Helicobacter pylori CagA determines the gp130-activated SHP2/ERK and JAK/STAT signal transduction pathways in gastric epithelial cells

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Directed by Professor Yong Chan Lee

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This certifies that Doctoral Dissertation
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거의 5년이라는 학위 과정을 보내면서 지금 이 자리에 그 시간들을 다시 한번 돌이켜보게 되는데 힘든 시간보다 재미있고 즐거웠던 일들이 먼저 떠오르면서 미소를 짓게 합니다. 실험이라는 것을 전혀 몰랐던 내가 여기서 하나하나 배우고 그 배운걸 토대로 실험해서 나온 결과가 신기하기만 했었고 많은 실수와 실패를 거치면서 만들어진 데이터로 하나의 스토리를 만들어 갈 때 논문은 이렇게 해서 만들어지는구나 라고 느끼게 했던 시간들이었습니다. 인제 졸업을 앞둔 저는 제 인생의 한 챕터가 넘겨지고 새 다른 챕터를 맞이하게 합니다. 저는 이제까지 내 앞에 닫혀진 문을 멍하니 처다보면서 누군가 열어주기를 바래었지만 인제 주어진 문을 내 스스로 열려고 노력을 해야 하며 나아가야 할 때인 것 같습니다.

본 연구를 수행하는데 있어서 아낌없는 지도와 조언으로 이끌어 주시며 연구에 대한 뜨거운 열정으로 귀감이 되시는 이용찬 교수님께 진심으로 감사 드립니다. 바쁘신 와중에도 항상 귀중한 시간을 내어 소중한 가르침을 주셨고 실험 진행이 잘 되지 않을 때 충분히 수행해 낼 수 있다는 믿음을 저희들에게 심어주셨습니다.

미국 뉴욕의과대학에서 연수를 하면서 정말 실험자가 가져야 할 마음과 태도, 또한 실험을 하는데 있어서 단순히 하는 것이 아니라 할 것을 미리 랩 노트에 기록하고 그 기록된 계획에 맞추어 진행하고 정리해야 될 것등 무엇으로도 살 수 없는 아주 귀한 가르침과 제 인생에 있어서 소중한 기회를 허락해 주신 따스하고 열린 마음을 지니신 블레이져 교수님께 깊은 감사의 마음을 전합니다.

이 학위논문은 제 혼자의 힘으로 쓰여진 것이 아니라 항상 옆에 같이 일하고 시간을 보냈던 동료들의 도움으로 이루어진 결실입니다.

같이 학위과정을 보내면서 도와주고 웃어주었던 최연정 동생, 멋지고도 항상 우리 랩을 챙겨주시며 실험적 조언을 주신 김지현 선생님, 랩 일을 챙기고 이끌어가신 이상헌 선생님, 깔끔하고 꼼꼼하게 한편으로는 재미있는 소리로 우리를 즐겁게 해준 신현수 선생님, 새 석사과정으로 입학을 하게 되어 뒤 늦게 만나게 된 이여송 선생님께 이 자리를 빌어 감사의 말을 드립니다.

무엇보다 이 학위를 무사히 마칠 수 있도록 한결 같은 마음으로 지켜봐 주고 염려와 격려해준 가족들, 친구들 모두에게 감사 드리고 특히 언제나 저를 위해서 기도 해주시는 부모님께 사랑의 말씀을 전해드리고 싶습니다.

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ABSTRACT

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(Directed by Professor Yong Chan Lee)

The *Helicobacter pylori* oncoprotein, CagA may undergo tyrosine phosphorylation following its translocation into human gastric epithelial cells, with downstream effects on signal transduction. Recent studies of gp130 knock-out mice have shown that disruption of the gp130 receptor modulating the balance of the SHP2/ERK and JAK/STAT pathways enhanced peptic ulceration and gastric cancer. This study aimed to evaluate the effect of translocated CagA, in relation to its tyrosine phosphorylation status, on the gp130-mediated signal switch between the SHP2/ERK and STAT3 pathways. Human gastric epithelial cells (AGS) and a pair of naturally occurring cagA isogenic mutants of *H.pylori,* 147C and 147A (presence or absence of tyrosine phoshorylation activities) were used. CagA expression vectors with or without tyrosine phosphorylation activities were also

used. Immunoprecipitation assay was used to assess the interaction between CagA, SHP2, and/or gp130. Influence of phosphorylation status of CagA on the activation of STAT3 or ERK pathways was analyzed using western blotting. The results of this study showed that in the presence of CagA, SHP2 is recruited to gp130. Phosphorylated CagA showed enhanced SHP2-binding activity, and greater induction of ERK1/2 phosphorylation, whereas unphosphorylated or dephosphorylated CagA showed preferential STAT3 activation. These findings indicate that phosphorylation status of CagA affects the signal switch between the SHP2/ERK and STAT3 pathways through gp130, providing a novel mechanism to explain pathogenesis of *H.pylori*-induced diseases.

Key words : *Helicobacte pylori*, SHP2, STAT, gp130

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I. INTRODUCTION

Helicobacter pylori frequently colonizes the human stomach.[1] Hosts with cytotoxin associated antigen A (*cagA*)-positive *H. pylori* strains are at increased risks of gastric cancer and peptic ulceration.[2-10] *H. pylori* injects the CagA protein into host gastric epithelial cells via a type IV secretion system.[11-16] The injected CagA is tyrosine phosphorylated by Src family protein-tyrosine kinases and binds the SH2 domain-containing Src-homology tyrosine phosphatase (SHP2);[17-21] the CagA-SHP2 complex has been detected in human gastric mucosa.[22, 23]

The interleukin 6/glycoprotein 130/signal transducer and activation of transcription 3 (IL6/gp130/STAT3) pathway may also play a role in the development of gastric cancer.[24, 25] IL6 exerts its biological activities through receptor subunit gp130.[26] At least two functional

modules of gp130 have been characterized; one encompasses the four membrane-distal phospho-tyrosine (pY) binding sites for the SH2-domain of the latent transcription factors, STAT1 and STAT3, whereas the other comprises the membrane-proximal pY_{757} residue responsible for engagement of cytoplasmic Src-homology tyrosine phosphatase (SHP2).[26, 27] IL6 induces recruitment and homodimerization of gp130, leading to balanced signaling through both the JAK/STAT and SHP2/Ras/ERK signaling cascades.[28, 29] However, disrupting this balance in the gp130 'knock-in' mouse induces pre-malignant lesions as well as gastric cancer.[24] In addition, when the IL6 cytokine family signaling pathway is disrupted, increased STAT3 signaling may favor development of gastric adenomas,[25, 30] while increased SHP2/ERK signaling may lead to mucosal inflammation.[25, 30]

The fact that cellular factors up-regulated in response to *H. pylori* could modulate SHP2/ERK or JAK/STAT signaling suggests that gp130-mediated signal transduction through the JAK/STAT and SHP2/ERK pathways may be involved in the pathogenesis of *H.pylori*-induced gastric cancer.[30] The mechanism of CagA-induced signaling and the role of CagA tyrosine phosphorylation were examined in the activation of the SHP2/ERK and JAK/STAT pathways downstream of the gp130 receptor. These studies indicate that the CagA tyrosine phosphorylation status of a population of *H. pylori* cells directs the traffic of signals transduced through the two pathways.

II. MATERIALS AND METHODS

1. Bacteria, cell culture and co-incubation with *H. pylori*

A pair of naturally occurring isogenic *cagA* isolates (147C with a tyrosine phosphorylation motif and 147A without the motif) from the same host have been described.[31-33] 60190 (*cag*PAI$^+$, ATCC 49503), 88-22 (*cag*PAI$^-$), and Δ*cagA* [(an isogenic mutant lacking *cagA* (ATCC 49503)] were used. *H. pylori* strains were cultured on agar plates containing 10% horse serum at 37℃ in a microaerobic atmosphere, using the Campy Container System (BBL, Sparks, MD). Human gastric epithelial cells (AGS) were cultured in an RPMI-1640 medium (Gibco, Grand Island, NY, USA), containing 10% FBS (Gibco). The cells were incubated at 37℃ in a humidified atmosphere with 5% CO_2. On the day of experimentation, confluent cells were incubated overnight with fresh serum- and antibiotic-free media. AGS cells also were incubated with *H. pylori* up to multiplicities of infection (MOI) of 100:1 for different periods of time.

2. Immunoblotting and antibodies

Whole cell extracts were prepared with lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 mM PMSF, and protease inhibitors (Roche Molecular Biochemical, Indianapolis, IN, USA). Lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Immunodetection was performed using an electrochemiluminescece (ECL) reagent (Intron, Seoul, Korea), according to the manufacturer's

instructions. In experiments using kinase inhibitors, cells were incubated with PP2 (Calbiochem, San Diego, CA, USA), UO126 (Promega, Madison, WI, USA), or AG490 (Calbiochem) for 1 hr before co-incubation with *H. pylori*. α-SHP2, α-pY99, α-phospho STAT3, α-STAT3, α-gp130, α-phospho JAK2, and α-JAK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), α-ERK1/2 and α-phospho ERK1/2 were purchased from Cell Signaling (Danvers, MA, USA) and α-CagA antibody (HPP-5003-9) was purchased from ASTRAL Biologicals (San Ramon, CA, USA).

3. Co-immunoprecipitation

For immunoprecipitation, whole cell extracts prepared with lysis buffer were incubated overnight with appropriate antibodies for 4 hr at 4°C and immune complexes were trapped on protein G-sepharose beads (Amersham, Buckinghamshire, England). Beads were washed 5 times with cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and protease inhibitors (Roche Molecular Biochemical). The cell lysates and immunoprecipitated materials were subjected to SDS-PAGE, and then proteins transferred to PVDF membranes and incubated with the α-SHP2, α-CagA, and α-gp130 antibodies. The proteins were then visualized using a chemiluminescence reagent (Intron).

4. Expression vectors and transient transfection

CagA expression vectors with or without CagA tyrosine phosphorylation activities (kindly provided by Prof M.Hatakeyama,

Hokkaido University) were used.[34] For transient transfection, AGS cells were cultured in RPMI 1640 medium containing 10% FBS, and transfected with 30 μg of plasmid DNA with 20 μl of LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. In experiments using kinase inhibitors, cells were incubated with PP2 (5 uM) (Calbiochem, CA, USA) for 2 hr before harvest. SHP2-specific small interfering RNA (siRNA) (kindly provided by Prof. M. Hatakeyama) was used to silence the expression of SHP2.[35] To selectively knock down human SHP2 expression, AGS cells were co-transfected with pSUPER-SHP2 (21μg) and pBabePuro (7μg). After 12 hr, cells were incubated in RPMI 1640/10 % FBS containing 0.3 μg/ml puromycin (Sigma) to select transfectants. The SHP2 expression of the cloned cells was then examined by α-SHP2 immunoblotting.

5. Immunofluorescence microscopy

AGS cells co-cultured with *H. pylori* were washed twice with cold PBS, fixed in methanol for 10 min, rinsed with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 4 min. Nonspecific binding was blocked with 3% BSA in 0.1% Triton X-100-PBS for 30 min, followed by incubation with an α-phospho-STAT3 mouse monoclonal antibody in 2% BSA-0.1% Triton X-100-PBS at 4℃ overnight. After rinsing with PBS, α-phospho-STAT3 (1:200) was visualized by treatment with FITC-conjugated goat anti-mouse immunoglobulin (Santa Cruz, CA, USA) for 60 min at room temperature. Cells were rinsed with PBS, mounting medium for

fluorescence was added, and slides were sealed with cover slips and examined for immunofluorescence using a confocal laser scanning microscope (LSM 510; Carl Zeiss, Inc., German).

6. Nuclear protein extraction and immunoblotting

AGS cells co-cultured with *H. pylori* were harvested and fractionated into cytoplasmic and nuclear fractions using CEB buffer (10 mM Tris-Cl, pH 8; 60 mM KCL; 1 mM EDTA; 1 mM DTT), containing 0.5% Nonidet P-40 and protease inhibitors (Roche). The cytoplasmic fraction was clarified by centrifugation at 1,200 g for 5 min, and the nuclear pellet was washed with CEB buffer containing 0.2% Nonidet P-40 to remove cytoplasmic contamination. The nuclear pellet was lysed with NEB buffer (20 mM Tris-Cl, pH 8; 0.4 M NaCl; 1.5 mM $MgCl_2$; 1.5 mM EDTA; 1 mM DTT) containing 0.5% Nonidet P-40 and protease inhibitors (Roche). Nuclear proteins were analyzed by immunoblotting as described above.

7. Evaluation of phosphorylation of STAT3 after the inhibition of IL-6 receptor binding by α -IL6R and α -gp130 monoclonal antibodies

AGS cells were cultured with monoclonal antibodies to IL6R or gp130 (R&D Systems, Minneapolis, MN, USA) for 24 hr in a serum-free medium and co-incubated with *H. pylori* for 6 hr at 37℃ in 5% CO_2. Whole cell extracts were prepared with lysis buffer and analyzed by immunoblotting with the antibody specific for phospho-STAT3. Equal loading of lanes was confirmed by comparison with lanes that had been resolved using the α-STAT3 antibody.

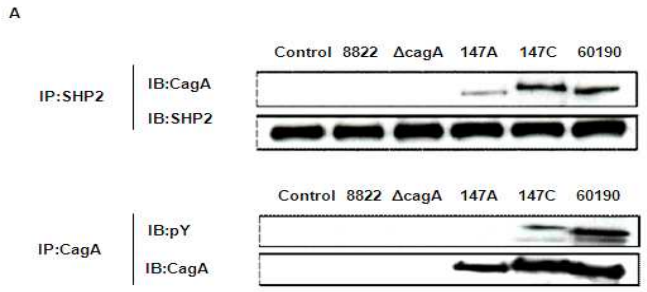
III. RESULTS

1. Role of CagA tyrosine phosphorylation status on SHP2 binding

Both *H.pylori* strains that possess tyrosine phosphorylated CagA (147C and 60190) showed higher SHP2 binding affinity than strain 147A, which has CagA that lacks the domain and thus is unphosphorylated (Figure 1A). The *cagA* PAI negative 88-22 and Δ*cagA* strains showed no detectable SHP2 binding.

2. Interaction between SHP2 and gp130 according to CagA status

To examine whether *H. pylori* affects SHP2 recruitment to gp130, a constituent of the IL-6 receptor complex, cellular lysates from AGS cells co-incubated with *H. pylori* were immunoprecipitated and immunoblotted using antibodies to CagA, gp130, and SHP2 (Figure 1B). For AGS cells co-incubated 147C and 60190 strains, immunoprecipitation showed that SHP2 was associated with both gp130 and with CagA. However, for the AGS cells co-incubated with the 88-22 or 147A strains, no association with gp130 was detected. These data indicate that the injected phosphorylated CagA plays a role in the recruitment of SHP2 to gp130.



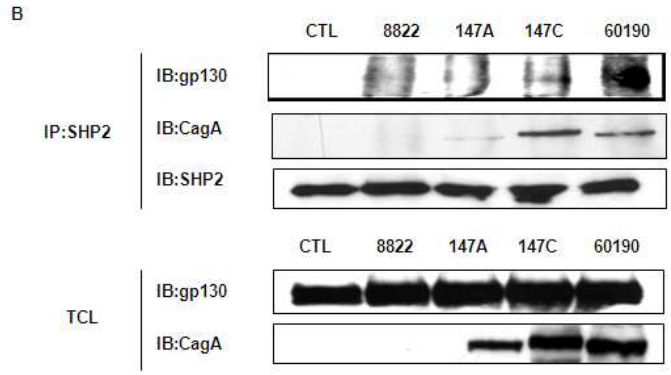


Figure 1. Relation of *H. pylori* CagA status to interaction with SHP2 and gp130. (A) Interaction between SHP2 and CagA as detected by immunoprecipitation. Lysates of AGS cells co-incubated with *H. pylori* cells were immunoprecipitated with α-SHP2 or α-CagA and immunoblotted (IB) with the converse antibodies to α-SHP2 and α-CagA. CagA from strains 147C and 60190 showed greater SHP2 binding activity than that from 147A, whereas control *cagA*$^{-}$ *H. pylori* (Δ*cagA*, 8822) strains showed no immunoprecipitation or binding (B) Co-immunoprecipitation of gp130 and SHP2 in relation to CagA status. Lysates of AGS cells co-incubated with *cagA*$^{-}$ or *cagA*$^{+}$ *H. pylori* strains for 60 min were immunoprecipitated with the antibody to α-SHP2. The immunoprecipitates (IP) and total cell lysates (TCL) were immunoblotted with antibodies to α-phosphotyrosine, α-gp130, or α-SHP2. There was an association of gp130 with SHP2, only in the presence of phosphorylated CagA.

3. Influence of CagA tyrosine phosphorylation status on *H.pylori*-medicated gp130 receptor phosphorylation

To determine whether the epithelial cell gp130 receptor becomes activated by tyrosine phosphorylated CagA, AGS cells were co-incubated with the *H. pylori* strains and subjected to immunoprecipitation with an antibody to α-gp130. Both 147A and 147C strains induced similar activation of the gp130 receptor, indicating that activation is independent of tyrosine phosphorylation status of CagA (Figure 2) and phosphorylated gp130 provides a docking site for downstream STAT3 and SHP2 activation.

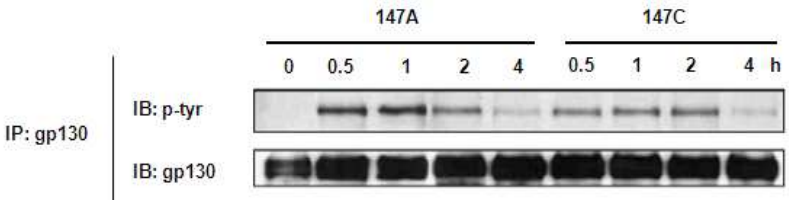


Figure 2. Phosphorylation of the gp130 receptor in relation to *H. pylori* CagA status. AGS cells were co-incubated with *H. pylori* for 60 min. The gp130 receptor was immunoprecipitated (IP) with α-gp130. Phosphorylated gp130 receptor was detected by immunoblotting with monoclonal antibody α-phosphotyrosine (α-p-tyr) (PY99) (upper panel). The gp130 receptor levels then were assessed by reprobing the blot with a polyclonal antibody to α-gp130 receptor (lower panel).

4. STAT3 activation by *H. pylori* in relation to CagA status

To analyze STAT3 activation in relation to CagA tyrosine

phosphorylation status, AGS cells were co-incubated for 6 hr with *H. pylori* strains varying in *cagA* genotype. Immunoblotting using an antibody to α-p-STAT3 showed that STAT3 became phosphorylated in AGS cells co-incubated with phosphorylation-negative strain 147A. Two phosphorylation-positive strains (147C and 60190) and two strains lacking *cagA* demonstrated considerably reduced phosphorylation of STAT3 (Figure 3A). The role of CagA phosphorylation was tested using the CagA expression vectors with or without CagA tyrosine phosphorylation functions (Figure 3B). With the WT vector, Phosphorylated CagA was expressed more than 9 hr after transfection, but there was no STAT3 activation. In contrast, the PR CagA transfection yielded CagA expression as well as STAT3 activation at 9 hr. These results indicate that CagA molecules lacking the tyrosine phosphorylation domain (aP-CagA) preferentially activate STAT3.

5. Influence of JAK2 inhibition on CagA-induced STAT3 phosphorylation

To determine whether CagA-induced STAT3 phosphorylation is mediated via JAK2, AGS cells were pretreated for 60 min with AG490, a JAK2 inhibitor, and then co-incubated with *H. pylori* (Figure 3C). AGS cells pretreated with AG490 showed greatly reduced 147A-mediated STAT3 phosphorylation. These results indicated that aP-CagA-induced STAT3 activation is influenced by JAK2.

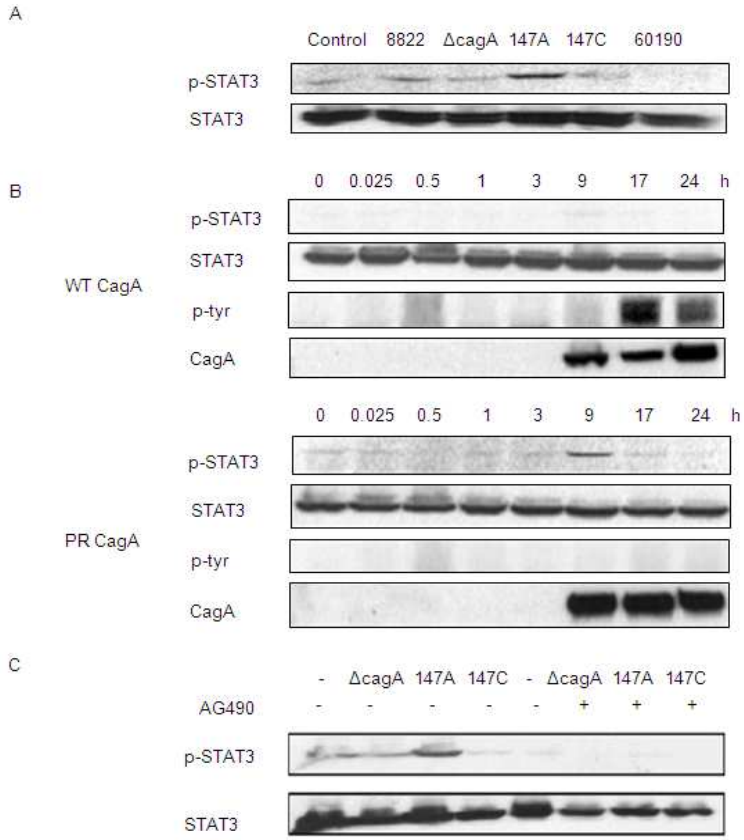


Figure 3. Influence of *H. pylori* CagA phosphorylation status on STAT3 phosphorylation. (A) AGS cells were co-incubated with either *cagA*$^{-}$ or *cagA*$^{+}$ *H. pylori* strains for 6 hr, and STAT3 activation was determined by immunoblotting using an antibody recognizing STAT3 phosphorylated at tyrosine 705 (p-STAT). As a loading control, blots where stripped and reprobed with an antibody to α-STAT3. (B) AGS cells were transfected with WT CagA or PR CagA expression vectors, and phosphorylation of STAT3 and CagA were assessed by immunoblotting. (C) Two groups of AGS cells,

pretreated or not pretreated with 40 uM of JAK2 inhibitor (AG490) for 60 min, were co-incubated with *H.pylori* strains for 6 hr STAT3 phosphorylation was analyzed by immunoblotting using an antibody to α-phospho-STAT3.

6. Influence of Src family kinases on CagA-induced STAT3 phosphorylation

Since CagA is phosphorylated by the Src family of protein-tyrosine kinases, it was analyzed whether pretreatment with PP2, a specific inhibitor of Src family kinases, would affect the CagA-induced STAT3 activation in AGS cells co-incubated with *H. pylori*. First, morphological changes in AGS cells after incubation with *H.pylori* for 24 hr with or without PP2 pretreatment was examined (Figure 4A). As expected, strain 147C induced hummingbird phenotype to a significantly greater extent than that induced by 147A, while PP2 pretreatment significantly reduced the 147C-induced effect. Next, AGS gastric epithelial cells were pretreated with PP2 (5 uM) for 2 hr prior to infection and STAT3 activation was examined. PP2 pretreatment did not affect the 147A induced activation of STAT3, but 147C induced STAT3 activation (Figure 4B). To confirm that aP-CagA is responsible for STAT3 activation, AGS cells were transfected with the WT CagA and PR CagA expression vectors, and incubated with PP2 for 2 hr before cell harvest (Figure 4C). For AGS cells transfected with the WT CagA vector, pretreatment with PP2 induced STAT3 activation to a similar extent to those with PR CagA transfection. These results

indicate that unphosphorylated CagA is essential for STAT3 activation.

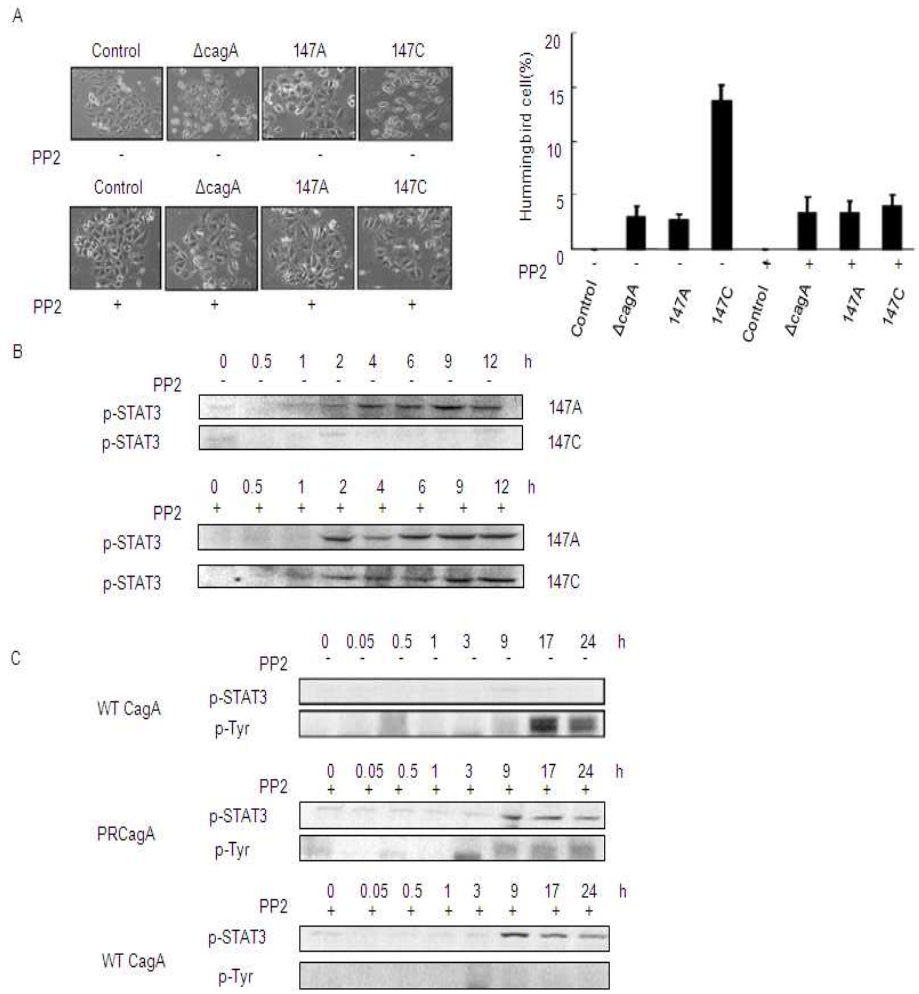


Figure 4. Influence of Src kinase inhibition on STAT3 phosphorylation. (A) AGS cells were co-incubated with *H. pylori* strains for 24 hr with or without pretreatment with PP2, and cells were examined for hummingbird phenotype by microscopy

(Olympus IX70, ×100). Hummingbird phenotypes per 100 AGS cells were enumerated in triplicate. (B) AGS cells were co-incubated with *H. pylori* strains 147A or 147C with or without PP2 pretreatment and were examined for STAT3 phosphorylation by immunoblotting. (C) AGS cells were transfected with WT CagA or PR CagA expression vectors and incubated with or without 5 uM of PP2 for 2 hr before harvest. STAT3 activation and CagA tyrosine phosphorylation status were assessed by immunoblotting.

7. Influence of phosphorylated CagA on ERK1/2 activation

To investigate whether CagA tyrosine phosphorylation status affects the ERK1/2 pathway downstream of the gp130 receptor, AGS cells were co-incubated with strains 147A or 147C. There was early ERK activation by both 147A and 147C, however activation was more sustained for 147C (Figure 5A). Control cells in the absence of *H. pylori* showed no detectable levels of phosphorylated ERK1/2 (data not shown). Taken together, these data indicate that phosphorylated CagA facilitates signal transduction through ERK1/2 to a greater extent than unphosphorylated CagA.

8. Role of ERK in STAT3 dephosphorylation induced by phosphorylated CagA

Since that higher ERK activation in AGS cells co-incubated with 147C might lead to STAT3 inhibition was hypothesized, it has been next investigated whether the ERK pathway downstream from SHP2

is involved in the STAT3 dephosphorylation that occurred in AGS cells co-incubated with strain 147C. Pretreatment of AGS cells with the ERK inhibitor U0126 prior to *H. pylori* co-incubation had no effect on STAT3 activity, indicating that 147C CagA suppresses STAT3 activation via an ERK-independent mechanism (Figure 5B).

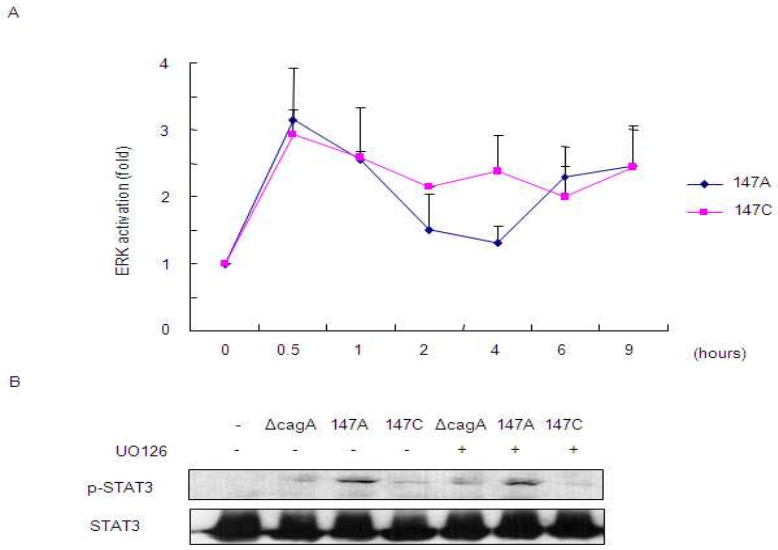


Figure 5. ERK1/2 activation in AGS cells co-incubated with *H. pylori* cells. (A) AGS cells were co-incubated with *H. pylori* strains 147A or 147C. The phosphorylated ERK1/2 was assessed by immunoblotting. Blots were reprobed with an antibody to control ERK1/2 to minimize differences in loading. Densitometric ratios between p-ERK and ERK immunoblotting were shown. (B) AGS cells were pretreated with U0126 (20 uM) for 1 hr prior to co-incubation with *H. pylori*. Phosphorylation of STAT3 was analyzed by immunoblotting.

9. Influence of IL-6R neutralizing antibodies on the phosphorylation of STAT3 in relation to *H. pylori* CagA status

To examine whether *H. pylori*-induced STAT3 activation in AGS cells is mediated through the gp130 receptor, monoclonal antibodies to IL-6R and gp130 were used. These antibodies function as a specific receptor antagonist, abrogating activation of the IL-6/IL-6R/gp130 complex.[36] AGS cells were incubated with the monoclonal antibodies to IL-6R and gp130 for 24 hr in serum-free medium and then co-incubated with *H. pylori* for 6 hr (Figure 6A). The antibody treatment substantially inhibited the STAT3 activation in AGS cells induced by 147A, indicating that the predominant signaling by EPIYA$^-$ CagA is via gp130.

10. Relation of STAT3 nuclear translocation to CagA phosphorylation status

Dimeric STAT3 translocates into the cell nucleus, where it binds to defined DNA elements within the promoter regions of target genes, activating their transcription.[37-39] Therefore, it was necessary to investigate the effects of *H. pylori*, according to CagA status, on STAT3 translocation in AGS cells. It was found that Co-incubation with strain 147A (aP-CagA) induced greater STAT3 nuclear translocation compared with other strains (Figure 6B). Confocal microscopy of AGS cells after immunofluorescent staining for STAT3 was employed to determine the STAT3 subcellular localization after *H. pylori* co-incubation. Co-incubation of AGS cells with 147A for 9 hr induced greater phospho-STAT3 nuclear

translocation than with either 147C or the CagA-negative strain (Figure 6C).

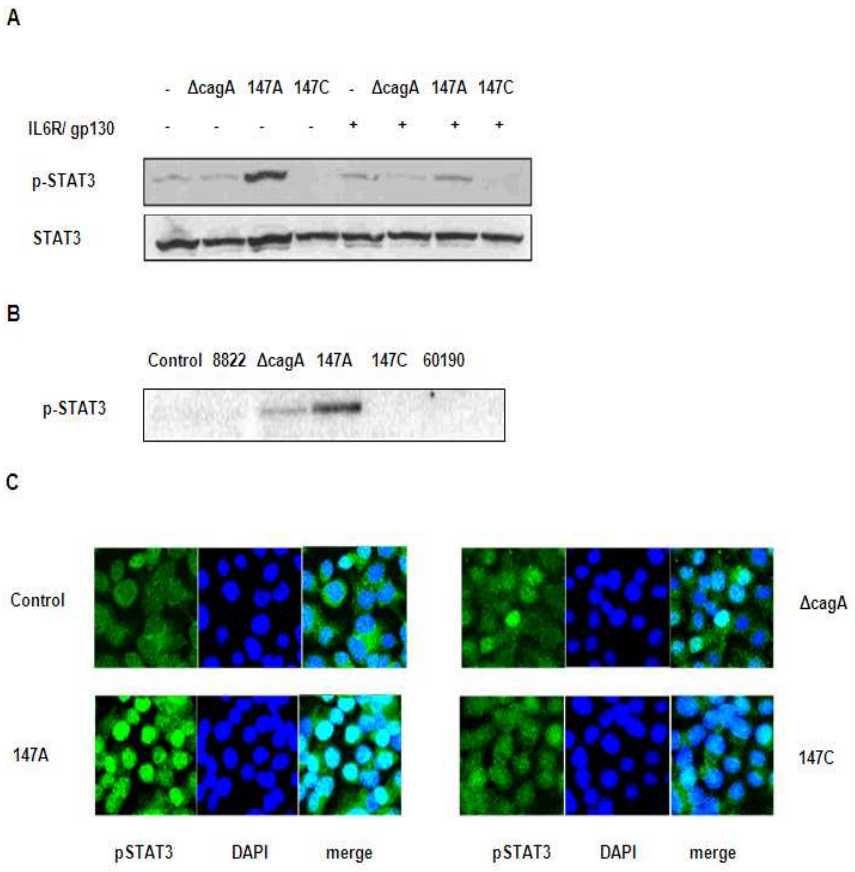


Figure 6. gp130-activated STAT3 phosphorylation and nuclear localization, according to *H. pylori* CagA status. (A) STAT3 phosphorylation was down-regulated by co-treatment of AGS cells with monoclonal antibodies to both IL-6R (α-IL-6R) and gp130 (α-gp130). The AGS cells pretreated with IL-6R mAb and gp130 mAb for 24 hr in serum-free medium were co-incubated with *H.*

pylori cells for 6 hr and STAT3 phosphorylation was detected by an antibody to phospho-STAT3. Immunoblotting of total STAT3 served as control. (B) AGS cells were co-incubated with *H. pylori* strains for 6 hr, then their cytoplasmic and nuclear fractions were separated, and the nuclear proteins were analyzed by immunoblotting with an antibody to α-phospho-STAT3. (C) AGS cells were co-incubated with *H. pylori* cells for 9 hr and examined using confocal microscopy. α-phospho STAT3 was stained in green (× 100).

11. Effect of phosphorylated SHP2 on JAK2 activation

To determine the role of SHP2 in JAK/STAT3 activation and the reason why phosphorylated CagA does not induce STAT3 activation, the involvement of SHP2 in JAK2/STAT3 activation in AGS cells co-incubated with *H. pylori* was investigated. SHP2 activity has been reported to correlate with its own phosphorylation, and phosphorylated SHP2 can catalyze the tyrosine phosphorylation of JAKs, receptors, or other cellular proteins.[40] In cells co-incubated with 147C, SHP2 was phosphorylated, as expected, but JAK2 was not activated (Figure 7A and 7B). Since the SHP2 recruitment site within gp130 is involved in negative regulation of IL-6-induced STAT3 activation and gene induction[41-43], SHP2 expression using SHP2 siRNA pretreatment was selectively inhibited. As expected, treatment of AGS cells with SHP2 siRNA resulted in strong reduction of SHP2 levels (Figure 7C). In AGS cells co-incubated with 147A, STAT3 activation was not affected by pretreatment with

SHP2 siRNA. In contrast, JAK2 and STAT3 activity were increased with siRNA pretreatment in AGS cells co-incubated with 147C. The result that reduced SHP2 activity induced STAT3 activation through JAK2 activation provides further evidence that phosphorylated SHP2 controls JAK2/STAT3 signaling pathways after injection of tyrosine-phosphorylation competent EPIYA present CagA molecules.

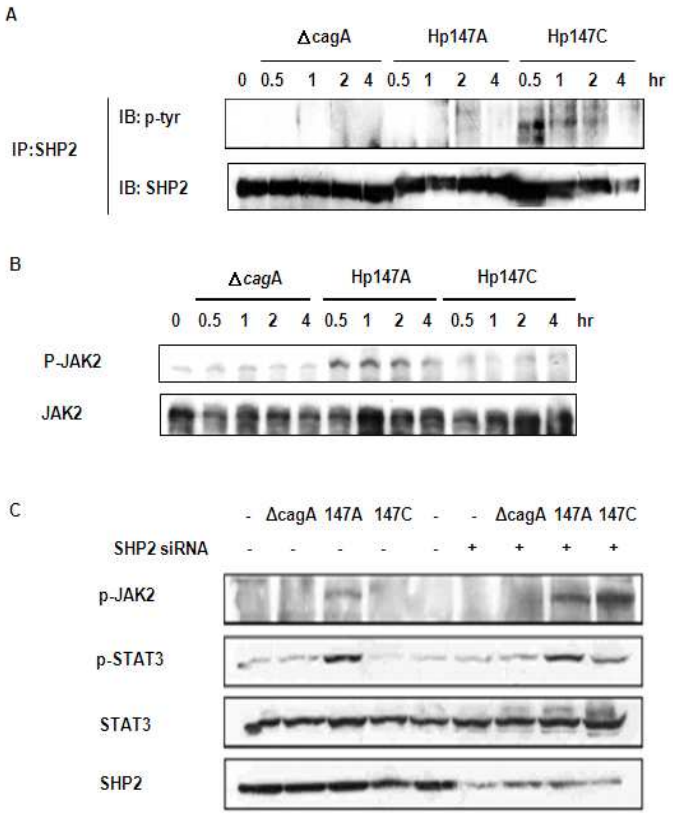


Figure 7. Effect of SHP2 on JAK/STAT3 activation in AGS cells.

(A) Lysates from AGS cells co-incubated with *H. pylori* strains were immunoprecipitated with SHP2, then immunoblotting was done with

PY99 (α-p-tyr). The membrane then was stripped and analyzed with α-SHP2 to standardize loading. (B) The level of phosphorylated JAK2 was determined by α-phospho-JAK2, and loading was standardized using antibodies to JAK2. (C) AGS cells were transfected with SHP2 siRNA or control empty vector for 12 hr and primarily selected in RPMI 1640 medium with 10% FBS containing 0.3 ug/ml puromycin for 3 days. Selected cells with SHP siRNA were co-incubated with *H. pylori*. Total cell lysates were immunoblotted with α-phospho-JAK2, α-phospho-STAT3, or α-SHP2.

IV. DISCUSSION

Gastric colonization with *cagA* positive-*H. pylori* strains increases risks for atrophic gastritis, peptic ulcer disease, and gastric cancer,[2-6] but the molecular mechanisms involved are uncertain. In this study, a pair of isogenic strains (147A and 147C) with identical CagA proteins, differing only by the presence of a single EPIYA motif[31-33] was used to elucidate the mechanisms by which tyrosine phosphorylated CagA affects signaling within gastric epithelial cells. The phosphorylated CagA (from 147C) has greater binding affinity to SHP2, as shown previously[23, 34] and confirmed in the co-immunoprecipitation experiments, the extent of this interaction could affect downstream signaling pathways. For example, SHP2 positively regulates ERK activity, which is necessary for CagA induction of the hummingbird phenotype.[23] Consistent with this pathway, it now has been shown that phosphorylated 147C CagA induced greater ERK1/2 activation than did unphosphorylated 147A aP-CagA, confirming our prior observations.[33]

Since gp130 has bifunctional domains, its activation can lead to signaling through either the SHP2/ERK and JAK/STAT pathways.[26, 27] It has been investigated whether signaling through these competing transduction pathways is affected by CagA in tyrosine phosphorylation dependent manner. First it has been shown that the gp130 receptor is phosphorylated by CagA, regardless of its tyrosine phosphorylation status. Thus, in addition to its induction of IL-6 in the human gastric mucosa,[37] enhanced by its cag PAI, *H.*

pylori also affects gp130-regulated signal transduction from the interior of epithelial cells. Next, it was found that aP-CagA induced substantially more STAT3 than did phosphorylated CagA, and confirmed the inverse relationship for ERK activation. Since STAT3 is activated via the JAK signaling pathway, it has been investigated whether the aP-CagA-induced STAT3 activation is mediated through the gp130 receptor. Use of the JAK2 inhibitor AG490 showed that STAT3 activation by 147A requires JAK2 activation. Use of PP2, a specific inhibitor of Src family kinases, which blocked CagA phosphorylation, restored induction of STAT3 phosphorylation by 147C CagA. The results indicate that STAT3 activation is dependent on aP-CagA. The results that monoclonal antibodies to IL-6R and to gp130 significantly inhibited STAT3 phosphorylation in the presence of aP-CagA confirmed the role of gp130 in the process. The findings consistently indicate that the gp130/STAT3 pathway is preferentially activated by aP-CagA, whereas tyrosine phosphorylated CagA preferentially activates the SHP2/ERK pathway. Thus, the relative proportion of the two forms of CagA in an *H. pylori* population[31, 32, 44] may determines the type of signaling pathways in the gastric mucosal cells.

Next, it has been examined why phosphorylated CagA failed to activate the STAT3 pathway. The JAK/STAT signal transduction pathway is down-regulated by several families of proteins, including SHP2, which inhibits IL-6 signal transduction.[45] After SHP2 binds to gp130, it may dephosphorylate gp130 and its associated factors, including JAKs and STATs.[41] SHP2 may act soon

after signal initiation,[26, 27] explaining the effects of SHP2 on downstream targets of JAK/STAT recruitment. Suppression of the JAK/STAT pathway by SHP2 provides an explanation for the negative feedback induced by phosphorylated CagA on the JAK-related signal pathway. The prolonged STAT3 activity induced by aP-CagA also can be explained by prolonged STAT3 phosphorylation through receptor-associated kinases that are not suppressed by SHP2, as well as by reduced dephosphorylation of receptor-recruited STATs by SHP2.[46]

These findings indicate that regulation of host signal transduction pathways induced by translocated CagA vary depending on its tyrosine phosphorylation status. Variation in intracellular CagA phosphorylation status thus may be responsible for different gastric mucosal phenotypic changes. These experimental data show that tyrosine phosphorylated CagA preferentially activates the SHP2/ERK pathways, while the dephosphorylated CagA results in preferential activation of the JAK/STAT pathway.

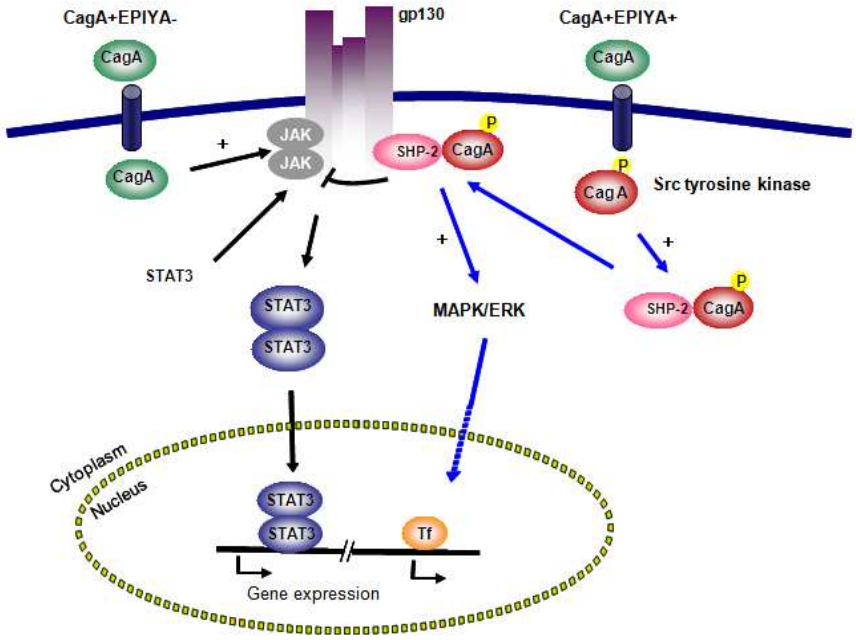


Figure 8. Signal switching between the JAK/STAT3 and SHP2/ERK pathways via the gp130 receptor in AGS cells. AGS cells incubated with CagA^{+} EPIYA^{-} *H. pylori* have preferential activation of the JAK/STAT3 pathway, whereas cells incubated with CagA^{+} EPIYA^{+} *H. pylori* undergo tyrosine phosphorylation (dotted line), then have preferential activation of the SHP2/ERK pathway. This results in inhibition of gp130-mediated JAK activation due to the interaction of phosphorylated CagA with SHP2. Dashed lines indicate recruitment to the cell membrane gp130 complex. Solid lines indicate signals that originate from the activated gp130 complex.

Ⅴ. CONCLUSION

In conclusion, *H. pylori* CagA tyrosine phosphorylation status in part determines the traffic flowing from the opposing gp130-activated SHP2/ERK and JAK/STAT signal pathways in gastric epithelial cells. The active role of unphosphorylated CagA in JAK/STAT pathway induction explains the ability of *H. pylori* to modify CagA tyrosine phosphorylation status, with the polar forms (varying in EPIYA content) inducing separate and inhibitory pathways. This study provide one evidence that the *H. pylori* population biology with respect to CagA status, creates a "rheostat," under selective pressure, by which the divergent forms (CagA-negative, phosphorylated CagA, unphosphorylated CagA, multi phosphorylated CagA) affect host cell phenotypes, and ultimately the downstream risk of disease.

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ABSTRACT (IN KOREAN)

헬리코박터 파이로리 암단백 CagA에 의한 SHP2 활성화에 따른 신호전달 교란기전

<지도 교수 이용찬>

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이 인 옥

헬리코박터 암단백 CagA는 위 상피세포내로 주입되어 타이로신 인산화를 거쳐 활성화되면 SHP2와 결합하여 일련의 신호전달 체계를 교란시키게 된다. 또한 Il6 의 세포 수용체인 gp130은 bifunctoinal domain을 지니고 있어 두 신호전달체계의 균형을 조절하게 되는데 이 두 신호전달계계의 균형이 깨졌을 경우 즉, STAT1/3 신호전달계의 활성화가 월등히 나타날 경우 위종양을, SHP2-Ras-ERK 활성화가 월등히 나타날 경우 심한 소화관점막의 궤양성 변화를 유도하는 것이 보고 되었다. 본 연구의 목적에서는 CagA 단백의 활성화가 숙주 위상피 세포내 성장인자의 신호전달에 중요한 역할을 하는 것으로 알려진 SHP-2 효소의 활성화를 유도한다는 데에 기반을 두고 gp130 경유 SHP-2/Ras/ERK 신호전달체계와 STAT1/3-JAK 신호전달체계의 차별적 활성화를 비교함으로써 CagA 단백의 인산화 유무에 따른 영향을 연구하고자 하였다. 이 실험에서 위암상피세포로 AGS를 사용하였고 헬리코박터 균주로는 한쌍의 아형균주인 147A 와 147C 균주를 사용하였다. 또한 인산화유무를 지닌 CagA 발현벡터를 사용하였다. CagA, SHP2 또는 gp130 단백 사이의 결합능을

immunoprecipitation 실험기법을 통해 확인하였다. CagA 인산화에 따른 STAT3와 ERK 활성도를 웨스턴 기법을 통해 확인하였다. 이 연구에서 세포 내로 주입된 CagA는 인산화 유무에 상관없이 gp130 수용체를 활성화시켰음을 확인하였다. CagA 단백의 인산화가 immunoprecipitation을 통해 SHP2 단백과의 결합능을 증가시키는 것을 확인하였고 immunoblot을 통해 하위신호전달체계인 ERK 활성화도 증가시키는 것을 확인하였다. 반면, STAT3 활성화를 immunoblot으로 분석한 결과 인산화가 일어나지 않는 CagA 균쥬에서는 STAT3 활성화가 나타나는 반면 인산화가 일어나는 CagA 균주에서는 STAT3 활성도가 현저히 낮았다. 따라서 균주의 CagA 단백의 인산화 여부에 따라서 대표적 두 가지 신호전달체계의 활성화가 다르게 나타났으며 이는 변화하는 숙주환경에서 *H. pylori* 유전형의 끊임없는 변이가 다양한 병태생리를 유발할 수 있는지를 보여주는 한 기전을 설명 할 수 있는 결과로 판단된다.

핵심되는 말 : *Helicobacte pylori*, SHP2, STAT, gp130