Effects of the *Helicobacter pylori* Virulence Factor CagA and Ammonium Ion on Mucins in AGS Cells

Xiaoyu Zhang[1], Ding Shi[2], Yong-pan Liu[3], Wu-jie Chen[2], and Dong Wu[2]

[1]Department of Gastroenterology, Henan University of Chinese Medicine, Zhengzhou;
[2]Department of Gastroenterology, Ningbo No. 2 Hospital, Ningbo;
[3]Department of Gastroenterology, the First People's Hospital of Yuhang District, Hangzhou, China.

Purpose: To investigate the effects of *Helicobacter pylori* (*H. pylori*)-CagA and the urease metabolite NH_4^+ on mucin expression in AGS cells.

Materials and Methods: AGS cells were transfected with CagA and/or treated with different concentrations of NH_4CL. Mucin gene and protein expression was assessed by qPCR and immunofluorescence assays, respectively.

Results: CagA significantly upregulated *MUC5AC*, *MUC2*, and *MUC5B* expression in AGS cells, but did not affect E-cadherin and *MUC6* expression. *MUC5AC*, *MUC6*, and *MUC2* expression in AGS cells increased with increasing NH_4^+ concentrations until reaching a peak level at 15 mM. *MUC5B* mRNA expression in AGS cells (NH_4^+ concentration of 15 mM) was significantly higher than that at 0, 5, and 10 mM NH_4^+. No changes in E-cadherin expression in AGS cells treated with NH_4^+ were noted, except at 20 mM. The expression of *MUC5AC*, *MUC2*, and *MUC6* mRNA in CagA-transfected AGS cells at an NH_4^+ concentration of 15 mM was significantly higher than that at 0 mM, and decreased at higher concentrations. The expression of *MUC5B* mRNA increased with increases in NH_4^+ concentration, and was significantly higher compared to that in untreated cells. No significant change in the expression of E-cadherin mRNA in CagA-transfected AGS cells was observed. Immunofluorescence assays confirmed the observed changes.

Conclusion: *H. pylori* may affect the expression of *MUC5AC*, *MUC2*, *MUC5B*, and *MUC6* in AGS cells via CagA and/or NH_4^+, but not E-cadherin.

Key Words: Mucin, *Helicobacter pylori*, CagA, ammonium chloride, stomach

INTRODUCTION

Mucins are the major glycoprotein components of mucus and play an important role in maintaining mucosal integrity.[1] It has been shown that changes in the expression of mucins might contribute to cancer cell growth, immune recognition, and cellular adhesion of gastric epithelial cells, which in turn might increase the risk of their malignant transformation[2,3] and influence their invasive and metastatic capabilities. *Helicobacter pylori* (*H. pylori*) infection is the major risk factor for gastric cancer (GC), and *H. pylori* has been classified as a class I carcinogen by the World Health Organization.[4] It is able to bind to specific mucins in the gastric mucosa,[5] and it has been reported that it can elicit changes in the expression of mucins in the stomach.[2,6-8] In addition, *H. pylori* can modulate the expression of various mucin components via urease in order to facilitate its movement in viscous surroundings.[7] In view of the significance of *H. pylori* for GC tumorigenesis,[9] the identification of virulence factors potentially involved in changes in the expression of mucins is an important topic. It has been shown previously that CagA is associated with the modification of intracellular signal transduction pathways and the cellular phenotype transformation of gastric cells.[10,11] Indeed, the risk of GC is significantly higher in individuals infected with *H. pylori*

strains expressing the oncoprotein CagA.[12] A previous study[13] showed that *H. pylori* may cause changes in mucin expression through its virulence factors (CagA and urease). Urease mainly produces ammonia by hydrolyzing urea, and plays a role in preventing stomach acid from stabilizing *H. pylori*. Therefore, we speculate that CagA and the urease metabolite ammonium (NH₄⁺) may mediate the expression of mucin in AGS cells. However, the previous study did not exclude the effect of other pathogenic factors of *H. pylori* on mucin in AGS cells. In this study, after excluding the interference of other pathogenic factors of *H. pylori*, we respectively investigated the effects of CagA and urease metabolites on mucin expression in AGS cells by transfecting CagA overexpression plasmid into AGS cells or treating AGS cells (transfected CagA and untransfected) with different concentrations of ammonium ions, and detected changes in the expression of mucin in AGS cells in each group.

MATERIALS AND METHODS

This experimental study was divided into two parts: 1) First, the human GC cell line AGS was transfected with a CagA expression vector, and the expression of mucins in these cells was examined. In this part, control cells (CK), empty-plasmid transfected cells (pCDNA3.1), and cells transfected with CagA expression vector (pCDNA3.1-CagA) were established. 2) In the second part, different concentrations of ammonium chloride were added to the AGS cell culture medium, and the expression of mucins in AGS cells was detected.

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 (Library of the Shanghai Academy of Biological Sciences, Shanghai, China) supplemented with 10% fetal calf serum, and were maintained at 37°C under a 5% CO_2 atmosphere.

CagA transfection experiments
The vector used for the expression of CagA protein was the pcDNA 3.1 plasmid obtained from Life Technologies (Carlsbad, CA, USA). The CagA-coding insert was established by total gene synthesis. For transfection of AGS cells, 4 µg of the CagA plasmid and 6 µL of Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) were added to 200 µL of F12 medium. After mixing and incubation for 20 min, the mixture was added to AGS cells that had reached 75% cell confluence.

Verification of CagA protein expression
Protein expression of the CagA transgene in AGS cells was verified by western blotting. In brief, total proteins were extracted from nontransfected AGS cells, vector control-transfected AGS cells, and AGS cells transfected with CagA-encoding plasmid.

The CagA levels were assessed by electrophoresis on a polyacrylamide gel, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). After washing three times with Tris-buffered saline containing Tween 20 for 15 min, the PVDF membrane was incubated with the secondary antibody (sc-25766; Invitrogen) for 1 h at room temperature, followed by repeated washing and enhanced chemiluminescence detection (Beyotime Biotechnology, Shanghai, China), according to the manufacturer's specifications and routine procedures using a gel imaging analyzer (Chemidoc XRS+, Bio-Rad, Hercules, CA, USA).

Table 1. Primer Sequences Used in qPCR Analysis of mRNA Expression

|	Forward	Reverse
GAPDH	5'-AGAAGGCTGGGGCTCATTTG-3'	5'-AGGGGCCATCCACAGTCTTC-3'
CagA	5'-ATTCACAATAACGCTCTG-3'	5'-ACCACCTGCTATGACTAA-3'
MUC5AC	5'-ACGGGAAGCAATACACGG-3'	5'-GGTCTGGGCGATGATGAA-3'
MUC5B	5'-CTGTGGTCACCCGTGTTGT	5'-GGCGTTGTGGGCATAGAA-3'
MUC2	5'-CTGTGGTCACCCGTGTTGT-3'	5'-GGCATCGCTCTTCTCAA-3'
MUC6	5'-CCTTCGGGTGCTTCTGC-3'	5'-CCTGGCGTCAAATGTGGTA-3'
E-cadherin	5'-CCGCCATCGCTTACAC-3'	5'-CGTTAGCCTCGTTCTCA-3'
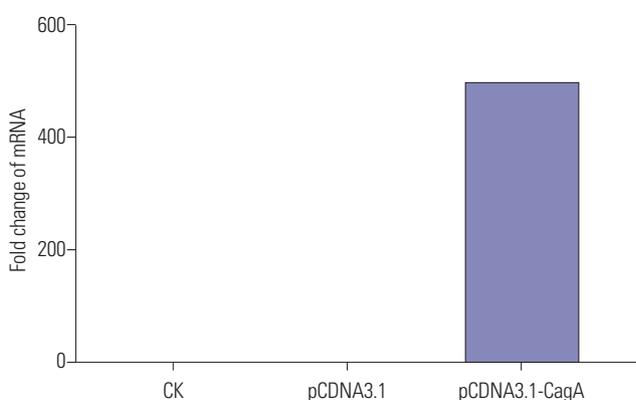
Fig. 1. CagA mRNA expression examined by qPCR in differently treated AGS cells. CagA mRNA expression was not detected in the CK and pCDNA3.1 cells, and was significantly expressed in pCDNA3.1-CagA cells. pCDNA3.1-CagA cells vs. pCDNA3.1 cells (p<0.001); pCDNA3.1-CagA cells vs. CK cells (p<0.001). CK, control cells; pCDNA3.1, empty-plasmid transfected cells; pCDNA3.1-CagA, cells transfected with CagA expression vector.
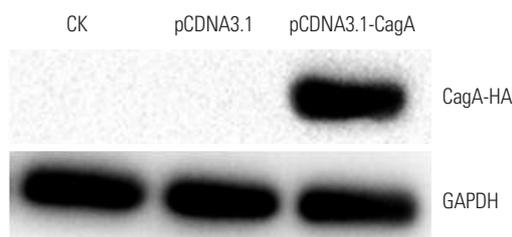

Fig. 2. CagA protein expression examined by western blotting in AGS cells. CagA protein was expressed in pCDNA3.1-CagA cells but not in CK and pCDNA3.1 cells. CK, control cells; pCDNA3.1, empty-plasmid transfected cells; pCDNA3.1-CagA, cells transfected with CagA expression vector.

Treatment of AGS cells and CagA- transfected AGS cells with ammonium chloride

Different concentrations of ammonium chloride (5, 10, 15, and 20 mM) were added into the culture medium of nontransfected or transfected AGS cells. The culture conditions were the same as described above. After 24 h, expression of mucins in these cells was detected.

qPCR analysis of mRNA expression

The Gene IDs for GAPDH, *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin were 2597, 4586, 727897, 4583, 4588, and 999, respectively. The expected lengths of the GAPDH, *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin PCR products were 258, 281, 293, 399, 242, and 298 bp, respectively. The primer sequences used in the qPCR analysis are listed in Table 1.

For the qPCR amplification reaction, 10 μL of IQ SYBR Green Supermix (Tiangen Biotech Co., Ltd., Beijing, China) was used in conjunction with 1 μL of the forward primer (10 μM), 1 μL of the reverse primer (10 μM), and 8 μL of water. The CFX connect Real-Time PCR System (ver. 3.0: Bio-Rad) was used to perform qPCR reaction as follows: 50.0°C for 3 min, 95.0°C for 15 min, 95.0°C for 10 s, 59°C for 25 s, and 72°C for 25 s for 40 cycles, followed by melting curve analysis, which was performed from 70°C to 95°C at a 0.5°C increment for 0.05 s, followed by plate reading.

Immunofluorescent analysis of mucin expression

Cells exposed to the described transfection and treatments were grown for 24 h in 24-well plates under standard conditions, washed three times with phosphate-buffered saline (PBS)
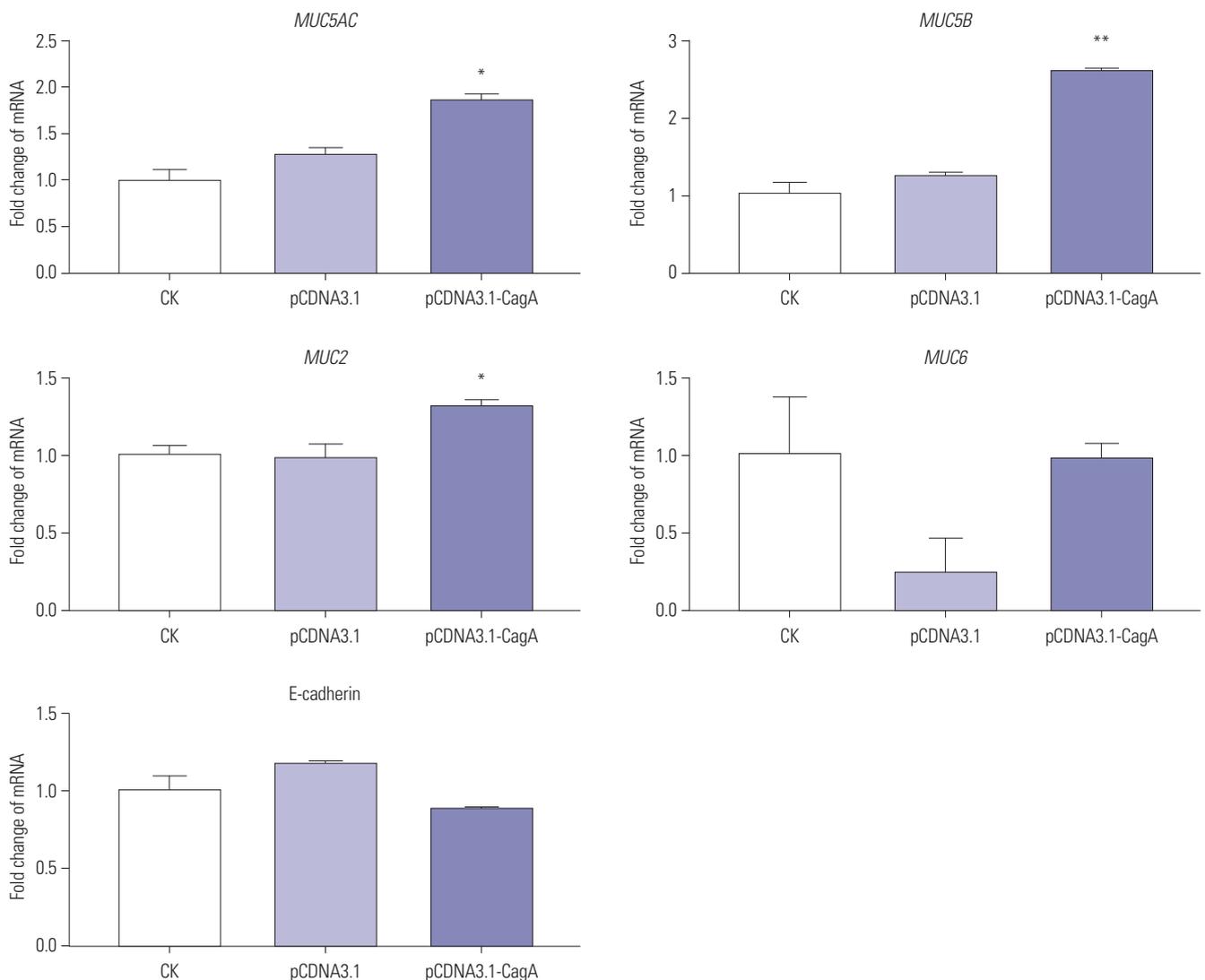

Fig. 3. *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin mRNA expression levels examined by qPCR. *MUC5AC*, *MUC2*, and *MUC5B* expression levels in pCDNA3.1-CagA AGS cells were significantly higher than those in pCDNA3.1 and CK cells, respectively (*$p<0.01$; **$p<0.001$). *MUC6* mRNA expression in pCDNA3.1-CagA cells was higher than that in pCDNA3.1 cells ($p<0.001$), and was similar to that in CK cells ($p>0.05$). E-cadherin expression in pCDNA3.1-CagA cells did not differ significantly from that in CK cells ($p>0.05$) or pCDNA3.1 cells ($p>0.05$). CK, control cells; pCDNA3.1, empty-plasmid transfected cells; pCDNA3.1-CagA, cells transfected with CagA expression vector.

for 5 min, and fixed with a 4% paraformaldehyde solution for 20 min. Subsequently, the samples were washed with PBS twice for 5 min and permeabilized using a 20-min incubation with 0.1% Triton X-100 in PBS. After two further washes for 5 min with PBS, nonspecific immunoreactivity was blocked with a 30-min incubation in 2% fetal bovine serum in 0.3 M glycine at 37°C (plates were sealed). Next, the appropriate primary antibody for a specific mucin (1:50; Santa Cruz Biotechnology) was added, and samples were incubated overnight at 4°C. The next day, fluorescently labeled (Alexa Fluor® 555) secondary antibody (1:800; Invitrogen) was added, and the experimental samples were incubated under humidified conditions for 1 h at 37°C. For staining nuclei, 4′,6-diamidino-2-phenylindole was added, and samples were incubated for 30 min before being washed three times with PBS for 5 min each. Finally, cells were mounted on glass slides and analyzed by fluorescence microscopy (IX73, Olympus, Tokyo, Japan) to assess the subcellular distribution of mucins.

Statistical analysis

The results of qPCR analysis of CagA and mucin mRNA expression in AGS cells were calculated and analyzed using the CFX Manager PCR analysis software from Bio-Rad. Following densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA), CagA protein levels as detected by western blotting were determined. Statistical analyses were performed using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA). The data were then analyzed using Student's t test or by the one-way analysis of variance test for selected data pairs using the Tukey post hoc test. p-values less than 0.05 were considered statistically significant.

RESULTS

Establishment of forced CagA expression in gastric AGS cancer cells

To establish a model of GC epithelium, AGS cells were transfected with a CagA-expressing plasmid, and the results of all performed analyses were compared to those in AGS cells transfected with an empty vector or not transfected. This strategy successfully established heterologous CagA expression at the RNA level. CagA expression levels in all experiments as detected by qPCR are shown in Fig. 1. From these experiments, we have observed that CagA mRNA expression in pCDNA3.1-CagA cells was significantly higher than that in cells transfected with empty pCDNA3.1 vector or in untreated AGS cells ($p<$ 0.01 and $p<0.001$, respectively). At the protein level, the induction of forced CagA expression was also successful. Fig. 2 shows a western blot of all experimental groups, revealing only CagA expression in the CagA-transfected cells. Based on these results, we concluded that our experimental set-up allowed us to assess the effect of forced CagA expression on mucin levels in AGS cells.

CagA expression changes the expression of functional mucins

Next, AGS cells transfected with CagA were probed for *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin expression using qPCR, and the results were compared to the appropriate controls. As evident in the results presented in Fig. 3, heterologous CagA expression increased *MUC5AC*, *MUC5B*, and *MUC2* expression at the RNA level. *MUC5AC* ($p<0.01$), *MUC5B* ($p<0.001$), and *MUC2* ($p<0.01$) expression levels in pCDNA3.1-CagA-transfected cells were significantly higher than those in the pCDNA3.1 and CK cells. The mRNA expression of *MUC6* in pCDNA3.1-CagA cells was higher than that in pCDNA3.1 cells ($p<0.001$). No significant differences in *MUC6* and E-cadherin expression were observed between pCDNA3.1-CagA and CK cells ($p>0.05$ for both).

As shown in Fig. 4, these results were further confirmed by immunofluorescence assays, which demonstrated substantial changes in the expression of mucins following transfection of
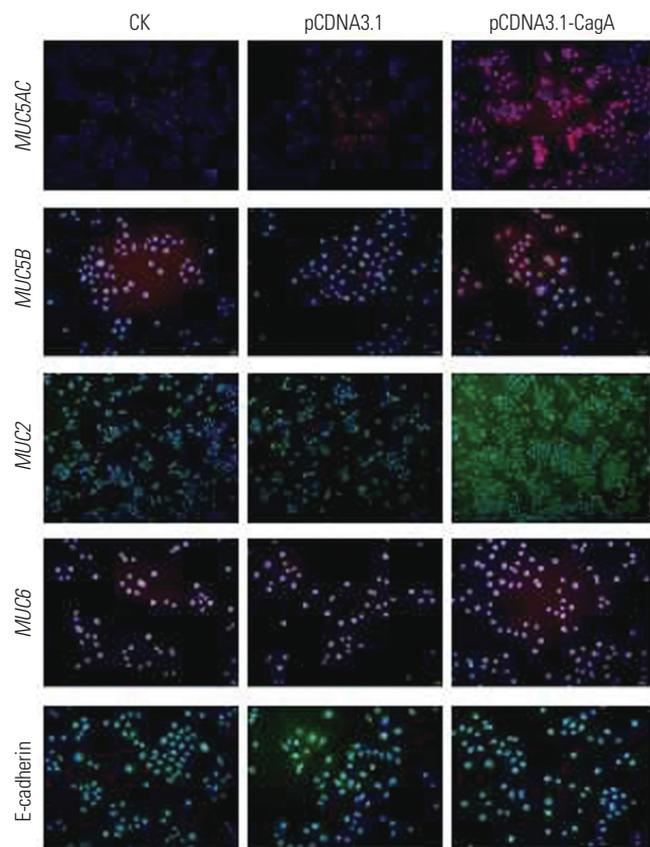

Fig. 4. Expression of mucins as detected by immunofluorescence assays (×200). The intensities of *MUC5AC*, *MUC5B*, and *MUC2* in pCDNA3.1-CagA cells were significantly higher than those in CK and pCDNA3.1 cells. The intensity of *MUC6* in pCDNA3.1-CagA cells was significantly higher than that in the pCDNA3.1 cells, but similar to that in CK cells. The intensity of E-cadherin protein in pCDNA3.1-CagA cells was similar to that in pCDNA3.1 and CK cells. CK, control cells; pCDNA3.1, empty-plasmid transfected cells; pCDNA3.1-CagA, cells transfected with CagA expression vector.
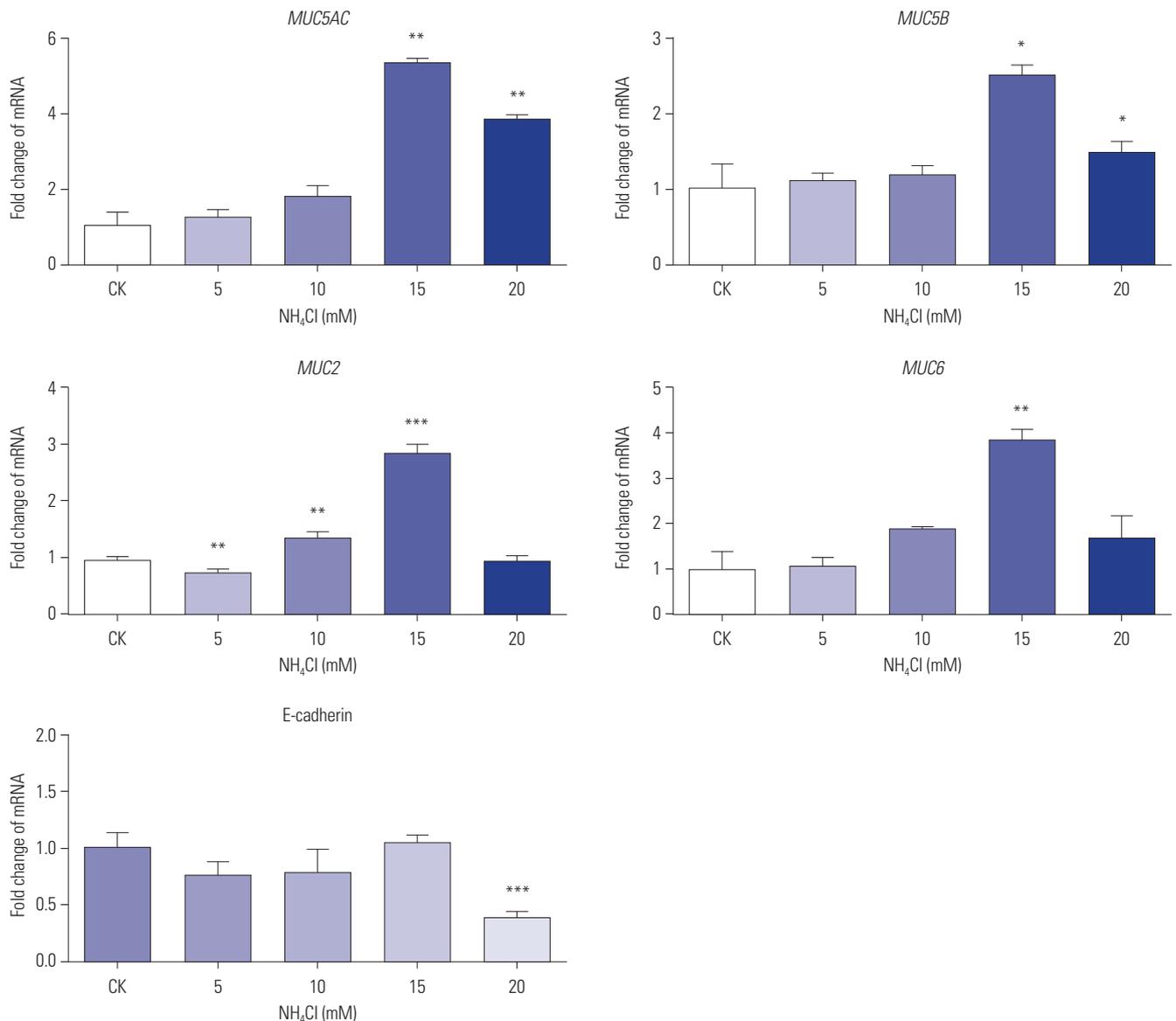
Fig. 5. qPCR analysis of mRNA expression of mucins in AGS cells treated with different NH_4^+ concentrations. *MUC5AC*, *MUC6*, and *MUC2* expression increased with increases in NH_4^+ concentration, reaching peak expression at 15 mM, which was significantly higher than that in untreated cells (*p<0.05, **p<0.01, ***p<0.001). The expression level of *MUC5B* mRNA in AGS cells at 15 mM of NH_4^+ was significantly higher than those at 0, 5, and 10 mM (*p<0.05). No significant change in the expression of E-cadherin mRNA was observed with NH_4^+ concentrations below 20 mM. CK, control cells.

AGS cells with the CagA expression-driving plasmid, as compared to the relevant controls. We, therefore, concluded that CagA is sufficient to provoke changes in the expression of mucins in GC cells.

NH_4^+ induced changes in functional mucins

AGS cells cultured with different concentrations of NH_4^+ were examined for *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin mRNA expression using qPCR. As evident from the results presented in Fig. 5, the mRNA expression of most mucins (*MUC5AC*, *MUC6*, *MUC2*) increased with increases in ammonium ion concentrations, reaching peak levels at 15 mM NH_4^+, followed by decreased mRNA expression with any fur-

ther increase in the ammonium ion concentration. The expression of *MUC5B* mRNA in AGS cells increased at an NH_4^+ concentration of 15 mM, and then decreased. *MUC5AC* (p< 0.01), *MUC6* (p<0.01), *MUC2* (p<0.001), and *MUC5B* (p<0.05) mRNA expression in ASG cells treated with the ammonium ion concentration of 15 mM were significantly higher than these levels in untreated cells. No significant change in the mRNA expression of E-cadherin was observed with an ammonium concentration below 20 mM.

As shown in Fig. 6, these results were further confirmed by immunofluorescence assays, which demonstrated substantial changes of mucin expression following treatment of AGS cells cultured in the presence of different NH_4^+ concentrations.
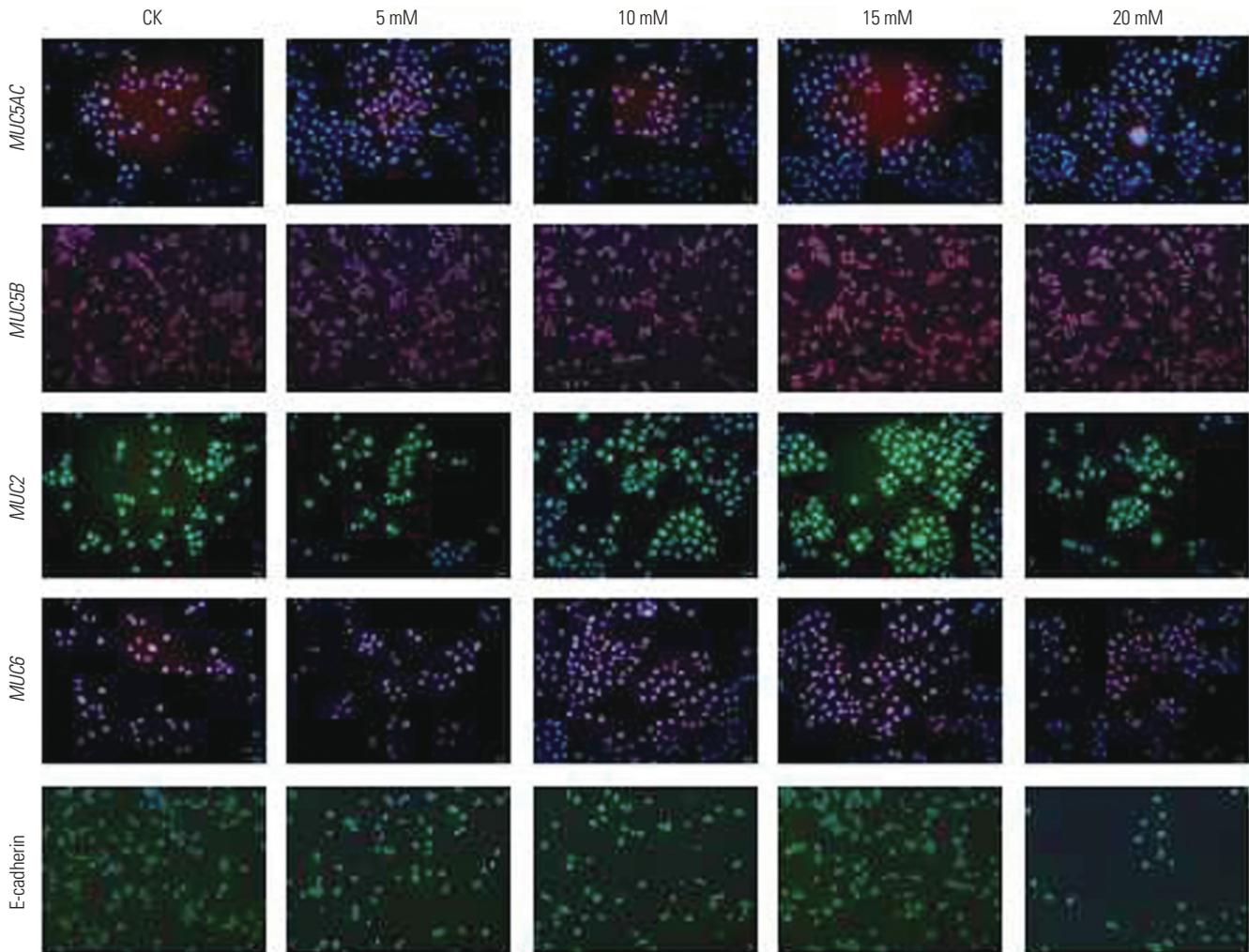
Fig. 6. Protein expression of different mucins in AGS cells exposed to 0, 5, 10, 15, and 20 mM NH₄Cl as detected by immunofluorescence assays (×200). CK, control cells.

Effect of NH_4^+ on the expression of mucins in CagA-transfected AGS cells

CagA-transfected AGS cells cultured with different concentrations of NH_4^+ were examined for *MUC5AC*, *MUC5B*, *MUC2*, *MUC6*, and E-cadherin mRNA expression using qPCR. The expression of *MUC5AC* ($p<0.01$), *MUC2* ($p<0.05$), and *MUC6* ($p<0.01$) mRNA in CagA-transfected AGS cells at NH_4^+ concentration of 15 mM were significantly higher than those at 0 mM. The expression of *MUC5B* mRNA increased with increases in NH_4^+ concentration, and was significantly higher than that in untreated cells (5 mM vs. 0 mM, $p<0.05$; 10 mM vs. 0 mM, $p<0.01$; 15 mM vs. 0 mM, $p<0.01$; 20 mM vs. 0 mM, $p<0.01$). No significant change in the expression of E-cadherin mRNA was observed under different NH_4^+ concentrations (Fig. 7).

As shown in Fig. 8, these results were further confirmed by immunofluorescence assays, which demonstrated substantial changes of mucin expression following the treatment of CagA-transfected AGS cells cultured in the presence of different NH_4^+ concentrations.

DISCUSSION

H. pylori has been implicated as one of the major causative agents of GC. Previous studies have indicated a potential link between *H. pylori*, mucins and GC[7,14]; however, the exact mechanism by which *H. pylori* contributes to changes in the expression patterns of mucins has not been clarified. It has been reported previously that *H. pylori* infection is correlated with a decrease in *MUC5AC* protein expression in GC tissue,[3] and that it may further accelerate the GC progression by decreasing *MUC5AC* expression.[6] *MUC5B* is expressed *de novo* in gastric mucosa only in gastric tumors,[15] and infection with *H. pylori* was correlated with increased *MUC5B* expression.[16] Moreover, it has also been reported that *MUC6* is aberrantly expressed in response to *H. pylori* infection,[17] and that its expression is lower in GC, compared with normal gastric mucus tissue.[18] In GC, expression of *MUC2* is increased[2,19,20] and may be activated by proinflammatory cytokines expressed after *H. pylori* infection, leading to its overexpression.[21] E-cadherin methylation is an early event in gastric carcinogenesis and is
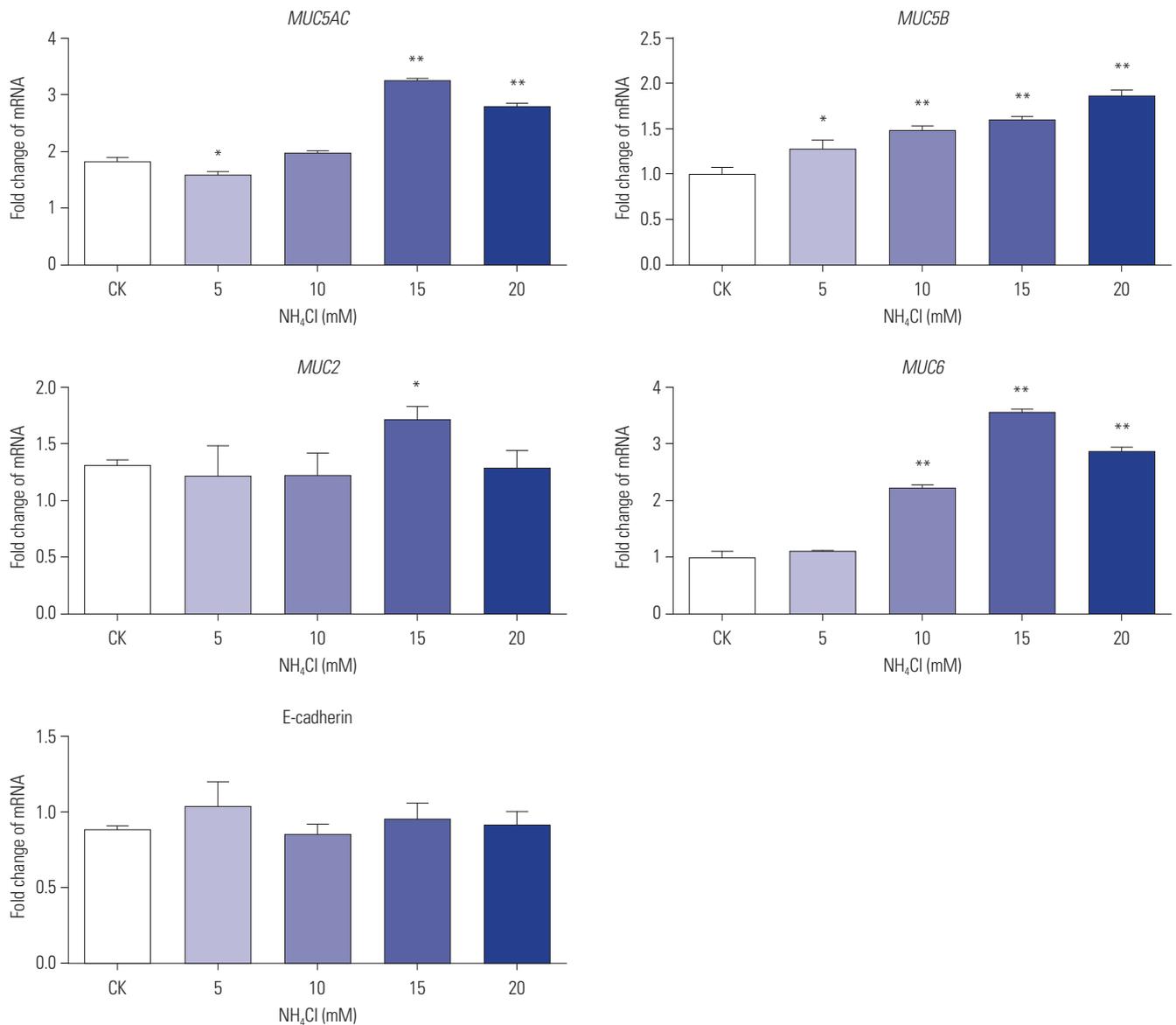
Fig. 7. qPCR analysis of mRNA expression of mucins in CagA-transfected AGS cells treated with different NH_4^+ concentrations. The expression levels of *MUC5AC*, *MUC2*, and *MUC6* mRNA in AGS cells transfected with CagA at 15 mM of NH_4^+ were significantly higher than those at 0 mM (*$p<0.05$, **$p<0.01$). The expression level of *MUC5B* mRNA increased with the increase in NH_4^+ concentration, and was significantly higher than that in untreated cells (*$p<0.05$, **$p<0.01$). No significant change in the expression of E-cadherin mRNA was observed under different NH_4^+ concentrations. CK, control cells.

initiated by *H. pylori* infection.[22]

Collectively, these data demonstrate that the occurrence and development of GC caused by *H. pylori* is often determined by its virulence factors.[23] In addition, it has been shown that *H. pylori* infection causes alterations in the expression patterns of mucins in gastric mucosa, and it is suspected that these alterations are involved in *H. pylori*-induced gastric carcinogenesis.[24,25] However, little is known about whether *H. pylori* causes changes in the mucin expression patterns through its virulence factors, CagA and urease. Previous reports[13] showed that alterations in mucin expression likely involve bacterial CagA and urease, as evident from experiments involving AGS GC cells infected with wild-type *H. pylori* strains and the UreB-isogenic mutant. Still, the exact interaction be-

tween *H. pylori* infection and mucin expression remained only partly resolved. We, therefore, decided to examine which of the two virulence factors, CagA or urease, influence the gene expression of different mucins in AGS GC cells.

In our study, *MUC5AC* expression was significantly upregulated upon the introduction of cellular CagA and/or in the ammonium chloride environment, which is consistent with individual literature reports,[13] but contrary to other reports.[3,6,26,27] However, these two results may not be contradictory. Presumably, injection of CagA into the gastric epithelial cytosol and the release of urease to produce NH_4^+ present two obvious countermeasures of *H. pylori* against gastric epithelial defensive downregulation of the *MUC5AC* glycoprotein in response to bacterial infection. Evidence that *MUC5AC* not only colo-
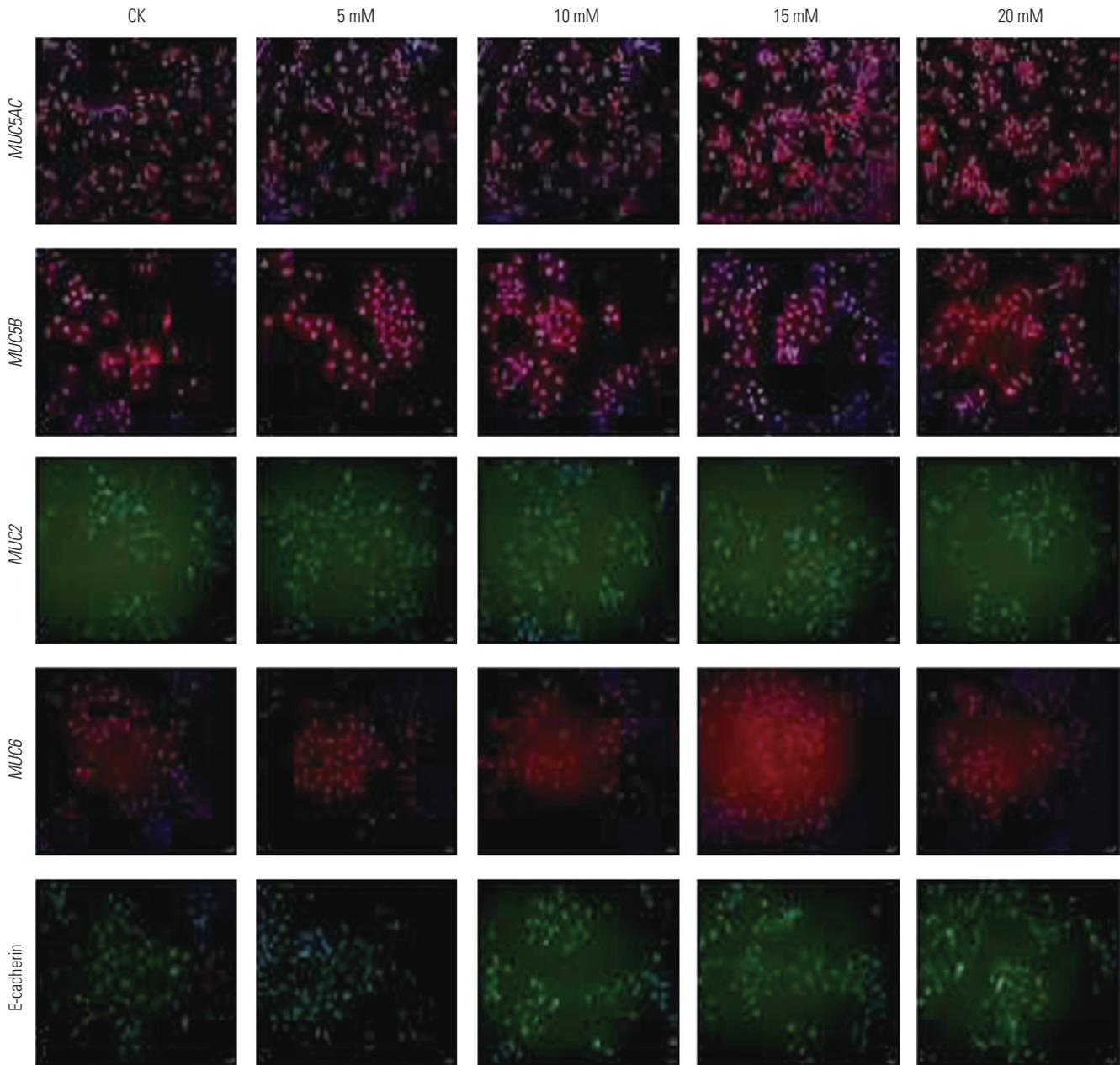
Fig. 8. Protein expression of different mucins in CagA-transfected AGS cells exposed to 0, 5, 10, 15, and 20 mM NH₄Cl as detected by immunofluorescence assays (×200). CK, control cells.

calizes with *H. pylori* but also functions as an important *H. pylori* receptor tethering it to the gastric mucosa[5] is overwhelming and not disputed. Thus, it is reasonable to speculate that *H. pylori* creates a favorable environment by stimulating gastric epithelial *MUC5AC* production, which facilitates bacterial adherence. In this sense, injection of CagA into gastric epithelial cells and the release of urease to produce NH_4^+[28] may stimulate these cells to upregulate *MUC5AC* early in the infectious process, causing a transient increase in *MUC5AC* that facilitates further colonization. A subsequent interaction between the bacterium and the gastric cell provokes morphological changes in the cell and host-cell epithelial barrier injury that

is associated with *H. pylori* infection.[29]

Previous studies indicated that human *H. pylori* infection increased *MUC5B* expression in the gastric mucosa,[16,30] which is consistent with our results. However, these studies did not explain how *H. pylori* induces the upregulation of *MUC5B* expression. Our results demonstrated that the expression of *MUC5B* was significantly upregulated in CagA-transfected AGS cells, as well as in AGS cells (non-transfected or CagA-transfected) exposed to ammonium salts. *MUC5B* is expressed only during a brief period of fetal life in the human stomach, and its expression is later detected only in GC.[15] Therefore, *MUC5B* expression may be one of the events in the initial pro-

cess of gastric carcinogenesis induced by *H. pylori*. Furthermore, in this process, CagA and ammonium ions are involved in the upregulation of *MUC5B* expression. In addition, *MUC5B* expression has previously been shown to be associated with the coexpression of *MUC5AC*,[15] which may also allow for increased binding of *H. pylori*.[30] Similarly, it can be speculated that CagA and ammonium ions are also involved in the upregulation of *MUC2* expression, since its expression is increased in GC, compared to normal gastric mucosa,[2,19,20] and altered *MUC2* expression has been reported in *H. pylori*-infected intestinal metaplasia.[31] Previous studies have shown that *MUC2* up-regulation is associated with the *H. pylori* virulence factors CagA and urease.[13] In addition *MUC2* might be activated by proinflammatory cytokines expressed after *H. pylori* infection, leading to its overexpression.[14] However, CagA is just one of the proinflammatory cytokines of *H. pylori*,[32] and ammonium ions are the final metabolite of urease function. Therefore, our results support the previous conclusions and support that CagA and ammonium ions might be factors inducing activation of *MUC2* expression in AGS cells.

Interestingly, we found no effect of CagA on *MUC6* expression, which is in contradiction with the upregulation or downregulation of *MUC6* expression induced by *H. pylori* that has been reported previously.[7,8] However, when AGS cells (nontransfected or CagA-transfected) were exposed to ammonium salt in our study, the expression of *MUC6* was significantly upregulated. Our results, therefore, support the conclusion that *H. pylori* may induce the upregulation of *MUC6* expression through its urease metabolites, ammonium irons, rather than CagA. Furthermore, increased *MUC6* expression may help inhibit colonization, using *MUC6* antibiotic properties.[7]

Downregulation of E-cadherin induced by *H. pylori* is a key step in the pathogenesis of GC. Indeed, few studies have shown that the downregulation of E-cadherin induced by *H. pylori* is regulated by its virulence factor, CagA.[33,34] However, our results do not corroborate these previous reports. In fact, in our study, AGS cells transfected with CagA or AGS cells (non-transfected or CagA-transfected) exposed to ammonium salts did not show any changes in E-cadherin expression. It is well known that *H. pylori* plays an important role in the carcinogenesis of intestinal type of GC. E-cadherin is closely related to the hereditary diffuse GC, which is caused by a genetic mutation rather than by *H. pylori* infection. In intestinal type GC cases, no significant difference in E-cadherin expression between *H. pylori* negative and positive cases was observed.[35] Therefore, we speculate that CagA might have no effect on E-cadherin expression in AGS cells predominately exhibiting the intestinal phenotype.[25]

A limitation of this study is that we did not examine the influence of *H. pylori* VacA virulence factors or whether CagA and ammonium ions have an effect on expression of mucins of other GC cell lines. These issues will need be addressed in future studies.

In conclusion, *H. pylori* may affect the expression of mucins via CagA and/or urease ammonium ion. CagA was found to upregulate *MUC5AC*, *MUC2*, and *MUC5B* expression, but to have no effect on *MUC6* and E-cadherin expression in AGS cells. Ammonium ions increased *MUC5AC*, *MUC2*, *MUC5B*, and *MUC6* expression and had no effect on E-cadherin expression in AGS cells.

ORCID

Xiaoyu Zhang   https://orcid.org/0000-0001-9664-2371
Ding Shi   https://orcid.org/0000-0002-3310-7854

REFERENCES

1. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 2004;4:45-60.
2. Babu SD, Jayanthi V, Devaraj N, Reis CA, Devaraj H. Expression profile of mucins (MUC2, MUC5AC and MUC6) in Helicobacter pylori infected pre-neoplastic and neoplastic human gastric epithelium. Mol Cancer 2006;5:10.
3. Shi D, Qiu XM, Bao YF. Effects of Helicobacter pylori infection on MUC5AC protein expression in gastric cancer. Future Oncol 2013;9:115-20.
4. Gomceli I, Demiriz B, Tez M. Gastric carcinogenesis. World J Gastroenterol 2012;18:5164-70.
5. Van de Bovenkamp JH, Mahdavi J, Korteland-Van Male AM, Büller HA, Einerhand AW, Borén T, et al. The MUC5AC glycoprotein is the primary receptor for Helicobacter pylori in the human stomach. Helicobacter 2003;8:521-32.
6. Shi D, Qiu XM, Yan XJ. The changes in MUC5AC expression in gastric cancer before and after Helicobacter pylori eradication. Clin Res Hepatol Gastroenterol 2014;38:235-40.
7. Niv Y. Helicobacter pylori and gastric mucin expression: a systematic review and meta-analysis. World J Gastroenterol 2015;21: 9430-6.
8. Kang HM, Kim N, Park YS, Hwang JH, Kim JW, Jeong SH, et al. Effects of Helicobacter pylori Infection on gastric mucin expression. J Clin Gastroenterol 2008;42:29-35.
9. McLean MH, El-Omar EM. Genetics of gastric cancer. Nat Rev Gastroenterol Hepatol 2014;11:664-74.
10. Zhang BG, Hu L, Zang MD, Wang HX, Zhao W, Li JF, et al. Helicobacter pylori CagA induces tumor suppressor gene hypermethylation by upregulating DNMT1 via AKT-NFκB pathway in gastric cancer development. Oncotarget 2016;7:9788-800.
11. Tohidpour A. CagA-mediated pathogenesis of Helicobacter pylori. Microb Pathog 2016;93:44-55.
12. Figura N, Marano L, Moretti E, Ponzetto A. Helicobacter pylori infection and gastric carcinoma: not all the strains and patients are alike. World J Gastrointest Oncol 2016;8:40-54.
13. Perrais M, Rousseaux C, Ducourouble MP, Courcol R, Vincent P, Jonckheere N, et al. Helicobacter pylori urease and flagellin alter mucin gene expression in human gastric cancer cells. Gastric Cancer 2014;17:235-46.
14. Wen R, Gao F, Zhou CJ, Jia YB. Polymorphisms in mucin genes in the development of gastric cancer. World J Gastrointest Oncol 2015; 7:328-37.
15. Pinto-de-Sousa J, Reis CA, David L, Pimenta A, Cardoso-de-Oliveira M. MUC5B expression in gastric carcinoma: relationship with clinico-pathological parameters and with expression of mucins MUC1, MUC2, MUC5AC and MUC6. Virchows Arch 2004;444: 224-30.

16. Van De Bovenkamp JH, Korteland-Van Male AM, Büller HA, Einerhand AW, Dekker J. Infection with Helicobacter pylori affects all major secretory cell populations in the human antrum. Dig Dis Sci 2005;50:1078-86.

17. Byrd JC, Yan P, Sternberg L, Yunker CK, Scheiman JM, Bresalier RS. Aberrant expression of gland-type gastric mucin in the surface epithelium of Helicobacter pylori-infected patients. Gastroenterology 1997;113:455-64.

18. Zheng H, Takahashi H, Nakajima T, Murai Y, Cui Z, Nomoto K, et al. MUC6 down-regulation correlates with gastric carcinoma progression and a poor prognosis: an immunohistochemical study with tissue microarrays. J Cancer Res Clin Oncol 2006;132:817-23.

19. Reis CA, David L, Carvalho F, Mandel U, de Bolós C, Mirgorodskaya E, et al. Immunohistochemical study of the expression of MUC6 mucin and co-expression of other secreted mucins (MUC5AC and MUC2) in human gastric carcinomas. J Histochem Cytochem 2000;48:377-88.

20. Ho SB, Shekels LL, Toribara NW, Kim YS, Lyftogt C, Cherwitz DL, et al. Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. Cancer Res 1995;55:2681-90.

21. Mejóas-Luque R, Lindén SK, Garrido M, Tye H, Najdovska M, Jenkins BJ, et al. Inflammation modulates the expression of the intestinal mucins MUC2 and MUC4 in gastric tumors. Oncogene 2010; 29:1753-62.

22. Chan AO, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, et al. Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer. Gut 2003;52:502-6.

23. Meng W, Bai B, Sheng L, Li Y, Yue P, Li X, et al. Role of Helicobacter pylori in gastric cancer: advances and controversies. Discov Med 2015;20:285-93.

24. Chung WC, Jung SH, Joo KR, Kim MJ, Youn GJ, Kim Y, et al. An inverse relationship between the expression of the gastric tumor suppressor RUNX3 and infection with Helicobacter pylori in gastric epithelial dysplasia. Gut Liver 2013;7:688-95.

25. Matsuda K, Yamauchi K, Matsumoto T, Sano K, Yamaoka Y, Ota H. Quantitative analysis of the effect of Helicobacter pylori on the expressions of SOX2, CDX2, MUC2, MUC5AC, MUC6, TFF1, TFF2, and TFF3 mRNAs in human gastric carcinoma cells. Scand J Gastroenterol 2008;43:25-33.

26. Byrd JC, Yunker CK, Xu QS, Sternberg LR, Bresalier RS. Inhibition of gastric mucin synthesis by Helicobacter pylori. Gastroenterology 2000;118:1072-9.

27. Kocer B, Ulas M, Ustundag Y, Erdogan S, Karabeyoglu M, Yldrm O, et al. A confirmatory report for the close interaction of Helicobacter pylori with gastric epithelial MUC5AC expression. J Clin Gastroenterol 2004;38:496-502.

28. Hayashi T, Senda M, Morohashi H, Higashi H, Horio M, Kashiba Y, et al. Tertiary structure-function analysis reveals the pathogenic signaling potentiation mechanism of Helicobacter pylori oncogenic effector CagA. Cell Host Microbe 2012;12:20-33.

29. Wu J, Xu S, Zhu Y. Helicobacter pylori CagA: a critical destroyer of the gastric epithelial barrier. Dig Dis Sci 2013;58:1830-7.

30. Schmitz JM, Durham CG, Ho SB, Lorenz RG. Gastric mucus alterations associated with murine Helicobacter infection. J Histochem Cytochem 2009;57:457-67.

31. Reis CA, David L, Correa P, Carneiro F, de Bolós C, Garcia E, et al. Intestinal metaplasia of human stomach displays distinct patterns of mucin (MUC1, MUC2, MUC5AC, and MUC6) expression. Cancer Res 1999;59:1003-7.

32. Li N, Xu X, Xiao B, Zhu ED, Li BS, Liu Z, et al. H. pylori related proinflammatory cytokines contribute to the induction of miR-146a in human gastric epithelial cells. Mol Biol Rep 2012;39:4655-61.

33. Yu H, Zeng J, Liang X, Wang W, Zhou Y, Sun Y, et al. Helicobacter pylori promotes epithelial-mesenchymal transition in gastric cancer by downregulating programmed cell death protein 4 (PDCD4). PLoS One 2014;9:e105306.

34. Wagih HM, El-Ageery SM, Alghaithy AA. A study of RUNX3, E-cadherin and β-catenin in CagA-positive Helicobacter pylori associated chronic gastritis in Saudi patients. Eur Rev Med Pharmacol Sci 2015;19:1416-29.

35. Yu XW, Xu Q, Xu Y, Gong YH, Yuan Y. Expression of the E-cadherin/β-catenin/tcf-4 pathway in gastric diseases with relation to Helicobacter pylori infection: clinical and pathological implications. Asian Pac J Cancer Prev 2014;15:215-20.