

Transfection of the *Helicobacter pylori* CagA gene alters MUC5AC expression in human gastric cancer cells

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Abstract. *Helicobacter pylori*, the primary causative agent of stomach cancer, is known to affect gastric mucin expression. However, the underlying molecular mechanisms mediating this *H. pylori*-dependent effect remain unknown. In the present study, the effect of exogenous expression of the *H. pylori* virulence factor, CagA, on mucin 5AC oligomeric mucus/gel-forming (MUC5AC) expression was investigated using an *in vitro* model of the gastric mucosa. AGS cells were either untreated or transfected by a vector control (pCDNA3.1) or heterologous DNA, which induced CagA overexpression (pCDNA3.1-CagA). The expression and functionality of MUC5AC was analyzed using the reverse transcription-quantitative polymerase chain reaction and immunofluorescence assays. The expression of *H. pylori*-CagA in AGS cells was able to significantly upregulate MUC5AC expression compared to the vector control. In addition, immunofluorescence assays were able to validate increased MUC5AC expression following exogenous expression of *H. pylori*-CagA. The results of the present study revealed that the *H. pylori*-derived virulence factor CagA was able to increase the expression of MUC5AC. As this mucin constitutes an important ecological niche for *H. pylori*, this response may be involved in *H. pylori* colonization of the stomach.

Introduction

Mucin 5AC oligomeric mucus/gel-forming (MUC5AC) is a principal component of the gastric mucosa (1,2) and constitutes an important part of the ecological niche, where the stomach bacterium *Helicobacter pylori* is located (3). Adhesion to the gastric mucosa is important in the life cycle of *H. pylori* (4). As MUC5AC is an important receptor for gastric mucosa adhesion (5-7), efficient colonization of the stomach requires high expression of MUC5AC by gastric epithelial cells (8,9). Previous studies have demonstrated that the stomach epithelium reacts to *H. pylori* infection by activating pro-inflammatory signaling pathways (10), thereby activating innate defense mechanisms against *H. pylori*, which may include the downregulation of MUC5AC expression in the human gastric mucosa (2,6,11,12). Downregulation of MUC5AC expression in the gastric mucosa is evident following *H. pylori*-associated transformation of the gastric epithelium into cancer (13-15). As MUC5AC expression is key for the ecological niche required for *H. pylori* (6,7), it is to be expected that, in response to evolutionary pressure, the bacterium may have evolved compensatory mechanisms to combat the downregulation of MUC5AC. However, to date, such mechanisms have been incompletely characterized.

The association between *H. pylori* infection and gastric epithelial MUC5AC expression is well-recognized (6-9,16), there are a limited number of studies regarding the molecular mechanisms that mediate *H. pylori*-dependent compensatory responses, with respect to host downregulation of MUC5AC mucin gene expression. The downregulation of MUC5AC expression appears to be derived from an epithelial reaction towards the urease virulence factor of *H. pylori* (17). Therefore, it may be hypothesized that other virulence factors may mediate compensatory responses. In view of the importance of *H. pylori* for gastric oncological transformation and its involvement in a number of types of non-malignant disease (18), the identification of virulence factors potentially involved is required. Of these virulence factors, cytotoxin-associated gene A (CagA) has been associated with the interaction and modification of cellular phenotype and intracellular signaling pathways e.g., CagA induction of tumor suppressor gene hypermethylation via AKT-NFκB pathway in gastric cancer development, and is likely to mediate these effects (19-21).

The aforementioned considerations prompted the present study to investigate the interaction between *H. pylori*-derived

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Abbreviations: PVDF, polyvinylidene difluoride; CK, control group; pCDNA3.1-CagA, CagA overexpression group; pCDNA3.1, empty plasmid group

Key words: mucin 5AC oligomeric mucus/gel-forming, *Helicobacter pylori*, cytotoxin-associated gene A, stomach

CagA and MUC5AC expression in the gastric epithelium. It has already been established that upon CagA injection into the gastric epithelial cytosol by the bacteria, a number of cellular responses facilitating bacterial propagation and pathological responses are initiated (22-24). Therefore, CagA expression may affect MUC5A expression. It has been well established that CagA is injected directly from the bacteria into the gastric epithelial cytosol through its needle-like structure (23). In the present study, to isolate the effects of CagA from other *H. pylori*-induced molecules, in particular the urease virulence factor, a CagA expression construct was created that allows the cell-autonomous introduction of the bacterial protein into gastric epithelial cells. The results of the present study indicated that CagA is able to drive MUC5AC expression in gastric epithelial cells. Therefore, CagA insertion into host cells by *H. pylori* may constitute a bacterial defense mechanism against host downregulation of MUC5AC.

Materials and methods

Cell lines and cell culture. The AGS gastric adenocarcinoma cell line was purchased from the Shanghai Cell Bank (Shanghai, China). AGS cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum and were maintained at 37°C in an atmosphere containing 5% CO₂ (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (25).

Transfection of the CagA gene. Control group (CK), empty-plasmid group (pCDNA3.1) and overexpression CagA group (pCDNA3.1-CagA) were set up. The vector used for expressing CagA protein was the pcDNA 3.1 plasmid from Life Technologies (Thermo Fisher Scientific, Inc.). The CagA-coding insert was established by total gene synthesis. For transfection, 4 µg CagA plasmid and 6 µl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) were added to 200 µl F12 medium. Following mixing and incubated at room temperature for 20 min, the mixture was added to AGS cells that had been plated and had reached 75% cell confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for CagA, GAPDH and MUC5AC expression in the AGS model of the gastric epithelium. The AGS cells were harvested and RNA was extracted with RNeasy RLT Total RNA Isolation Solution (Generay Biotech Co., Ltd., Shanghai, China). Total RNA (1 µg) was mixed with 2 µl reagent 5X Prime Script RT Master mix (Vazyme Biotech Co., Ltd., Nanjing, China) and RNase Free dH₂O up to 10 µl, prior to being reverse transcribed in a one-step process at 50°C for 15 min and the introduction of inactivated enzymes at 85°C for 5 sec, each for once cycle according to the manufacturer's protocols. The cDNA product is finally stored at -80°C. The mRNA levels of MUC5AC of all groups were assessed using qPCR. The forward and reverse primer sequences used for determining *H. pylori*-CagA expression were 5'-ATTCAACAATAACGCTCTG-3' and 5'-ACCACCTGCTATGACTAA-3', respectively. The forward and reverse primer sequences for establishing Homo *GAPDH* (Gene ID, 2597; product size, 258 bp) expression were 5'-AGAAGGCTGGGGCTCATTG-3' and 5'-AGG

GGCCATCCACAGTCTTC-3', respectively. The forward and reverse primer sequences for determining Homo MUC5AC (Gene ID, 4586; product size, 281 bp) expression were 5'-ACG GGAAGCAATACACGG-3' and 5'-GGTCTGGGCGATGATGAA-3', respectively. For the qPCR amplification reaction, 10 µl IQ SYBR-Green Supermix (Tiangen Biotech Co., Ltd., Beijing, China) was used in conjunction with 1 µl cDNA product, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 8 µl water. The thermocycling conditions used were as follows: 50.0°C for 3 min, 95.0°C for 15 min, followed by 40 cycles of 95.0°C for 10 sec, 59°C for 25 sec and 72°C for 25 sec. Quantification was performed using the 2^{-ΔΔC_q} method (26).

Verification of CagA expression at the protein level. The protein expression of the CagA transgene in AGS cells was validated using western blot analysis. Total protein was extracted from non-transfected AGS cells, vector control-transfected AGS cells and AGS cells transfected with CagA-encoding plasmid. The cells were lysed by protein extraction buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was determined by the BCA method. The CagA levels were assessed by adding 30 µg of protein per lane, electrophoresis using a 10% polyacrylamide gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was subsequently blocked with a solution containing 5% skimmed milk powder and 5% bovine serum albumin (BSA (Shanghai Biyuntian Bio-Technology Co., Ltd.) for 1 h at room temperature, followed by washing and the addition of the CagA antibody (catalog no. sc-25766; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Following washing three times with Tris-buffered saline containing Tween-20 for 15 min, the PVDF membrane was exposed to the goat anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (catalog no. A16113; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 1 h at room temperature, followed by repeated washing. Subsequently, the membrane was visualized using BeyoECL Plus (Shanghai Biyuntian Bio-Technology Co., Ltd.), according to the manufacturer's protocol and routine procedures using a gel imaging analyzer (Chemidoc XRS+; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescent analysis of MUC5AC expression. The protein levels of MUC5AC of all groups were assessed using fluorescence. Cells (3x10⁴ per well) exposed to the appropriate transfections and conditions were grown for 24 h in 24-well plates under routine conditions, washed three times with PBS and fixed with a 4% paraformaldehyde solution for 15 min at 37°C. Subsequently, the samples were washed with PBS twice for 5 min and permeabilized using a 10-min incubation with 0.1% Triton X-100 in PBS. Following two washes for 5 min with PBS, non-specific immunoreactivity was blocked via a 90-min incubation with 1% BSA in 0.3 M glycine at 37°C (plates were sealed). Subsequently, MUC5AC antibody (catalog no. sc-398985, dilution 1:100; Santa Cruz Biotechnology, Inc.) was added and the samples were incubated overnight at 4°C. The following day, the fluorescence-labeled (Alexa Fluor® 555) Alexa Fluor® 555 goat anti-mouse IgG (H+L) antibody (catalog no. A21424;

Table I. CagA mRNA levels in CK, pCDNA3.1 and pCDNA3.1-CagA groups.

Group	mRNA expression level (mean \pm standard deviation; n=3)
CK	0.00 \pm 0.00
pCDNA3.1	0.00 \pm 0.00
pCDNA3.1-CagA	491.11 \pm 0.41

CagA, cytotoxin-associated gene A; CK, control group; pCDNA3.1, empty-plasmid; pCDNA3.1-CagA, CagA expression plasmid.

Table II. MUC5AC mRNA levels in CK, pCDNA3.1 and pCDNA3.1-CagA groups.

Group	mRNA expression level (mean \pm standard deviation; n=3)
CK	1.00 \pm 0.12
pCDNA3.1	1.29 \pm 0.06
pCDNA3.1-CagA	1.87 \pm 0.07

MUC5AC, mucin 5AC oligomeric mucus/gel-forming; CK, control group; pCDNA3.1, empty-plasmid; pCDNA3.1-CagA, CagA expression plasmid.

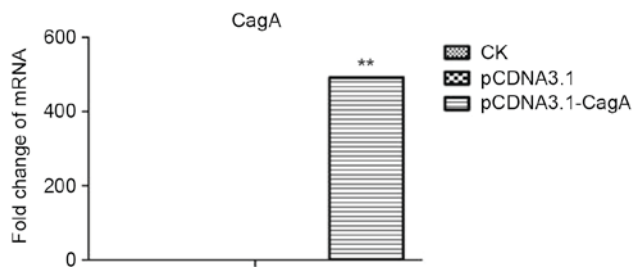


Figure 1. CagA expression levels in CK, pCDNA3.1 and pCDNA3.1-CagA groups as determined using reverse transcription-quantitative polymerase chain reaction. mRNA for CagA was not detected in the CK group or the pCDNA3.1 group. The level of CagA expression was significantly increased compared with CK and pCDNA3.1 groups. pCDNA3.1-CagA group vs. pCDNA3.1 group (**P<0.01); pCDNA3.1-CagA group vs. the CK group (**P<0.01). CagA, cytotoxin-associated gene A; CK, control group; pCDNA3.1, empty plasmid; pCDNA3.1-CagA, CagA expression plasmid.

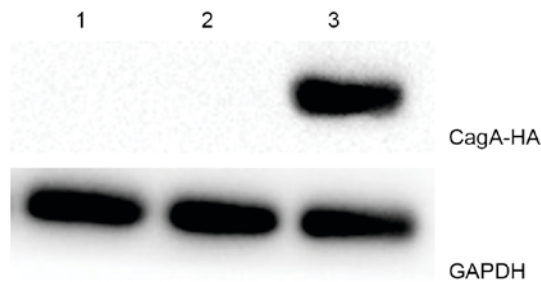


Figure 2. CagA expression levels of all groups as detected by western blotting. CagA protein was detected in the pCDNA3.1-CagA group and was not detected in the CK group or the pCDNA3.1 group. 1, control group; 2, pCDNA3.1 group (empty plasmid); 3, CagA expression plasmid; CagA, cytotoxin-associated gene.

dilution 1:800; Invitrogen; Thermo Fisher Scientific, Inc.) was added, and the experimental samples were incubated under humidified conditions for 1 h at 37°C. For staining of nuclei, 4',6-diamidino-2-phenylindole was added, and the samples were incubated for 10 min. Subsequently, the samples were washed three times with PBS for 5 min and mounted on glass slides and analyzed using magnification, x200 fluorescence microscopy (IX73; Olympus Corporation, Tokyo, Japan), to assess the subcellular distribution of MUCAC immunoreactivity.

Statistical analysis. The RT-qPCR results of CagA and MUC5AC expression in AGS cells were statistically analyzed

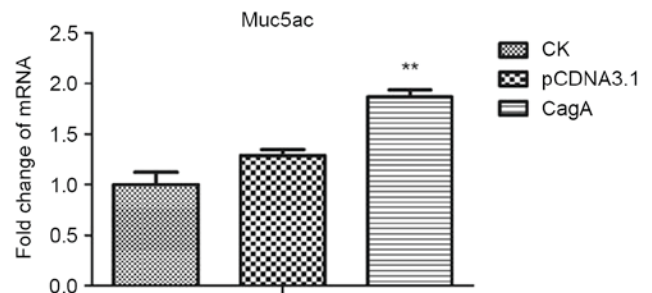


Figure 3. Levels of MUC5AC expression in CK, pCDNA3.1 and pCDNA3.1-CagA groups as determined using reverse transcription-quantitative polymerase chain reaction. The level of MUC5AC expression in the pCDNA3.1-CagA group was significantly increased compared with the pCDNA3.1 and CK groups. pCDNA3.1-CagA group vs. pCDNA3.1 group (**P<0.01); pCDNA3.1-CagA group vs. the CK group (**P<0.01). MUC5AC, mucin 5AC oligomeric mucus/gel-forming; CagA, cytotoxin-associated gene A; CK, control group; pCDNA3.1, empty-plasmid; pCDNA3.1-CagA, CagA expression plasmid.

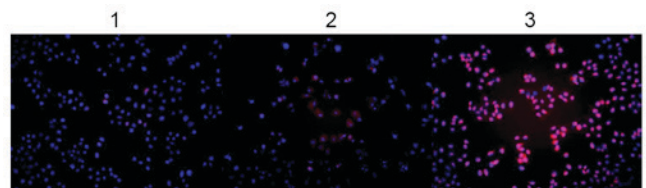


Figure 4. MUC5AC expression status of CK, pCDNA3.1 and pCDNA3.1-CagA groups as determined using immunofluorescence assays. The pCDNA3.1-CagA group revealed markedly more intense MUC5AC protein staining, compared with the CK and pCDNA3.1 groups. 1, CK (control group); 2, pCDNA3.1 group (empty plasmid); 3, CagA expression plasmid; MUC5AC, mucin 5AC oligomeric mucus/gel-forming; CagA, cytotoxin-associated gene A.

and calculated using the CFX Manager PCR analysis software (version 3.0; Bio-Rad Laboratories, Inc.). Following densitometric analysis using Image J software (National Institutes of Health, Bethesda, MD, USA), the CagA protein levels, determined using western blot analysis, were established. The data were subsequently analyzed using the Student's t-test or by one-way analysis of variance test for selected data pairs, following by the Tukey post-hoc test. P<0.05 was considered to indicate a statistically significant difference. The experiments were repeated 3 times.

Results

Exogenous expression of CagA in an experimental model of gastric epithelium. To investigate the effects of exogenous CagA expression in the gastric epithelium, AGS cells were transduced with a CagA-expressing plasmid, and the results were compared with cells transduced with an empty vector or non-transduced cells. This strategy successfully established heterologous CagA expression at the RNA level. The level of CagA expression in all groups as detected by RT-qPCR are shown in Table I and Fig. 1. The level of CagA expression in the pCDNA3.1-CagA group was significantly increased compared with the pCDNA3.1 group and the untreated AGS cells group ($P < 0.01$, $P < 0.01$; Fig. 1). In addition, at the protein level, the induction of exogenous CagA expression was successful. Western blots of all experimental groups are shown in Fig. 2, and CagA protein was only detected in the pCDNA3.1-CagA group. Therefore, this experimental set-up enabled the present study to analyze the effect of exogenous CagA expression on the levels of MUC5AC in AGS cells.

CagA expression upregulates MUC5AC. AGS cultures transduced with CagA were analyzed for MUC5AC expression using RT-qPCR and compared with the appropriate controls. As evident from the results presented in Table II and Fig. 3, heterologous CagA expression increased MUC5AC expression at the RNA level, compared with the control group. The level of MUC5AC expression in the pCDNA3.1-CagA group was significantly increased compared with that of the pCDNA3.1 and CK groups ($P < 0.01$, $P < 0.01$, respectively). As presented in Fig. 4, this result was validated using immunofluorescence assays, demonstrating substantial upregulation of MUC5AC, following the transfection of AGS cells with the CagA expression plasmid, compared with the control. Therefore, the results of the present study demonstrated that CagA was able to increase MUC5AC expression.

Discussion

At present, it is typically accepted that the gastric epithelium reacts to *H. pylori* infection by downregulating MUC5AC (2,6,11-15). This may be a protective response towards *H. pylori*, which enables the removal of this organism from its ecological niche. However, the precise interaction between *H. pylori* and MUC5AC remains unclear. The gastric epithelial downregulation of MUC5AC may involve bacterial urease, as evident from experiments involving AGS gastric cancer cells that were infected with the UreB-isogenic mutant of *H. pylori* (17). It is speculated that evolutionary pressure has favored the emergence of *H. pylori* variants that may counteract this effect. In the present study, it was demonstrated that the CagA virulence factor may exhibit a function in this process. The results of the present study revealed that the expression of MUC5AC, determined using RT-qPCR and immunofluorescence assays, was significantly upregulated upon the introduction of cellular CagA. Therefore, the injection of CagA into the gastric epithelial cytosol by *H. pylori* represents a countermeasure of *H. pylori* against gastric epithelial defensive downregulation of the MUC5AC glycoprotein in response to bacterial infection.

MUC5AC has been demonstrated to co-localize with *H. pylori* and functions as an important *H. pylori* receptor, which tethers *H. pylori* to the gastric mucosa (5,6). Therefore, it may be hypothesized that *H. pylori* aims at creating a favorable environment by stimulating gastric epithelial MUC5AC production, which facilitates bacterial adherence. In this sense, injection of CagA into gastric epithelial cells may stimulate these cells to produce MUC5AC early in the infection process, causing a transient increase in MUC5AC expression that facilitates additional colonization (27). A subsequent interaction between the bacterium and gastric cells leads to morphological alterations in the cell and the injury of host-cell epithelial barrier that is associated with *H. pylori* infection (28). MUC5AC is only produced by the stomach surface epithelium (29), which is consistent with this cell type being the important target for *H. pylori* during the infection process (30). These findings indicated that CagA has a role as a MUC5AC-targeting bacterial effector. The molecular mechanisms underlying how the virulence factor may result in transactivation of the MUC5AC promoter remain unknown. However, mucin production is typically a protective response against bacterial infection, and therefore, it may be exploited by the CagA protein (31-33). MUC5AC, as a principal component of the mucosal layer, protects the stomach surface from chemical, enzymatic, mechanical and microbial challenge, and its upregulation constitutes an intuitive response of the epithelium to infection (1,2,7,11,29,34-36). Additional studies are required to delineate the molecular mechanisms, which mediate the effects of CagA on MUC5AC transcription. However, the results of the present study have revealed that CagA is sufficient for cell-autonomous upregulation of MUC5AC and have therefore, to the best of our knowledge, revealed a novel mechanism employed by this bacterium for its colonization of the stomach.

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