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Overexpression of EFGR as Prognostic Factor and Effect of EGF in the Progression of Hypopharyngeal Cancer

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하인두암에서 예후인자로서의 표피성장인자수용체(EGFR) 과발현과 하인두암의 진행에 있어 표피성장인자(EGF)의 역할 분석

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= 국문초록 =

연구배경 및 목적

표피성장인자수용체(EGFR)는 HER2/neu(erbB2), HER3(erbB3), HER4(erbB4)를 포함하는 receptor tyrosine kinase의 erbB 그룹에 속하는 수용체이다. 표피성장인자수용체의 과발현은 다양한 종류의 암, 특히 두경부편평세포암에서 예후를 악화시킨다고 알려져 있다. 이에 저자들은 하인두편평세포암에서 표피성장인자수용체의 발현 및 분포를 확인하고, 하인두암에서 표피성장인자(EGF)가 암세포의 증식과 침습에 미치는 영향에 대해 알아보고자 하였다.

대상 및 방법

57명의 하인두편평세포암 환자의 조직에서 표피성장인자수용체의 발현을 면역화학적염색을 통해 확인하고, 이에 대해 임상병리학적 요인과 생존율에 대한 분석을 시행하고, 일부 환자의 정상 및 암조직에서 Western blot을 시행하였다. 하인두편평세포암 세포주인 FaDu에서 proliferative assay, colony dispersion, wound healing assay, invasion assay를 시행하여, 하인두암의 진행에서 표피성장인자의 역할에 대하여 분석하였다. 또한 RT-PCR과 Zymography를 통하여 Matrix metalloproteinase(MMP)-2, 9의 발현을 확인하였다.

결 과

63.2%의 하인두편평세포암 조직에서 표피성장인자수용체의 발현이 확인되었다. 표피성장인자수용체의 발현은 정상조직에서 비하여 하인두암 조직에서 유의하게 증가되어 있었으며, 병리학적 병기(p=0.022)가 올라갈수록 유의하게 증가하였으나, 증례수의 제한으로 생존율에서는 통계적 유의성을 얻지는 못했다(p=0.053). *in vitro*의 결과로 표피성장인자를 FaDu 세포주에 처리하였을 때, FaDu 세포주의 증식이 유의하게 증가되었으며(p<0.05), Transwell invasion chamber상 침습의 증가가 확인되었다(p<0.05). RT-PCR과 zymogram 실험상 표피성장인자처리시 FaDu

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세포주의 MMP-2, 9이 발현이 증가되고 활성화되는 것이 확인하였다.

결 론

본 연구에서 표피성장인자수용체의 과발현이 하인두암의 예후 인자로서의 가능성을 확인하였고, 표피성장인자가 하인두편평세포암의 증식과 침습에 관여하는 것을 확인하였다.

중심 단어 : 표피성장인자수용체 · 표피성장인자 · 하인두편평세포암 · 침습 · 예후.

Introduction

With over 500,000 cases worldwide and a high mortality rate, head and neck squamous cell carcinoma(HNSCC) is the sixth most common cancer in men. Even with improved treatments, the overall survival rate has been less than 50% for the past 30 years.[1] Among cancers of the head and neck, hypopharyngeal carcinoma has very high invasion and metastasis rates, making locoregional control of these cancers difficult. The 5-year overall survival rate for all patients with hypopharyngeal carcinoma is only 30%, which is essentially unchanged from the rate recorded two decades ago in spite of various treatment trials. An understanding of the mediators of the critical steps in the metastatic process is essential for the development of targeted therapies for hypopharyngeal carcinoma.

Patients with advanced hypopharyngeal carcinoma are at greatest risk for recurrence, but the tumor, node, metastasis (TNM) system for tumor staging is inadequate for distinguishing outcome and tailoring therapy in these patients. The characterization of the molecular mechanisms involved in the pathogenesis of hypopharyngeal carcinoma has been undertaken to develop new approaches for identifying patients at highest risk for progression or recurrence of disease and to select patients who could benefit from multimodal and molecularly targeted therapies.

Recent research efforts have attempted to exploit biologic differences that may exist between normal and malignant cells to develop tumor-specific therapies. The epidermal growth factor(EGF) and its receptor(EGFR, ErbB-1, or HER-1) were not only shown to play an influential role in cellular growth and differentiation in healthy tissues, but also in tumorigenesis and the progression of malignant disease.[2] In addition to expression on the surface of healthy cells, the EGFR is commonly expressed at high levels in a variety of epithelial tumors, including HNSCC. The aberrant activation of the EGFR leads to enhanced proliferation and other tumor-promoting activities, which provides a strong rationale to target this receptor. During the past decade, intense research has initiated a new era of cancer treatment, that of molecular therapeutics. Today, the EGFR is a prime target for new anticancer therapies, with a broad range of inhibitors currently under investigation.[3] Promising preclinical studies have prompted the development of clinical trials testing EGFR inhibitors as single-agent therapy or in combination with conventional cytotoxic therapies, with response rates lower than anticipated in the advanced disease setting. The clearest benefit of EGFR-inhibitor treatment to date is noted when it is combined with radiation therapy to treat locally advanced HNSCC.[4]

In normal cells, the expression of EGFR ranges from 40,000 to 100,000 receptors per cell.[5] In HNSCC, EGFR and its ligand, EGF, are overexpressed in 80−90% of cases ; the corresponding magnitudes of increase are 1.7-fold(p=0.005) and 1.9-fold(p=0.006), respectively compared to controls.[6] The nature of this overexpression is thought to result from enhanced transcription, with no apparent change in mRNA stability ; gene amplification has been observed less frequently. Overexpression of EGFR is observed in 42% to 80% of HNSCCs studied.[7] Grandis et al. demonstrated that EGFR overexpression provided independent prognostic value for both local control and survival in 91 head and neck squamous carcinoma patients treated with surgical resection-postoperative radiotherapy.[8] EGFR overexpression was also associated with both an increased risk of local relapse and an adverse overall survival, independent of tumor stage.

Therefore, we investigated the expression and distribution of the EGFR in hypopharyngeal squamous cell carcinoma by immunohistochemistry and the effect of EGF in progression of hypopharyngeal SCC in a hypopharyngeal SCC cell line.

Materials and Methods

1. Patients

Fifty-seven patients admitted for hypopharyngeal squamous cell carcinoma between 1994 and 2002 at Ajou University Hospital and Yonsei University Medical Center in Korea were enrolled in this study. Surgically removed specimens of hypopharyngeal squamous cell carcinoma, excluding carcinoma *in situ* and verrucous carcinoma, were used. None of the patients had undergone radiotherapy or chemotherapy before surgical excision. The tumors were classified according to the

2002 AJCC TNM classification system. The average age at clinical onset was 60 years, with a range of 37−76 years. Six patients had stage I or II disease and 51 patients had stage III or IV cancer. The histologic typing was based on the World Health Organization(WHO) system. Of the 57 tumors, 13 were well differentiated, 29 were moderately differentiated, and 15 were poorly differentiated.

2. Cell culture

FaDu cells, a hypopharyngeal cancer cell line, were obtained from the American Type Culture Collection(ATCC, Manassas, VA, USA) and cultured in Eagle's minimum essential medium (EMEM) containing 25mM $NaHCO_3$, 10% fetal bovine serum (FBS), glutamine, 1% essential amino acids, vitamin E, and streptomycin at 37℃ under a 5% CO_2 atmosphere.

3. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was cut into 5-μm sections and then dewaxed and rehydrated via serial passage through xylene and a graded series of ethanol. Endogenous peroxidase activity was blocked by incubation for 45min in 6% hydrogen peroxide in methanol. The primary antibodies were human EGFR polyclonal antibodies(Cell Signaling Technology, Danvers, MA, USA) used at a concentration of 50μg/mL. Primary antibody incubations were carried out for 2h at 25℃. The sections were extensively washed in phosphate-buffered saline(PBS) and then incubated with the appropriate biotinylated secondary antibody followed by avidin-peroxidase. Goat and rabbit Vectastain-Elite Immunoperoxidase Kits(Vector Laboratories, Burlingame, CA, USA) were purchased for this purpose and used in accordance with manufacturer's instructions. The chromogenic reaction was carried out with 3-3'-diaminobenzidine in a peroxidase substrate solution and lasted 4min. Hematoxyline was used for nuclear staining. For each experiment, negative controls omitting either primary or secondary antibodies were included to examine nonspecific staining. The slides were reviewed by at least two pathologists and scored semiquantitatively as follows : - no staining, ±definite but weak staining, + moderate staining, and ++ strong staining. Tumor samples scored positive if given a + or ++ and if more than 30% of the cancer cells were stained.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA from fresh hypopharyngeal cancer tissues and FaDu cells was extracted using TRIzol®(Invitrogen BV, Groningen, The Netherlands). cDNA was prepared using the Omniscript Reverse Transcriptase kit(Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the effects of EGF, cells were treated with EGF(0, 10, 30ng/mL). The sequences of PCR primers were as follows : EGFR-F, 5'- CAAATAAAACCGGACTGAAG-3' ; EGFR-R, 5'-GTCAGTTTCTGGCAGTTCTC-3' ; EGF-F, 5'-TGGAAGCCTTTATAGAGCAG-3' ; EGFR, 5'-TTGGCTATCCAAATTGTCTT-3'. PCR products were separated by electrophoresis in 1.5% agarose gels and were detected under UV light(Bio-Rad, Hercules, CA, USA).

5. Western blot analysis

Cells were washed with PBS, placed in radioimmunoprecipitation(RIPA) buffer containing 150mM NaCl, 1% NP-40, 50mM Tris-HCl(pH 8.0), 1mM EDTA, 0.5% deoxycholate, 100μg of phenylmethylsulfonyl fluoride, and 1μg/mL leupeptin, and the mixture was homogenized. The reaction mixture was centrifuged at 12,000 rpm for 5 min at 4℃, and the supernatant was used for Western blot analysis. Protein concentration was measured using the Bio-Rad protein assay. Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate 20μg of protein per well, and the bands were transferred to a nitrocellulose filter(Amersham, Piscataway, NJ, USA). The membrane was incubated with specific antibodies overnight at 4℃. The membrane was then washed with Tris-buffered saline(TBS) containing 0.1% Tween 20 and reacted with peroxidase-conjugated donkey anti-rabbit antibody(Amersham) or donkey anti-mouse antibody (Amersham). The membrane was developed using the Enhanced Chemiluminescence Detection System(ECL ; Amersham) and X-ray film.

6. Proliferation assay

FaDu cells were plated in culture plates(Costar, Cambridge, MA, USA) at a density of approximately 1×10^5/well in the absence of serum. EGF(0, 10, 30ng/mL) was added, and cells were cultured for 5 days. Cells were counted using a hemocytometer on days 1, 3, and 5. Seven separate experiments were performed to obtain statistical significance.

7. Colony dispersion analysis

FaDu cells were subcultured and maintained in growth medium until colonies containing more than 16 cells were established. Cultures were then deprived of growth factors and serum for 48h prior to treatment with EGF(0, 10, 30ng/mL) for the indicated times ; EGF treatment of the cells was preceded by a 2h pretreatment with mitomycin C(8μg/mL). Colony dispersion was documented by photography at 6, 12, 18, and 24h.

8. Wound healing assay

FaDu cells were plated in culture plates at a density of

approximately 1×10^5/well in the absence of serum. Confluent cell monolayers were then deprived of growth factors for 48h, and a cell-free area was introduced by scraping the monolayer with a sterile pipette tip followed by extensive washing to remove cellular debris. *In vitro* reepithelialization was monitored by the repopulation of the cleared area with cells over time. To determine the effect of EGF, cells were treated with 0, 10, and 30ng/mL EGF. Wound healing was documented by photography at 12, 24, 36 and 48h. For objective measurements, the distance between both sides was measured by a computer calculator at five points(per one field) at 48h. The average distance at seven fields was compared with each other(control vs. EGF treatment group ; EGF 10ng/mL vs. EGF 30ng/mL).

9. Invasion assay

Transwell chambers(Costar) were used to verify the degree of invasiveness depending on the administration of EGF. First, type I collagen(6μg/filter) melted in 100μL EMEM was poured into the upper part of a polyethylene filter(8-μm pore size) ; coating was carried out in a laminar flow hood for one night. Then 500μL of 0.5% FBS medium was placed into the lower part of each well, and the wells were adjusted to EGF concentrations of 0, 10, and 30ng/mL. After pretreatment with mitomycin C(8μg/mL) for 30min, 10^5 cells(in 100μL of growth medium) were allowed to attached to the top of the filter of the upper well. After this chamber was incubated in 5% CO_2 at 37℃ for 48h, the filter of the upper well was removed and the cells were passed through the pore. The attached cells on the lower part were dyed with hematoxylin and counted under a light microscope.

10. RT-PCR of MMP-2 and MMP-9

Using 50−100mg of frozen FaDu cells, RNA was prepared as described above. The primer pairs used for MMP-2 and MMP-9 were as follows : MMP-2-F, 5'-ACCTGG ATGCCGTCGTGGAC-3' ; MMP-2-R, 5'-TGTGGCAGCACCAGGGCAGC-3' ; MMP-9-F, 5'-GGGGAAGATGCTGCTGTTCA-3' ; and MMP-9-R, 5'-GGTCCCAGTGGG GAT TTACA-3'. After denaturation for 3min at 96℃, the samples were amplified by PCR for 30 cycles of 30 s at 96℃, 30 s at 55℃, and 30 s at 72℃, with extension for 5min at 72℃.

11. Zymography of MMP-2 and MMP-9

Cultures were deprived of growth factors and serum for 48 h prior to treatment with EGF(0, 10, 30ng/mL) for the indicated periods of time. After quantification of the protein in the supernatant, 30μg of protein from each sample was mixed with 15μL p-aminophenylmercuric acetate(APMA), and the samples were activated for 1h at 37℃. Cytoplasm samples were obtained after 24h using the MightySlim™ SX 250(Hoefer, San Francisco, CA, USA) process. Each 10μL sample was placed in sample buffer for 10min and then electrophoresed in a polyacrylamide gel at 125V for 120min at 4℃ using the Novex XCell II. The gel was incubated in renaturation buffer for 60min at room temperature, and then incubated in 100mL of developing buffer at 37℃ for 18 h with light shaking. The gel was stained with Coomassie blue for 3h and washed with water. After decolorization in 400mL methanol, 100mL acetic acid, and 500mL distilled water, cell images were taken every 10min using an image analyzer.

12. Statistical methods

Pearson's chi-square test and Fisher's exact test were used for statistical analyses of immunohistochemical data. Patient survival rates were calculated using the Kaplan-Meier method, and statistically significant differences in survival were identified using the log-rank test. Student's *t*-test and one-way analysis of variance(ANOVA ; for the invasion assay) were also used for analyses. All statistical analyses were conducted using SPSS 10.0 software(SPSS Inc., Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

Results

1. Expression of EGFR in human hypopharyngeal cancer tissue

EGFR overexpression was observed in 63.2%(36/57) of hypopharyngeal cancer tissues. In most of the specimens, EGFR expression was primarily observed in the cell membrane and cytoplasm of cancer cells(Fig. 1A). EGFR expression was observed in 52.9% of patients with tumors less than 4cm in diameter(stage T1 or T2) and in 67.5% of patients with tumors larger than 4cm in diameter(stage T3 and T4). Although EGFR expression appeared to increase with tumor size, these results were not statistically significant(Table 1). In cases when no lymph node metastasis occurred, EGFR expression was observed in nine out of 16 cases(56.3%). However, a statistically nonsignificant increase in EGFR expression was observed in 27 of 41 patients(65.9%) with lymph node metastasis. EGFR expression also appeared to increase in cases of distant metastasis(90%), with a significant difference compared to those without distant metastasis(57.4 %). EGFR expression was positive in 16.7%(1/6) and 68.6%(35/51) of early and advanced stage carcinomas, respectively, demonstrating a significant increase in EGFR expression according to tumor

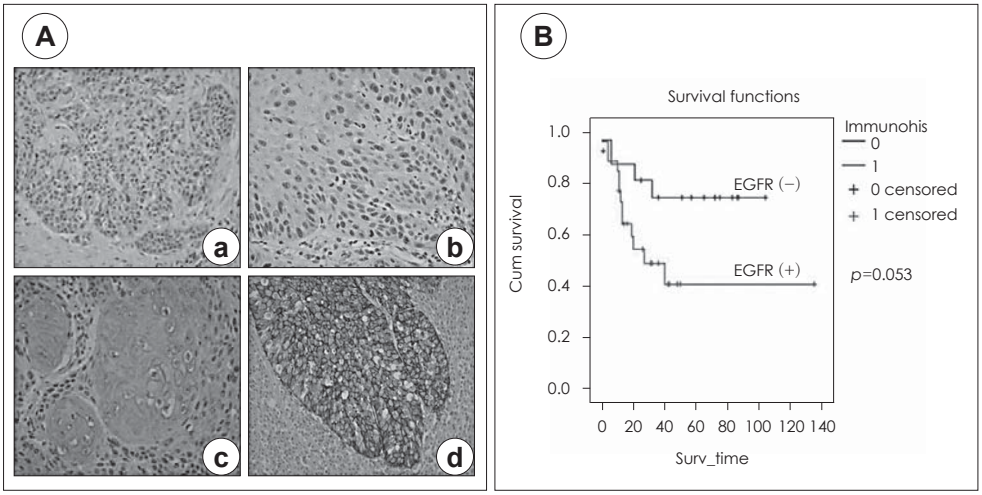

Fig. 1. (A) Immunohistochemistry : Paraffin sections of hypopharyngeal carcinomas immunostained for EGFR. EGFR staining was primarily observed in the membrane of the cancer cells, but was observed occasionally in both the membrane and cytoplasm of cancer cells(200× magnification). (a) no staining, (b) definite but weak staining(±), (c) moderate staining(+), (d) strong staining(++). (B) Survival rate : Five-year survival of 59 patients with hypopharyngeal carcinomas according to EGFR expression. Survival was significantly poorer in patients with positive EGFR expression compared to those with negative EGFR expression(p=0.053).

Table 1. Correlation between the expression pattern of EGFR and clinicopathologic factors

Variables		Expression of EGFR(%)		p-value
		Negative(–/+36.8)	Positive(++/+++ 63.2)	
T	1, 2(n=17)	8(47.1)	9(52.9)	0.372*
	3, 4(n=40)	13(32.5)	27(67.5)	
N	−(n=16)	7(43.8)	9(56.3)	0.551*
	+(n=41)	14(34.1)	27(65.9)	
M	−(n=47)	20(42.6)	27(57.4)	0.074**
	+(n=10)	1(10.0)	9(90.0)	
Pathologic stage	I, II(n=6)	5(83.3)	1(16.7)	0.022**
	III, IV(n=51)	16(31.4)	35(68.6)	
Pathologic grades	Well(n=13)	4(30.8)	9(69.2)	0.620**
	Moderate(n=29)	12(41.4)	17(58.6)	
	Poor(n=15)	4(26.7)	11(73.3)	
Recurrence	Yes(n=15)	6(40)	9(60)	0.742*
	No(n=24)	12(50)	12(50)	

* : Calculated by Pearson's chi-square test(p<0.05), ** : Calculated by Fisher's exact test(p<0.05)

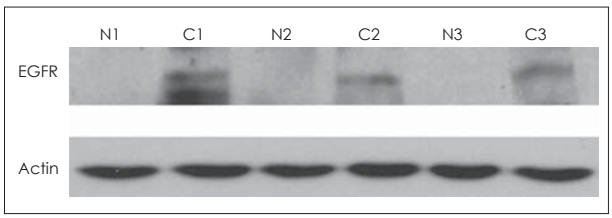

Fig. 2. Expression of EGFR in human hypopharyngeal cancer tissues. Western blotting demonstrated that EGFR was strongly expressed in carcinomas compared to normal tissues. N : Normal tissue, C : Cancer tissue.

stage(p=0.022). Pathologic cell differentiation did not seem to affect EGFR expression. No statistically significant difference was observed between groups with and without recurrence(60% and 50%, respectively). Of the 57 patients, 20(35.1%) died during the follow-up period. The cause of death was local recurrence in 11 cases and distant metastasis in nine cases. Survival analysis was performed using the Kaplan-Meier method, and the statistical significance was evaluated by the log-rank test(Fig. 1B). Although a difference in survival rates appeared to exist between the groups with negative and positive EGFR expression, the p value was 0.053.

2. Western blot analysis of hypopharyngeal cancer tissues and RT-PCR and Western blot analysis of the human hypopharyngeal cancer cell line

Western blotting was performed on three patients, and EGFR was strongly expressed in carcinoma cells and not in normal tissues(Fig. 2). The expression of EGFR mRNA in FaDu cells was detected by RT-PCR, and the EGFR protein was detected by Western blotting. However, EGF was not de-

tected by either RT-PCR or Western blotting(Fig. 3).

3. Effect of EGF on proliferation of FaDu cells

EGF stimulates the proliferation of various cancer cells. {Arteaga, 2003 #2} Therefore, we performed a proliferation assay to determine whether EGF also stimulates the proliferation of FaDu cells. As shown in Fig. 4, EGF at 10ng/mL and 30ng/mL stimulated the proliferation of FaDu cells on the third and fifth days($p<0.05$), respectively. Moreover, EGF at 30ng/mL increased proliferation to a greater extent than cells treated with 10ng/mL EGF on the third day(Fig. 4).

4. Effect of EGF on colony dispersion

EGF is one of the most potent tumor motility factors and contributes to metastasis by stimulating tumor motility.[9] In the control, no significant colony dispersion occurred in the FaDu cells. In EGF-treated groups, the FaDu cells dissociat-

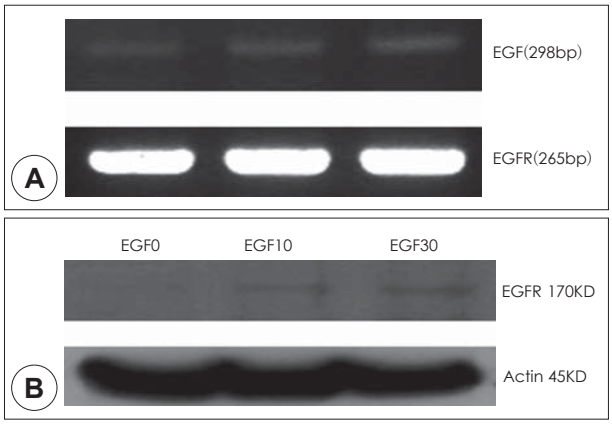

Fig. 3. Analysis of EGF and EGFR expression in the FaDu cell line. (A) RT-PCR : The expression of EGF and EGFR mRNA was measured by RT-PCR in the hypopharyngeal cancer line, FaDu. (B) Western blotting : Detection of the EGFR protein in FaDu cells by Western blotting. EGF was not detected by either RT-PCR or Western blot analysis.

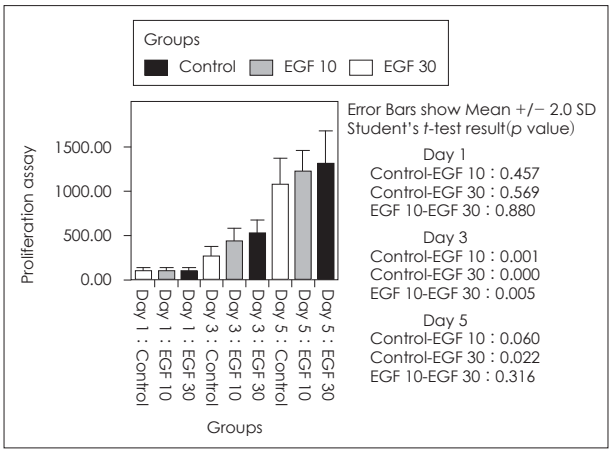

Fig. 4. Proliferative activity of EGF. Proliferative assay of FaDu cells after treatment with EGF for 5 days. Exogenous EGF significantly enhanced the growth of FaDu cells in a dose-dependent manner (*$p<0.05$).

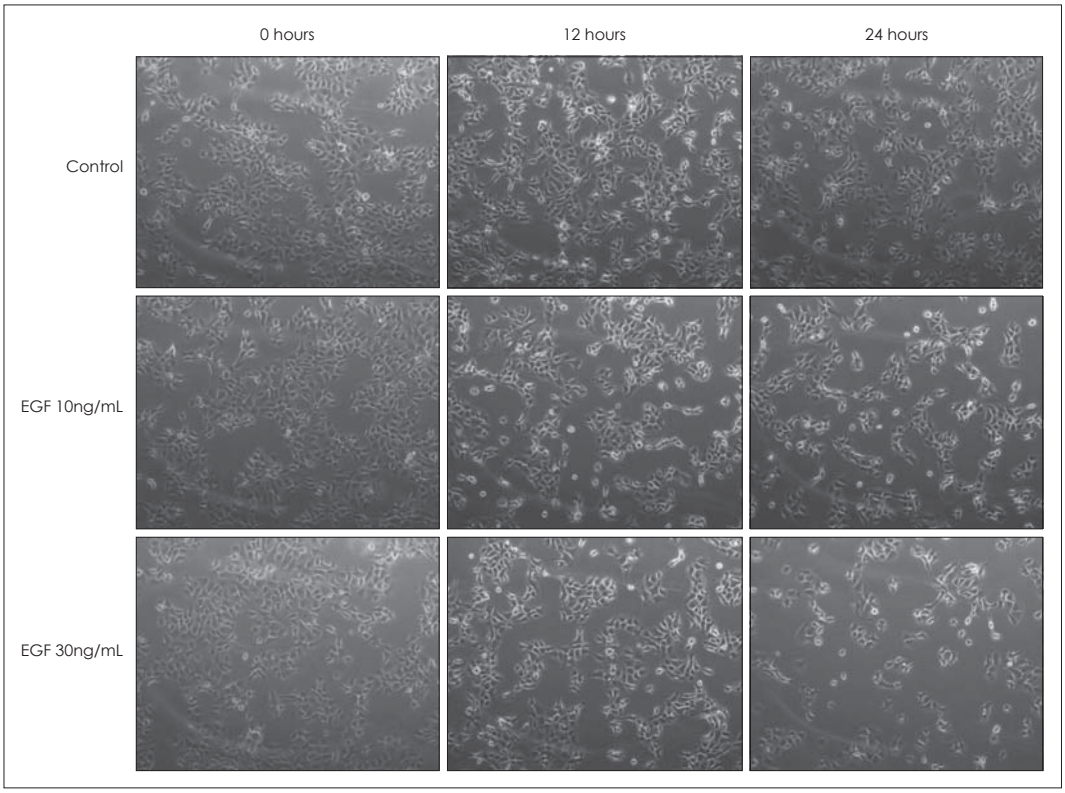

Fig. 5. Scatter activity of EGF. FaDu cells were cultured(A) without treatment(control), (B) with 10ng/mL EGF, or (C) with 30ng/mL EGF for 24h(original magnification, 150×). In the control group, significant colony dispersion of FaDu cells did not occur. In EGF -treated groups, EGF significantly increased dispersion of FaDu cells. EGF at 30ng/mL appeared to have a more potent scattering effect than EGF at 10ng/mL 24h after EGF stimulation.

ed 24h after treatment(Fig. 5). After 6h, the formation of actin microspikes(filopodia) and membrane ruffling(lamellipodia) were observed, and cell morphology showed spindle-like features. This result suggests that EGF may be associated with scattering. Although we did not measure the objective scattering effect according to the concentration of EGF, EGF at 30ng/mL appeared to produce a more potent scattering effect than EGF at 10ng/mL 24h after EGF stimulation.

5. Effect of EGF on wound healing

To assess the contributions of EGF to both migratory and proliferative activities, we performed an *in vitro* wound healing assay in FaDu cells using EGF at 0, 10, and 30ng/mL concentrations. Exogenous EGF significantly enhanced the migration and proliferation of FaDu cells in dose-dependent manner(Fig. 6). EGF at 10ng/mL led to a 1.65±0.93-fold increase in cell migration($p<0.05$ vs. control), and EGF at 30 ng/mL increased cell migration 4.42±2.59-fold($p<0.05$ vs. control). EGF at 30ng/mL increased cell migration 2.67± 1.22-fold compared to cells treated with 10ng/mL EGF($p<$ 0.05).

6. Effect of EGFR on cell invasion

A Transwell chamber invasion assay was performed using the FaDu cell line to determine whether EGF contributes to cell invasiveness. Fig. 7 demonstrates that EGF treatment dose-dependently increased the number of cells that invaded, compared to untreated controls. EGF at 10ng/mL increased cell invasion by 4±1.5-fold($p<0.05$ vs. control), and EGF at 30ng/mL led to an 8.5±2-fold increase in cell invasion ($p<0.05$ vs. control). EGF at 30ng/mL increased cell invasion by 2.5±0.5-fold compared to the 10ng/mL EGF treatment group($p<0.05$)(Fig. 7).

7. RT-PCR of MMP-2 and MMP-9

To confirm whether EGF induces MMP gene expression in FaDu cells, we performed RT--PCR after treatment of the cells with EGF(10ng/mL or 30ng/mL). MMP-2 mRNA was markedly elevated after treatment of FaDu cells with EGF for 24h, but no significant difference was detected between EGF at 10ng/mL and 30ng/mL. RT-PCR showed a slight increase in the level of MMP-9 with EGF treatment compared to the control(Fig. 8A).

8. Zymography of MMP-2 and MMP-9

MMP-2 activity increased after 24h of EGF treatment compared to the control, but no significant difference was observed between the 10ng/mL and 30ng/mL EGF groups. MMP-9 activity after EGF treatment was markedly elevated at 24h, although no significant difference was seen between EGF at 10ng/mL and 30ng/mL(Fig. 8B).

Discussion

EGFR is a glycoprotein of 170 kDa, encoded by a gene located on chromosome 7p12.[10] The EGFR is a member of the erbB family of receptor tyrosine kinase proteins, which also includes HER2/neu(erbB2), HER3(erbB3), and HER4(erbB4). These receptors are composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and an intracellular tyrosine kinase domain, and, with the exception of HER2, all bind to receptor-specific ligands. Phosphorylation of the tyrosine kinase domain followed by homodimerization or heterodimerization between different receptors of

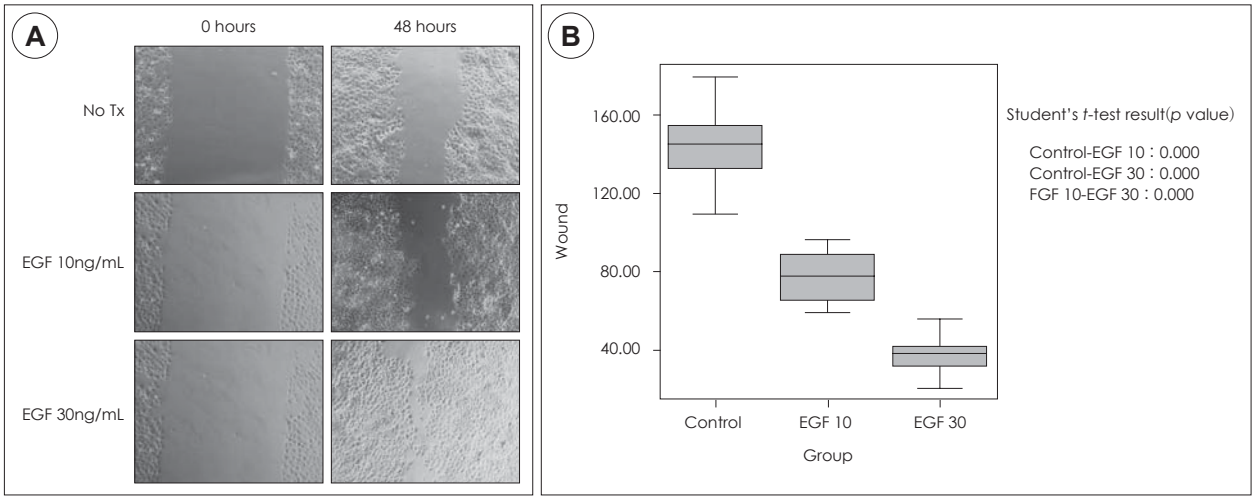

Fig. 6. Wound healing assay in EGF-treated cells. FaDu cells were plated in a 6-well plate and grown to confluency in serum containing media. The cells were starved of growth factors for 48h. The monolayer was scratched with a pipette tip and washed with PBS. To evaluate the effect of EGF on both migratory and proliferative activities, FaDu cells were treated with EGF at 0, 10, and 30ng/mL concentrations. Exogenous EGF enhanced the migration and proliferation of FaDu cells in a dose-dependent manner($p<0.05$). (A) Microscopic findings ; (B) graph.

the same family leads to protein activation.[11] Receptor dimerization is promoted by ligand binding, high receptor density due to overexpression, and mutations in the kinase domain. Protein activation on the cell surface of cancer cells is believed to promote signaling cascades, cell growth, differentiation, cell survival(apoptosis), drug and radiation sensitivity, cell cycle progression, and angiogenesis.[9]

Growth and differentiation of HNSCC are regulated by sev-

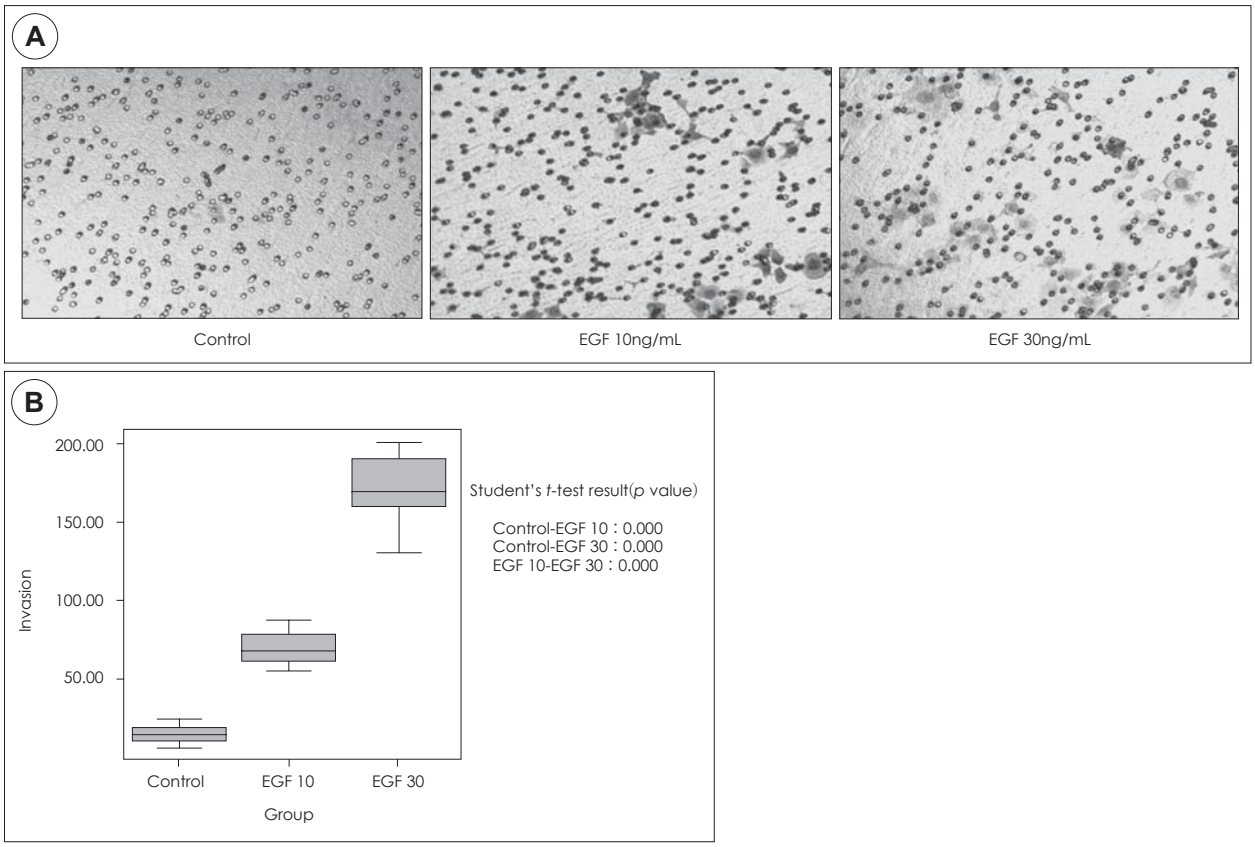

Fig. 7. Invasion assay of EGF-treated cells. Transwell chambers were used to verify the level of cell invasiveness. FaDu cells were seeded on the upper membrane in EGF at 0, 10, and 30ng/mL concentrations. After 48h of incubation, plugged cells in the 8-μm pore or cells attached to the undersurface membrane were counted, and the cells attached in the lower section were stained with hematoxylin and counted under a light microscope. The bars show the SD of triplicate samples. Data are representative of three separate experiments with similar results. EGF significantly promoted the invasion of FaDu cells in a dose-dependent manner.

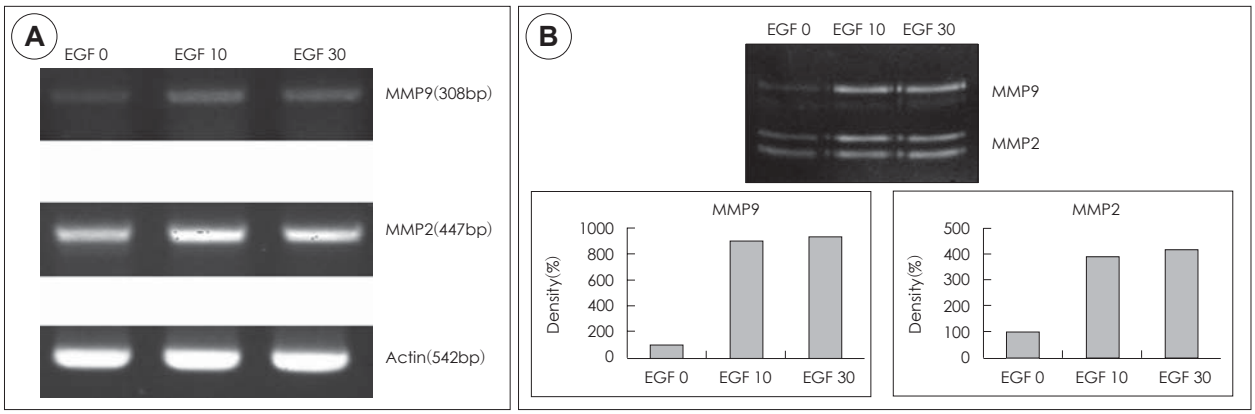

Fig. 8. Induction of MMP-2 and MMP-9 activities by EGF. A : RT-PCR of MMP-2 and MMP-9 in FaDu cells. Detection of MMP-2 and MMP-9 expression in FaDu cells treated for 24h with 0, 10, and 30ng/mL EGF. Exogenous EGF enhanced MMP-2 and MMP-9 expression. B : Zymography. FaDu cells were serum-deprived for 48h and then incubated with fresh medium containing EGF at 10 or 30ng/mL concentrations. The conditioned media were collected after 24 and 48h. The samples were fractionated on a polyacrylamide gel containing 0.1% gelatin, and a zymogram was developed as described in the Materials and methods. The 92- and 72-kDa gelatinase activity bands are indicated by the solid arrows. The level of MMP-2 and MMP-9 activity increased after EGF treatment for 24h compared to the control group. However, no significant difference was observed between the 10 and 30mL EGF treatment groups.

eral growth factors and their surface receptors. EGFR is upregulated in several carcinomas and is encoded by the *c-erb-B2* proto-oncogene. This protein is expressed at a low levels in many normal human tissues, but activation of the *c-erb-B2* oncogene results in its overexpression and is observed in many human cancers.[12-14] In HNSCC, EGFR is not only an independent prognostic factor of outcome in multivariate analysis, but also a first choice therapeutic target.[4] The recent demonstration of a significant survival benefit when combining cetuximab with external radiation therapy is a major breakthrough in the management of HNSCC[4,15] and establishes a new treatment option for locally advanced HNSCC. This trial also provided an important proof -of -principle that targeting a pertinent signaling pathway can enhance the radiation response of tumors. However, the improvement in the locoregional control rate has been modest(within the range achieved with concurrent radiotherapy and chemotherapy), and more than half of patients receiving radiotherapy plus cetuximab still experienced locoregional relapse.[4] Therefore, a need exists to further improve the outcome. Ongoing clinical efforts are devoted to address whether the addition of cetuximab to concurrent chemoradiation can yield a better outcome(i.e., RTOG study 0522). Cancer cells rely on several, sometimes, redundant, activation pathways ; EGFR is only one of them. The risk of treatment failure is higher if only one receptor is targeted ; hence, combining broader -range tyrosine kinase inhibitors such as CI-1033, which targets all four members of the Erb family(pan ErbB), could increase treatment success.[16] In head and neck cancer patients receiving 5-fluorouracil, EGFR was shown to be a tumor parameter with the highest prognostic impact.[17] The relationship between EGFR overexpression and increased tumor size, and/or the local extent of primary tumors(T classification), has been described in previous studies.[18] In another study, multivariate analysis showed that N status was the most powerful clinical prognostic factor, and that the EGFR level of expression was a major prognostic factor in the population of patients with resectable larynx and hypopharynx cancers treated by induction chemotherapy and radiotherapy. In several studies, EGFR expression was suggested to be correlated with prognosis in advanced laryngeal carcinoma(stage III and IV).[19] Conversely, studies have also reported no significant correlation between EGFR expression and tumor prognosis in HNSCC.[20]

In this study, immunohistochemical staining was performed on 57 cases of hypopharynx cancer to analyze the expression of EGFR. Overexpression of EGFR was noted in 63.2% of the cases. EGFR expression was significantly elevated in advanced stage tumors. Furthermore, the rate of distant metastasis increased when EGFR was overexpressed, and we believe that a statistically significant correlation may be found with a larger study population. Because EGFR was more strongly expressed in the cancer cells, EGFR activation is thought to be associated with the progression of cancer. In this study, even in the cases when EGFR expression was not observed, EGFR was presumed to be present, but could not be observed because it was expressed at levels that were undetectable by immunohistochemistry. Further studies measuring the concentration of EGFR in the tissue or using Western blotting to determine the relationship between the concentration of EGF and EGFR, and their expression are warranted. Although no statistical significance was detected between the expression of EGFR and the survival rate in this study(p=0.053), a correlation may be revealed in a larger study population.

Degradation of the extracellular matrix(ECM), which is a necessary step in tissue remodeling processes such as wound healing and embryonal development, has been attributed to proteolytic activity of MMPs. The role of EGFR -mediated signals in regulating matrix degradation is not completely understood, but EGFR inactivation has been shown to impair MMP activity during pancreatic and craniofacial development. Wound healing is another remodeling process, in which properly controlled stromal-epithelial interactions are crucial to the outcome. In EGFR -null mice, early proliferation and migration of keratinocytes is impaired resulting in delayed wound healing, which provides insight into the mode of action of EGFR in tissue remodeling processes.[21] In this study, RT-PCR after EGF treatment for 24 and 48h revealed a marked increase in the level of MMP-2 and MMP-9. In addition, we confirmed by zymography that the activities of MMP-2 and MMP-9 increased in the EGF treatment group. These results suggest that exogenous EGF enhances the expression and activity of MMP-2 and MMP-9.

In summary, high EGFR expression was observed in hypopharyngeal cancer tissue, and the level of EGFR significantly increased with tumor pathologic stage. Moreover, EGFR protein expression increased in hypopharyngeal cancer cells compared to normal cells, and exogenous EGF significantly enhanced the growth and invasiveness of cancer cells. MMP expression was detected in the hypopharyngeal cancer cells, and exogenous EGF enhanced the expression and activity of MMP-2 and MMP-9. Furthermore, the expression of EGFR was associated with tumor progression and the prognosis of patients with hypopharyngeal cancer. Further research pertaining to the effect of EGF/EGFR on hypopharynx cancer in a larger study population could aid in the diagnosis and man-

agement of hypopharyngeal cancer.

Acknowledgments

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