Epithelial-Mesenchymal Transition in Cervical Cancer: Correlation with Tumor Progression, Epidermal Growth Factor Receptor Overexpression, and Snail Up-Regulation

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Abstract

Purpose: Acquisition of epithelial-mesenchymal transition (EMT) by primary carcinoma cells is associated with disrupted epithelial integrity, local invasion, and ultimately metastasis. Little is known about the existence and function of EMT in cervical cancer. This study aims to investigate the regulation of EMT in cervical squamous cell carcinoma.

Experimental Design: We investigated the molecular events of EMT in surgical specimens, which present the progression of cervical carcinoma. Two cervical cancer cell lines and the primary culture of normal cervical epithelia were used to study the regulatory mechanisms of EMT.

Results: The chronic epidermal growth factor (EGF) treatment induces the elongation of cell shape, increases cell scattering, and enhances cell invasion. EGF treatment down-regulates E-cadherin and up-regulates vimentin in cervical cancer cells. These characteristics are consistent with the morphologic changes, molecular events, and functional significance of EMT. EGF receptor (EGFR) signaling inactivates glycogen synthase kinase-3β, which results in the nuclear accumulation of up-regulated Snail and then leads to EMT program. α5β1 integrin signaling and extracellular matrix fibronectin can modulate EGF-induced EMT. Importantly, the immunofluorescent stainings of surgical specimens indicate that cervical carcinoma progression is accompanied by EGF overexpression, which is in parallel with decreased E-cadherin and increased vimentin. Up-regulation and nuclear accumulation of Snail correlate with EMT program in tumor tissues.

Conclusion: EGF cooperates with α5β1 integrin signaling to induce EMT in cervical cancer cells via up-regulated Snail. Blockade of EGFR activity or expression may provide a potential target for the treatment of cervical cancer progression.

During metastatic progression, polarized epithelial tumor cells convert into motile mesenchymal cells, invade the basement membrane beneath, enter blood vessels, and disseminate into secondary organ. The initial stage of these processes is associated with morphogenetic changes referred to as epithelial-mesenchymal transition (EMT), which is characterized by morphologic changes from epithelial cells to fibroblast-like cells, disassembly of intercellular junctions, and increased cell motility (1, 2). Down-regulation of E-cadherin was considered as a critical step in EMT process, and the down-regulation of which can be achieved by transcriptional suppression mediated by transcription factors Snail, Slug, or Twist (3–5). This results in the disruption of intracellular adhesion junctions formed by E-cadherin/β-catenin complexes.

Cervical cancer is the second most common cancer among women worldwide (6). A growing body of evidence has accumulated to indicate that oncogenic types of human papillomavirus serve as an important factor in the development of the precursors of cervical cancer. However, only a small fraction of those infected by human papillomavirus develops cancer, indicating that other factors contribute to the progression to cervical cancer (6, 7). How cervical cancer cells acquire the ability to invade surrounding tissue is unclear, but EMT likely plays a role. Little information is available on the regulation of EMT in cervical cancer cells and on the association of EMT program with clinical outcome of cervical cancer patients. Diverse growth factors secreted by the cancer cells and host cells in their local microenvironments may trigger the molecular events that are associated with the EMT program (8). Epidermal growth factor (EGF) has been reported as a potent stimulator of cervical cancer cell invasiveness (9). The expression of EGF receptor (EGFR) is up-regulated in the lymph node metastases and recurrence of cervical cancer (10).

This study aims to investigate the effects of EGF on the EMT program of cervical cancer cells. Three specific questions are asked. (a) Will EGF induce the transition of epithelial to...
mesenchymal-like phenotype in cultured cervical cancer cell lines? (b) Is the overexpression of EGFR accompanied by the EMT program in vivo? (c) Can specific adhesion receptors of integrin and extracellular matrix modulate EGF-induced EMT in cervical cancer cells? The results indicate that EGFR signaling induces the EMT program, which is mediated by up-regulation of transcription factor Snail.

Materials and Methods

Primary antibodies and kinase inhibitors. Monoclonal antibodies against E-cadherin, β-catenin, ZO-1, fibronectin, α-tubulin, and β-actin were purchased from BD Transduction Laboratories. Polyclonal antibodies against EGFR, phospho-glycogen synthase kinase-3β (GSK-3β; Ser9), Snail, Twist, and Slug were obtained from Santa Cruz Biotechnology. The antibodies against vimentin and various types of integrin were from Chemicon. The antibody against α-smooth muscle actin was from Sigma-Aldrich. The EGFR kinase inhibitor AG-1478 (Calbiochem) was solubilized in DMSO, in which the final concentration of DMSO was <0.5%.

Cell cultures and surgical specimens. Normal human cervical epithelial cells and cervical cancer SiHa and Caski cell lines were prepared as described previously (11). For coating extracellular matrix, 10 μg/ml fibronectin was used to cover the dish surface by shaking at 4°C overnight. We selected 36 consecutive patients with squamous cell carcinoma of the uterine cervix from March 1999 to December 2000 who were scheduled for radical hysterectomy and pelvic lymphadenectomy at National Cheng Kung University Hospital, Taiwan. Squamous cell carcinoma is the most common form (>80%) of cervical cancer. The clinical staging was based on the criteria of International Federation of Gynecology and Obstetrics classification.

Immunofluorescent staining of surgical specimens. Tissue sections were immunostained with primary antibodies against interesting proteins followed by exposure to Alexa 488– or Alexa 594–labeled secondary antibodies (Molecular Probes) and/or by Hoechst 33258 for nuclei (Sigma-Aldrich). At least five sections from each patient’s specimen were analyzed for histology and the expression of interesting proteins. Each section was examined blindly by two investigators trained in gynecologic pathology. The staining was observed by confocal microscope (FV-1000, Olympus) over 15 to 20 high-power fields. The intensity and distribution of immunofluorescent staining of interesting proteins were graded as high (++), moderate (+), or low (-) in each specimen. Low grade (-) indicates the distribution of positive staining is less than one-third area of tumor tissues or normal/ noncancerous squamous epithelia, whereas high grade (+++) indicates that the distribution of positive staining is more than two-thirds of tumor tissues or normal/noncancerous squamous epithelia. Others were classified as the moderate grade (+).

Immunofluorescent analysis and immunoblotting of cultured cells. For immunofluorescent staining, cells were seeded into two-well chamber slide, treated for the indicated conditions, fixed by 4% paraformaldehyde, permeabilized by PBS containing 0.25% Triton X-100, blocked with goat blocking serum (Vector Laboratories), incubated with primary antibody at 4°C overnight, washed, and then incubated with Alexa 488– or Alexa 594–labeled secondary antibodies and Hoechst 33258. After that, cells were washed and mounted, and the fluorescent images were examined by confocal microscope (FV-1000, Olympus). For immunoblotting, cells were seeded at 2 × 10^5 per 10 cm dish and clustered as epithelial islands after being cultured for 2 days. Cells were then cultured with medium containing 1% fetal bovine serum in the absence or presence of 50 ng/ml EGF for the indicated period. Equal amounts of protein lysates were separated on SDS-polyacrylamide gel and then transferred to nitrocellulose blotting membranes (Pall). Immunoblots were blocked, incubated with the primary antibody, washed, and incubated with the corresponding horseradish peroxidase–conjugated secondary antibody (BD Transduction Laboratories).

Invasion assay. Cervical cancer CaSki cells were incubated with or without EGF for 24 h before the invasion assay. Invasive migration was done in the BD Matrigel invasion chamber (BD Biosciences) for 12 h in serum-free culture medium at 37°C as an index of invasive activity (12). Cells were then fixed with paraformaldehyde, stained with Giemsa, and counted immediately after staining.

Statistics. All values in the present study were reported as mean ± S.E. Student’s paired or unpaired t test was used for statistical analyses. Differences between values were considered significant when P < 0.05.

Results

Progression of cervical carcinoma is associated with EMT program. Cervical cancer is usually pictured as spreading beyond the uterine cervix through direct involvement of the lateral parametrium and spread to the pelvic wall. We selected 10 cases of cervical cancer with clinical stage Ib-IIa, in which each case contained the following samples: (a) normal or noncancerous squamous epithelia, (b) superficial tumor tissues, (c) tumor tissues in the parametrium, and (d) tumor tissues in the pelvic lymph nodes (Fig. 1A). These samples represent the progression of cervical carcinoma. To evaluate the EMT program in vivo, these surgical specimens were triple-immunofluorescent stainings with E-cadherin, vimentin, and nucleos. E-cadherin protein, an epithelial marker, was obvious in the normal or noncancerous cervical squamous epithelia but was absent in the underlying stromal tissues (Fig. 1A). E-cadherin protein was significantly decreased in tumor tissues and almost disappeared when tumor cells invaded deeply into the parametrium or spread to pelvic lymph nodes. In contrast, vimentin, the mesenchymal marker, was absent in the normal or noncancerous cervical squamous epithelia but was clearly expressed in the adjacent stromal tissues. However, the expression of vimentin in tumor tissues became abundant as tumor cells invaded deeply into the parametrium or pelvic lymph nodes. These data indicate that the progression of cervical carcinoma was accompanied by down-regulated E-cadherin and up-regulated vimentin (summarized in Fig. 1B).

We also studied the expression of β-catenin, another epithelial marker, in the surgical specimens. Similar to the expression pattern of E-cadherin, β-catenin protein was abundant in the normal or noncancerous cervical squamous epithelia but was progressively decreased in the tumor tissues (Supplementary Fig. S1).

EGF induces EMT in cultured cervical cancer cell lines. We studied if EGF triggers the molecular events that are associated with the EMT program. In the absence of EGF stimulation, cervical cancer CaSki and SiHa cells exhibited well-organized cell-cell association and islet-like structure, which are the characteristics of squamous cell carcinoma (Fig. 2A). EGF treatment induced the elongation of cell shape and increased the cell scattering, which were similar to the morphology of mesenchymal cells. We then studied the possible alterations of molecular markers of EMT program. EGF significantly decreased the abundance of E-cadherin and β-catenin in a time-dependent manner (Fig. 2B). The immunofluorescent stainings also showed that EGF down-regulated E-cadherin/β-catenin complex as well as ZO-1 in cell-cell adhesion junction (Fig. 2C and D). In contrast, EGF significantly increased the abundances of vimentin, α-smooth muscle actin, and fibronectin (Fig. 2D).
These results indicate that EGF exhibits a profound effect on the EMT program of cervical cancer cells by down-regulation of epithelial markers and up-regulation of mesenchymal markers.

Transcription factors involved in EGF-induced EMT of cervical cancer cells. Twist, Snail, and Slug are three transcription repressors of E-cadherin expression (3–5). These three molecules are frequently detected in metastatic cancer cells and are required for EMT in breast cancer (13). To determine whether EGF promoted EMT via these transcription factors, we examined the effect of EGF treatment on the expression of Snail, Twist, and Slug. As shown in Fig. 3A and B, Snail protein was up-regulated by EGF stimulation and accumulated in nuclei. We then asked via which downstream pathways of EGFR Snail became abundant. GSK-3β, an important EGFR downstream signal module, is known to phosphorylate Snail and promote its nuclear export and further proteolysis (14). The phosphorylation of GSK-3β at Ser9 site indicates the inhibition of GSK-3β activity, which will be accompanied by increasing Snail abundance due to block its degradation (14). As shown in Fig. 3A and B, EGF increased the phosphorylation level of GSK-3β (inactive form, phospho-GSK-3β Ser9), which was accompanied by increased Snail expression. The inhibitor of EGFR tyrosine kinase activity (5 μmol/L AG1478) abolished
EGF-induced GSK-3β phosphorylation and EGF-stimulated Snail up-regulation. As a consequence, EGF-induced EMT program (down-regulated E-cadherin and up-regulated vimentin) was therefore inhibited (Fig. 3A). These data imply that EGFR signaling inactivates GSK-3β. Snail is thus up-regulated, accumulates in the nuclei, and performs its transcriptional function on EMT markers. In contrast, the amount and cellular distribution of Twist protein were insensitive to EGF treatment (Fig. 3C and D). However, we cannot detect the protein expression of Slug in cervical cancer cells.

Normal cervical epithelial cells expressed much less amount of EGFR compared with cervical cancer cells (Supplementary Fig. S2A). We then tested if EGF shows differential effect on EMT program between normal cervical epithelial cells and cervical cancer cells. As shown in Supplementary Fig. S1B, EGF did not change either the cell morphology or markers of EMT program in normal cervical epithelial cells.

**Association between EGFR abundance and EMT program in vivo.** To evaluate the *in vivo* condition, we examined the association of EGFR expression and EMT program in the surgical specimens of cervical carcinoma (*n* = 36) by immunoﬂuorescent staining. EGFR is scarce in noncancerous squamous epithelia, whereas E-cadherin is abundant in noncancerous squamous epithelia (Fig. 4A). On the other hand, EGFR expression is up-regulated in the tumor tissues, which is in parallel with decreased E-cadherin and increased vimentin (Fig. 4A). The phosphorylation of GSK-3β is remarkably increased in the tumor tissues (Fig. 4B). Snail is obviously up-regulated and translocates into nuclei in the tumor tissues (Fig. 4C). The overexpression of Snail in the tumor tissues is associated with down-regulated E-cadherin and up-regulated vimentin (Fig. 4C). These data indicate that EGFR overexpression is accompanied by the EMT program in the surgical specimens of cervical carcinoma (summarized in Fig. 4D).

**α5β1 integrin and fibronectin modulate EGF-induced EMT.** The cross-talks between EGFR and specific integrins (e.g., β1 and α5β1) have been reported to play an important role in EGF-dependent cellular function and tumor progression (15–17). Expressions of integrin family, such as α5β1, α5β3, αvβ3, αv, and αvβ6, have been shown in human cervical cancer cells (9, 18–20). To study if integrin signaling...
modulates EGF-dependent EMT, cervical cancer CaSki cells were preincubated with integrin blocking antibody of α5β1, α5β3, α5β4, or β4 to inhibit integrin-extracellular matrix interaction. Inhibition of α5β1 integrin signaling by blocking antibody resulted in a more epithelial morphology in EGF-treated cells (Supplementary Fig. S3). In contrast, anti-α2β1, anti-α4, anti-β1, and anti-αvβ3 integrin antibodies showed no effects on EGF-induced mesenchymal morphology of cervical cancer cells. Importantly, EGF stimulated cervical cancer cell invasiveness in a dose-dependent manner, which was abolished by α5β1 integrin blocking antibody (Supplementary Fig. S4).

EGF-induced EMT program was remarkably inhibited by α5β1 integrin blocking antibody. For example, the effect of EGF on the reduction of E-cadherin expression was almost abolished by α5β1 integrin blocking antibody (Fig. 5A). This blocking antibody also inhibited 40% of EGF-induced increase of vimentin expression. EGF-induced up-regulation and nuclear accumulation of Snail were also significantly inhibited by α5β1 integrin blocking antibody (Fig. 5B).

The main extracellular ligand of α5β1 integrin is fibronectin (21). To confirm the interaction between extracellular matrix and adhesion receptors of integrin would affect EGF-induced EMT, we applied fibronectin as extracellular matrix in the cell culture system. As shown in Fig. 5C and D, extracellular fibronectin alone could not induce EMT but augmented EGF-induced E-cadherin down-regulation, vimentin up-regulation, and morphologic change. These data indicate that α5β1 integrin signaling and extracellular matrix fibronectin could modulate EGF-induced EMT program of cervical cancer cells.

**Discussion**

Here, we identify EGF as a novel EMT inducer in cervical cancer cells. The conclusion is supported by the following evidences. (a) The chronic EGF treatment induces the elongation of cell shape, increases cell scattering, and enhances cancer cell invasion. (b) EGF decreases the abundances of epithelial markers such as E-cadherin and β-catenin. (c) EGF increases the abundances of mesenchymal markers, including vimentin, α-smooth muscle actin, and fibronectin. These characteristics are consistent with the morphologic changes, molecular events, and functional significance of EMT program. Normal cervical epithelial cells express much less amounts of...
EGFR, and the morphology and molecular markers of EMT are not affected by EGF stimulation. Evidences from surgical specimens of cervical carcinoma suggest that cervical carcinoma progression is accompanied by overexpressed EGFR in parallel with E-cadherin down-regulation and vimentin up-regulation. To our knowledge, this is the first study providing both in vivo and in vitro evidences to show that EGF induces the EMT program in cervical cancer cells.

Little is known about the regulation of EMT in cervical cancer cells. Up to date, only membrane KCl cotransporter-3, human papillomavirus, and transforming growth factor-β1 were reported as EMT inducers in cervical cancer cell lines (22–24). Here, we show that EGFR signalings lead to GSK-3β Ser9 phosphorylation and Snail up-regulation, which are abolished by the inhibition of EGFR activation. Snail triggers EMT by repressing the expression of epithelial markers and inducing the expression of mesenchymal markers (3). Snail is highly unstable, with a short half-life of ~25 min (14). GSK-3β is a multitasking kinase involved in the modulation of several growth factor receptor downstream signalings. GSK-3β binds to and phosphorylates Snail at two consensus motifs to dually regulate the function of this protein. Phosphorylation of the first motif regulates the ubiquitination of Snail, whereas phosphorylation of the second motif controls the subcellular localization of Snail (14). In addition, GSK-3β is regarded as an endogenous inhibitor of Snail transcription (25). The phosphorylation of GSK-3β at Ser9 site inhibits its activity, which results in Snail up-regulation and EMT induction (14). Our results in the cell culture systems suggest that EGFR signalings lead to GSK-3β inactivation, Snail up-regulation, and EMT program. Consistent with the observations in cell cultures, this regulation exists in cervical cancer tissues. Up-regulation and nuclear accumulation of Snail significantly correlated with the inactive GSK-3β, the down-regulation of E-cadherin, and the up-regulation of vimentin in the tumor tissues. This suggests that there is likely a cause and effect between EGFR overexpression, nuclear accumulation of up-regulated Snail, and EMT program in vivo. Thus, the evidences from the cell cultures and surgical specimens support the hypothesis that GSK-3β and Snail function together as a

![Fig. 4. Association between EGFR abundance and EMT program in vivo. A, EGFR is overexpressed in the tumor tissues in parallel with down-regulated E-cadherin and up-regulated vimentin. The surgical specimens of the same patient with cervical cancer were triple-immunofluorescent stainings with (a) E-cadherin, EGFR, and nucleus or (b) EGFR, vimentin, and nucleus. Representative images of the same patient. Bar, 20 μm. B, phospho-GSK-3β at Ser9 is up-regulated in the tumor tissues, which is associated with down-regulated E-cadherin and up-regulation of Snail and vimentin in the tumor tissues of cervical cancer. The surgical specimens of the same patient were triple-immunofluorescent stainings with (a) E-cadherin, Snail, and nucleus or (b) vimentin, Snail, and nucleus. Representative images of one case. Bar, 20 μm. Inset, up-regulated Snail accumulates in the nucleus. C, summary of the immunofluorescent stainings (n = 36). The stains of interesting proteins were graded as high (++), moderate (+), or low (−) in each sites. The grading system was described in Materials and Methods.](https://www.aacrjournals.org/clinanceres/article-pdf/14/15/4748/659227/clincancerres-08-05-04748.pdf)

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molecular switch for EGFR signalings that lead to EMT in cervical cancer.

Epithelial cells receive important stimuli from the environment through soluble growth factors and insoluble extracellular matrix proteins. The receptors responsive for the binding are growth factor receptors and integrins, respectively. Extracellular matrices in concert with growth factors can profoundly influence cell phenotypes and behaviors (26). This study shows that the signaling of specific adhesion receptor of integrin could modulate EGF-induced EMT. To abolish $\alpha_5\beta_1$ integrin signaling by blocking antibody inhibits EGF-induced EMT. One of the functions of EGF is to stimulate the expression of extracellular matrix proteins (27). We show that EGF increases the abundance of fibronectin, which in turn augments EGF-induced EMT in cervical cancer cells, indicating a positive feedback loop in the axis of EGFR signaling-fibronectin expression-EMT program.

Taken together, the results of cell culture systems and surgical specimens, the regulatory mechanisms of EGFR signaling on EMT of cervical cancer cells, can be summarized as follows (Fig. 6). Cervical cancer tissues overexpress EGFR. The activation of EGFR by its ligand increases the abundance of the transcription factor Snail, which accumulates in the nucleus. The up-regulated Snail represses the expression of E-cadherin and induces the expression of vimentin. Polarized epithelial tumor cells therefore convert into motile mesenchymal-like tumor cells, which invade deeply into the local tissues and disseminate into distal tissues. EGF-induced EMT can be modulated by the signalings from $\alpha_5\beta_1$ integrin and its extracellular ligand fibronectin. Thus, blockade of EGFR activity or expression may provide a potential target for the treatment of cervical cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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Fig. 6. Schematic diagram indicating that EGF promotes EMT that is important for cervical cancer cell invasiveness. Cervical cancer tissues overexpress EGF. The activation of EGFR by its ligand increases the abundance of Snail, which accumulates in the nucleus. The up-regulated Snail represses E-cadherin expression and induces vimentin up-regulation. Polarized epithelial tumor cells therefore convert into mobile mesenchymal-like tumor cells, which leave the primary sites, invade deeply into the local tissues, and disseminate into distal tissues. EGF-induced EMT can be modulated by the signalings from α5β1 integrin and its extracellular ligand fibronectin.

References

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