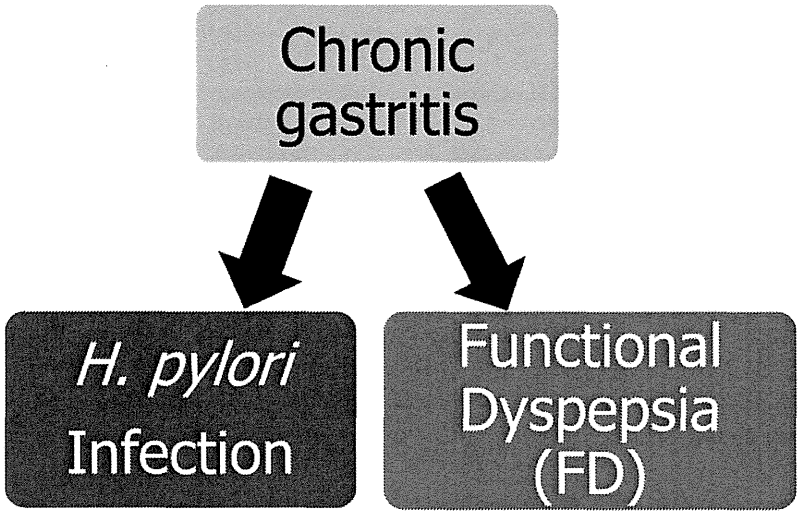


**Fig. 2** New disease entity concept clarifying the difference between *H. pylori*-associated and non-*H. pylori*-associated dyspepsia.[48,53]

pathogenesis of gastric MALT lymphomas, and determination of the DNA methylation pattern may hold promise as a clinical tool for surveillance of gastric MALT lymphomas.

### *H. pylori and idiopathic thrombocytopenia purpura – molecular approach*

Idiopathic thrombocytopenic purpura (ITP) is a bleeding disorder in which platelet-specific autoantibodies cause platelets to be lost. In a subset of *H. pylori*-infected patients with ITP, the number of platelets recovers after the eradication of *H. pylori*.[41,42] According to our previous research into the role of *H. pylori* infection in the pathogenesis of ITP,[43] a significant increase in platelet numbers was observed after *H. pylori* eradication in 61% of patients. In this study, at baseline, monocytes from *H. pylori*-positive patients exhibited an enhanced phagocytic capacity and low levels of the inhibitory Fcγ receptor IIB (FcγRIIB). This activated monocyte phenotype was suppressed 1 week after the start of the *H. pylori* eradication regimen, and this suppression was followed by improvements in other autoimmune and platelet kinetic parameters. *H. pylori* infection was also associated with an altered monocyte FcγR balance in individuals without ITP; a similar result was also found in mice. Our findings strongly suggest that the recovery in platelet numbers observed in ITP patients after *H. pylori* eradication is mediated through a shift of the FcγR balance toward inhibitory FcγRIIB.

Veneri et al. reported that the host factors HLADRB111, HLA-DRB 114, and HLA-DQB 103 occurred at high frequencies in patients with *H. pylori*-positive ITP, while the frequency of the host factor HLA-DRB 103 was higher in the *H. pylori*-negative ITP group than in the *H. pylori*-positive ITP group. HLA-DQB 103 showed favorable platelet reactivity after *H. pylori* eradication therapy in *H. pylori*-positive ITP patients.[44]

### *Functional dyspepsia and H. pylori infection*

Functional dyspepsia (FD) is a condition in which upper abdominal symptoms occur in the absence of any explanatory organic disease.[45] *H. pylori* infection is not an exclusion criterion for FD under the current Rome III classification.[45] However, recent advances in basic and clinical research have revealed that *H. pylori* infection plays an important role in the development of gastroduodenal dysmotility and hypersensitivity and also in dyspeptic symptoms.[46–48] For example, the authors revealed that in mice, chronic *H. pylori* infection downregulates expression of muscle-specific miRNAs (*miR-1*, *miR-133a*, and *miR-133b*) and upregulates expression of their targets, such as histone deacetylase 4 and serum response factor. These transcriptional changes cause hyperplasia of the muscular layer of the stomach and gastric emptying deregulation.[49] In addition, *H. pylori* eradication therapy appears to have a small but statistically significant effect on FD.[50,51] Therefore, *H. pylori*-associated dyspepsia (HpD) should be excluded from the umbrella category of FD to better elucidate the pathophysiological mechanisms of FD and to establish more precise diagnostic markers or criteria (**Fig. 2**).[46,48,52] In Japan, for national insurance claim purposes, *H. pylori*-positive histological gastritis, NSAID-induced gastritis, and neurotic gastritis (or stress-induced gastritis) have all been combined under the diagnosis of "chronic gastritis" and have therefore been treated with the same drugs. Of these groups of diseases, FD would be categorized as classical stress-induced gastritis. However, as each type of gas-

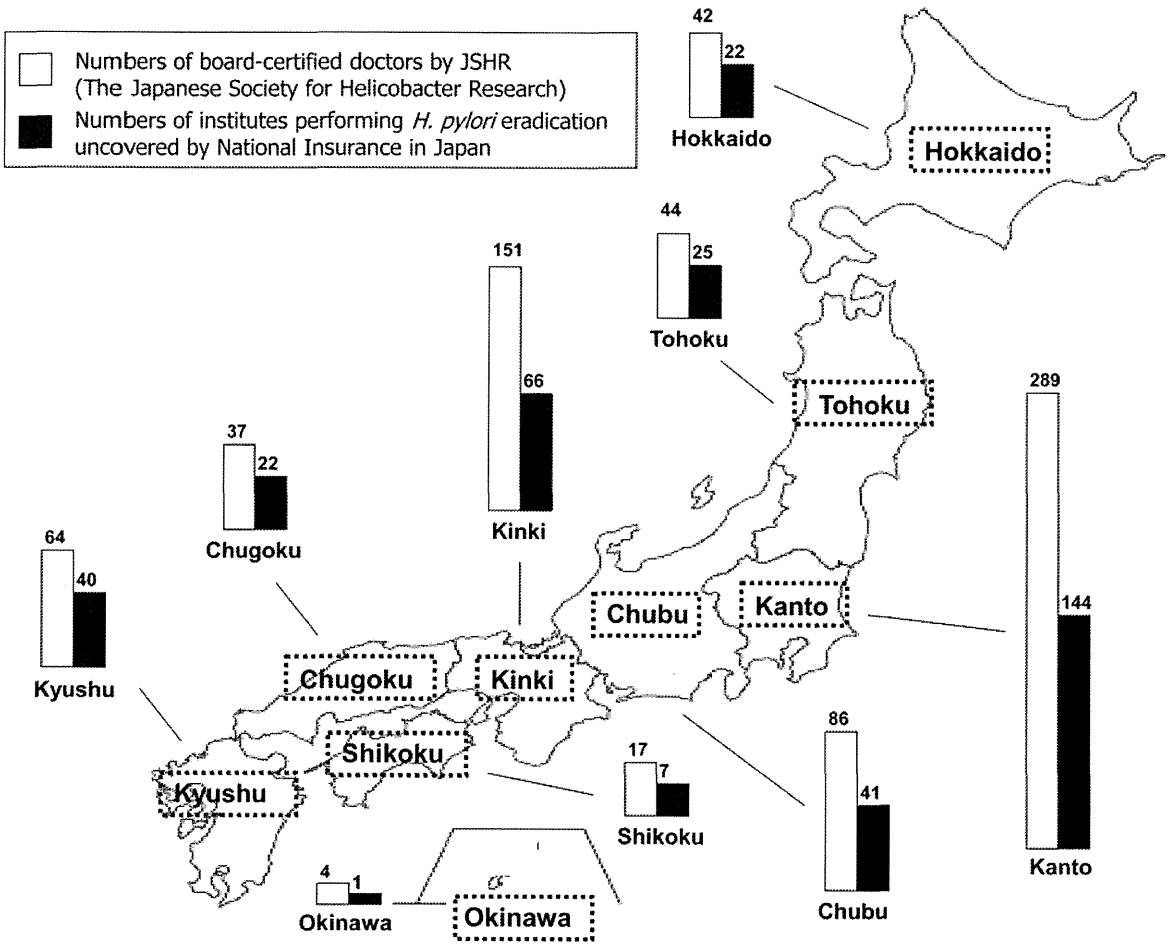


**Fig. 3** Numbers of board certified doctors and institutes performing *H. pylori* eradication therapy uncovered by the Japanese national insurance, at the time point of March 2012.
(adopted from the homepage of the JSHR with permission; http://www.jshr.jp/index.php?page=medic_list, http://www.jshr.jp/index.php?page=medic_facility)

tritis has a different etiology, only diseases produced by known pathogens, such as *H. pylori,* should be treated by targeting the pathogen directly. In addition, *H. pylori*-negative FD should not be categorized as so-called "chronic gastritis" because it is histologically negative for inflammation. As the pathophysiology underlying the disturbances of gastroduodenal motor and sensory functions and dyspepsia symptoms caused by *H. pylori* infection is gradually being elucidated, *H. pylori*-positive FD (*i.e.,* HpD) should be considered an organic disease and dealt with as a disease entity distinct from FD[53] (**Fig. 3**). Separating HpD from FD may reconcile the conflicting results of previous studies on drug therapy for FD. The differences between the most effective therapeutic strategies against HpD and *H. pylori*-negative FD also require further investigation.

## Indications for *H. pylori* Eradication in Japan

At present, *H. pylori* eradication is used not only for the treatment of peptic ulcer disease but also for prophylaxis and treatment of *H. pylori*-associated diseases such as gastric cancer, gastric MALT lymphoma, and ITP, as well as for inhibiting the spread of this bacterial infection.

*H. pylori* infection was approved as an official disease name in the 2003 edition of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). *H. pylori* infection itself is soon expected to be accepted by the Japanese national health scheme as a disease entity; this will make the diagnosis and treatment of *H. pylori*-associated diseases a part of routine medical practice, resulting in the successful prevention of gastric cancer. In addition, *H. pylori* eradication therapy could

**Table 2** Indications for *H. pylori* eradication therapy[54]

| | Diseases | Minds recommendation grade | Level of evidence classification | Coverage by the Japanese national health insurance system |
|---|---|---|---|---|
| *H. pylori* infection | Peptic ulcer (gastric/duodenal ulcer) | A | I | Yes |
| | Gastric MALT lymphoma | | III | Yes |
| | Idiopathic thrombocytopenic purpura (ITP) | | I | Yes |
| | Patients after endoscopic treatment of early gastric cancer | | II | Yes |
| | Atrophic gastritis | | I | No |
| | Gastric hyperplastic polyps | | II | No |
| | Functional dyspepsia (FD) | | I | No |
| | Reflux esophagitis | | II | No |
| | Iron-deficiency anemia | | III | No |
| | Chronic urticaria | | III | No |

prevent the spread of this infection, which would lead to dramatic savings in medical costs.

According to the Guidelines for the Management of *Helicobacter pylori* Infection in Japan: 2009 Revised Edition of the Japanese Society of Helicobacter Research (JSHR),[54] *H. pylori* eradication has, based on strong evidence, been strongly recommended as level A (Minds Recommendation Grades) for all *H. pylori* infections. In the guidelines, the level of evidence for each recommendation was classified from Level I to Level VI. For example, peptic ulcer, ITP, atrophic gastritis, and FD were categorized as Level I [supported by systematic review and meta-analysis (**Table 2**)].[54] In contrast, early gastric cancer post-endoscopic treatment, gastric hyperplastic polyp, and reflux esophagitis were categorized as Level II (supported by at least one randomized controlled clinical trial), while gastric MALT lymphoma, iron-deficiency anemia, and chronic urticaria were categorized as Level III (supported by non-randomized controlled clinical studies) (**Table 2**).

## Diagnosis of *H. pylori* Infection

*H. pylori* infection is investigated before and after eradication therapy using the following tests. Tests that require endoscopic biopsy include (i) the rapid urease test, (ii) histology, and (iii) culture, whereas tests not requiring endoscopic biopsy include (i) the $^{13}$C-urea breath test (UBT), (ii) detection of anti-*H. pylori* antibodies in the serum or urine, and (iii) measurement of *H. pylori* stool antigen. The national health insurance scheme now covers the use of two separate tests.[54]

The rapid urease test has the advantages of low cost, ease of use, and speed, whereas the advantages of culture include 100% specificity (direct demonstration of the presence of *H. pylori*) and the ability to further characterize the organism (e.g., determination of its antibiotic susceptibility and investigation of its virulence factors). On the other hand, the UBT is a practical and readily available test with a diagnostic accuracy of >95%.

The detection of anti-*H. pylori* antibodies in the serum is a widely available and inexpensive test, but the diagnostic accuracy is low (80%–84%). Some highly accurate (90%) serology kits are recommended in validated settings.[55] The anti-urease activity of proton pump inhibitor (PPI) treatment can result in false-negative results for invasive and noninvasive diagnostic tests; therefore, PPI treatment should be withheld for at least 2 weeks before testing. However, this consideration does not apply to serology.

A systematic review of 89 studies evaluating the stool antigen test found an aggregate sensitivity and specificity of 91% and 93%, respectively.[56] Stool samples must be stored at −20°C prior to testing for this test to be accurate; the sensitivity of the stool antigen test decreases to 69% after storage of the samples at room temperature for 2–3 days. Stool antigen tests have low specificity in patients presenting with acute bleeding peptic ulcers, as there is cross-reactivity with blood products in the stool.

## *H. pylori* Eradication Therapy

In Japan, 1 week of triple therapy using a PPI combined with amoxicillin (750 mg, b.i.d.) and clarithromycin (400 mg or 200 mg, b.i.d.) is recommended as the first-choice treatment for eradication of *H. pylori*.[57] At the 2008 meeting of the JSHR, the mean national clarithromycin resistance rates from 2002 to 2006 were reported to be 18.9%, 21.2%, 27.7%, 29.0%, and 27.2%. The mean nationwide clarithromycin resistance rate determined by the Japanese Society of Chemotherapy in 2000 was 7.0%, so the resistance rate has increased by approxi-

mately 20% over several years. Given the recent surge in clarithromycin resistance, new strategies for first-line treatment of *H. pylori* infection should be considered.[58] Recently, a European group performed a randomized, open-label, non-inferiority, phase 3 trial comparing the efficacy and safety of first-line quadruple therapy with omeprazole plus a single three-in-one capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline (quadruple therapy) for 10 days versus omeprazole, amoxicillin, and clarithromycin for 7 days (standard therapy) in adults with known *H. pylori* infection.[59] According to this study, the eradication rate in the intent-to-treat (ITT) population (n =440) was 80% (174 of 218 participants) in the quadruple therapy group versus 55% (123 of 222) in the standard therapy group ($P < 0.0001$). This suggests that quadruple therapy would be considered for first-line treatment of *H. pylori* infection, especially in view of the rising prevalence of clarithromycin-resistant *H. pylori* and the fact that quadruple therapy provides superior eradication, with safety and tolerability similar to those of standard therapy, even though this study showed an extraordinarily low eradication rate in the standard therapy arm.[59] However, several issues, including the optimal doses of bismuth and amoxicillin and the duration of treatment, must be also considered before quadruple therapy can be established as the standard first-line therapy for *H. pylori* eradication.[60]

In Japan, the recommended second-choice treatment for *H. pylori* infection is 1 week of triple therapy using a PPI combined with amoxicillin (750 mg, b.i.d.) and metronidazole (250 mg, b.i.d.). The prevalence of resistance to metronidazole in *H. pylori* in Japan has been reported to range between 5% and 12%.[61] The Tokyo *H. pylori* Study Group examined the rate of eradication in response to the second-line regimen consisting of PPI, amoxicillin, and metronidazole in a multicenter study in the Tokyo metropolitan area.[62] ITT and per-protocol (PP) analyses revealed eradication rates of 87.6% and 90.6%, respectively. Murakami et al. reported that eradication rates following second-line treatment of *H. pylori* infection with the PPI + amoxicillin + metronidazole regimen were 97% for infections with metronidazole-sensitive strains and 82% for infections with metronidazole-resistant strains. Two explanations are available for the discrepancy between the MICs for metronidazole and the eradication rates for PPI-amoxicillin-metronidazole triple therapy: (i) metronidazole is relatively stable under anaerobic conditions, possibly making its antimicrobial activity stronger in the stomach than *in vitro*, and (ii) the mechanism by which *H. pylori* acquires resistance to metronidazole remains unclear (mutations in the nitroreductase *rdxA* chromosome have been implicated, but there are other possibilities), making it difficult to predict the effects of metronidazole-containing second-line treatment regimens from the MIC of metronidazole alone. To improve prediction of eradication success by second-line treatment, we developed and reported an eradication resistance index, calculated as: [Pre-treatment urea breath test result (‰)] × [amoxicillin MIC (μg/mL)] × [metronidazole MIC (μg/mL)].[63] When a cutoff value of 3 was used, the eradication resistance index predicted the response to therapy with a specificity of 81.8%, a sensitivity of 93.8%, and an accuracy of 92.5%. However, because the superiority of the first-line eradication regimen (PPI + amoxicillin + clarithromycin) to the second-line regimen (PPI + amoxicillin + metronidazole) in Japan has recently been called into question, we retrospectively confirmed that the metronidazole-containing second-line regimen could be a superior primary eradication regimen for *H. pylori* in Japan.[58]

There is at present no standard third-line treatment regimen for eradication of *H. pylori*, and European guidelines recommend culture before the selection of a third-line treatment to tailor the regimen to the antibiotic sensitivity of the isolate. *H. pylori* isolated after the failure of first- and second-line eradication regimens is often resistant to both metronidazole and clarithromycin.[46] Therefore, these two drugs are not recommended for inclusion in third-line regimens. In Japan, a PPI + amoxicillin + fluoroquinolone combination or high-dose PPI/amoxicillin therapy is recommended if second-line eradication therapy fails.[54,64] However, we recently reported that even amoxicillin resistance (MIC ≥0.5 μg/mL) is enhanced after multiple eradication failures (1st: 0%, 2nd: 0.9%, 3rd: 6.1%, and 4th: 18.2%) due to the accumulation of *PBP1* mutations.[46] Clinicians should therefore be aware of the possibility of resistance to amoxicillin along with that to other antibiotics.

Novel fluoroquinolones have recently been developed, and sitafloxacin has been reported to have superior *in vitro* activity against *H. pylori* compared with levofloxacin.[65,66] We investigated the efficacy of sitafloxacin-based triple therapy as a third-line treatment administered after assessment of the isolates for sitafloxacin susceptibility and the presence of *gyrA* mutations. Sitafloxacin-based triple therapy achieved 83.6% (PP) or 78.2% (ITT) success among 78 Japanese patients. Even among the 47 patients with *gyrA* mutation-positive *H. pylori*, the eradication rates were 74.4% (PP) and 68.1% (ITT)[67]. In that study, the position of the *gyrA* mutation (N87 or D91) was found to be superior to the MIC value for predicting the eradication outcome.[67] A randomized, open-label, parallel study comparing two sitafloxacin-based triple therapies in patients with multiple eradication failures is now underway in Keio University Hospital (Tokyo, Japan) (UMIN000006483) (http://kompas.hosp.keio.ac.jp/contents/medical_info/presentation/201201.html).

## Summary

*H. pylori* infection is now known to be the main cause of peptic ulcer disease, chronic atrophic gastritis, and gastric MALT lymphoma, as well as non-cardia gastric cancer. In addition, an association between *H. pylori* infection and some extra-gastrointestinal diseases, such as ITP, has been indicated. We summarized recent basic research and clinical data on the link between *H. pylori* infection and *H. pylori*-associated diseases. Accurate diagnostic methods for *H. pylori* detection are available, allowing ready detection of the infection. Although the recommended first-line therapy regimens are effective and well tolerated, their wide use throughout the country over the years has led to failure of these regimens in 20%–30% of patients. Treatment failure is due mainly to increasing antibiotic resistance, in particular clarithromycin resistance. Although novel therapeutic strategies should be considered for first-line treatment, local monitoring of antimicrobial resistance must be implemented as well. In 2009, the JSHR established a board certification system for doctors treating *H. pylori* infection, and 734 doctors have been registered as a board-certified doctor by the JSHR after four board certification examinations on March 2012 (http://www.jshr.jp/index.php?page=medic_list) (**Fig. 3**). The JSHR also reported the list of institutes performing *H. pylori* eradication not covered by national insurance in Japan (http://www.jshr.jp/index.php?page=medic_facility) (**Fig. 3**).

While medical qualifications, scientific knowledge, and techniques in the field of *H. pylori* infection all continue to improve, the total control of these silent organisms in the stomach remains a challenge.



## Acknowledgments

The present study was supported by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (No. 22300169 to H.S.), a grant of the Adaptable and Seamless Technology Transfer Program through Target-Driven R & D (A-STEP) (AS231Z00132G to H.S.) from the Japan Science and Technology Agency (JST), a grant from the Smoking Research Foundation (to H.S.), the Keio Gijuku Academic Development Fund (to H.S.), and the Nateglinide Memorial Toyoshima Research and Education Fund (to H.S.).


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Dig Dis Sci (2012) 57:3308–3309
DOI 10.1007/s10620-012-2451-6

CORRESPONDENCE

# Enhanced Gastric Ghrelin-Induced c-Kit Protein Expression in Rats with Gastric Outlet Obstruction

**Eisuke Iwasaki · Hidekazu Suzuki · Sachiko Mogami · Toshifumi Hibi**

Received: 1 October 2012 / Accepted: 3 October 2012 / Published online: 23 October 2012


Dear Editor,

We reported in the April issue of *Digestive Diseases and Sciences* that rats with gastric outlet obstruction (GOO) had an enhanced mRNA expression of c-kit, a marker of interstitial cells of Cajal (ICC), in the stomach with an increased level of ghrelin production [1]. However, the distribution of c-Kit protein expression in the stomach was not shown. Now, we have completed the additional experiments to clarify this point.

For histochemical staining of c-Kit, stomach tissue specimens were fixed with cold acetone for 30 min and embedded in Tissue-Tek OCT compound 4583 (Sakura Finetechnical, Tokyo, Japan), frozen in liquid isopentane cooled with liquid nitrogen, and cut into 5-μm sections with a cryostat (CM1850; Leica, Nussloch, Germany). The sections were placed on a microslide glass (Matsunami, Osaka, Japan) and incubated with Protein Block (DAKO Japan, Tokyo, Japan) containing 0.5 % Triton X-100 for 1 h at room temperature and incubated with rabbit anti-c-Kit polyclonal antibody (A4502; 1:200, Daco Japan) overnight at 4 °C. Immunoreactivity was detected using Alexa Fluor 488 donkey anti-rabbit IgG (1:1,000; Invitrogen, Eugene, OR, USA). Coverslips were mounted with Permafluor (Beckman Coulter, Fullerton, CA, USA) and immunofluorescence was examined using a keyence BIOZERO (Keyence, Osaka, Japan) [2].

Now, we showed the enhanced levels of c-Kit immunoreactive cells in rats with GOO (Fig. 1a). The areas of the c-Kit-positive cells were quantified in 12 microscopic fields from each individual rat using the ImageJ program (National Institutes of Health). For the measurement of ICC-IM (intramuscular ICC)-positive levels, the area of the c-Kit-positive cells in the muscular layers was normalized with the whole area of inner circular muscular layers. For the measurement of ICC-MY (myenteric ICC)-positive levels, the area of the c-Kit-positive cells in the myenteric plexus was normalized with the whole area of intermuscular space. By the morphometric analyses, in the GOO group, the levels of ICC-IM as well as ICC-MY, in both the antrum and the corpus, were quantified (Fig. 1b). After intraperitoneal injection of [D-Lys3] GHRP-6, a ghrelin receptor antagonist, (6.0 mg/kg; Bachem, PA, USA), both the levels of ICC-IM and ICC-MY were decreased in the antrum and the corpus. It is concluded that GOO enhances c-Kit expression through the enhancement of ghrelin in the stomach.

In the present experiment, we performed histopathologic sampling of the specimen 30 min after administration of ghrelin receptor antagonist, since Miyazawa et al. [3] reported that the biological half-time of c-Kit protein was 30–60 min in the cultured cells.

The enhancement of immunoreativity of c-Kit protein was shown not only in the ICC-IM but also in the ICC-MY. Our results indicate that enhanced ghrelin keeps the expression of the mRNA and the protein expression of c-Kit at high levels in rats with GOO.

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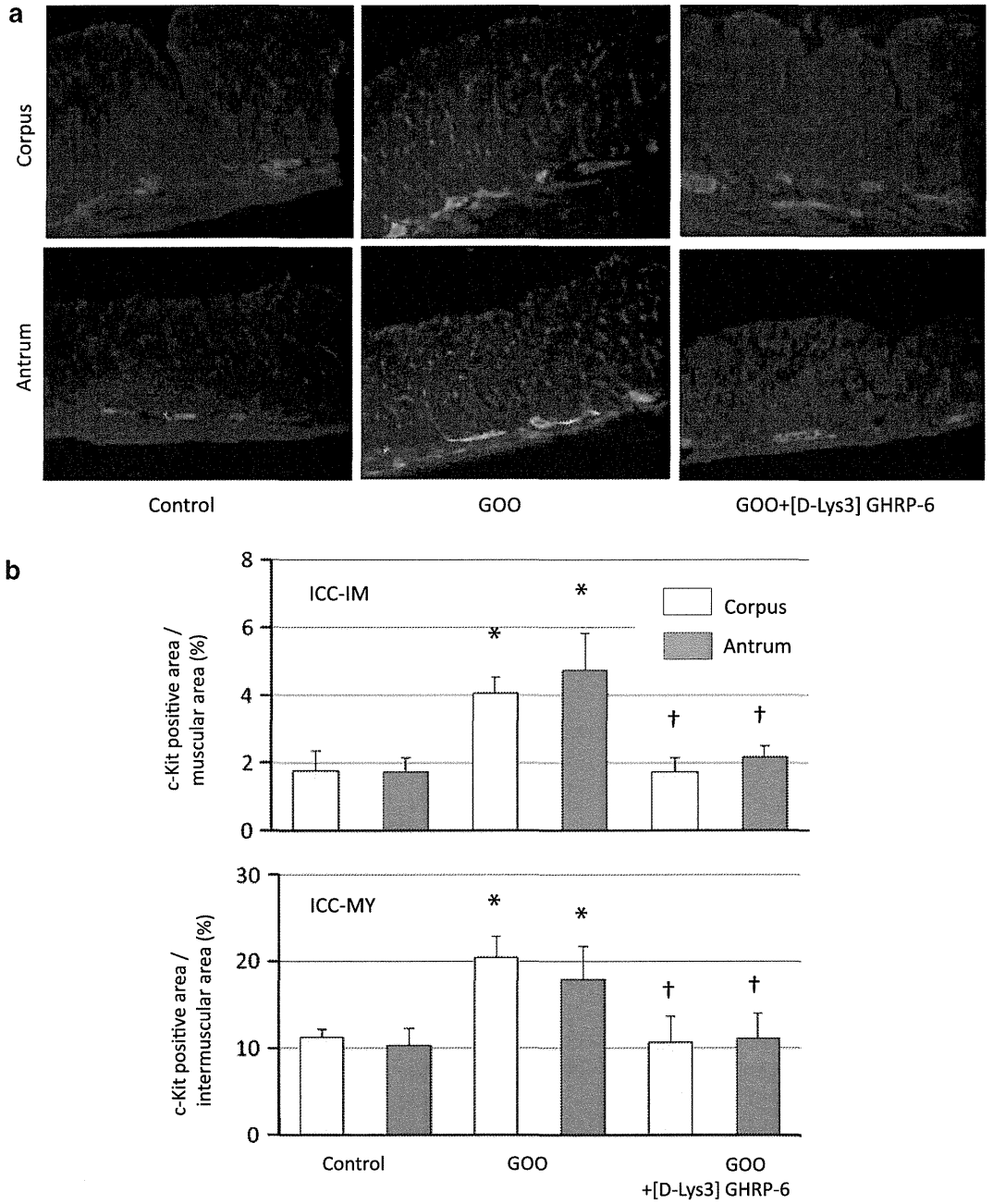


**Fig. 1** **a** Immunofluorescent staining of c-Kit in the corpus and the antrum of sham-operated control rats and GOO rat and GOO rats with [D-lys3] GHRP-6. **b** Areas of ICC-IM and ICC-MY were quantified by image analysis and are shown ($n = 6$, each group). Data are mean ± SD. $^{*}P < 0.05$ compared to control rats, $^{\dagger}P < 0.05$ compared to GOO rats

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12012

HEPATOLOGY

# Effect of ursodeoxycholic acid and endoscopic sphincterotomy in long-term stenting for common bile duct stones

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**Key words**
biliary stent, common bile duct (CBD) stones, endoscopic sphincterotomy, ursodeoxycholic acid.

Accepted for publication 3 October 2012.

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## Abstract

**Background and Aim:** We investigated the patency rate of a biliary stent and the effects of ursodeoxycholic acid (UDCA) therapy and endoscopic sphincterotomy (EST) for difficult-to-remove common bile duct stones.

**Method:** A total of 63 endoscopic retrograde cholangiopancreatographies (ERCPs) were performed in 36 patients (mean age, 86.0 years; male–female, 17:19) for stenting. Among the 63 subjects, 28 were further treated with EST; 20, with UDCA therapy; and 43, without UDCA therapy.

**Results:** The mean patency time was significantly longer in the UDCA treatment group (1,012 days) than in the "stent without UDCA" group (354 days; $P = 0.0002$; hazard ratio, 0.253). The mean patency time was significantly longer in the patients who had stent placement with EST (1074 days) than in those who had stent placement without EST (279 days; $P = 0.001$; hazard ratio, 0.439). The mean patency time was significantly longer in the patients who had stent placement with UDCA therapy and EST (1211 days) than in the patients who had stent placement with either UDCA therapy or EST (425 days; $P = 0.031$; hazard ratio, 0.3292). The mean patency time was significantly longer in the patients who had stent placement with either UDCA therapy or EST than in those who had stent placement without UDCA therapy or EST (263 days; $P = 0.0465$; hazard ratio, 0.5124).

**Conclusion:** Biliary stenting combined with UDCA therapy and EST may be considered as an effective treatment method for cases of common bile duct stones in elderly patients that are difficult to remove.

## Introduction

Stone extraction after endoscopic sphincterotomy (EST) is considered the current standard treatment for common bile duct (CBD) stones. The treatment success rate is approximately 90% when using a combination of basket and balloon catheter and lithotripsy.[1] However, in some cases in elderly patients who are clinically unfit for the complicated endoscopic retrograde cholangiopancreatography (ERCP) procedure, biliary stenting is an option, and its short-term effect has been proven.[2] However, the long-term benefit of stenting is unclear. In the present study, we investigated the long-term outcome and patency rate of biliary stents, and the effects of ursodeoxycholic acid (UDCA) therapy and additional EST for CBD stones.

## Method

We retrospectively reviewed the medical case charts of patients who underwent stent placement for CBD stones between 1 January 2003 and 30 April 2012. Patients were identified from a computerized database listing of all patients who underwent an endoscopic procedure at the National Tokyo Medical Center.

Thirty-six patients had difficult-to-remove CBD stones. The CBD stones were too large for successful endoscopic extraction or the patients too frail and elderly for further frequent attempts. Biliary drainage was achieved by inserting a single 7- (7 or 10 cm) or 6-Fr (7 or 10 cm) double pigtail stent (Cook Japan, Tokyo) with the proximal end at the portion just above the stones and the distal end in the duodenum. In the patients who were receiving antiplatelet or anticoagulant therapy and those with intradiverticular papilla, EST was not performed. The UDCA medication was chosen at the clinician's discretion. After the endoscopic procedures, all the patients were informed of the possible complications related to long-term biliary stent placement and were advised to contact our hospital if symptoms suggestive of cholangitis or jaundice occurred. Stent replacement was performed when symptoms of cholangitis occurred and/or elevated alkaline phosphatase or

bilirubin levels recurred. The period until the symptoms occurred was defined as stent patency time.

Differences between the groups were compared using the Student *t*-test for continuous variables and the Fisher exact test or chi-squared test for categorical variables. The patency rate and time to treatment failure were analyzed using the log-rank test and presented as Kaplan–Meier estimates with the hazard ratio and 95% confidence intervals (CIs).

## Results

During the study period, a total of 63 ERCP procedures for stenting were performed in 36 patients (age, 86.0 ± 6.78 years; male–female, 17:19), of whom 28 also underwent EST. One ERCP procedure was performed in 21 patients, two ERCP procedures were performed in 10 patients, three ERCP procedures were

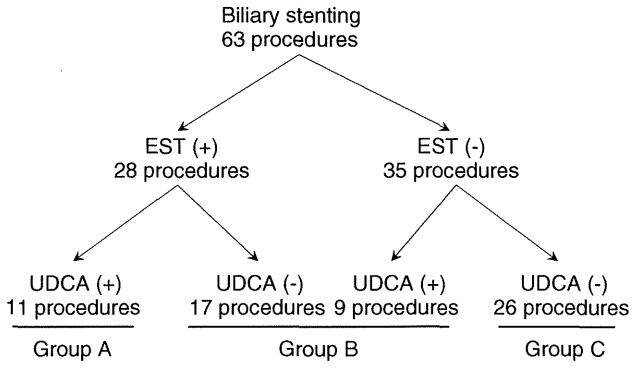


**Figure 1** Patients' demographic data.

**Table 1** Characteristics of the patients who underwent stent placement with or without ursodeoxycholic acid (UDCA) therapy

| | Stent with UDCA (20 procedures) | Stent without UDCA (43 procedures) | *P* value |
|---|---|---|---|
| Age (mean ± SD, years) | 87.6 ± 4.1 | 85.5 ± 7.4 | 0.25 |
| Male–female | 11/9 | 23/20 | 0.91 |
| Stent, 6 Fr/7 Fr | 7/13 | 14/29 | 0.84 |
| Stent, 7 cm/10 cm | 11/9 | 26/17 | 0.68 |
| With/without EST | 11/9 | 17/26 | 0.38 |
| Size of stones (mm) | 15.3 ± 8.1 | 14.0 ± 7.8 | 0.56 |
| Number of stones | 4.3 ± 2.3 | 3.6 ± 1.6 | 0.14 |
| Mean patency time (days) | 1012.0 | 354.1 | 0.0002 |
| 1-year patency rate | 62.5% | 31.7% | |
| 6-month patency rate | 80% | 48.3% | |

EST, endoscopic sphincterotomy.

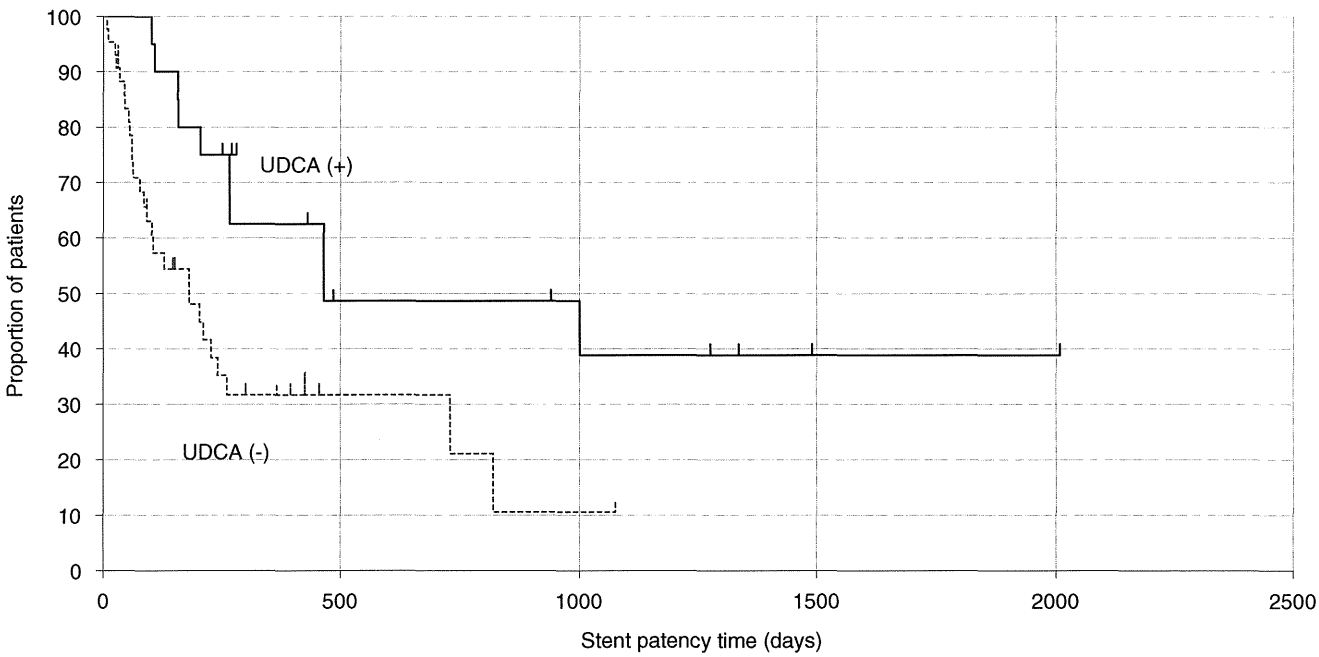


**Figure 2** Biliary stent patency in the patients who did or did not receive ursodeoxycholic acid (UDCA) therapy.

**Table 2** Characteristics of the patients who underwent stent placement with or without endoscopic sphincterotomy (EST)

| | Stent with EST (28 procedures) | Stent without EST (35 procedures) | *P* value |
|---|---|---|---|
| Age (mean ± SD, years) | 84.9 ± 7.4 | 87.1 ± 5.7 | 0.19 |
| Male/female | 12/16 | 22/13 | 0.11 |
| Stent, 6 Fr/7 Fr | 8/20 | 13/22 | 0.47 |
| Stent 7 cm/10 cm | 13/15 | 24/11 | 0.08 |
| With/without UDCA | 11/17 | 9/26 | 0.25 |
| Size of stones (mm) | 15.0 ± 9.5 | 14.0 ± 6.4 | 0.64 |
| Number of stones | 3.5 ± 2.0 | 4.0 ± 1.8 | 0.26 |
| Mean patency time | 1074.2 | 279.0 | 0.001 |
| 1-year patency rate | 59.0% | 33.4% | |
| 6-month patency rate | 69.9% | 47.9% | |

UDCA, ursodeoxycholic acid.

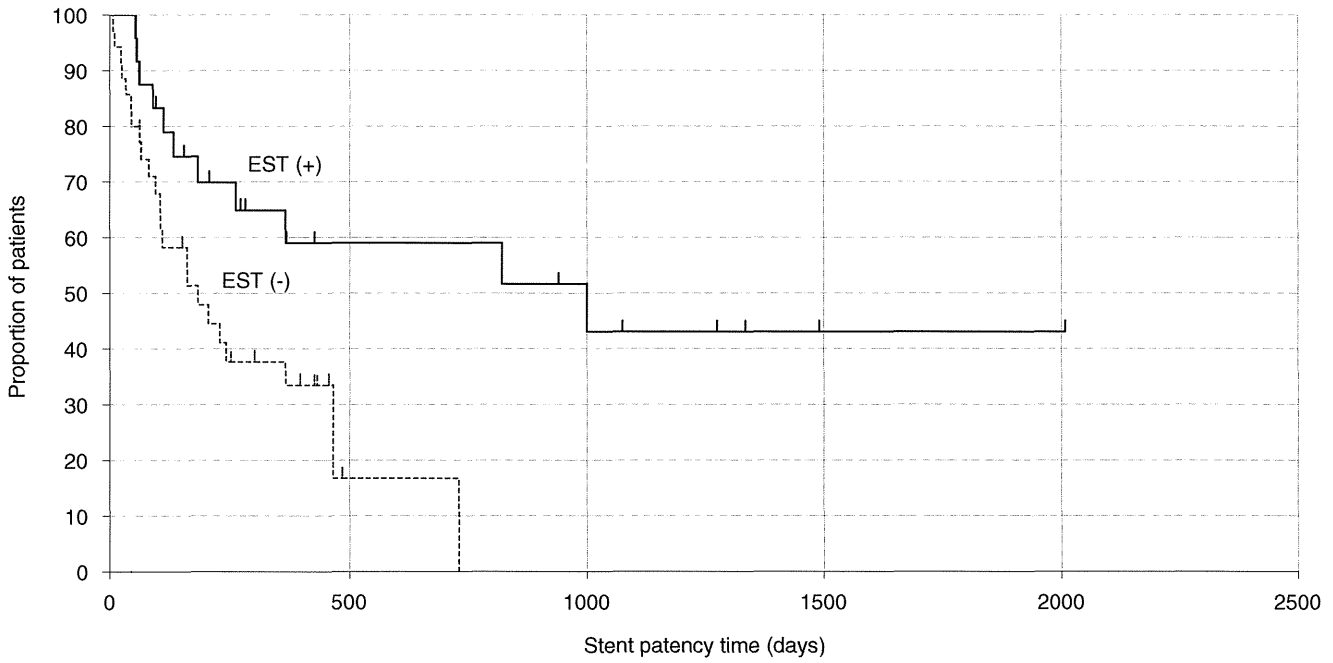


**Figure 3** Biliary stent patency in the patients who underwent or did not undergo endoscopic sphincterotomy (EST).

performed in 2 patients, four ERCP procedures were performed in 2 patients, and eight ERCP procedures were performed in 1 patient (mean 1.75 sessions of ERCP). After undergoing ERCPs for stenting, 20 subjects were treated with UDCA (600 and 300 mg/day in 12 and 8 subjects, respectively), whereas 43 did not receive UDCA (Fig. 1). There was no stent dislodge. There were eight deaths due to unrelated causes. Four patients dropped out of the study because they were transferred to other hospitals.

The baseline characteristics of the patients who had stent placement with and without UDCA treatment were similar (Table 1). The mean patency time was significantly longer in the UDCA treatment group (1,012 days) than in the "stent without UDCA" group (354.1 days; $P = 0.0002$; hazard ratio, 0.253; 95% CI, 0.0283–0.3319; Fig. 2).

The baseline characteristics of the patients who had stent placement with and without EST were similar (Table 2). The mean patency time was significantly longer in the "stent with EST" group (1,074.2 days) than in the "stent without EST" group (279 days; $P = 0.001$; hazard ratio, 0.439; 95% CI, 0.2121–0.8155; Fig. 3).

The characteristics of the patients who had stent replacement with UDCA therapy and EST, with either UDCA therapy or EST, or without UDCA therapy or EST are outlined in Table 3. The mean patency time was significantly longer in the "stent with UDCA therapy and EST" group (1211.9 days) than in the "stent with either UDCA therapy or EST" group (425.8 days; $P = 0.031$; hazard ratio, 0.3292; 95% CI, 0.1381–0.90734). The mean patency time was significantly longer in the "stent with either UDCA therapy or EST" group (425.8 days) than in the "stent without UDCA therapy or EST" group (263.4 days; $P = 0.0465$; hazard ratio, 0.5124; 95% CI, 0.2352–0.988915; Fig. 4).

**Table 3** Impact of ursodeoxycholic acid (UDCA) therapy and endoscopic sphincterotomy (EST) on the biliary stents

| | Stent with UDCA and EST (11 procedures) | Stent with either UDCA or EST (26 procedures) | Stent without UDCA or EST (26 procedures) | *P* value |
|---|---|---|---|---|
| Age (mean ± SD, years) | 86.5 ± 4.9 | 85.6 ± 7.4 | 86.6 ± 6.4 | 0.84 |
| Male/female | 4/7 | 17/9 | 8/18 | 0.03 |
| Stent 6 Fr/7 Fr | 5/6 | 5/21 | 11/15 | 0.14 |
| Stent 7 cm/10 cm | 4/7 | 16/10 | 17/9 | 0.24 |
| Size of stones (mm) | 16.2 ± 8.9 | 14.2 ± 9.1 | 14.0 ± 6.1 | 0.14 |
| Number of stones | 4.3 ± 2.3 | 3.2 ± 1.8 | 3.9 ± 1.8 | 0.52 |
| Mean patency time (days) | 1211.9 | 425.8 | 263.4 | 0.031† 0.047‡ |
| 1-year patency rate | 70.1% | 44.7% | 26.0% | |
| 6-month patency rate | 81.8% | 60.6% | 41.6% | |

†Stent with UDCA and EST versus stent with either UDCA therapy or EST.
‡Stent with either UDCA or EST versus stent without UDCA therapy or EST.

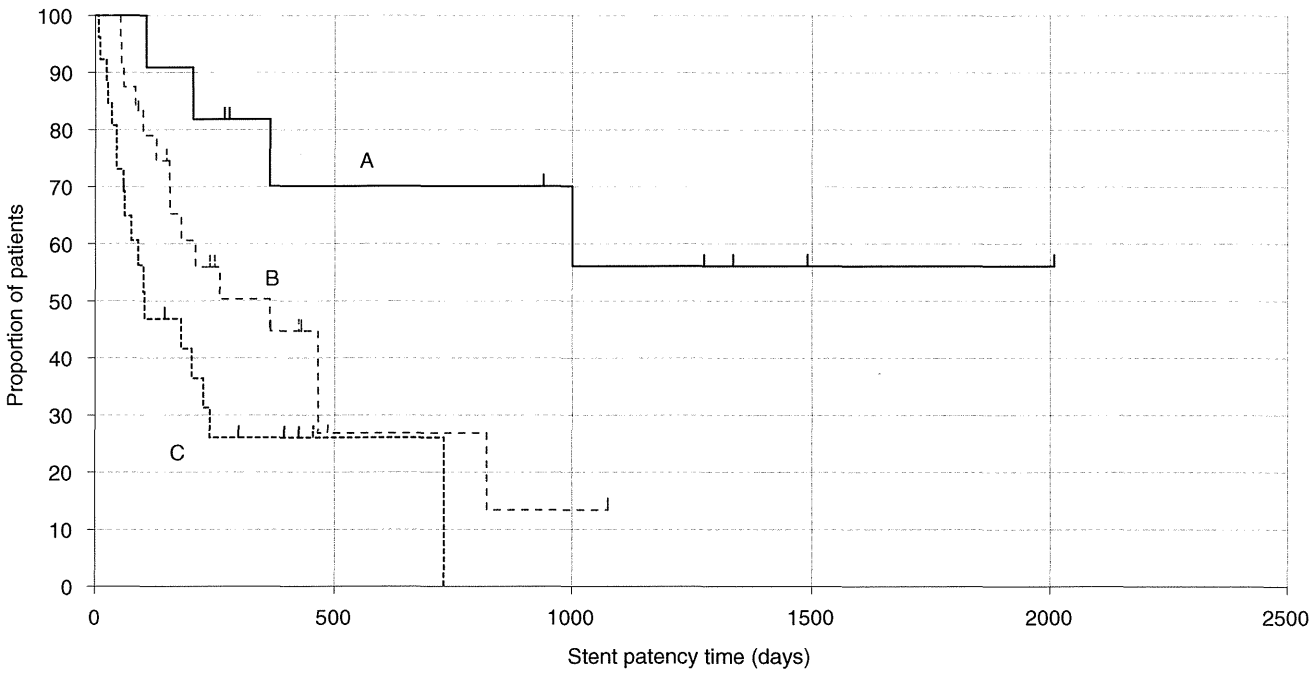


**Figure 4** Biliary stent patency in the patients who underwent stent placement with ursodeoxycholic acid (UDCA) therapy and endoscopic sphincterotomy (EST). (A) Patients who underwent stent placement with UDCA therapy and EST. (B) Patients who underwent stent placement with either UDCA therapy or EST. (C) Patients who underwent stent placement without UDCA therapy or EST.

## Discussion

This is the first report indicating that the combination of UDCA therapy and EST with biliary stenting is effective for treating elderly patients with CBD stones that are difficult to remove.

UDCA has been used to dissolve gallstones. UDCA, a hydrophilic dihydroxy bile acid (3α,7β-dihydroxy-5β-cholanoic acid), reduces the lithogenicity of bile by decreasing the biliary lipid output composed of cholesterol.[3] A report by Johnson *et al.*[4] emphasized the supplementary use of UDCA in cleaning the bile duct. Nine of 10 patients who were treated with concomitant UDCA therapy showed complete clearance of CBD stones 9 months after biliary stenting, whereas 10 patients who received biliary stent alone showed only partial clearance. However, in a recent randomized blinded trial of the combination of plastic biliary stents and 750-mg/day oral UDCA or placebo for 6 months, there was no significant difference in the clearance of CBD stones (76.9% *vs* 75.0%, respectively) and stone size reduction (0.40 *vs* 0.37 cm, respectively) between the two groups.[5] However, in long-term biliary stenting, our data showed a beneficial effect of the UDCA therapy on the stent patency rate.

The biliary stent is not considered as the sole conduit for bile flow when used for CBD stones. As expected, a free space remains in the CBD after biliary stent placement alongside the stones,

possibly providing a pathway for bile flow even when the biliary stent is completely obstructed.[6] EST may provide an additional outflow pathway. In all patients requiring stenting for CBD stones, we suggest that EST should be performed because complications such as cholangitis are more frequent in patients who had not undergone EST.

The limitations of this study include the small number of patients and nonrandomized retrospective study design. To validate our results, a randomized prospective study comparing the results of biliary stenting only with those of biliary stenting combined with UDCA therapy and EST is required.

In conclusion, based on our results, biliary stenting combined with UDCA therapy and EST may be considered a safe and effective method for treating elderly patients with CBD stones that are difficult to remove.

## Acknowledgment

This study was supported by a grant from the National Hospital Organization (No. H24-NHO [gastroenterology]-01, to T.N.).

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Cell Host & Microbe
Article

# Reactive Oxygen Species-Induced Autophagic Degradation of *Helicobacter pylori* CagA Is Specifically Suppressed in Cancer Stem-like Cells

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http://dx.doi.org/10.1016/j.chom.2012.10.014

## SUMMARY

**Sustained expression of CagA, the type IV secretion effector of *Helicobacter pylori*, is closely associated with the development of gastric cancer. However, we observed that after translocation, CagA is degraded by autophagy and therefore short lived. Autophagy and CagA degradation are induced by the *H. pylori* vacuolating cytotoxin, VacA, which acted via decreasing intracellular glutathione (GSH) levels, causing reactive oxygen species (ROS) accumulation and Akt activation. Investigating this further, we found that CagA specifically accumulated in gastric cells expressing CD44, a cell-surface marker associated with cancer stem cells. The autophagic pathway in CD44-positive gastric cancer stem-like cells is suppressed because of their resistance to ROS, which is supported by increased intracellular GSH levels. These findings provide a molecular link between *H. pylori* and gastric carcinogenesis through the specific accumulation of CagA in gastric cancer stem-like cells.**

## INTRODUCTION

A possible link has been demonstrated between *Helicobacter pylori* infection and development of gastric cancer by epidemiological (Uemura et al., 2001) and animal studies (Suzuki et al., 2009). Although long-term *H. pylori* infections of the gastric mucosa might cause gastric cancer from severe inflammation, no direct molecular link was demonstrated until Hatakeyama reported that the transfer of *H. pylori*-derived CagA to epithelial cells through a bacterial type IV secretion system promoted an early event of gastric carcinogenesis (Hatakeyama, 2004). Ohnishi et al. (2008) also demonstrated that systemic expression of CagA in *cagA*-transgenic mice induced gastrointestinal malignancies, indicating the oncogenic potential of bacterial CagA in mammals. However, it was also reported that CagA, after translocation to gastric epithelial cells, does not persist for a long period (Ishikawa et al., 2009).

Recently, it has been reported that autophagy—a system for bulk protein degradation and the elimination of invaded pathogens (Deretic and Levine, 2009)—is induced in *H. pylori*-infected cells (Raju et al., 2012; Terebiznik et al., 2009). In the present study, we observed that intracellular CagA was degraded by autophagy induced by the accumulation of reactive oxygen species (ROS), suggesting that CagA may not promote carcinogenesis. Even if CagA escapes the autophagy system, intracellular CagA could never be transferred to daughter cells and would be lost after cell division. If translocated CagA does indeed trigger gastric carcinogenesis, it should be transferred to slow-cycling master-regulator cells and escape from autophagic degradation.

CD44 is a cell-surface marker associated with cancer stem cells in various tumors (Dalerba et al., 2007). Gastric cancer stem-like cells expressing the variant isoform of CD44 (CD44v9) suppress ROS accumulation by control of intracellular glutathione (GSH) levels by stabilizing xCT, a cystine transporter (Ishimoto et al., 2011). In the present report, we used CD44v9-expressing gastric cancer stem-like cells to study the ability of intracellular CagA to escape from autophagy and show a direct molecular link between *H. pylori*-derived CagA and gastric cancer stem-like cells.

## RESULTS

### CagA Is Degraded by Autophagy

To investigate the stability of intracellular CagA, we constructed an in vitro *H. pylori* infection model using a gastric cancer cell line (AGS). After 5 hr of *H. pylori* ATCC700392 infection, the AGS cells were incubated with kanamycin to kill extracellular bacteria. In AGS cells after *H. pylori* eradication, the levels of intracellular CagA and tyrosine-phosphorylated CagA (p-CagA) decreased in a time-dependent manner (Figure 1A). Therefore, intracellular CagA did not persist for a prolonged period in gastric epithelial cells and was soon degraded by host cell defenses.

To examine the mechanism of CagA degradation in host epithelial cells, we used proteasome inhibitors (MG132 and lactacystin [Lact]) and autophagy inhibitors (3-methyladenine [3MA] and wortmannin [Wort]). At 24 hr after *H. pylori* ATCC700392 eradication, while intracellular CagA and p-CagA levels were

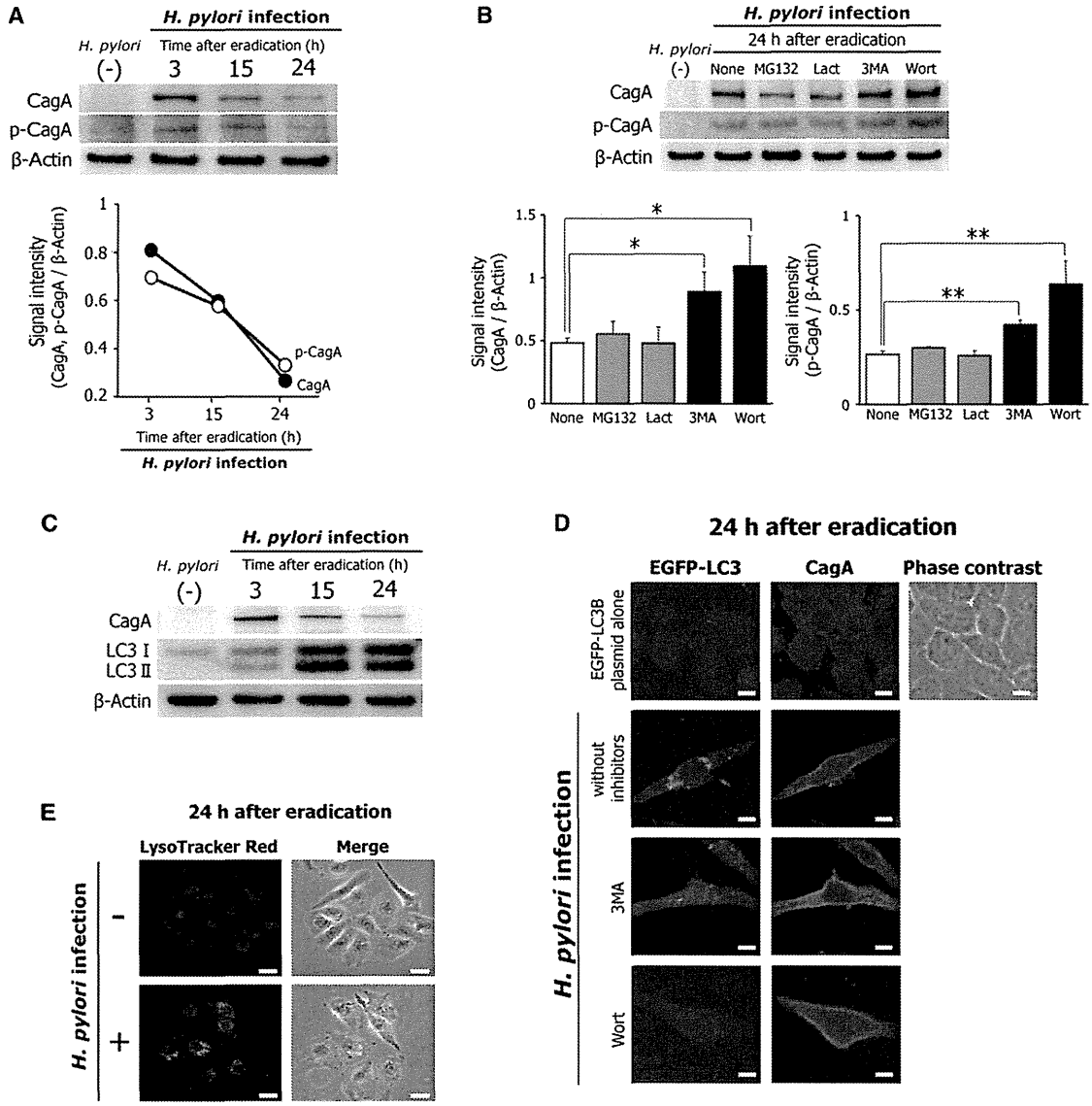



**Figure 1. Autophagy Induction Associated with Intracellular CagA Stability**

(A) AGS cells infected with *H. pylori* ATCC700392 for 5 hr were incubated at indicated times in a medium containing antibiotic to kill extracellular bacteria. Intracellular CagA and phosphorylated CagA (p-CagA) levels were quantified. Data represent the mean of three independent assays.

(B) AGS cells infected with *H. pylori* for 5 hr were incubated in a medium containing antibiotic with or without a proteasome inhibitor (10 μM MG132 or 20 μM lactacystin [Lact]) or autophagy inhibitor (5 mM 3-methyladenine [3MA] or 50 nM wortmannin [Wort]) for 24 hr. Intracellular CagA and p-CagA levels were quantified. Data represent the mean ± SD of three independent assays; *$p < 0.05$, **$p < 0.01$.

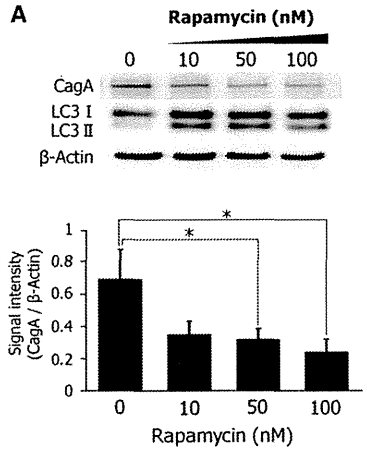
(C) AGS cells infected with *H. pylori* ATCC700392 for 5 hr were incubated in a medium containing antibiotic for the indicated times, and intracellular CagA and LC3-I to LC3-II conversion were examined.

(D) After transfection of AGS cells with the EGFP-LC3B plasmid, cells infected with *H. pylori* for 5 hr were incubated with a medium containing antibiotic for 24 hr with or without an autophagy inhibitor (10 μM MG132 or 20 μM Lact), and intracellular CagA was stained. EGFP-LC3B plasmid alone indicates the absence of *H. pylori* infection. Scale bar = 25 μm.

(E) AGS cells infected with *H. pylori* ATCC700392 for 5 hr were incubated with a medium containing antibiotic for 24 hr, and LysoTracker Red DND-99 staining was performed. Scale bar = 50 μm.

not affected by proteasome inhibitors (10 μM MG132 or 20 μM Lact), they were significantly increased by exposure of the cells to autophagy inhibitors (5 mM 3MA or 50 nM Wort), as compared with cells not exposed to these inhibitors (None) (Figure 1B). These results indicated that autophagy contributed to CagA degradation in host epithelial cells.

We then examined whether autophagy was activated within AGS cells after *H. pylori* ATCC700392 eradication. A hallmark of autophagy is the carboxyl terminus modification of microtubule-associated protein light chain 3 (LC3), which becomes linked to phosphatidylethanolamine and associates with the autophagosomal membrane. LC3-I to LC3-II conversion was



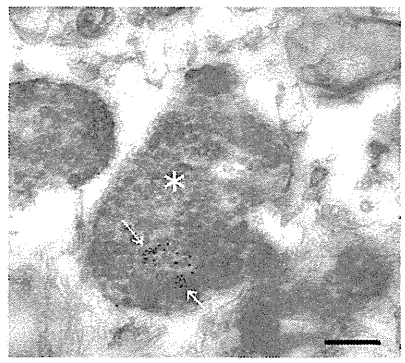




**Figure 2. Degradation of Intracellular CagA by the Induction of Autophagy**

(A) CagA expression in WT-A10 cells was induced by removal of Dox for 24 hr (CagA-expressing WT-A10 cells). CagA-expressing WT-A10 cells were stimulated with rapamycin for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; $^*p < 0.05$.

(B) CagA-expressing WT-A10 cells stimulated with 100 nM rapamycin for 24 hr were reacted with a 15 nm immunogold-labeled antibody; immunogold-labeled CagA was detected by electron microscopy. Arrows indicate immunogold-labeled CagA. Label is autophagolysosomal components. Scale bar = 200 nm.

detected most clearly at 15 and 24 hr after *H. pylori* eradication (Figure 1C). In addition, 24 hr after eradication, EGFP-LC3B-positive puncta were clearly detected within the cytoplasm of AGS cells transfected with the EGFP-LC3B plasmid, unlike AGS cells exposed to autophagy inhibitors (5 mM 3MA, 50 nM Wort) (Figure 1D), suggesting that LC3 is activated and localized to autophagosomes. LysoTracker Red stains late autophagic vacuoles (autolysosomes), but not early autophagosomes. LysoTracker Red staining was clearly detected within AGS cells at 24 hr after *H. pylori* eradication, consistent with the formation of autolysosomes (Figure 1E). These results demonstrate that autophagy was activated in AGS cells at 15 and 24 hr after eradication.

To evaluate whether autophagy was specifically associated with degradation of intracellular CagA, CagA-expressing WT-A10 cells—in which CagA expression was induced through the pTet-off-*cagA* expression vector by the absence of doxycycline (Dox) for 24 hr—was used. When these cells were incubated with rapamycin, which promotes autophagy by inhibiting mammalian target of rapamycin (mTOR), intracellular CagA decreased in a dose-dependent manner, and LC3-I to LC3-II conversion was clearly detected (Figure 2A). In addition, electron immunocytochemical examination following immunogold labeling for CagA in CagA-expressing, rapamycin-stimulated WT-A10 cells revealed the presence of labeled CagA in autophagic vesicles (Figure 2B). From these results, we conclude that intracellular CagA is degraded by autophagy.

### CagA Degradation via Autophagy Is Activated by m1VacA

Although autophagy was activated in AGS cells after *H. pylori* eradication (Figures 1C–1E), no LC3-I to LC3-II conversion was detected in CagA-expressing WT-A10 cells without rapamycin (Figure 2A; rapamycin [0 nM] lane). These results indicate that the induction of autophagy was independent of intracellular CagA, but was dependent on *H. pylori* infection. According to Terebiznik et al. (2009), autophagy was induced by VacA in *H. pylori*-infected AGS cells; therefore, we tested whether VacA participated in induction of autophagy associated with CagA degradation. In CagA-expressing WT-A10 cells exposed to culture supernatant from *H. pylori* ATCC700392 (s1m1VacA), intracellular CagA levels were significantly decreased in the culture supernatant in a dose-dependent manner with LC3-I to LC3-II conversion (Figure 3A). In addition, in AGS cells at 24 hr after *H. pylori* ATCC700392 (s1m1VacA) eradication, intracellular CagA levels were significantly decreased, as compared to 15 hr after eradication; conversion of LC3-I to LC3-II was clearly evident (Figure 3B). Conversely, in WT-A10 cells exposed to *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) culture supernatant, there was no decrease in intracellular CagA levels, and no LC3-I to LC3-II conversion was detected (Figure S1A). Moreover, in AGS cells at 24 hr after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) eradication, there was no decrease in intracellular CagA, and no LC3-I to LC3-II conversion was detected (Figure S1B). To investigate the function of CagA from each strain, we examined the tyrosine phosphorylation level of each CagA protein. All the CagA proteins were phosphorylated (Figure S1C), suggesting that those CagA species behaved similarly in delivered host cells.

In CagA-expressing WT-A10 cells incubated with m1VacA for 24 hr, a significant m1VacA-dependent decrease in intracellular CagA levels was observed along with LC3-I to LC3-II conversion (Figure 3C). Autophagy inhibitors (5 mM 3MA or 50 nM Wort) repressed the LC3-I to LC3-II conversion induced by m1VacA and significantly increased intracellular CagA levels (Figure 3D). In CagA-expressing WT-A10 cells incubated with m2VacA, intracellular CagA was not degraded and LC3-I to LC3-II conversion was not observed (Figure S1D). In addition, at 24 hr after *H. pylori* F57 (VacA-negative) eradication, there was a significant increase in CagA, as compared with cells infected with *H. pylori* ATCC700392 (s1m1VacA) (Figure S1E). The increase of intracellular CagA produced by *H. pylori* F57 (VacA-negative) was reduced by the addition of 60 nM m1VacA, in contrast to the addition of 60 nM m2VacA (Figure S1E). To evaluate the biological activity of VacA, we examined the vacuolation activity of m1VacA and m2VacA. Both proteins induced vacuolation in CagA-expressing WT-A10 cells in a dose-dependent manner, although m1VacA induced stronger vacuolation activity than m2VacA (Figure S1F). Our observations demonstrate that the autophagy responsible for CagA degradation is induced by m1VacA in gastric epithelial cells, independent of vacuolating cytotoxicity.

We recently found that low-density lipoprotein receptor-related protein-1 (LRP1) was one of the VacA receptors that

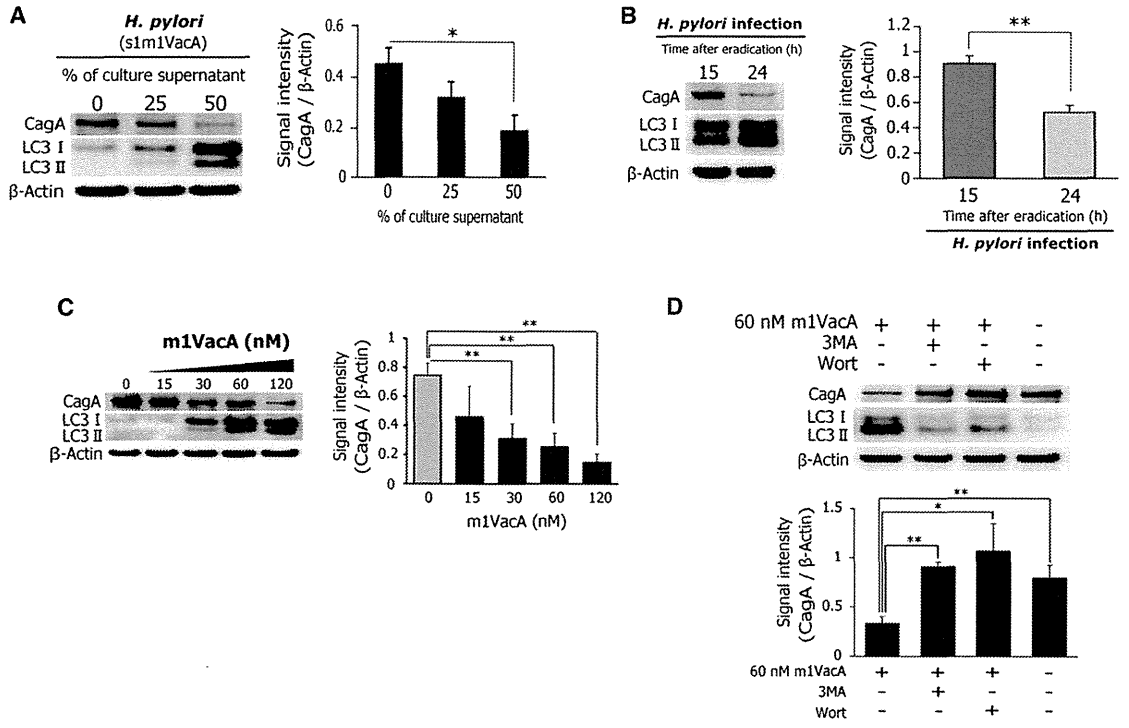



**Figure 3. Autophagy, Causing CagA Degradation, Is Induced by m1VacA**

(A) CagA-expressing WT-A10 cells were stimulated with *H. pylori* ATCC700392 (s1m1VacA) culture supernatant, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *$p < 0.05$.

(B) AGS cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for 15 and 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; **$p < 0.01$.

(C) CagA-expressing WT-A10 cells were incubated with m1VacA for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; **$p < 0.01$.

(D) CagA-expressing WT-A10 cells, stimulated by m1VacA, were incubated with an autophagy inhibitor (5 mM 3MA or 50 nM Wort) for 24 hr, and intracellular CagA and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; *$p < 0.05$, **$p < 0.01$. See also Figure S1.

mediate induction of autophagy (Yahiro et al., 2012). Then, to examine the relevance of LRP1 for the induction of autophagy-mediated CagA degradation, we constructed specific LRP1-knockdown AGS cells using small interfering RNAs (siRNAs) (Figure S1G). The LRP1 knockdown repressed the LC3-I to LC3-II conversion, resulting in the inhibition of CagA degradation (Figure S1H). This result indicates that LRP1 is required for the induction of autophagy-mediated CagA degradation in response to m1VacA. Next, to compare the binding ability of m1VacA and m2VacA to LRP1, we performed an immunoprecipitation assay with anti-LRP1. An 87 kDa fragment of VacA was detected by western blotting with an anti-VacA antibody in the anti-LRP1 immunoprecipitates from AGS cells infected with *H. pylori* ATCC700392 (s1m1VacA) (Figure S1I). In contrast, VacA was not detected in the anti-LRP1 immunoprecipitates from AGS cells infected with *H. pylori* ot210 (s1m2VacA) (Figure S1I). This result demonstrates that m1VacA, but not m2VacA, has a binding potential to LRP1.

### p53 Downregulation via Increased MDM2-Phosphorylation Induces Autophagy, Causing CagA Degradation

p53 inactivation by chemical inhibition or knockdown induces autophagy via the inhibition of mTOR. To investigate the induction of autophagy associated with CagA degradation, we examined p53 expression in AGS cells after *H. pylori* infection. In AGS cells at 15 and 24 hr after eradication of *H. pylori* ATCC700392 (s1m1VacA), p53 expression was significantly decreased and LC3-I to LC3-II conversion was clearly detected (Figure 4A). We then examined the mechanisms of p53 downregulation, focusing on posttranslational mechanisms, since *H. pylori*-infected AGS cells have been reported to show no change in *p53* mRNA expression (Wei et al., 2010). It is well known that p53 can be degraded by ubiquitination and proteasomal degradation pathways and that murine double minute 2 (MDM2) is the main E3 ubiquitin ligase that mediates p53 degradation. MDM2 expression was unaltered in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection (Figure 4B). MDM2 is activated by phosphorylation at Ser166 (pMDM2) (Zhou et al., 2001). At 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), pMDM2 levels were significantly increased (Figure 4B). Conversely, in AGS cells after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) infection, neither a decrease in p53 expression nor an increase in pMDM2 was noted (Figure S2).

We then examined the relationship between p53 downregulation and intracellular CagA stability. The specific

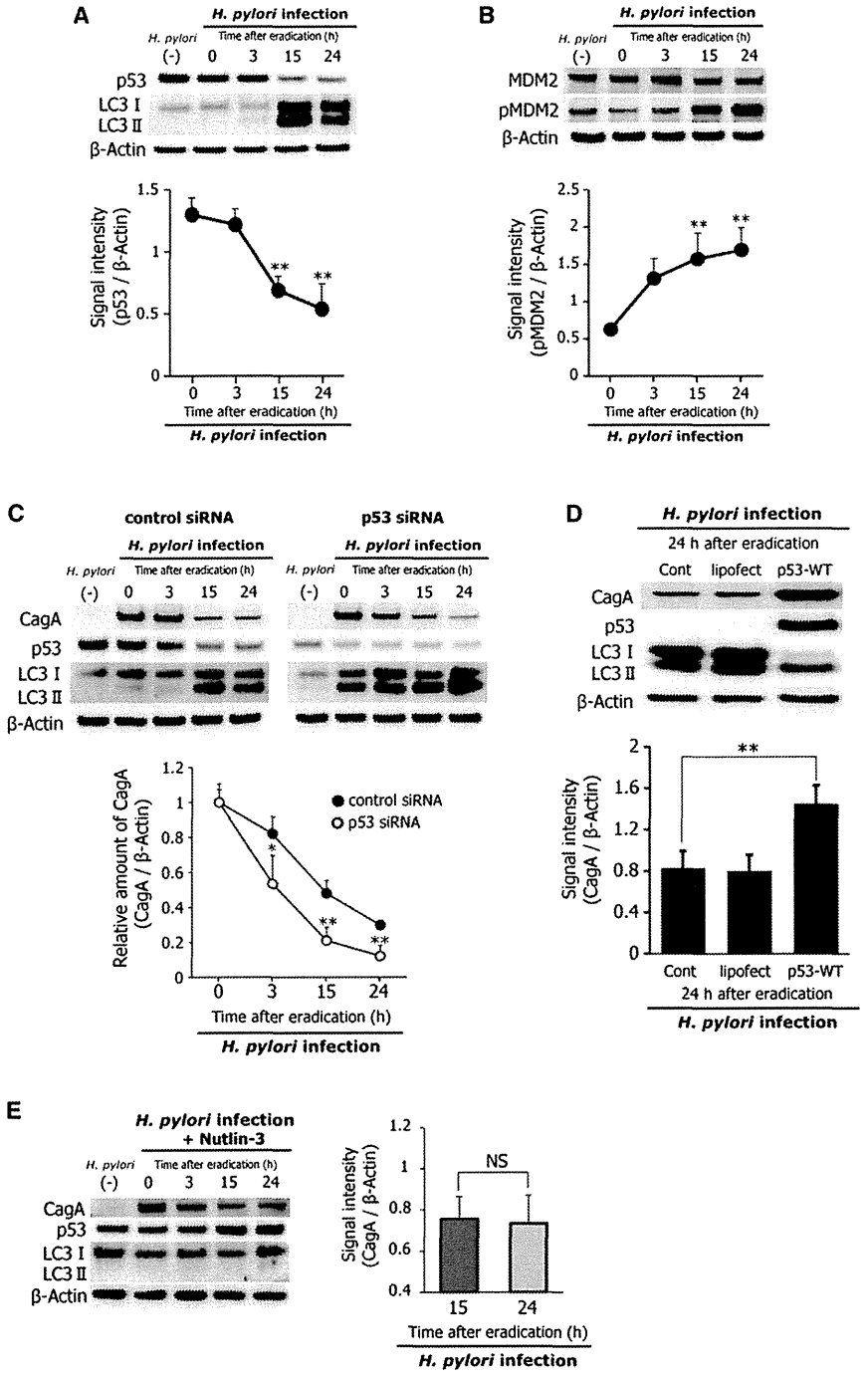



**Figure 4. Autophagy, Causing CagA Degradation, Is Induced through MDM2-Mediated p53 Degradation**

(A) AGS cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated time, and p53 expression and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; $^{**}p < 0.01$, compared to AGS cells at 0 hr after *H. pylori* (s1m1VacA) eradication.

(B) AGS cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated times, and the levels of MDM2 and phosphorylated-MDM2 (pMDM2) were examined. Data represent the mean ± SD of three independent assays; $^{**}p < 0.01$, compared to AGS cells at 0 hr after *H. pylori* (s1m1VacA) eradication.

(C) After AGS cells were transfected with control siRNA or p53 siRNA, cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated times, and the levels of CagA, p53 expression, and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; $^{**}p < 0.01$, compared to AGS cells transfected with control siRNA at each time point after *H. pylori* (s1m1VacA) eradication.

(D) KATOIII cells were transfected with the pCMV-Neo-Bam WT *p53* plasmid (p53-WT) or without (Cont). Each cell after *H. pylori* (s1m1VacA) infection for 5 hr was incubated in a medium containing antibiotic for the indicated times. CagA levels and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; $^{**}p < 0.01$; lipofect indicates KATOIII cells treated with only Lipofectamine 2000.

(E) AGS cells infected with *H. pylori* (s1m1VacA) for 5 hr were incubated in a medium containing antibiotic for the indicated times with 10 μM nutlin-3, and the levels of CagA, p53 expression, and LC3-I to LC3-II conversion were examined. Data represent the mean ± SD of three independent assays; NS, not significant. See also Figure S2.

p53-knockdown using small interfering RNAs (siRNAs) accelerated LC3-I to LC3-II conversion, thereby enhancing CagA degradation in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection (Figure 4C). Moreover, in KATOIII cells, which are genetically deficient of p53 ($p53^{-/-}$ KATOIII cells), LC3-I to LC3-II conversion was clearly detected at 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), and intracellular CagA levels were significantly decreased, as compared with $p53^{-/-}$ KATOIII cells transfected with the WT *p53* expression plasmid (Figure 4D). In addition, we examined the effect of nutlin-3—an inhibitor of MDM2-phosphorylation—on CagA stability. Treatment with 10 μM nutlin-3 repressed p53 downregulation and LC3-I to LC3-II conversion (Figure 4E), resulting in the inhibition of CagA degradation (Figure 4E). These results show that p53 downregulation, through the acceleration of MDM2-phosphorylation by m1VacA, induces autophagy, causing CagA degradation.

### ROS Accumulation Is Necessary for the Induction of Autophagy, Causing CagA Degradation

An accumulation of intracellular ROS induces autophagy, and the generation of intracellular ROS is enhanced in gastric epithelial cells during *H. pylori* infection (Ding et al., 2007). We hypothesized that the enhanced generation of intracellular ROS participates in induction of autophagy, causing CagA

degradation. AGS cells at 15 and 24 hr after the eradication of infected *H. pylori* were analyzed using fluorescence microscopy and flow cytometry after staining with CM-$H_2$DCFDA, an ROS-sensitive fluorescent probe. Hydrolyzed CM-$H_2$DCFDA is oxidized to dichlorofluorescein (DCF) by intracellular ROS (Suzuki et al., 1994). DCF fluorescence was apparent in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), as compared with AGS cells without *H. pylori* exposure (Figure 5A). The intensity of DCF fluorescence in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) was significantly increased, as compared to AGS cells without *H. pylori* exposure (Figure 5B). Conversely, in AGS cells after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) infection, no increase in DCF fluorescence was observed (Figure S3A). These results show that the accumulation of intracellular ROS was enhanced during the induction of autophagy.

NADPH oxidase (NOX)-generated ROS is a key regulator of autophagy (Huang et al., 2009), while mitochondrial-superoxide ($O_2^-$) production is involved in the induction of autophagy (Scherz-Shouval and Elazar, 2007). To identify the source of enhanced ROS generation associated with the induction of autophagy through p53 downregulation, we examined the effects of an NOX inhibitor (acetovanillone), an MnSOD mimic compound (MnTMPyP), and N-acetylcysteine (NAC). p53 downregulation was not inhibited by 250 μM acetovanillone or 20 μM MnTMPyP; therefore, LC3-I to LC3-II conversion was not repressed (Figure 5C). Conversely, p53 downregulation was inhibited by treatment with 10 mM NAC, and LC3-I to LC3-II conversion was repressed (Figure 5C). Moreover, intracellular CagA levels were significantly increased by treatment of AGS cells with 10 mM NAC at 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) (Figure 5D). These results show that the accumulation of intracellular ROS is necessary for induction of autophagy, causing CagA degradation, independent of NOX- and mitochondria-associated ROS generation.

Administration of NAC, a cysteine prodrug, replenishes intracellular GSH levels; therefore, NAC has been used to treat GSH deficiency (Atkuri et al., 2007). We hypothesized that the accumulation of intracellular ROS during the induction of autophagy was caused by decreased GSH levels. To prove this, we examined the change of GSH levels in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection. Intracellular GSH levels in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) were significantly decreased, as compared to AGS cells without *H. pylori* exposure (Figure 5E). Moreover, intracellular GSH levels in AGS and CagA-expressing WT-A10 cells were significantly decreased by m1VacA in a dose-dependent manner (Figure 5F). In AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), intracellular GSH was decreased, as compared to cells at 15 and 24 hr after eradication of *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) (Figure S3B). Moreover, intracellular GSH levels in AGS and CagA-expressing WT-A10 cells were not decreased by treatment with m2VacA (Figure S3C). These results show that the accumulation of intracellular ROS associated with the induction of autophagy was induced by decreased GSH levels caused by m1VacA. Next, to provide the relevance of LRP1 in the reduction of intracellular GSH levels, we measured intracellular GSH levels in specific LRP1-knockdown AGS cells; they were significantly increased at 15 or 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA), as compared with those in AGS cells transfected with control siRNA (Figure S3D). These results demonstrate that the binding of m1VacA to LRP1 is required for the reduction of intracellular GSH levels.

### Activation of the Akt Pathway Depends on the Accumulation of ROS for Autophagy Induction

Phosphorylated Akt enhances the ubiquitination-promoting function of MDM2 by phosphorylation, resulting in p53 downregulation (Ogawara et al., 2002). In addition, exogenous and endogenous ROS enhance Akt phosphorylation (Dong-Yun et al., 2003). We hypothesized that the accumulation of intracellular ROS by decreased GSH levels enhances Akt phosphorylation, leading to the induction of autophagy through p53 downregulation by the activation of MDM2. To investigate this hypothesis, we examined Akt phosphorylation in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection. Although Akt expression was unaltered, the levels of phosphorylated Akt at Thr308 and Ser473 were significantly increased in AGS cells after *H. pylori* ATCC700392 (s1m1VacA) infection (Figure 6A). To examine whether Akt phosphorylation depends on the accumulation of intracellular ROS, we examined the effect of NAC on Akt phosphorylation. Treatment with 10 mM NAC inhibited Akt phosphorylation at Ser473, but not at Thr308 (Figure 6A); therefore, Akt phosphorylation at Ser473 was dependent on accumulation of intracellular ROS after *H. pylori* ATCC700392 (s1m1VacA) infection. In addition, although Akt phosphorylation at Thr308 was increased in AGS cells after *H. pylori* F57 (VacA-negative), ot210 (s1m2VacA), or SS1 (s2m2VacA) infection, Akt phosphorylation at Ser473 was not increased (Figure S4A). Moreover, Akt phosphorylation at Thr308 and Ser473 was not increased in CagA-expressing WT-A10 cells, suggesting that Akt phosphorylation was independent of intracellular CagA (Figure S4B).

To examine the relevance of Akt phosphorylation at Ser473 to the induction of autophagy, causing CagA degradation, we examined the effect of LY294002, an inhibitor of Akt phosphorylation, on the stability of intracellular CagA and autophagy induction. Ten micromolar LY294002 inhibited intracellular CagA degradation, the increase in pMDM2, and p53 downregulation (Figure 6B). As a result, LC3-I to LC3-II conversion in AGS cells at 15 and 24 hr after the eradication of *H. pylori* ATCC700392 (s1m1VacA) was repressed by LY294002 with reduced accumulation of LysoTracker Red (Figure 6C). These results suggest that enhanced Akt phosphorylation at Ser473 induced MDM2 phosphorylation, leading to the induction of autophagy and causing CagA degradation through p53 downregulation.

### Accumulation of Translocated CagA in CD44v9-Expressing Gastric Cancer Stem-like Cells

Intracellular CagA produced by m1VacA *H. pylori*, but not m2VacA *H. pylori*, was degraded by autophagy. Although some studies indicated that m1VacA *H. pylori* infection was at a greater risk of gastric cancer compared with m2VacA *H. pylori* infection (Basso et al., 2008; Miehlke et al., 2000), others have indicated that there is no correlation between virulence and the *vacA*