiscent of cells of the mesenchymal lineage. To further evaluate the above observation, we wondered whether the expressions of critical epithelial protein E-cadherin as well as mesenchymal protein N-cadherin and vimentin, EMT markers, were altered in the EGF-stimulated cells. Both Western blot and/or immunocytofluorescent staining showed that activation of EGFR by EGF significantly down-regulated E-cadherin, and up-regulated N-cadherin and vimentin in the SCC10A cells in a dose dependent manner (Fig. 2B and C). As the induction of N-cadherin and vimentin with the concurrent attenuation of E-cadherin is an important cellular event observed during EMT, EGFR-activated SCC10A cells might occur EMT. Moreover, EMT frequently occurs during epithelial tumor progression to more aggressive metastatic tumors, thus activation of EGFR by EGF promotes the cell migration and invasive capability possibly by EMT in SCC10A cells.

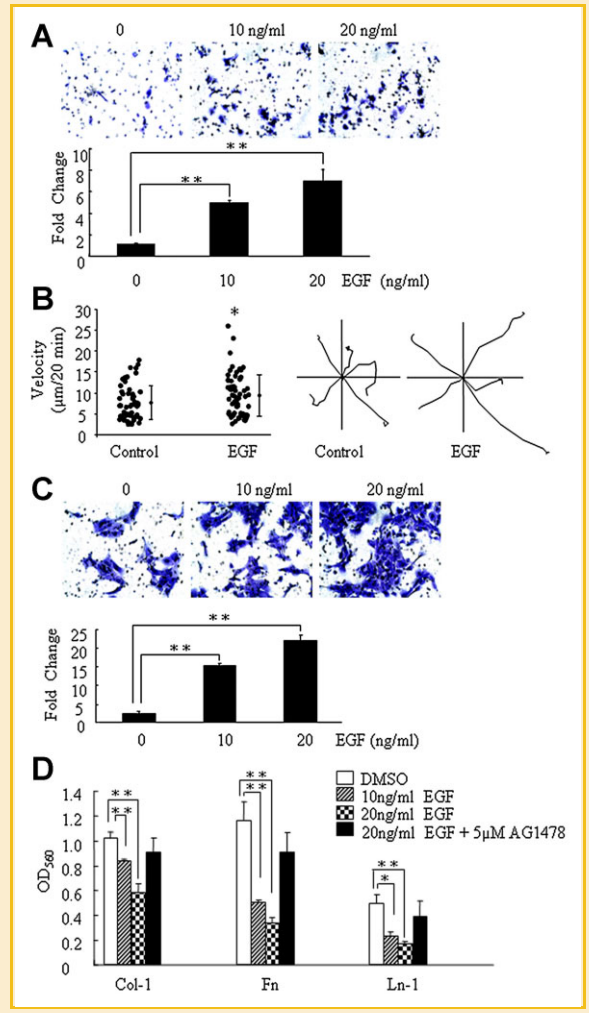

Fig. 1. Activation of EGFR by EGF promotes cell migration and invasion and reduces cell adhesion in SCCA10 cells. A: The EGF-stimulated SCCA10 cells and control cells were subjected to a transwell migration assay. The cells that migrated through the membranes were stained with 0.1% crystal violet and then representative photomicrographs were taken (top; original magnification × 200). The number of cells that migrated through the transwell membranes was estimated by counting at least 10 random microscopic fields (bottom). Columns, mean; bars, SD ($^{**}P < 0.001$). B: Cell velocity on Col-I-coated substrates was measured by time-lapse video microscopy (left; $^{*}P < 0.05$). Representative cell tracks on Col-I substrate are shown (right). C: The EGF-stimulated SCCA10 cells and control cells were subjected to a transwell invasion assay using the membranes coated with Matrigel. The cells that invaded through the membranes were stained with 0.1% crystal violet and then representative photomicrographs were taken. The cells that invaded (top; original magnification × 200). The number of cells that migrated through the transwell membranes was estimated by counting at least 10 random microscopic fields (bottom). Columns, mean; bars, SD ($^{**}P < 0.001$). D: The EGF-stimulated SCCA10 cells and control cells were plated on the different ECM proteins (collagen I, laminin, and fibronectin), and subjected to a cell adhesion assay as described in the Materials and Methods. The adherent cells were fixed using a 2% paraformaldehyde and stained with 0.1% crystal violet. A microplate reader was used to measure the bound dye (adherent cells) after eluting with 2% SDS at a wavelength of 560 nm. Three independent experiments were performed in triplicate. Columns, mean; bars, SD ($^{*}P < 0.05$, $^{**}P < 0.001$).

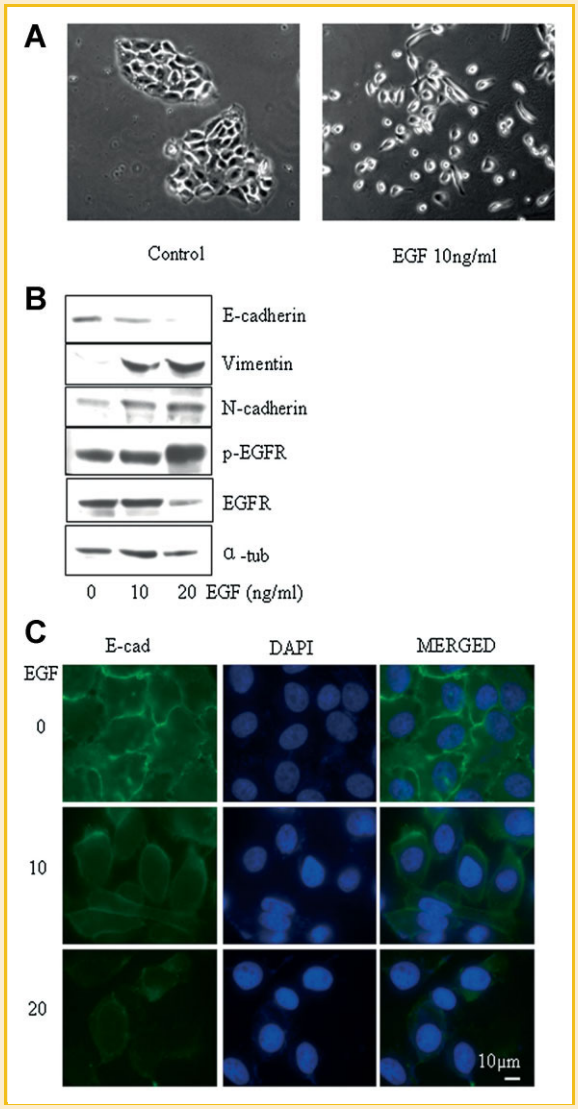

Fig. 2. Activation of EGFR by EGF induces an EMT-like phenotype change in SCCA10 cells. A: Representative phase-contrast images of control and EGF-stimulated SCC10A cells. EGF-stimulated cells show an EMT-like morphological change. B: A representative result of Western blot analysis shows the effect of EGFR activation on the expressions of E-cadherin, vimentin, and N-cadherin in the SCC10A cells. α-tublin serves as a loading control. C: A representative result of immunofluorescent staining shows the effect of EGFR activation by EGF on the E-cadherin expression in the SCC10A cells.

EGFR ACTIVATION PROMOTES CELL MIGRATION AND INVASION BY MMP-9-MEDIATED DEGRADATION OF E-CADHERIN INTO SE-CAD IN SCC10A CELLS

Previous studies have reported that MMPs could degrade cell surface associated molecules including E-cadherin. Therefore, we wondered whether EGFR activation lead to the degradation of E-cadherin into sE-cad in SCC10A cells by MMP-9. We first confirmed the induction of MMP-9 mRNA expression by EGFR activation using real-time quantitative PCR. As shown in Figure 3A, the level of MMP-9 mRNA expression in the EGF-treated SCC10A cells was enhanced by more than 20-fold compared with the control cells. We next detected the levels of MMP-9 and sE-cad proteins in the media from the EGF-treated cells and control cells by gelatinolytic zymography and Western blot, respectively. The results showed that EGFR activation increased the production of MMP-9 and sE-cad in the medium of SCC10A in a dose dependent manner (Fig. 3B). To validate MMP-9-mediated degradation of E-cadherin into sE-cad in the EGF-treated cells, we examined whether knockdown of MMP-9 by siRNA blocked degradation of E-cadherin into sE-cad. As shown in Figure 3C and D, knockdown of MMP-9 inhibited the expression of MMP-9 and degradation of E-cadherin into sE-cad induced by EGF, indicating that MMP-9 mediated degradation of E-cad into sE-cad induced by EGFR activation in SCC10A cells.

To examine if the increased migratory and invasive potentials of EGF-activated cells was related to MMP-9-mediated degradation of E-cadherin into sE-cad, we analyzed the effect of MMP-9 knockdown and E-cadherin overexpression on the cell migration and invasion in SCC10A cells. The results showed that both MMP-9 knockdown and E-cadherin overexpression reduced SCC10A cell migration and invasion (Figs. 3E and F and 4) owning to the activation of EGFR, which suggests that EGFR activation promoted cell migration and invasion through MMP-9-mediated degradation of E-cadherin into sE-cad in SCC10A cells.

EGFR ACTIVATION PROMOTES CELL MIGRATION AND INVASION THROUGH ERK-1/2 AND PI3K-REGULATED MMP-9/E-CADHERIN SIGNALING PATHWAYS IN SCC10A CELLS

To further define the mechanism of the increased cell migration and invasion by EGFR activation, we examined the activity of ERK-1/2 and PI3K of critical EGFR downstream signaling components known to regulate cell migration and invasion in EGFR-activated SCC10A cells. We found that phosphorylated levels of EGFR, ERK-1/2, and AKT were increased in the EGFR-activated cells compared with the control cells, and EGFR inhibitor AG1478, MEK inhibitor U0126 and PI3K inhibitor LY294002 inhibited phosphorylation of EGFR, ERK, and AKT induced by EGF, respectively (Fig. 5A). Moreover, AG1478, U0126, or LY294002 inhibited production of MMP-9 and sE-cad, and expressional change of EMT markers (E-cadherin and N-cadherin) induced by EGF (Fig. 5B). The above results indicated that EGFR activation induced degradation of E-cadherin into sE-cad and EMT-like cell phenotype change by ERK-1/2 and PI3K-regulated MMP-9 signaling pathways. Next, we tested whether ERK-1/2 and PI3K signaling pathways were contributing to the increased migration and invasion of EGFR-activated SCC10 cells. As expected, AG1478, U0126, or LY294002 could inhibit cell migration (Fig. 5C) and invasion (Fig. 5D) induced by EGF. Taken together, the data suggest that EGFR activation promotes cell migration and invasion via ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways in SCC10A cells.

DISCUSSION

The results presented here identify the potential mechanisms by which EGFR activation can lead to the enhanced migration and invasion potentials of HNSCC SCC10A cells. We show that EGFR activation (a) enhances cell migration and invasion, (b) induces an

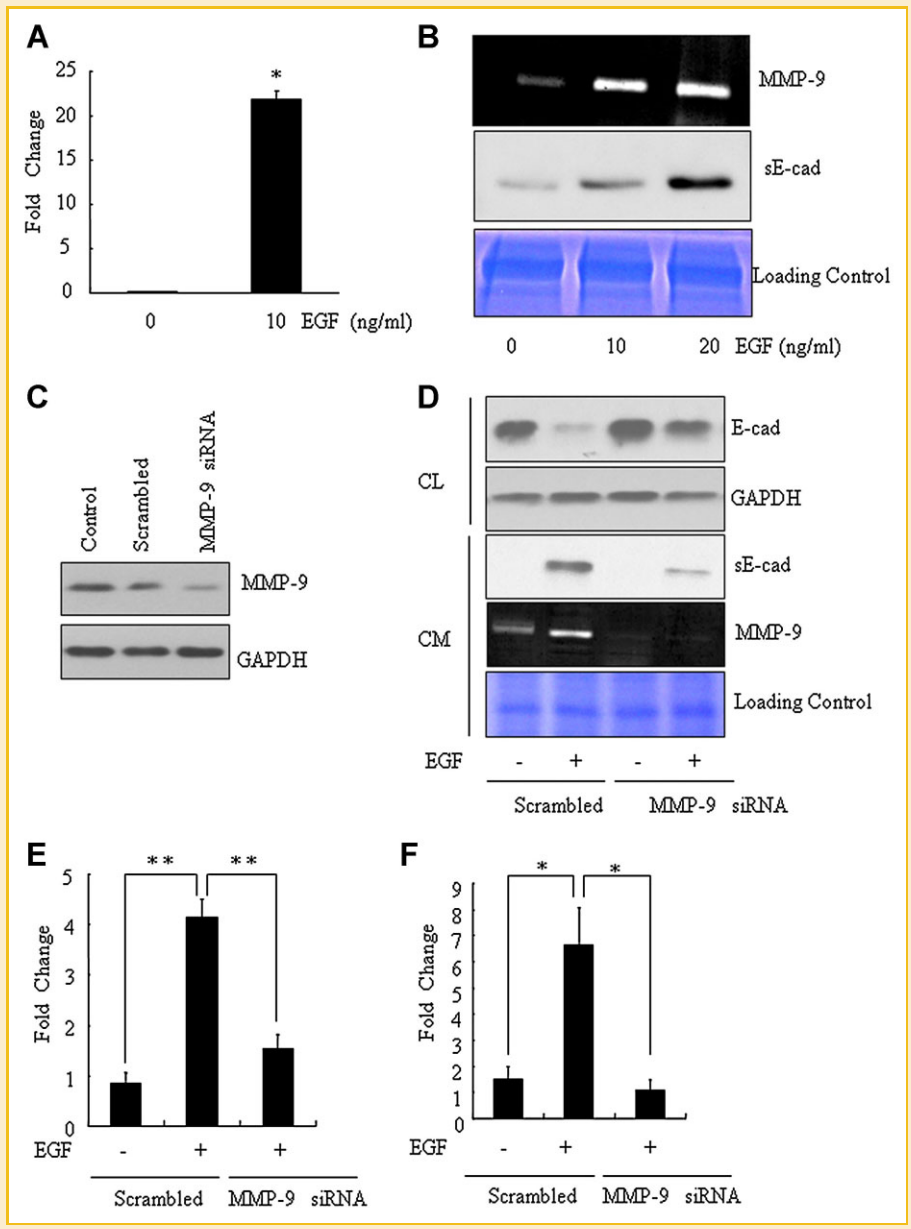

Fig. 3. EGFR activation promoted cell migration and invasion through inducing MMP-9-mediated degradation of E-cadherin into sE-cad in SCCA10 cells. A: Real-time PCR shows the expression of MMP-9 mRNA in the control and EGF-stimulated SCC10A cells. B: A representative result of gelatinolytic zymography shows MMP-9 activity in the conditioned medium from control and EGF-stimulated SCCA10A cells (top). A representative result of Western blot analysis shows the expression levels of sE-cad in the conditioned medium from control and EGF-stimulated SCC10A cells (middle). Loading control, nontarget protein bands in Coomassie blue staining-gel before transferring to a nitrocellulose membrane (bottom). C: Western blot analysis shows the expression levels of MMP-9 in MMP-9 siRNA and scrambled siRNA transfected SCC10A cells and control cells. D: The transfected cells were treated with 10 ng/mL EGF, and cell lysate (CL) or conditioned medium (CM) were used to detect the expression of E-cadherin, sE-cad, and MMP-9 by Western blot or gelatinolytic zymography. Loading control, nontarget protein bands in Coomassie blue staining-gel before transferring to a nitrocellulose membrane. E: The transfected cells were treated with 10 ng/mL EGF, and then cells were subjected to a transwell migration assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^{**}P < 0.001$). F: The transfected cells were treated with 10 ng/mL EGF, and then cells were subjected to a transwell invasion assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^{*}P < 0.05$).

EMT-like cell phenotype change, (c) promotes the production of MMP-9 and MMP-9-mediated degradation of E-cadherin into sE-cad through ERK-1/2 and PI3K signaling pathways, and (4) induces cell migration and invasion via ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways. These findings indicate that EGFR activation promotes the migration and invasion potentials of HNSCC SCC10A cells by an EMT-like cell phenotype change and MMP-9-mediated degradation of E-cadherin into sE-cad related to activation of ERK-1/2 and PI3K signaling pathways.

HNSCC usually highly expresses EGF and EGFR compared with normal squamous epithelium, which forms an autocrine stimulatory pathway in HNSCC [Timpson et al., 2007; Egloff et al., 2009].

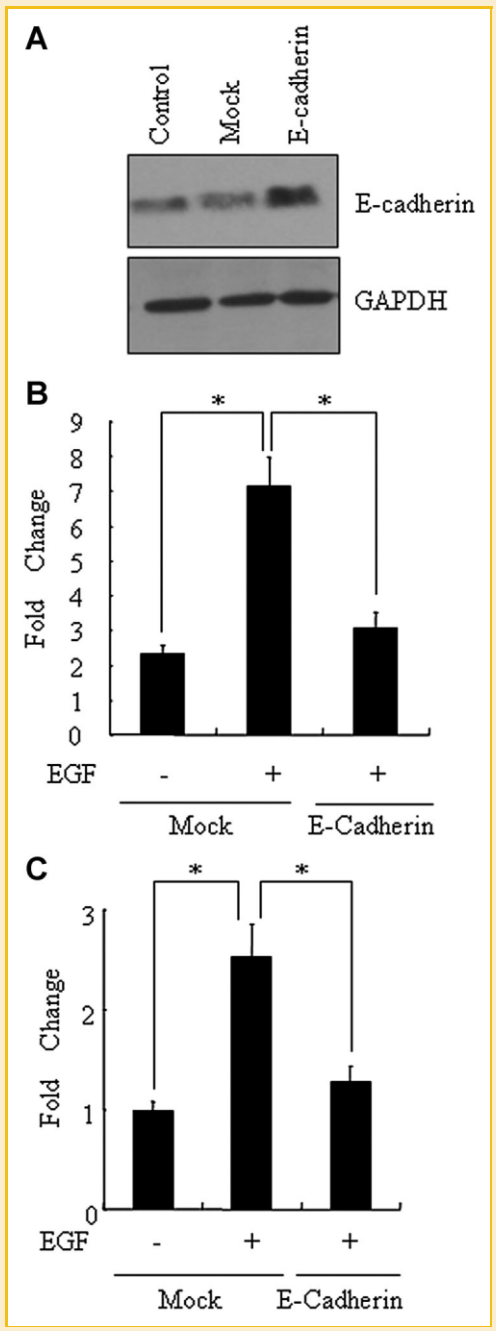

Fig. 4. E-cadherin overexpression inhibits cell migration and invasion induced by EGFR activation in SCCA10 cells. A: Western blot analysis shows the expression levels of E-cadherin in E-cadherin expression vector and empty vector transfected SCC10A cells and control cells. B: The transfected cells were treated with 10 ng/mL, and then cells were subjected to a transwell migration assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^{**}P < 0.001$). C: the transfected cells were treated with 10 ng/mL EGF, and then cells were subjected to a transwell invasion assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^{*}P < 0.05$).

Previous studies have shown that EGFR overexpression in HNSCC was associated with the tumor invasion, metastasis, recurrence and poor survival [Bernier et al., 2009; Egloff et al., 2009]. However, the mechanism by which EGFR may stimulate tumor cell invasion and metastasis is still largely unknown. Our study shows that EGFR activation enhanced cell migratory and invasive potentials and leaded to lose epitheloid morphology in HNSCC SCC10A cells. Moreover, we found that EGFR activation decreased cell adhesion, down-regulated epithelial protein E-cadherin, and upregulatded mesenchymal proteins N-cadherin and vimentin in HNSCC SCC10A cells, which are important cellular events observed during EMT. These results indicate that EGFR activation enhanced the invasion and metastatic potentials of HNSCC SCC10A cells possibly by EMT-like phenotype change.

E-cadherin, an epithelial specific protein marker, can retain an epithelial phenotype and inhibit invasion and metastasis in various tumor cell lines and tumor models in vivo, whereas loss of its expression or function diminishes cell–cell adhesion and leads to high tumor invasion and metastasis [Schmalhofer et al., 2009]. It has been reported that E-cadherin expression is frequently down-regulated or lose in many different types of tumors including HNSCC [Eriksen et al., 2004; Marsit et al., 2008; van Roy and Berx, 2008]. In our study, E-cadherin expression significantly decreased in the EGFR-activated cells, which might be associated with decreased cell adhesion, an EMT-like morphologic change and enhanced invasion and migration potentials of HNSCC SCC10A cells.

Proteolytic degradation of ECM and endothelial cell basement membrane by MMPs represents an important component of the invasion-metastasis cascade [Kohrmann et al., 2009; Libra et al., 2009]. In particular, MMP-9, which is expressed by many types of human carcinomas including HNSCC, has been closely associated with tumor invasion and metastasis [Kohrmann et al., 2009; Libra et al., 2009; Rosenthal and Matrisian, 2006]. Previous studies have reported that MMP-9 cleaves E-cadherin ectodomain into sE-cad [van Roy and Berx, 2008]. Our results showed that EGFR activation increased the production of MMP-9 and sE-cad in the SCC10A cells, and knockdown of MMP-9 by siRNA not only inhibited the expression of MMP-9 induced by EGF stimulation, but also decreased EGF-dependent degradation of E-cadherin into of sE-cad, which validated that MMP-9 mediated EGF-dependent degradation of E-cadherin into sE-cad. Moreover, both knockdown of MMP-9 and E-cadherin overexpression reduced the cell migration and invasion owning to the activation of EGFR. The results suggested that EGFR activation promoted cell migration and invasion through inducing MMP-9-mediated degradation of E-cadherin into sE-cad in the HNSCC SCC10A cells.

EGF binds to EGFR, and activates the receptor and its downstream signal pathways such as ERK-1/2, PI3K and Stat3 pathways, ultimately causing modulation of various cellular processes. Activation of ERK-1/2 and PI3K in tumor cells has been shown to be associated with enhanced MMP-9 expression and induction of invasive capacity [Thant et al., 2000; Ruhul et al., 2003; Chinni et al., 2006; Hulit et al., 2007; Lungu et al., 2008; Sen et al., 2011]. To further define the mechanism of the increased migration and invasion by EGFR activation, we examined whether EGFR activation regulates cell migration and invasion in the SCC10A by ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways. We found that phosphorylation of ERK-1/2 and AKT and production of MMP-9 and sE-cad were increased in the EGFR activated cells, and EGFR inhibitor AG1478, MEK inhibitor U0126 and PI3K inhibitor

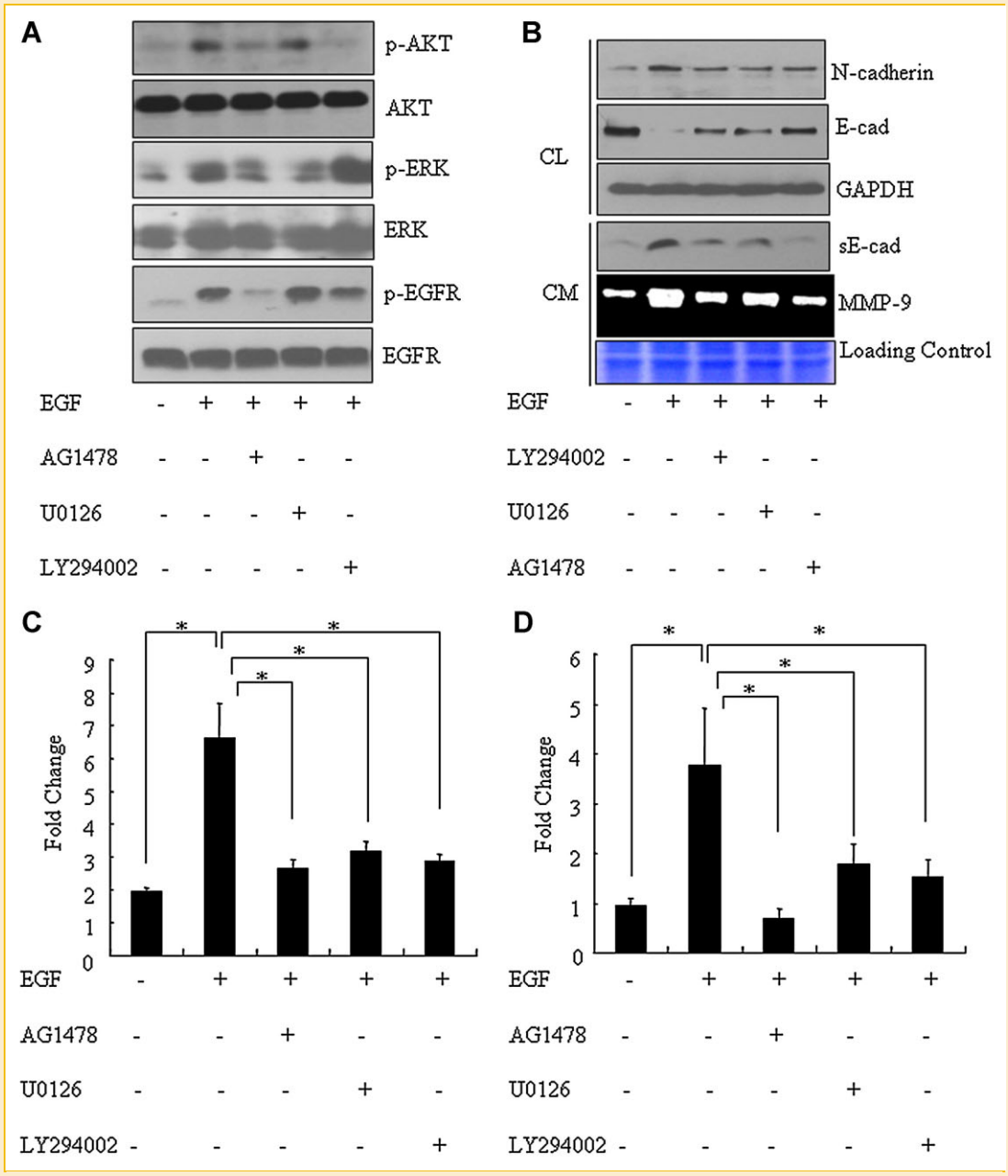

Fig. 5. EGFR activation promotes the cell migration and invasion via ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways in SCCA10 cells. A: Cell extracts were prepared from SCC10A cells in the presence and absence of 10 ng/mL EGF, as well as in the EGF stimulation combined with the pretreatment of 5 μmol/L AG1478 or U0126 or LY294002, and were used to detect the expressions of total ERK1/2, p-ERK1/2, total AKT, p-AKT, total EGFR, and p-EGFR by Western blot. B: Cell lysates (CL) and conditioned medium (CM) were prepared from SCC10A cells in the presence and absence of 10 ng/mL EGF, as well as in the EGF stimulation combined with the pretreatment of 5 μmol/L AG1478 or U0126 or LY294002, were used to detect the expressions of N-cadherin, E-cadherin and sE-cad by Western blot, and MMP-9 activity by gelatinolytic zymography. Loading control, nontarget protein bands in Coomassie blue staining-gel before transferring to a nitrocellulose membrane. C: Cells were incubated in the presence and absence of 10 ng/mL EGF, as well as in the EGF stimulation combined with the pretreatment of 5 μmol/L AG1478 or U0126 or LY294002, and then cells were subjected to a transwell migration assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^*P < 0.05$). D: Cells were incubated in the presence and absence of 10 ng/mL EGF, as well as in the EGF stimulation combined with the pretreatment of 5 μmol/L AG1478 or U0126 or LY294002, and then cells were subjected to a transwell invasion assay as described in the "Materials and Methods." Columns, mean; bars, SD ($^*P < 0.05$).

LY294002 inhibited phosphorylation of ERK-1/2 and AKT, production of MMP-9 and sE-cad, and expressional change of EMT markers induced by EGFR activation. Moreover AG1478, U0126, and LY294002 could also inhibit the migration and invasion enhanced by EGFR activation in the SCC10A cells. Taken together, the data suggest that EGFR activation promotes HNSCC SCC10A cell migration and invasion via ERK-1/2 and PI3K-regulated MMP-9/E-cadherin signaling pathways.

In summary, the present work identified that EGFR activation promoted SCC10A cell migration and invasion possibly by inducing

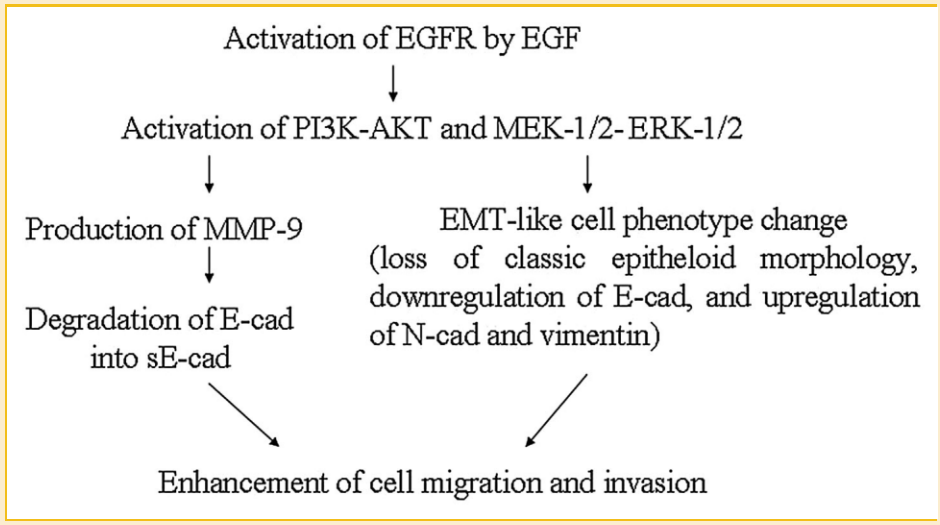

Fig. 6. Schematic representation of activation of EGFR signaling pathways promoting cell migration and invasion in SCCA10 cells.

an EMT-like cell phenotype change and MMP-9-mediated degradation of E-cadherin related to activation of ERK-1/2 and PI3K signaling pathways (Fig. 6). The results will be helpful for elucidating the mechanisms of EGFR-promoting cell invasion and metastasis and finding new molecularly targeted drugs in HNSCC.

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REFERENCES

Ballo H, Koldovsky P, Hoffmann T, Balz V, Hildebrandt B, Gerharz CD, Bier H. 1999. Establishment and characterization of four cell lines derived from human head and neck squamous cell carcinomas for an autologous tumor-fibroblast in vitro model. Anticancer Res 19:3827–3836.

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2:737–744.

Bernier J, Bentzen SM, Vermorken JB. 2009. Molecular therapy in head and neck oncology. Nat Rev Clin Oncol 6:266–277.

Berx G, Becker KF, Hofler H, van Roy F. 1998. Mutations of the human E-cadherin (CDH1) gene. Hum Mutat 12:226–237.

Chen Y, Ouyang GL, Yi H, Li MY, Zhang PF, Li C, Li JL, Liu YF, Chen ZC, Xiao ZQ. 2008. Identification of RKIP as an invasion suppressor protein in nasopharyngeal carcinoma by proteomic analysis. J Proteome Res 7: 5254–5262.

Chen LF, Cohen EE, Grandis JR. 2010. New strategies in head and neck cancer: Understanding resistance to epidermal growth factor receptor inhibitors. Clin Cancer Res 16:2489–2495.

Chinni SR, Sivalogan S, Dong Z, Filho JC, Deng X, Bonfil RD, Cher ML. 2006. CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: The role of bone microenvironment-associated CXCL12. Prostate 66:32–48.

Egloff AM, Rothstein ME, Seethala R, Siegfried JM, Grandis JR, Stabile LP. 2009. Cross-talk between estrogen receptor and epidermal growth factor receptor in head and neck squamous cell carcinoma. Clin Cancer Res 15: 6529–6540.

Eriksen JG, Steiniche T, Sogaard H, Overgaard J. 2004. Expression of integrins and E-cadherin in squamous cell carcinomas of the head and neck. Apmis 112:560–568.

Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeier W. 1991. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 113:173–185.

Fung C, Grandis JR. 2010. Emerging drugs to treat squamous cell carcinomas of the head and neck. Expert Opin Emerg Drugs 15:355–373.

Grandis JR, Pietenpol JA, Greenberger JS, Pelroy RA, Mohla S. 2004. Head and neck cancer: Meeting summary and research opportunities. Cancer Res 64:8126–8129.

Hoffmann T, Hafner D, Ballo H, Haas I, Bier H. 1997. Antitumor activity of anti-epidermal growth factor receptor monoclonal antibodies and cisplatin in ten human head and neck squamous cell carcinoma lines. Anticancer Res 17:4419–4425.

Hulit J, Suyama K, Chung S, Keren R, Agiostratidou G, Shan W, Dong X, Williams TM, Lisanti MP, Knudsen K, Hazan RB. 2007. N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation. Cancer Res 67:3106–3116.

Jetten AM, Kim JS, Sacks PG, Rearick JI, Lotan D, Hong WK, Lotan R. 1990. Inhibition of growth and squamous-cell differentiation markers in cultured human head and neck squamous carcinoma cells by beta-all-trans retinoic acid. Int J Cancer 45:195–202.

Kalyankrishna S, Grandis JR. 2006. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 24:2666–2672.

Kang Y, Massague J. 2004. Epithelial-mesenchymal transitions: Twist in development and metastasis. Cell 118:277–279.

Kohrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. 2009. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer 9:188.

Kupferman ME, Jiffar T, El-Naggar A, Yilmaz T, Zhou G, Xie T, Feng L, Wang J, Holsinger FC, Yu D, Myers JN. 2010. TrkB induces EMT and has a key role in invasion of head and neck squamous cell carcinoma. Oncogene 29:2047–2059.

Le Tourneau C, Faivre S, Siu LL. 2007. Molecular targeted therapy of head and neck cancer: Review and clinical development challenges. Eur J Cancer 43:2457–2466.

Libra M, Scalisi A, Vella N, Clementi S, Sorio R, Stivala F, Spandidos DA, Mazzarino C. 2009. Uterine cervical carcinoma: Role of matrix metalloproteinases (Review). Int J Oncol 34:897–903.

Lin MT, Chang CC, Lin BR, Yang HY, Chu CY, Wu MH, Kuo ML. 2007. Elevated expression of Cyr61 enhances peritoneal dissemination of gastric cancer cells through integrin alpha2beta1. J Biol Chem 282:34594–34604.

Lungu G, Covaleda L, Mendes O, Martini-Stoica H, Stoica G. 2008. FGF-1-induced matrix metalloproteinase-9 expression in breast cancer cells is mediated by increased activities of NF-kappaB and activating protein-1. Mol Carcinog 47:424–435.

Malemud CJ. 2006. Matrix metalloproteinases (MMPs) in health and disease: An overview. Front Biosci 11:1696–1701.

Mantha AJ, McFee KE, Niknejad N, Goss G, Lorimer IA, Dimitroulakos J. 2003. Epidermal growth factor receptor-targeted therapy potentiates lovastatin-induced apoptosis in head and neck squamous cell carcinoma cells. J Cancer Res Clin Oncol 129:631–641.

Marsit CJ, Posner MR, McClean MD, Kelsey KT. 2008. Hypermethylation of E-cadherin is an independent predictor of improved survival in head and neck squamous cell carcinoma. Cancer 113:1566–1571.

Momose F, Araida T, Negishi A, Ichijo H, Shioda S, Sasaki S. 1989. Variant sublines with different metastatic potentials selected in nude mice from human oral squamous cell carcinomas. J Oral Pathol Med 18:391–395.

Natalwala A, Spychal R, Tselepis C. 2008. Epithelial-mesenchymal transition mediated tumourigenesis in the gastrointestinal tract. World J Gastroenterol 14:3792–3797.

Nieuwenhuis EJ, Leemans CR, Kummer JA, Denkers F, Snow GB, Brakenhoff RH. 2003. Assessment and clinical significance of micrometastases in lymph nodes of head and neck cancer patients detected by E48 (Ly-6D) quantitative reverse transcription-polymerase chain reaction. Lab Invest 83:1233–1240.

Oc P, Modjtahedi H, Rhys-Evans P, Court WJ, Box GM, Eccles SA. 2000. Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. Cancer Res 60:1121–1128.

Reuter CW, Morgan MA, Eckardt A. 2007. Targeting EGF-receptor-signalling in squamous cell carcinomas of the head and neck. Br J Cancer 96:408–416.

Rosenthal EL, Matrisian LM. 2006. Matrix metalloproteases in head and neck cancer. Head Neck 28:639–648.

Ruhul Amin AR, Senga T, Oo ML, Thant AA, Hamaguchi M. 2003. Secretion of matrix metalloproteinase-9 by the proinflammatory cytokine, IL-1beta: A role for the dual signalling pathways, Akt and Erk. Genes Cells 8:515–523.

Schaaij-Visser TB, Graveland AP, Gauci S, Braakhuis BJ, Buijze M, Heck AJ, Kuik DJ, Bloemena E, Leemans CR, Slijper M, Brakenhoff RH. 2009. Differential proteomics identifies protein biomarkers that predict local relapse of head and neck squamous cell carcinomas. Clin Cancer Res 15:7666–7675.

Schmalhofer O, Brabletz S, Brabletz T. 2009. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev 28:151–166.

Sen T, Dutta A, Maity G, Chatterjee A. 2010. Fibronectin induces matrix metalloproteinase-9 (MMP-9) in human laryngeal carcinoma cells by involving multiple signaling pathways. Biochimie 92:1422–1434.

Sheikh Ali MA, Gunduz M, Nagatsuka H, Gunduz E, Cengiz B, Fukushima K, Beder LB, Demircan K, Fujii M, Yamanaka N, Shimizu K, Grenman R, Nagai N. 2008. Expression and mutation analysis of epidermal growth factor receptor in head and neck squamous cell carcinoma. Cancer Sci 99:1589–1594.

Stamenkovic I. 2000. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol 10:415–433.

Stamenkovic I. 2003. Extracellular matrix remodelling: The role of matrix metalloproteinases. J Pathol 200:448–464.

Swango PA. 1996. Cancers of the oral cavity and pharynx in the United States: An epidemiologic overview. J Public Health Dent 56:309–318.

Thant AA, Nawa A, Kikkawa F, Ichigotani Y, Zhang Y, Sein TT, Amin AR, Hamaguchi M. 2000. Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells. Clin Exp Metastasis 18:423–428.

Thomas SM, Coppelli FM, Wells A, Gooding WE, Song J, Kassis J, Drenning SD, Grandis JR. 2003. Epidermal growth factor receptor-stimulated activation of phospholipase Cgamma-1 promotes invasion of head and neck squamous cell carcinoma. Cancer Res 63:5629–5635.

Timpson P, Wilson AS, Lehrbach GM, Sutherland RL, Musgrove EA, Daly RJ. 2007. Aberrant expression of cortactin in head and neck squamous cell carcinoma cells is associated with enhanced cell proliferation and resistance to the epidermal growth factor receptor inhibitor gefitinib. Cancer Res 67:9304–9314.

Valkova C, Mertens C, Weisheit S, Imhof D, Liebmann C. 2010. Activation by tyrosine phosphorylation as a prerequisite for protein kinase C{zeta} to mediate epidermal growth factor receptor signaling to ERK. Mol Cancer Res 8:783–797.

van Roy F, Berx G. 2008. The cell–cell adhesion molecule E-cadherin. Cell Mol Life Sci 65:3756–3788.

Vleminckx K, Vakaet L Jr., Mareel M, Fiers W, van Roy F. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 66:107–119.

Yang LJ, Liu YQ, Gu B, Bian XC, Feng HL, Yang ZL, Liu YY. 2010. Effect of forced E-cadherin expression on adhesion and proliferation of human breast carcinoma cells. Zhonghua Bing Li Xue Za Zhi 39:842–847.

Zhou H, Kramer RH. 2005. Integrin engagement differentially modulates epithelial cell motility by RhoA/ROCK and PAK1. J Biol Chem 280:10624–10635.

Zuo J, Ishikawa T, Boutros S, Xiao Z, Humtsoe JO, Kramer RH. 2010. Bcl-2 overexpression induces a partial epithelial to mesenchymal transition and promotes squamous carcinoma cell invasion and metastasis. Mol Cancer Res 8:170–182.