의학석사 학위논문

The Effect of *H. pylori* on the EGFR-induced Signal Transduction and Preventive Effect of Celecoxib in the Gastric Cancer Cells

H. pylori 의 EGFR 신호전달에 미치는 영향 및 celecoxib 의 위암발생 억제기전 연구

2012 년 8 월

서울대학교대학원

의학과분자유전체전공

김재연

H. pylori 의 EGFR 신호전달에 미치는 영향 및 celecoxib 의 위암발생 억제기전 연구

The Effect of *H. pylori* on the EGFR-induced Signal Transduction and Preventive Effect of Celecoxib in the Gastric Cancer Cells

지도교수 김 나 영

이 논문을 의학석사 학위논문으로 제출함

2012 년 4 월

서울대학교 대학원

의학과 분자유전체전공

김 재 연

김재연의 의학석사 학위논문을 인준함

2012 년 7 월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

Background and aim: *Helicobacter pylori* (*H. pylori*) infection increases the risk of gastric cancer through disrupting the regulation of cell survival. *H. pylori* infection is associated with epithelial growth factor receptor (EGFR) activation. EGFR downstream targets, such as phosphatidyl inositol 3-OH kinase (PI3K)-Akt-glycogen synthase kinase-3 (GSK3) pathways, regulate cell survival and migration. *H. pylori* infection also induces cyclooxygenase-2 (COX-2) over-expression, and previous study suggested that selective COX-2 inhibitor, celecoxib, blocks Akt signaling pathways. COX-2 and EGFR may cross talk through Akt-signaling pathways. The aim of the present study was to evaluate the effect of *H. pylori* on EGFR signaling pathways and to find out whether celecoxib has inhibitory effect on the EGFR signaling pathway or not.

Methods: AGS gastric epithelial cell lines were co-cultured with the toxigenic *H. pylori cagA*+, *vacA*+ G27 and the *cagE*- mutant of G27. The expressions of COX-2, EGFR, TGF-ß, Snail, Slug and E-cadherin were measured by real-time PCR. In the next, western blot analyses of COX-2, EGFR, total Akt (tAkt), phosphorylated Akt (pAkt) and pGSK3ß were carried out at various concentrations (0, 10, 20, 30μmol/L) of celecoxib treatment for 24 hours in *H. pylori* treated AGS cell lines.

Results: *H. pylori* infection significantly up-regulated the mRNA levels of COX-2, EGFR,TGF-ß, Snail, Slug and down-regulated E-cadherin in RT-PCR. AGS cell lines treated with *cagE*-mutants, which have a defective type IV secretion system did not show EGFR up-regulation. Celecoxib had inhibitory effects on *H. pylori*-induced over-expression of COX-2 (p=0.015), EGFR (p=0.025), pAkt (p=0.025) and pGSK3ß (p=0.029) in AGS cell lines in Western blot analysis.

Conclusion: Infection by *H. pylori* with intact type IV secretion system activates EGFR signal pathways in AGS cell lines and celecoxib has inhibitory effect on this pathway. These finding provide insights into the anti-gastric cancer effect of celecoxib.

Keywords: Gastric carcinoma; *Helicobacter pylori*; Cyclooxygenase-2; EGFR; Celecoxib

***Student Number*: 2010-23711**

목차

List of Tables

.

List of Figures

Introduction

Helicobacter pylori (*H. pylori*) infection is a major risk factor of gastric cancer (1, 2). Infection with the organism is not the only factor associated with developing gastric cancer. Clinical outcome of the infection is the result of interaction of host genetics, environmental factors and the virulence of the bacteria such as vacuolating cytotoxin (*vacA*) and *cag* pathogenicity island (*cag*PAI) (3). VacA protein induces massive vacuolization in epithelial cells and *cag* PAI encodes CagA, major effector protein, and other proteins forming type IV secretion apparatus, which is used to penetrating the gastric epithelial cells and facilitating the translocation of CagA (4, 5).

Cyclooxygenase (COX) is enzyme that catalyzes the conversion of arachidonic acids to prostaglandins, plays important role in physiological and pathological pathways (6). COX-2 is inducible form and expressed in response to inflammation and carcinogenesis. Several epidemiological and clinical studies have shown the relationship of COX-2 expression to gastric cancer progression (7, 8). *H. pylori* infection causes COX-2 over-expression in the early step of gastric carcinogenesis (9, 10). Therefore, there have been efforts to target COX-2 to prevent the development of gastric cancer. Non-steroidal anti-inflammatory drugs (NSAID), especially, selective COX-2 inhibitors, such as celecoxib, have been suggested to reduce the risk of gastric cancer *in vivo* and *in vitro* (11-13). The anti-cancer effect of celecoxib is mediated by COX-2 independent pathways as well as by COX-2 dependent pathway (14-17). Our previous study showed that one of the anti-gastric cancer mechanisms of celecoxib is down-regulation of Akt signaling (16).

Epidermal growth factor receptor (EGFR) is a member of the ErbB family and plays important roles in cell survival, proliferation, differentiation and migration. EGFR over-expression is frequently detected in human gastric cancers (18). It has been proposed that *H. pylori* VacA up-regulates EGFR and the downstream target including Akt signaling pathways (19-21), which is

also proved to be downstream target of the COX-2 signaling pathway (16). In addition, *H. pylori* induced EGF activation has been reported to promote epithelial-mesenchymal transition (EMT) (22). Cells undergo EMT lose epithelial characteristics, such as, cell-to-cell adhesion, differentiation and acquire mesenchymal properties like mobility, invasiveness and resistance to apoptosis. It is regulated by transforming growth factor-ß (TGF-ß) mediated signal pathways; up-regulation of members of the EMT transcriptome, such as Snail and Slug, and down-regulation of E-cadherin (23, 24). There has been evidence that COX-2 is related to EMT in colon cancer cells (25) and EGFR signaling is required to TGF- ß mediated COX-2 induction in bronchial epithelial and hepatocellular carcinoma cell lines (26, 27). However, the relationship of COX-2, EGFR and EMT pathway has not been clarified in gastric cancer cells.

From this background, the aim of this study was to evaluate the effect on expression of COX-2, EGFR and the downstream targets after *H. pylori* infection in the gastric cancer cell line. In addition, we investigated whether celecoxib, COX-2 selective antagonist, has inhibitory effect on *H. pylori*-induced EGFR signal conduction pathway, thus finally to find out the chemopreventive mechanism of celecoxib in gastric cancer.

Materials and Methods

Materials and reagents

Purified celecoxib was provided by Pfizer Pharmaceuticals Korea (Seoul, Korea) and was dissolved in 100% dimethyl sulfoxide (DMSO); final DMSO concentrations in all cultures were below 0.1%. Cell was treated with increasing concentrations of celecoxib (0, 10, 20 and 30 μM) for 6 and 24 hours.

Cell culture and *H. pylori* strain

G27 strain (*cagA*+, *vacA*+ [s1, m1]) wild type and *cagE*- isogeneic mutant of the strain, obtained from Professor S. Falkow (Stanford University, Stanford, CA, USA) , G69a strain (*cagA*+, *vacA*+), expressing green fluorescence protein (A gift from Dr. Reiner Haas, Munich, Germany) and HP 99 strain (*cagA*+, *vacA*+), were used in this experiment. Bacteria were cultured under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37℃ on Chocolate agar plates for 5 days. Bacteria were harvested and resuspended in RPMI-1640 (Gibco, GrandIsland, NY, USA). Then, those were supplemented with 10% fetal bovine serum, and 100 U/mL penicillin and 100 mg/mL streptomycin for co-culture with the AGS cells (ATCC CRL 1739; obtained from American type culture collection, Bethesda, MD, USA) at a multiplicity of infection (MOI) of 100:1.

After 24 hours, the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4, 37℃) and various concentrations (0, 10, 20, 30 μmol/L) of celecoxib were added and incubated for another 24h with serum starvation. The controls were treated with a DMSO vehicle at a concentration equal to that of the drug-treated cells.

Real-time PCR

To extract total RNAs from AGS cell lines, we used TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer and the collected RNA was purified using RNeasy mini kits (Qiagen, Valencia, CA, USA). RNA samples were diluted to a final concentration of 0.5 mg/ml in RNase-free water and stored at 80℃, until use. Real-time PCR reaction was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in 20 L SYBR Premix Ex Taq™ (Takara Bio, Shiga, Japan) using 200 ng cDNA. The thermal cycler conditions were 10 sec hold at 95℃, followed by 40-45 cycles of 5 sec at 95℃ and 33 sec at 60℃. The following primers were used: COX-2 forward, TTCAAATGAGATTGTGGGAAAATTGCT; COX-2 reverse, AGATCATCTCTGCCTGAGTATCTT; HB-EGF forward, CTCTTTCTGGCTGCAGTTCTC;

HB-EGF reverse, AGCTGGTCCGTGGATACAGT; EGFR forward, CTATGAGATGGAGGAAGACG ; EGFR reverse, CAGAGGAGGAGTATGTGTGA;TGF-ß forward, GTATGGGGTCGCAGGGTGTT; TGF-ß reverse, CAGATGCGCTGTGGCTTTGC; Snail forward, CCCCAATCGGAAGCCTAACT; Snail reverse, GGTCGTAGGGCTG CTGGAA; Slug forward, AAAAGCCAAACTACAGCGAACTG; Slug reverse, AGAATCTCTGCTTGTGGTATGACA; Vimentin forward, AAAACACCCTGCAATCTTTCAGA; Vimentin reverse, CACTTTGCGTTCAAGGTCAAGAC; E-cadherin forward, GGCGCCACCTCGAGAGA; E-cadherin reverse, TGTCGACCGGTGCAATCTT; Homo sapiens actin, ß forward, TTCGAGCAAGAGATGGCCAC; Homo sapiens actin, ß reverse, CGGATGTCCACGTCACACTT. All equipment and reagents were purchased from Applied Biosystems and used according to their recommended protocols.

Western blotting

After washing twice with PBS (pH 7.4, 37℃), the AGS cells were treated with cell lyses buffer (Sigma Chemical Co, St. Louis, MO, USA), and the protein concentration was measured with the BCA TM protein assay kit (Pierce, Rockford, IL, USA). Cell extracts (20 mg protein) were subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to polyvinylidene difluoride membranes. After blocking the non-specific binding sites with non-fat dry milk, the membranes were incubated with anti COX-2, anti-EGFR, anti-phospho-Ser473-Akt, anti-Akt, pGSK3β (phosphorylated glycogen synthase kinase-3β), and actin. The COX-2 membrane incubation with COX-2 antibody (goat polyclonal IgG antibody, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was carried out at 4℃ overnight, then the blots were incubated with secondary antibody (donkey anti-goat antibody, 1:1000). The bound antibodies were detected using a chemiluminescence detection kit. Western blots were analyzed and quantified using the

Luminescent image analyzer LAS 1000-plus (Fuji Photo Film, Tokyo, Japan) and the Image Gauge ver. 3.12 (Fuji Photo Film).

Statistical analyses

SPSS for Windows (version 18.0; SPSS, Chicago, IL, USA) was used for the statistical analysis. The level of mRNA was expressed as fold changes (mean ± SEM) relative to the control groups. The level of protein was expressed as pg/ml (mean ± SEM). Mann-Whitney *U*-test was used for the comparison between two groups. Statistical significance was set at $p<0.05$.

Results

H. pylori stimulates COX-2 and EGFR expression

After 24-h incubation with G27 wild type, we could find over-expression of COX-2, EGFR, TGF-ß and Snail, Slug, Vimentin and down-regulation of E-cadherin in AGS cell lines (Table 1, Fig. 1). Similar patterns of mRNA expression were observed using different strains of *H. pylori*, such as G69a and HP99 (data not shown). However, AGS cells treated with *cagE*- mutant of G27 did not show significant difference compared to control (Table 1, Fig. 1). Comparing to AGS cells treated with G27 wild type, those treated with *cagE*- mutant showed significant lower level of mRNA expression in HB-EGF, EGFR, and Snail (Fig. 1).

Celecoxib inhibits COX-2 over-expression and EGFR up-regulation.

To assess whether celecoxib inhibits *H. pylori*-induced over-expression of those mRNAs or

Table 1. RT-PCR of COX-2. EGFR, HB-EGF, TGF-ß, Snail, Slug, Vimentin, and E-cadherin in AGS cell line treated with wild type toxigenic *H. pylori* and its isogenic *cagE*-mutant.

	AGS control	G27 wild type	*cagE*- mutant of G27
COX-2	1.00	**3.27 ± 0.92**†	1.21 ± 0.19
EGFR	1.00	**2.55 ± 0.31**†	1.43 ± 0.33
HB-EGF	1.00	**6.23 ± 1.21**†	1.69 ± 0.47
TGF-ß	1.00	**1.68 ± 0.42***	1.18 ± 0.28
Snail	1.00	**7.65 ± 3.01**†	1.96 ± 0.92
Slug	1.00	**6.79 ± 1.83**†	2.52 ± 0.65
Vimentin	1.00	**2.33 ± 0.75***	1.32 ± 0.36
E-cadherin	1.00	**0.62 ± 0.03***	0.76 ± 0.37

Data were shown as fold increase(mean± SE) of mRNA.

$^{*}p<0.05$, $^{\dagger}p<0.005$ compared to AGS cells.

not, we incubated AGS cell lines with *H. pylori* strain in the presence or absence of celecoxib. The effect of treatment with 0, 10, 20 and 30 µM of celecoxib for 6 hours on mRNA expression was evaluated. AGS cell lines were treated with G69a (n=6) and mRNA levels of COX-2, EGFR, TGF-ß, Snail, Slug, Vimentin and E-cadherin were measured by RT-PCR. Over-expressions of COX-2, Snail and Slug were shown after *H. pylori* treatment compared to AGS control. Celecoxib had inhibitory effect on overexpression of COX-2 ($p=0.026$) and Snail ($p=0.041$) (data not shown). However, we could not find significant difference in expressions of the other mRNAs after *H. pylori* infection with any other dose of celecoxib.

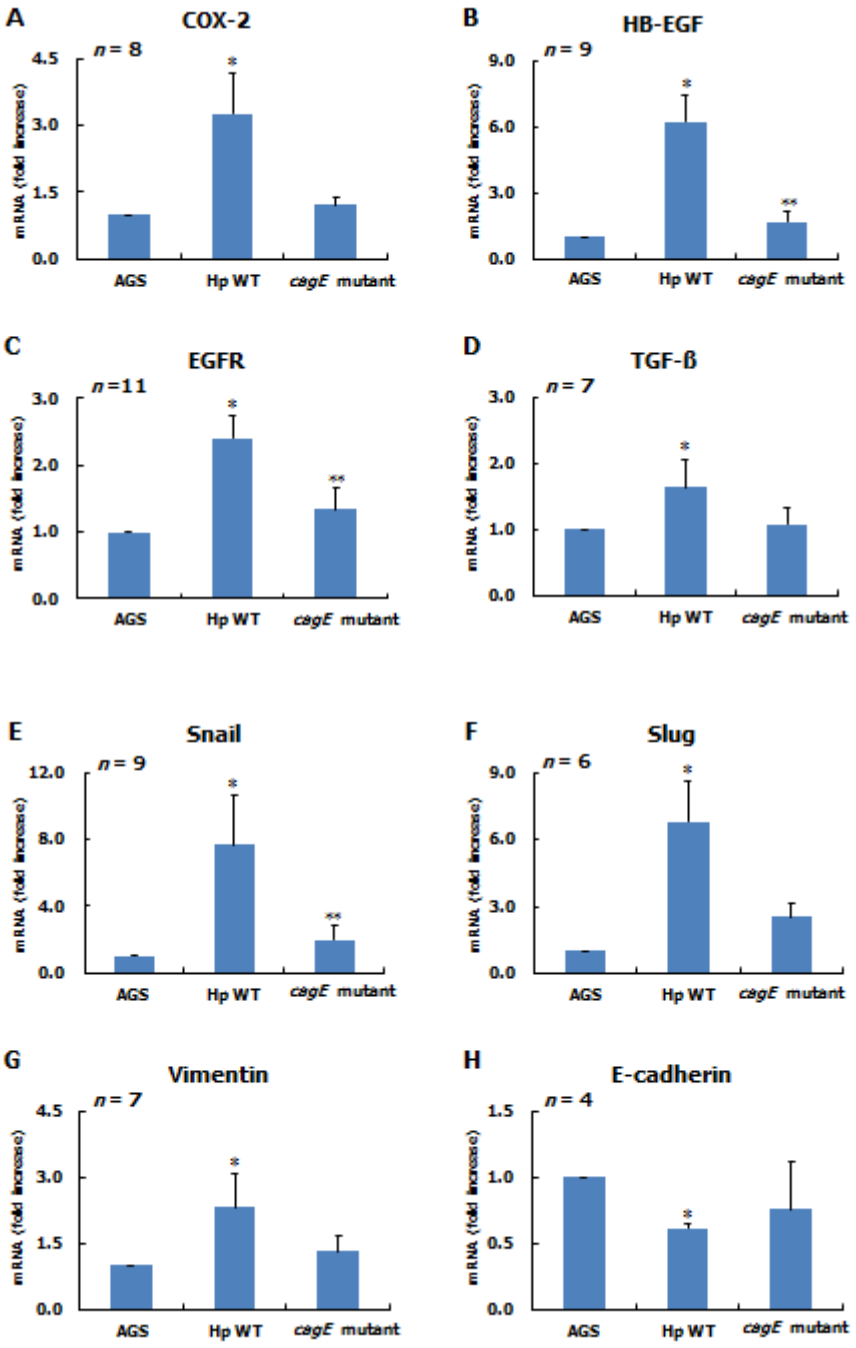

Figure 1. Effect of *H. pylori* infection on mRNA expressions of COX-2, EGFR, HB-EGF, TGF-ß, Snail, Slug, Vimentin, and E-cadherin in AGS cell line by RT-PCR. Incubation of AGS cells treated with *H. pylori* strain G27 wild type and *cagE*- isogenic mutant. (A) COX-2, (B) EGFR, (C) HB-EGF, (D) TGF-β, (E) Snail, (F) Slug, (G) Vimentin and (H) E-cadherin. Over-expressions of COX-2, EGFR, HB-EGF, TGF-β, Snail, Slug, Vimentin and down-regulation of E-cadherin were observed in AGS cell lines treated

with *H. pylori* strain G27 wild type. There was no significant difference in the levels of the eight mRNA in those treated with *cagE*- isogenic mutant and AGS control. Comparing to AGS cell lines treated with G27 wild type, expression of HB-EGF (p=0.001), EGFR (p=0.010) and Snail (p=0.013) was significant lower in those treated *with cagE*- isogenic mutant.

Hp WT: *H. pylori* wild type, *cagE* mutant: *cagE*- isogenic mutant of *H. pylori*

$^{*}p<0.05$ compared to AGS cells. $^{**}p<0.05$ compared to G27 wild type in *cagE*- isogenic mutants.

Western blotting for COX-2, EGFR, Akt, GSK3ß with or without celecoxib treatment

To assess whether *H. pylori*-induced protein expressions of COX-2 and EGFR-Akt pathway are suppressed by celecoxib, we incubated AGS cell lines with *H. pylori* strain G69a in the presence or absence of celecoxib. The effects of 0, 10, 20 and 30 μM of celecoxib treatment in AGS cells on the expression of COX-2, EGFR, Akt and pGSK3ß were evaluated. The 10, 20 and 30 μmol/L concentrations of celecoxib showed significant inhibitory effects on the expression of COX-2 at 24 h of incubation in the AGS cell lines (Fig. 2A). The 20 μmol/L concentration of celecoxib showed significant inhibitory effects on the over-expression of COX-2 by *H. pylori* infection (Fig. 2A). For EGFR, *H. pylori* infection induced EGFR over-expression with G69a strain (Fig. 2B) and with G27 wild type (data not shown). However, over-expression of EGFR was not observed in AGS cell lines treated with *cagE*- mutant of G27 strain (data not shown). The 20 and 30 μmol/L concentrations of celecoxib showed significant inhibitory effects on the expression of EGFR in *H. pylori* treated AGS cell lines with G69a strain (Fig. 2B).

Akt, a threonine protein kinase, found at the multiple signaling pathways, plays a role as a regulator of cell proliferation apoptosis, glycogen metabolism, migration and cell survival (28). It is downstream target of EGFR-PI3K pathway and its complete activation need phosphorylation of regulate sites. Phosphorylated Akt (pAkt) targets glycogen synthase kinase 3(GSK3), subsequently phosphorylates GSK3ß and GSK3α. We measured total Akt (tAkt), pAkt, and pGSK3ß as downstream targets of Akt signaling. AGS cell lines treated with *H. pylori* G69a strain showed significant over-expression of pAkt, not tAkt ($p<0.001$, Fig. 2C, Fig. 2D). Celecoxib showed significant inhibitory effects on the expression of pAkt in AGS control and *H. pylori* infected AGS cell lines (Fig. 2D). For pGSK3ß, we could observe that *H. pylori* induced over-expression of the protein and inhibitory effects of celecoxib (Fig. 2E).

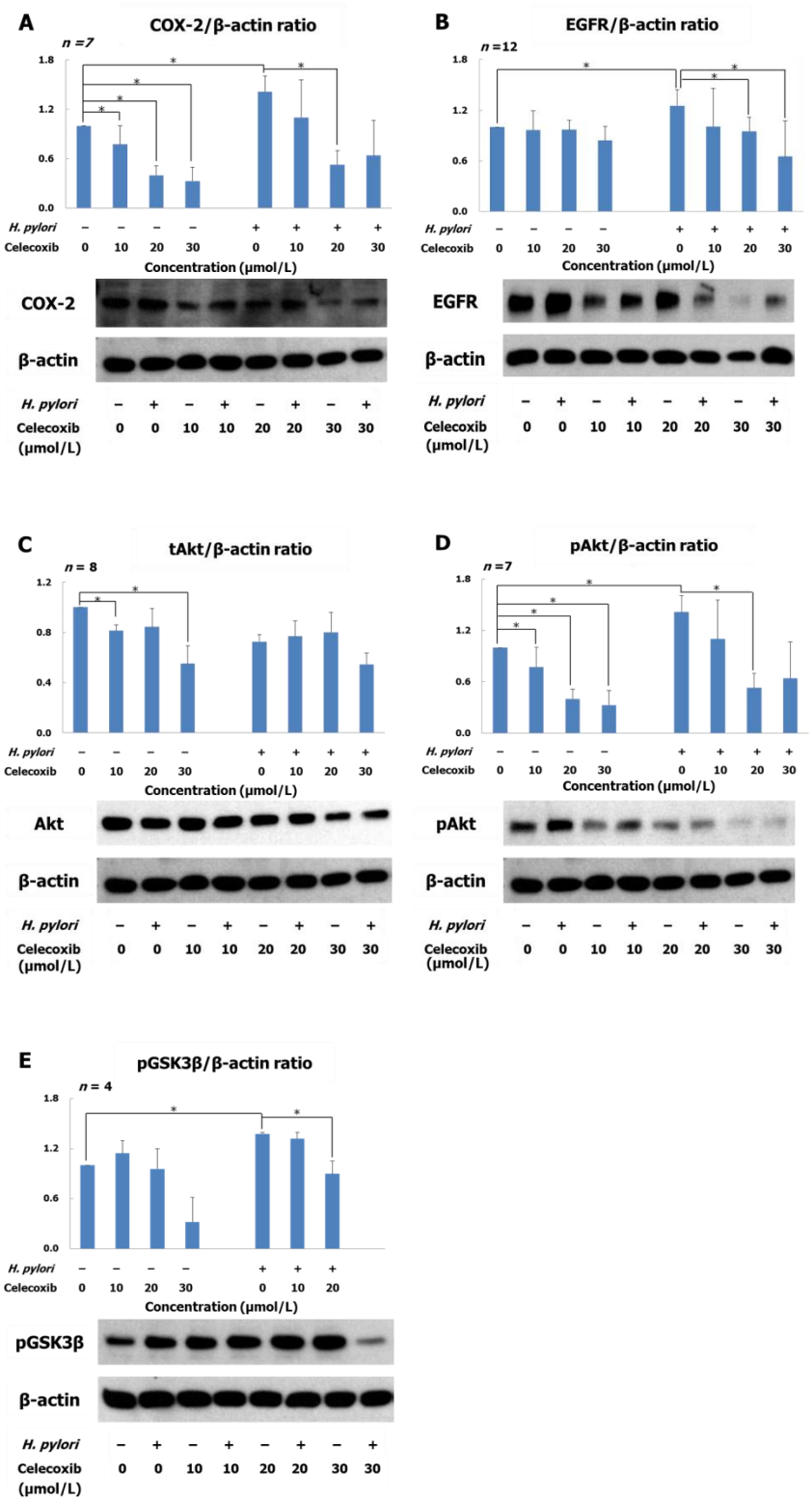

Figure 2. Western blotting for (A) COX-2, (B) EGFR, (C) tAkt, (D) pAKt and (E) pGSK3β in *H. pylori*

infected AGS cell line. Incubation of AGS cells infected by *H. pylori* strain G69a and AGS cell control with various concentrations (0, 10, 20, 30 μmol/L) of celecoxib for 24 hours show different protein expression. (A) AGS cell lines treated with *H. pylori* G69a strain showed COX-2 over-expression (p=0.001). The 10, 20 and 30 μmol/L concentrations of celecoxib showed inhibitory effects on the protein expression of COX-2 in the AGS control (p=0.026. p=0.001, p=0.017, respectively). The 20 μmol/L concentration of celecoxib showed inhibitory effects on the protein expression of COX-2 in the *H. pylori* treated AGS cells (p=0.015). (B) AGS cell lines treated with *H. pylori* G69a strain showed significant EGFR over-expression (p<0.001). The 20 and 30 μmol/L concentrations of celecoxib showed inhibitory effects on the protein expression of EGFR in the *H. pylori* treated AGS cells (p=0.025, p=0.004 respectively). (C), (D) AGS cell lines treated with *H. pylori* G69a strain showed over-expression of pAkt (p<0.001), not tAkt. The 10 and 30 μmol/L concentrations of celecoxib showed inhibitory effects on the expression of tAkt in the AGS control (p=0.010, p=0.020, respectively). There was significant increase in pAkt expression after *H. pylori* infection (p=0.001). The 10, 20 and 30 μmol/L concentrations of celecoxib inhibited the over-expression of pAkt at 24 h of incubation in the AGS control (p=0.026, p=0.001, p=0.017, respectively). The 20 μmol/L concentration of celecoxib showed inhibitory effect on the expression of COX-2 at 24h of incubation in the *H. pylori* treated AGS cell lines with G69a strain (p=0.015). (E) AGS cell lines treated with *H. pylori* G69a strain showed significant over-expression pGSK3ß (p<0.029). The 10 μmol/L concentrations of celecoxib showed significant inhibitory effect on the expression of pGSK3ß at 24 h of incubation in the AGS cell lines (p=0.029).

*p<0.05 compared to AGS cells.

Discussion

In this study, *H. pylori* infection in AGS cell lines up-regulated COX-2, EGFR. PI3K/Akt pathway, TGF-β, and EMT related genes such as Slug, Snail, and Vimentin. Importantly, from the observation that selective COX-2 antagonist, celecoxib, had inhibitory effect on EGFR and its downstream targets; we suggest that COX-2 may have important role in EGFR pathway (19, 20). Interestingly, AGS cells treated with *cagE*- strains did not show overexpression of EGFR compared to those treated with wild type. There have been numerous studies that *H pylori* strains that possess the *cag* PAI induce more severe gastritis and increase the risk of peptic ulcer disease gastric adenocarcinoma (29, 30). CagE, encoded in *cag* PAI, is one of structural protein of type IV secretion system that delivers the CagA protein into the host cell. This process is accomplished by specialized adhesin activates host cell integrins for subsequent delivery of CagA. Recently there have been studies that type IV secretion system, not only CagA paly important role in various signal pathways (31-33). The present study well corresponds with the previous studies that *cag* PAI, especially *cagE* is important in *H. pylori* induced EGFR transactivation.

We could find over-expression of COX-2, EGFR, TGF-ß and Snail, Slug, Vimentin and down-regulation of E-cadherin in AGS cell lines after *H. pylori* infection by RT-PCR. In the next step, we tried to prove the inhibitory effects of celecoxib on those mRNAs. However, there was no significant over-expression of mRNAs after *H. pylori* treatment except COX-2, Slug and Snail; we could observe the inhibitory effect of celecoxib on those over-expressed mRNAs. Small sample size may contribute to the result. Although, we failed to observe inhibitory effect of celecoxib on EGFR mRNA expression, we could find celecoxib could inhibit EMT transcriptomes, such as Slug and Snail. This is new finding in gastric cancer cells. We could observe the over-expression of COX-2 and EGFR-pAkt-pGSK3β after *H. pylori* infection, and

celecoxib inhibited over-expression of those proteins in Western blotting. It well coincide with the previous study that show inhibitory effect of celecoxib onpAKt-pGSK3β in AGS cell line without *H. pylori* infection (16). We could observe the inhibitory effect of celecoxib on EGFR protein over-expression after *H. pylori* infection. The present study implies that *H. pylori*-induced EGFR activation cross talk with COX-2 and plays important role in antiapoptotic pathway and EMT. Given that EGFR signaling is required to TGF-ß mediated COX-2 induction in bronchial and hepatocellular carcinoma cell lines (26, 27), COX-2 might involve in EMT as a downstream of EGFR pathway in gastric epithelial cell line (Fig.3).

COX-2 over-expression is frequently detected in human cancers, including lung, prostate and colon cancers (34-36). Thus, COX-2 inhibitors including NSAIDs and selective COX-2 inhibitors have been the target of prevent cancers including gastric cancer (37, 38). However, several COX-2 inhibitors, such asvaldecoxib (Bextra) and rofecoxib (Vioxx), were removed from the market previously due to increased risk of cardiovascular events, especially myocardial infarction (39, 40). For celecoxib, there have been controversies in cardiovascular events. Although, CLASS study found the risk of cardiovascular events in celecoxib users (41), other studies showed that celecoxib exposure did not elevate the risk of cardiovascular events (40, 42). Recently, a population-based intervention trial in China proved that celecoxib treatment alone could prevent progression of premalignant lesions to gastric cancer (43). In addition, there have been various combination of conventional chemotherapy with celecoxib have been studied (44-48), some of them proved the effect of celecoxib co-treatment (44-46). Furthermore, there have been studies to target COX-2 and EGFR synergistically in metastatic colon cancer or recurred head and neck cancers (49, 50). Given that COX-2 play a role not only in the early stage of gastric cancer involving apoptosis, but also in the late stage of EMT, selective COX-2 inhibitor, celecoxib could be resurfaced as an anti-cancer agent with combination of molecular target chemotherapy.

In summary, infection by *H. pylori* with intact type IV secretion system activates EGFR signal pathways in AGS cell lines and celecoxib has inhibitory effect on this pathway. These findings provide insights into the anti-gastric cancer effect of celecoxib.

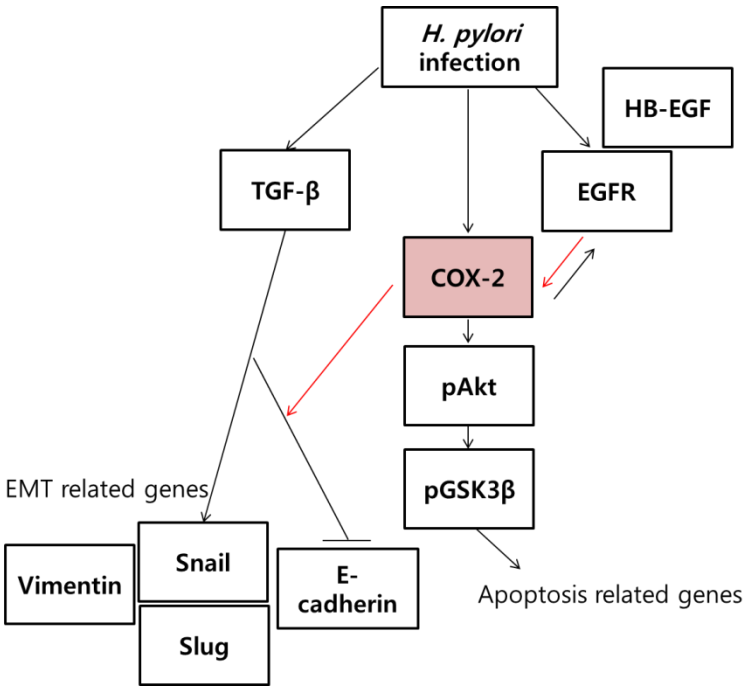

Figure 3. A schematic role of COX-2 in gastric cancer cell lines after *H. pylori* infection

References

1. Correa P. Helicobacter pylori and gastric cancer: state of the art. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 1996;5(6):477-81. Epub 1996/06/01.

2. Peek RM, Jr., Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nature reviews Cancer. 2002;2(1):28-37. Epub 2002/03/21.

3. Stoicov C, Saffari R, Cai X, Hasyagar C, Houghton J. Molecular biology of gastric cancer: Helicobacter infection and gastric adenocarcinoma: bacterial and host factors responsible for altered growth signaling. Gene. 2004;341:1-17. Epub 2004/10/12.

4. Cover TL, Blanke SR. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nature reviews Microbiology. 2005;3(4):320-32. Epub 2005/03/11.

5. Fischer W, Puls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Molecular microbiology. 2001;42(5):1337-48. Epub 2002/03/12.

6. Williams C, Shattuck-Brandt RL, DuBois RN. The role of COX-2 in intestinal cancer. Annals of the New York Academy of Sciences. 1999;889:72-83. Epub 2000/02/11.

7. Yamac D, Ayyildiz T, Coskun U, Akyurek N, Dursun A, Seckin S, et al. Cyclooxygenase-2 expression and its association with angiogenesis, Helicobacter pylori, and clinicopathologic characteristics of gastric carcinoma. Pathology, research and practice. 2008;204(8):527-36. Epub 2008/05/09.

8. Tatsuguchi A, Matsui K, Shinji Y, Gudis K, Tsukui T, Kishida T, et al. Cyclooxygenase-2 expression correlates with angiogenesis and apoptosis in gastric cancer tissue. Human pathology. 2004;35(4):488-95. Epub 2004/04/30.

9. Cho SO, Lim JW, Kim KH, Kim H. Involvement of Ras and AP-1 in Helicobacter pylori-induced expression of COX-2 and iNOS in gastric epithelial AGS cells. Digestive diseases and sciences. 2010;55(4):988-96. Epub 2009/06/06.

10. Wu WK, Sung JJ, Lee CW, Yu J, Cho CH. Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. Cancer letters. 2010;295(1):7-16. Epub

2010/04/13.

11. Hu PJ, Yu J, Zeng ZR, Leung WK, Lin HL, Tang BD, et al. Chemoprevention of gastric cancer by celecoxib in rats. Gut. 2004;53(2):195-200. Epub 2004/01/16.

12. Cho SJ, Kim N, Kim JS, Jung HC, Song IS. The anti-cancer effect of COX-2 inhibitors on gastric cancer cells. Digestive diseases and sciences. 2007;52(7):1713-21. Epub 2007/03/30.

13. Wang WH, Huang JQ, Zheng GF, Lam SK, Karlberg J, Wong BC. Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. Journal of the National Cancer Institute. 2003;95(23):1784-91. Epub 2003/12/05.

14. Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2003;17(3):590-603. Epub 2003/03/21.

15. Liu H, Huang P, Xu X, Liu J, Guo C. Anticancer effect of celecoxib via COX-2 dependent and independent mechanisms in human gastric cancers cells. Digestive diseases and sciences. 2009;54(7):1418-24. Epub 2008/10/17.

16. Kim N, Kim CH, Ahn DW, Lee KS, Cho SJ, Park JH, et al. Anti-gastric cancer effects of celecoxib, a selective COX-2 inhibitor, through inhibition of Akt signaling. Journal of gastroenterology and hepatology. 2009;24(3):480-7. Epub 2008/10/01.

17. Caputo R, Tuccillo C, Manzo BA, Zarrilli R, Tortora G, Blanco Cdel V, et al. Helicobacter pylori VacA toxin up-regulates vascular endothelial growth factor expression in MKN 28 gastric cells through an epidermal growth factor receptor-, cyclooxygenase-2-dependent mechanism. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(6):2015-21. Epub 2003/06/11.

18. Zheng L, Wang L, Ajani J, Xie K. Molecular basis of gastric cancer development and progression. Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association. 2004;7(2):61-77. Epub 2004/06/30.

19. Keates S, Keates AC, Nath S, Peek RM, Jr., Kelly CP. Transactivation of the epidermal growth factor receptor by cag+ Helicobacter pylori induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells. Gut. 2005;54(10):1363-9. Epub 2005/05/03.

20. Tabassam FH, Graham DY, Yamaoka Y. Helicobacter pylori activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. Cellular microbiology. 2009;11(1):70-82. Epub 2008/09/11.

21. Yan F, Cao H, Chaturvedi R, Krishna U, Hobbs SS, Dempsey PJ, et al. Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. Gastroenterology. 2009;136(4):1297-307, e1-3. Epub 2009/03/03.

22. Yin Y, Grabowska AM, Clarke PA, Whelband E, Robinson K, Argent RH, et al. Helicobacter pylori potentiates epithelial:mesenchymal transition in gastric cancer: links to soluble HB-EGF, gastrin and matrix metalloproteinase-7. Gut. 2010;59(8):1037-45. Epub 2010/06/30.

23. Maehara Y, Kakeji Y, Kabashima A, Emi Y, Watanabe A, Akazawa K, et al. Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1999;17(2):607-14. Epub 1999/03/18.

24. Vagenas K, Spyropoulos C, Gavala V, Tsamandas AC. TGFbeta1, TGFbeta2, and TGFbeta3 protein expression in gastric carcinomas: correlation with prognostics factors and patient survival. The Journal of surgical research. 2007;139(2):182-8. Epub 2007/02/03.

25. Jang TJ, Jeon KH, Jung KH. Cyclooxygenase-2 expression is related to the epithelial-to-mesenchymal transition in human colon cancers. Yonsei medical journal. 2009;50(6):818-24. Epub 2010/01/05.

26. Liu M, Yang SC, Sharma S, Luo J, Cui X, Peebles KA, et al. EGFR signaling is required for TGF-beta 1 mediated COX-2 induction in human bronchial epithelial cells. American journal of respiratory cell and molecular biology. 2007;37(5):578-88. Epub 2007/06/30.

27. Ogunwobi OO, Wang T, Zhang L, Liu C. Cyclooxygenase-2 and Akt mediate multiple growth-factor-induced epithelial-mesenchymal transition in human hepatocellular carcinoma. Journal of gastroenterology and hepatology. 2012;27(3):566-78. Epub 2011/11/22.

28. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nature reviews Cancer. 2002;2(7):489-501. Epub 2002/07/03.

29. Saadat I, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, et al. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. Nature. 2007;447(7142):330-3. Epub

2007/05/18.

30. Peek RM, Jr., Moss SF, Tham KT, Perez-Perez GI, Wang S, Miller GG, et al. Helicobacter pylori cagA+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. Journal of the National Cancer Institute. 1997;89(12):863-8. Epub 1997/06/18.

31. Backert S, Selbach M. Role of type IV secretion in Helicobacter pylori pathogenesis. Cellular microbiology. 2008;10(8):1573-81. Epub 2008/04/16.

32. Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(25):14648-53. Epub 1996/12/10.

33. Shibata W, Hirata Y, Maeda S, Ogura K, Ohmae T, Yanai A, et al. CagA protein secreted by the intact type IV secretion system leads to gastric epithelial inflammation in the Mongolian gerbil model. The Journal of pathology. 2006;210(3):306-14. Epub 2006/08/26.

34. Chang HC, Weng CF. Cyclooxygenase-2 level and culture conditions influence NS398-induced apoptosis and caspase activation in lung cancer cells. Oncology reports. 2001;8(6):1321-5. Epub 2001/10/18.

35. Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. The Journal of biological chemistry. 2000;275(15):11397-403. Epub 2001/02/07.

36. Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2001;15(14):2742-4. Epub 2001/10/19.

37. Futagami S, Suzuki K, Hiratsuka T, Shindo T, Hamamoto T, Ueki N, et al. Chemopreventive effect of celecoxib in gastric cancer. Inflammopharmacology. 2007;15(1):1-4. Epub 2007/02/27.

38. Yang P, Zhou Y, Chen B, Wan HW, Jia GQ, Bai HL, et al. Aspirin use and the risk of gastric cancer: a meta-analysis. Digestive diseases and sciences. 2010;55(6):1533-9. Epub 2009/08/13.

39. Juni P, Nartey L, Reichenbach S, Sterchi R, Dieppe PA, Egger M. Risk of cardiovascular events and rofecoxib: cumulative meta-analysis. Lancet. 2004;364(9450):2021-9. Epub 2004/12/08.

40. Kimmel SE, Berlin JA, Reilly M, Jaskowiak J, Kishel L, Chittams J, et al. Patients exposed to rofecoxib and celecoxib have different odds of nonfatal myocardial infarction. Annals of internal medicine. 2005;142(3):157-64. Epub 2005/02/03.

41. Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. The New England journal of medicine. 2005;352(11):1071-80. Epub 2005/02/17.

42. Graham DJ, Campen D, Hui R, Spence M, Cheetham C, Levy G, et al. Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non-selective non-steroidal anti-inflammatory drugs: nested case-control study. Lancet. 2005;365(9458):475-81. Epub 2005/02/12.

43. Wong BC, Zhang L, Ma JL, Pan KF, Li JY, Shen L, et al. Effects of selective COX-2 inhibitor and Helicobacter pylori eradication on precancerous gastric lesions. Gut. 2012;61(6):812-8. Epub 2011/09/16.

44. Arjona-Sanchez A, Ruiz-Rabelo J, Perea MD, Vazquez R, Cruz A, Munoz Mdel C, et al. Effects of capecitabine and celecoxib in experimental pancreatic cancer. Pancreatology. 2010;10(5):641-7. Epub 2010/11/06.

45. Aruajo AM, Mendez JC, Coelho AL, Sousa B, Barata F, Figueiredo A, et al. Phase II study of celecoxib with cisplatin plus etoposide in extensive-stage small cell lung cancer. Cancer investigation. 2009;27(4):391-6. Epub 2009/03/07.

46. Csiki I, Morrow JD, Sandler A, Shyr Y, Oates J, Williams MK, et al. Targeting cyclooxygenase-2 in recurrent non-small cell lung cancer: a phase II trial of celecoxib and docetaxel. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11(18):6634-40. Epub 2005/09/17.

47. El-Rayes BF, Zalupski MM, Manza SG, Rusin B, Ferris AM, Vaishampayan U, et al. Phase-II study of dose attenuated schedule of irinotecan, capecitabine, and celecoxib in advanced colorectal cancer. Cancer chemotherapy and pharmacology. 2008;61(2):283-9. Epub 2007/04/13.

48. Pan CX, Loehrer P, Seitz D, Helft P, Juliar B, Ansari R, et al. A phase II trial of irinotecan, 5-fluorouracil and leucovorin combined with celecoxib and glutamine as first-line therapy for advanced colorectal cancer. Oncology. 2005;69(1):63-70. Epub 2005/08/10.

49. Chan E, Lafleur B, Rothenberg ML, Merchant N, Lockhart AC, Trivedi B, et al. Dual blockade of the EGFR and COX-2 pathways: a phase II trial of cetuximab and celecoxib in patients with chemotherapy refractory metastatic colorectal cancer. American journal of clinical oncology. 2011;34(6):581-6. Epub 2011/01/11.

50. Kao J, Genden EM, Chen CT, Rivera M, Tong CC, Misiukiewicz K, et al. Phase 1 trial of concurrent erlotinib, celecoxib, and reirradiation for recurrent head and neck cancer. Cancer. 2011;117(14):3173-81. Epub 2011/01/20.

국문 초록

H. pylori 의 EGFR 신호전달에 미치는 영향 및 celecoxib 의 위암발생 억제기전 연구

배경 : *Helicobacter pylori* (*H. pylori*)의 감염은 세포의 생존 조절능력을 저해하여 위암의 발생위험을 증가시킨다. *H. pylori* 감염은 세포내 EGFR 의 활성화와 관련이 있으며, 하부단계의 phosphatidylinositol 3-OH kinase (PI3K)-Akt-Glycogen synthase kinase-3 (GSK3) 와 같은 경로를 활성화시켜 세포의 생존과 이동을 조절하는 것으로 알려져 있다. 또한 *H. pylori* 감염은 COX-2 의 과발현을 유발하는데, 이전 연구에서 COX-2 억제제인 celecoxib 가 Akt 신호전달경로를 억제한다고 밝힌 바 있다. EGFR 과 COX-2 는 Akt 신호전달경로를 공유하며 긴밀한 영향을 주고받을 가능성이 있다. 이에 본 연구는 *H. pylori* 가 EGFR 신호전달에 미치는 영향을 확인하고 celecoxib 이 이를 억제하는지 확인하고자 하였다.

연구재료 및 방법 : AGS 위암세포주를 *H. pylori* (*cagA*+, *vacA*+) G27과 *cagE* 돌연변이를 가진 G27에 24시간동안 감염시킨 후 mRNA와 단백질의 발현을 확인하였다. COX-2, EGFR, TGF-ß, Snail, Slug, E-cadherin의 mRNA발현을 RT-PCR로확인하였다. 그리고 다양한 농도(0, 10, 20, 30 μmol/L)의 ceclecoxib를 처리하여 COX-2, EGFR, tAkt, pAkt와 pGSK3ß의 단백질 발현을 확인하였다.

결과 : AGS 위암세포주에서 wild type의 *H. pylori* 감염은 COX-2, EGFR, TGF-ß, Snail, Slug의 mRNA 발현을 증가시켰으며 E-cadherin의 mRNA발현을 감소시켰다. 또한 wild type의 *H. pylori* 감염은 COX-2, EGFR, pAkt, pGSK3ß 의 단백질 발현을 증가시킨

반면, *cagE* 돌연변이로 인해 IV형 분비계에 결함이 있는 *H. pylori*에 의한 감염은 EGFR을 활성화시키지 못하였다. Celecoxib은 *H. pylori*에 의해 과발현되는 COX-2 (p=0.015), EGFR (p=0.025), pAkt (p=0.025)와 pGSK3ß (p= 0.029)를 억제시켰다.

결론 : AGS 위암세포주에서 IV 형 분비계가 정상인 *H. pylori* 에 의한 감염은 EGFR 신호전달체계를 활성화시켰으며, celecoxib 은 이를 억제하는 효과를 보였다.

주요어: 위암; *Helicobacter pylori*; COX-2; EGFR; Celecoxib

학번: 2010-23711