Published in final edited form as:
Scand J Gastroenterol. 2008 January ; 43(1): 25–33.

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

Kazuyuki Matsuda[1], **Kazuyoshi Yamauchi**[1], **Takehisa Matsumoto**[1], **Kenji Sano**[1], **Yoshio Yamaoka**[2], and **Hiroyoshi Ota**[3]

[1]Department of Laboratory Medicine, Shinshu University Hospital, Asahi, Matsumoto, Japan

[2]Department of Medicine/Gastroenterology, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas, USA

[3]Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University School of Medicine, Asahi, Matsumoto, Japan

Abstract

Objective—To investigate the phenotypic characters of carcinoma cells and the response of gastric epithelial cells to *Helicobacter pylori* (*H. pylori*) infection using the gastric carcinoma cell lines.

Material and methods—Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to assess the effect of *H. pylori* infection on mRNA levels of transcription factors (*SOX2* and *CDX2*), mucin core proteins (*MUC2*, *MUC5AC*, and *MUC6*), and trefoil factor family peptides (TFF) (*TFF1*, *TFF2*, and *TFF3*) in gastric carcinoma cells (AGS, MKN45, and KATO III cells). *H. pylori* ATCC 43504 and its isogenic *cag* pathogenicity island (PAI) deleted mutant were used.

Results—These cell lines expressed mixed gastric and intestinal phenotypes. The intestinal phenotype predominated in AGS cells and gastric phenotypes in MKN45 and KATO III cells. In all three cell lines, *H. pylori* infection inhibited *SOX2* mRNA expression, but induced the three *TFFs* mRNAs. In AGS cells, *H. pylori* induced *cag* PAI-dependent mRNA expression of *CDX2*, *MUC2*, *MUC5AC*, and *MUC6*. mRNA expressions of *CDX2*, *MUC5AC*, and *MUC6* were inhibited in KATO III cells, whereas *MUC2* mRNA expression was unchanged. In MKN45 cells, *H. pylori* induced the three *MUCs* mRNAs but inhibited *CDX2* mRNA expression.

Conclusions—This study provides a useful platform for selecting appropriate cell lines to model *H. pylori*-related changes in the gastric epithelium that mirror the changes seen *in vivo*. The outcome of *H. pylori* infection may reflect changes in the mucus gel layer caused by altered expression of mucins and TFF peptides.

Keywords

CDX2; Helicobacter pylori; MUC; PDX1; real-time PCR; TFF

Correspondence: Hiroyoshi Ota, MD, PhD, Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Tel: +81 263 37 2389. Fax: +81 263 37 2389. hohta@shinshu-u.ac.jp.

Introduction

Helicobacter pylori (*H. pylori*) infection is identified as the major etiologic factor in gastritis, gastroduodenal ulcer, gastric atrophy, intestinal metaplasia, and gastric cancer [1,2]. A pathogenicity island (PAI) has been identified within the *H. pylori* genome which contains a cluster of genes, the *cag* (cytotoxin-associated gene) PAI. The *cag* PAI, a 40 kb stretch of DNA, encodes a type IV secretory apparatus which injects the CagA protein and possibly other unknown proteins into the eukaryotic cells and induces intracellular responses in the *H. pylori* infected cells [3–5].

H. pylori infection is associated with alteration in the secretion of mucins and the trefoil factor family (TFF) peptides from the gastric epithelial cells [6] and these changes may contribute to *H. pylori*-associated gastric mucosal damage. The mucins secreted by gastric mucous cells form a mucous gel layer covering the gastric mucosa. This gel layer is considered to be the first line of gastric mucosal defense against luminal noxious agents [7–9] and damage to the mucous gel is thought to precede gastric mucosal injury. The gastric surface mucous cells and gland mucous cells express secretory mucin, MUC5AC and MUC6, respectively [10]. In contrast, the secretory mucin MUC2 is aberrantly expressed in gastric carcinoma, its closely related lesion (intestinal metaplasia) [10], and in the intestinal goblet cells [11]. In addition to secreting mucins, the mucous cells secrete TFF peptides, TFF1, TFF2, and TFF3, which are mucin-associated and along with mucins, provide a structural mucosal barrier function and also participate in the repair and healing of the damaged mucosa [12–14]. TFF1 (formerly designated as pS2) is produced by the gastric surface mucous cells [15] and TFF2 (human spasmolytic peptide: hSP) is produced by the gastric gland mucous cells [16].TFF3 (intestinal trefoil factor: ITF) is mainly produced by the intestinal goblet cells [17] and has also been demonstrated in surface mucous cells in the gastric pyloric mucosa [18].

Cell differentiation is controlled by the transcription factors encoded by the homeobox genes [19]. *SOX2* is one of the candidate factors for controlling gastric differentiation. *SOX2* mRNA expression in the human gastrointestinal tract is confined to the gastric mucosa [20] and strong immunoreactivity of SOX2 has been reported in the nuclei of the gastric surface mucous cells [20,21]. In addition, gastric carcinoma with a gastric phenotype expresses *SOX2* mRNA and SOX2 protein [21,22]. In contrast, the caudal homeobox 2 (*CDX2*) gene, which regulates the development of the intestine, is expressed in the intestinal epithelial cells, intestinal metaplastic cells, and gastric carcinoma cells [23,24].

Gastric carcinoma is histologically classified into two types, differentiated and undifferentiated type or intestinal and diffuse type, based on the prevalent gland formation [25,26]. Recently, human gastric carcinomas have been phenotypically classified into gastric, gastric and intestinal mixed, or intestinal type, depending on the expression of phenotypic markers of gastrointestinal epithelial cells. This phenotypic classification is useful for investigating carcinogenesis and the clinicopathological characteristics of gastric carcinoma [27–29].

A variety of gastric carcinoma cell lines have been used as *in vitro* systems to model gastric epithelial response to *H. pylori*. However, there is a lack of information on the quantitative analysis of the expression of mucins and TFF peptides in human gastric cancer cell lines. The present study was undertaken to characterize the phenotype expression in three gastric carcinoma cell lines: AGS, MKN45, and KATO III cells, which are widely used to model the gastric epithelial responses to *H. pylori* infection. We also examined the response of gastric epithelial cells to *H. pylori* infection by assessing the mRNA levels of the transcription factors (*SOX2* and *CDX2*), mucin core proteins (*MUC5AC, MUC6, and*

MUC2), and TFF peptides (*TFF1, TFF2,* and *TFF3*) in the three gastric carcinoma cell lines using wild-type *H. pylori* ATCC 43504 infection and its isogenic *cag* PAI totally deleted mutant.

Material and methods

Cell culture

The cell lines used in this experiment were derived from human gastric cancers and included AGS (American Type Culture Collection (ATCC), Manassas, Va., USA), MKN45 (Riken Cell Bank, Tsukuba, Japan) and KATO III (ATCC). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, N.Y., USA) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C under 5% CO_2. The cells were starved for 18 h with DMEM without any supplements before co-culture with *H. pylori*.

Bacterial strains and inoculation

The *H. pylori* strain ATCC 43504 (ATCC) and its isogenic *cag* PAI totally deleted mutant were cultured for 3 days on horse blood agar plates in a microaerophilic milieu (15% CO2) at 37°C. The strains were grown in 2 ml brucella broth (Becton Dickinson, Cockeysville, Md., USA) supplemented with 10% horse serum at 37°C under microaerophilic conditions with shaking at 120 rpm. After 40 h, the medium was removed, and the *H. pylori* cells were re-suspended in DMEM without any supplements and added to the AGS cells at a multiplicity of infection (MOI) described below.

To investigate the effect of the concentration of *H. pylori* on mRNA expression, the bacteria cells that had been re-suspended in DMEM were added to the 1×10^6 AGS cells at different MOIs (0, 50, 100, and 300), and mRNA expression was analyzed by real-time polymerase chain reaction (PCR) 12 h after *H. pylori* infection. To examine the cell viability under each condition, the number of viable cells was determined by the trypan-blue exclusion test.

The isogenic *cag* PAI totally deleted mutant was constructed according to the method previously reported [30]. Briefly, the regions upstream (hp0518-hp0519; 545,254-547,164 bp: hp number and location from *H. pylori* strain 26695: Genbank accession number: AE000511) and downstream (hp0549-hp0550; 584,570–586,563 bp) of the *cag* PAI were amplified to delete the entire *cag* PAI from the *H. pylori* chromosome. These fragments were separated by a chloramphenicol resistance cassette and cloned into the T7Blue vector (Novagen, Madison, Wisc., USA). All of the plasmids (1 mg) were used for inactivation of the chromosomal genes by natural transformation. Inactivation of the genes was confirmed by PCR amplification followed by Southern blot as well as by Western blot for CagA (Austral Biologicals, San Ramon, Calif., USA).

Real-time PCR analysis

The quantification of each of the transcription factors (*SOX2* and *CDX2*), mucin core proteins (*MUC2, MUC5AC,* and *MUC6*), and TFF peptides (*TFF1, TFF2,* and *TFF3*) mRNA levels in the AGS cells, MKN45 cells and KATO III cell was performed in the unstimulated state and 12 h after *H. pylori* infection. Total RNA was isolated using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, Calif., USA). The first-strand cDNA was synthesized from approximately 1 μg RNA by using a random primer and the Moloney murine leukemia virus reverse transcriptase. Additionally, 5 μl of the reverse transcription (RT) reaction mixture was used for quantitative PCR.

The primers and probe mixture that were used to amplify each of the targeted genes and the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*) were purchased from Applied Biosystems (Foster City, Calif., USA).

The reaction solution for real-time PCR was prepared by mixing 5 μl of synthesized cDNA solution with 25 μl TaqMan Universal PCR Master Mix (Applied Biosystems) and 2.5 μl of the reaction mixture. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, and at 60°C for 1 min. Each assay was done in triplicate. The expression of *GAPDH* was used to normalize that of the target genes. Before using the comparative threshold cycle (Ct) method for the relative quantification, a validation experiment was performed according to the manufacturer's instructions to verify that the efficiencies of the target and *GAPDH* genes were approximately equal. The change in the Ct (ΔCt) of the target genes was calculated as ΔCt = (Ct of target genes)–(Ct of*GAPDH*).We calculated the ratio of the target genes to *GAPDH* as $2^{-\Delta Ct}$, expressed as $2^{-\Delta Ct} \times 10^5$; this ratio was used to evaluate the expression level within each target gene in each gastric carcinoma cell in the unstimulated state. The abundance of the target genes relative to that of *GAPDH* was calculated as ΔΔCt = (ΔCt of target genes)–(ΔCt of *GAPDH*). The ratio was calculated as $2^{-\Delta\Delta Ct}$ to evaluate the alteration of the target genes 12 h after co-culture with the gastric cancer cells and *H. pylori*.

Statistics

The data were presented as mean values±standard deviation. We used the one-way analysis of variance, followed by *post hoc* contrasts using the Bonferroni limitation for the statistical analysis. *P*-values of less than 0.05 were considered significant.

Results

Characterization of human gastric cancer cell lines by RT-PCR

The mRNA levels of *SOX2*, *CDX2*, *MUCs*, and *TFFs* in the AGS, MKN45, and KATO III cells at the stage of early confluency are shown in Figure 1.

CDX2 mRNA levels were higher than those of the *SOX2* mRNA in all three cell lines (Figure 1A). *MUC2* mRNA levels were the highest in the AGS cells followed by *MUC5AC* mRNA and *MUC6* mRNA levels (Figure 1B). In both the MKN45 and KATO III cells, the *MUC5AC* mRNA levels (expression level; 1741±120 in MKN45 cells, 439±7.0 in KATO III cells) were higher than those of the *MUC6* (0.01±0.001 in MKN45 cells, 0.26±0.02 in KATO III cells) or *MUC2 mRNA* (3.07±0.05 in MKN45 cells, 1.16±0.24 in KATO III cells).

TFF3 mRNA levels were highest among three *TFF* mRNAs in the AGS cells and the MKN45 cells, followed by *TFF1* mRNA and the *TFF2* mRNA levels. In the KATO III cells, the *TFF2* mRNA levels were the highest, followed by the *TFF3* mRNA and the *TFF1* mRNA levels (Figure 1C). Both the MKN45 and KATO III cells showed much higher amounts of *MUC* mRNAs and *TFF* mRNAs than the AGS cells (Figure 1B, C).

Effect of H. pylori infection on mRNA expression of transcription factors, mucin core proteins, and TFF peptides

Wild-type *H. pylori* significantly inhibited *SOX2* mRNA expression in a concentration-dependent manner in all three cell lines (Figure 2A). In contrast, wild-type *H. pylori* significantly induced mRNA expression of the three *TFFs* in a concentration-dependent manner in all three cell lines (Figure 2C). Importantly, the expression patterns of *CDX2*,

MUC5AC, *MUC6*, and *MUC2* mRNA by wild- type *H. pylori* infection were gastric cell line specific. Wild-type *H. pylori* significantly induced the expression of these four mRNAs in AGS cells (Figure 2A–C), whereas *H. pylori* significantly inhibited the expression of these three mRNAs in KATOIII cells (except for *MUC2* mRNA, which was unchanged) (Figure 2A–C). In MKN45 cells, wild-type *H. pylori* significantly induced the expression of the three *MUC* mRNAs, but significantly inhibited that of *CDX2* mRNA (Figure 2A–C).

Under each condition, there was no significant alteration in the number of viable carcinoma cells determined by the trypan-blue exclusion test (data not shown).

Effect of cag-PAI on mRNA expression of transcription factors, mucin core proteins, and TFF peptides in AGS cells

We next examined the effects of the *cag* PAI on the expression of the transcription factors of interest, mucin core proteins, and TFF peptides in AGS cells. AGS cells were chosen because expression of *MUC5AC* mRNA and *MUC2* mRNA paralleled that of the corresponding transcription factors (*SOX2* and *CDX2*) suggesting that AGS cells should be ideal for investigating the effects of the *cag* PAI on expression of these mRNAs. With the exception of the *SOX2* mRNA and *MUC5AC* mRNA levels, infection with the *cag*-PAI mutant did not alter the mRNA levels of the transcription factors, mucin core proteins, or TFF peptides (Figure 2A–C). The *cag* PAI mutant also inhibited the expression of *SOX2* mRNA; however, the *SOX2* mRNA levels at a MOI of 100 and 300 were significantly higher in cells infected with the *cag* PAI mutant than in those infected with wild-type *H. pylori* suggesting that *SOX2* mRNA expression was partially *cag* PAI dependent ($p < 0.001$) (Figure 2A). The *MUC5AC* mRNA levels were significantly higher in cells infected with wild-type *H. pylori* than in those with the *cag* PAI mutant at a MOI of 50 ($p < 0.05$) (Figure 2B), whereas there was no difference in the levels between the strains at a MOI of 100 and 300, which suggests that *MUC5AC* mRNA expression was also partially *cag* PAI dependent.

Discussion

In this report, we showed that gastric cancer cell lines expressed mixed gastric and intestinal phenotypes. The intestinal phenotype predominated in AGS cells, whereas gastric phenotype predominated in MKN45 and KATO III cells. *H. pylori* infection altered the expression of the mRNA of both gastric and intestinal transcription factors as well as *MUCs* and *TFFs*.

In AGS cells, expression of the intestinal transcription factor *CDX2* was predominant compared with that of the gastric transcription factor *SOX2*. Accordingly, goblet cell-specific *MUC2* mRNA and *TFF3* mRNA levels were also predominant in AGS cells. These data clearly show that coordinated expression of transcription factor mRNAs, *MUCs* mRNAs, and *TFFs* mRNAs was observed in AGS cells under uninfected conditions, and are in agreement with previous studies that SOX2 and CDX2 are putative regulators for MUC5AC [21] and MUC2 [31], respectively. In addition, these co-expression patterns reflect the regulatory function of the transcription factors and correspond to those observed in *in vivo* expression [22].

In the MKN45 and KATO III cells, gastric surface mucous cell-specific *MUC5AC* mRNA levels were much higher than those of goblet cell-specific *MUC2* mRNA, whereas goblet cell-specific *TFF3* mRNA levels were higher than those of gastric surface mucous cell-specific *TFF1*. This discrepancy can be explained by the fact that human gastric carcinoma tissues express a gastric pyloric mucosal phenotype rather than a gastric fundic mucosal phenotype [32] and that *TFF3* mRNA and TFF3 protein are normally expressed in surface mucous cells in human normal gastric pyloric mucosa [18]. Thus, predominant expression of

the *MUC5AC* and *TFF3* mRNA in the MKN45 and KATO III cells shows that both cell lines express a predominantly gastric phenotype.

The mixed gastric and intestinal phenotype expression in these gastric carcinoma cell lines expands the results previously reported regarding mucins and mucin gene expression in gastric cancer cell lines [33,34]. For example, Carvalho et al. [33] used RT-PCR to show that MKN45 cells expressed *MUC5AC* mRNA but not *MUC2* or *MUC6* mRNA and that KATO III cells expressed both *MUC5AC* and *MUC2* mRNAs but not *MUC6* mRNA. Cornberg et al. [34] used RT-PCR and reported that both AGS and KATO III cells expressed both *MUC5AC* and *MUC2* mRNAs but not *MUC6* mRNAs. The lack of *MUC2* and *MUC6* mRNAs expression described in these reports could be explained by the relatively small amounts of *MUC2* and *MUC6* mRNAs expressed in these cells as confirmed in this study. A similar mixed gastric and intestinal phenotype expression has also been reported in human gastric adenocarcinoma tissues [22].

Interestingly, wild-type *H. pylori* strains inhibited *SOX2* mRNA expression in a dose-dependent manner in all three cell lines examined. In contrast, *H. pylori* at a MOI of 50 induced *MUC5AC* mRNA expression, whereas greater amounts of *H. pylori* inhibited the expression in AGS cells and MKN45 cells. Previously, Li et al. reported that the SOX2 protein was observed in the nuclei of the gastric surface mucous cells of normal human gastric mucosa and that *MUC5AC* mRNAwas induced in COS-7 cells transfected with a *SOX2* construct [21]. Taken together, the putative transcriptional regulator function of the SOX2 protein should influence the expression of the *MUC5AC* mRNA. Furthermore, there is a possibility that a regulatory factor other than SOX2 is also involved in the regulation of *MUC5AC* mRNA expression in AGS and MKN45 cells.

In contrast to AGS cells and MKN45 cells, *MUC5AC* mRNA levels were consistently inhibited by *H. pylori* infection irrespective of the MOI in KATO III cells. Similarly, *MUC6* mRNA levels were also induced by *H. pylori* infection of AGS cells and MKN45 cells, whereas the levels were inhibited in KATO III cells. The reason for the opposite function of *H. pylori* on the expression of *MUC5AC* mRNA and *MUC6* mRNA remains unknown. However, the data suggest that *H. pylori* infection activates different signaling pathways related to the expression of the *MUC5AC* mRNA and *MUC6* mRNA in the different cell lines. It is interesting that these differences appear independent of the original phenotype, since both MKN45 cells and KATO III cells were of gastric predominance. However, MKN45 cells and KATO III cells were derived from different types of carcinoma: MKN45 cells from a poorly differentiated adenocarcinoma of the medullary type [35] and KATO III cells from a signet-ring cell carcinoma [35,36]. This difference in histological type of the parent tumors might also be reflected in the differences in *MUC5AC* mRNA and *MUC6* mRNA expression patterns.

The down-regulation of MUC5AC in *H. pylori*-infected human gastric mucosa has been reported by using immunohistochemistry and *in situ* hybridization [37]. In contrast, the up-regulation of *MUC6* mRNA has been reported in the human gastric surface mucous cells of *H. pylori*-infected patients [38]. Since MUC5AC is a mucin core protein of the gastric surface mucous cells and a major mucin core protein of the mucins forming the gastric surface mucous gel layer covering the gastric mucosa [9], a decrease in MUC5AC synthesis after *H. pylori* infection may impair the protective gastric mucous gel layer. However, as shown in this study, different cell types showed different reactions by *H. pylori* infection. Further studies are necessary to investigate the MUC5AC induction patterns *in vivo* in the gastric mucosa.

Different reactions by *H. pylori* infection in different cells were also observed in *CDX2* mRNA levels. Wild-type *H. pylori* infection induced *CDX2* mRNA expression in the AGS cells, whereas it inhibited the expression in the MKN45 cells and KATO III cells. Our data for AGS cells were in agreement with a recent report that *H. pylori* induced *CDX2* mRNA expression in AGS cells [39]. In an *in vivo* study, immunohistochemistry was used to examine the expression of CDX2 and it was reported to be enhanced in *H. pylori*-infected human gastric mucosa [40]. Importantly, we found that wild-type *H. pylori* induced both *CDX2* mRNA and *MUC2* mRNA expressions in AGS cells. This coordinated upregulation of the expression of *CDX2* mRNA and *MUC2* mRNA in AGS cells could be explained by the putative transcriptional regulator function of *CDX2* on the *MUC2* mRNA expression [31]. In addition, the up-regulation of the *CDX2* and *MUC2* mRNA in the AGS cells incubated with *H. pylori* confirmed in this study suggests a close relationship between *H. pylori* infection and intestinal metaplasia, which is one of the lesions preceding the development of gastric carcinoma [41].

However, the expression of *MUC2* mRNA was independent of the expression of *CDX2* mRNA in both the MKN45 and KATO III cells. The mechanism involved in *MUC2* mRNA expression may be different between intestinal phenotype predominant AGS cells and gastric phenotype predominant MKN45 and KATO III cells.

In this study, wild-type *H. pylori* induced *TFF1*, *TFF2*, and *TFF3* mRNA expression in all three cell lines. These findings are consistent with those of previous reports on the increased immunohistochemical and mRNA expression of TFF1 in *H. pylori*-infected human gastric mucosa [6,42] and with the report of the increased *TFF1* mRNA levels in AGS cells following infection with *H. pylori* [43]. A recent study has reported that the up-regulation of *TFF1* and *TFF2* mRNA levels was carried out by incubation of MKN45 cells with peroxisome proliferator- activated receptor gamma (PPARγ) ligands [44]. PPARγ is a member of the nuclear hormone receptor superfamily that plays an important role in cell differentiation and regulation of metabolism [45]. It is notable that PPARγ expression was up-regulated by Kato III cells incubated with *H. pylori* [46]. Moreover, the hypothesis that strains containing the *cag* PAI augment PPARγ activation was reported [46]. We therefore hypothesize that the *cag* PAI-positive *H. pylori* could up-regulate PPARγ expression in gastric carcinoma cells and should contribute to the up-regulation of *TFF1* and *TFF2* mRNA levels in gastric carcinoma cells. This upregulation of TFF1 expression by *H. pylori* infection may facilitate the adhesion of *H. pylori* to the gastric mucosa and its colonization in the gastric surface mucous gel layer. On the one hand, TFF1 has been reported to act as a receptor for *H. pylori*, *in vivo*, and to explain the tropism of *H. pylori* for the gastric surface mucous cells and its co-localization with the gastric mucin MUC5AC [47]. On the other hand, from the perspective of a mucosal defense mechanism, this up-regulation of *TFF* mRNAs could be interpreted as a defensive reaction of the host cells against *H. pylori*-related gastric mucosal injuries. The mucosal protective functions of TFF peptides include stabilization of the gastric mucous gel layer, promotion of the gastric epithelial restitution, and suppression of apoptosis [12–14]. Interestingly, gender differences in *TFF1* mRNA expression in the gastric mucosa with *H. pylori* infection have been reported in the human stomach [42]. Differences in the expression of TFF peptide proteins as cellular responses to *H. pylori* infection in the stomach may be related to differences in disease outcomes of *H. pylori* infection.

In conclusion, we have shown that the intestinal phenotype predominated in AGS cells, whereas the gastric phenotype predominated in MKN45 and KATO III cells and that *H. pylori* cells alter the expression of the transcription factors mRNA, *MUCs* mRNA, and *TFFs* mRNA levels in *H. pylori*-infected cells in a pattern common to cell lines or in a pattern specific to cell lines. These alterations were largely *cag* PAI dependent and may contribute

to the outcomes of *H. pylori* infections. The present study provides a useful platform for selecting appropriate cell lines to model *H. pylori*-related changes in the gastric epithelium that mirror the changes seen *in vivo*.

Acknowledgments

We express our gratitude to Professor Tsutomu Katsuyama (Department of Laboratory Medicine, Shinshu University School of Medicine) and Professor David Y. Graham (Department of Medicine/Gastroenterology, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine) for their advice and encouragement. This report is based on work supported in part by a Grant-in-Aid for Scientific Research C-17590488 (to H.O.) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan and grants from the National Institutes of Health DK62813 (to Y.Y.).

References

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet. 1984; 1:1311–1315. [PubMed: 6145023]
2. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med. 1991; 325:1127–1131. [PubMed: 1891020]
3. Asahi M, Azuma T, Ito S, Ito Y, Suto H, Nagai Y, et al. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. J Exp Med. 2000; 191:593–602. [PubMed: 10684851]
4. Backert S, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, et al. Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell Microbiol. 2000; 2:155–164. [PubMed: 11207572]
5. Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science. 2000; 287:1497–1500. [PubMed: 10688800]
6. Van De Bovenkamp JH, Korteland-Van Male AM, Buller HA, Einerhand AW, Dekker J. Infection with *Helicobacter pylori* affects all major secretory cell populations in the human antrum. Dig Dis Sci. 2005; 50:1078–1086. [PubMed: 15986858]
7. Ota H, Katsuyama T. Alternating laminated array of two types of mucin in the human gastric surface mucous layer. Histochem J. 1992; 24:86–92. [PubMed: 1374372]
8. Allen A, Flemstrom G, Garner A, Kivilaakso E. Gastro-duodenal mucosal protection. Physiol Rev. 1993; 73:823–857. [PubMed: 8415927]
9. Ho SB, Takamura K, Anway R, Shekels LL, Toribara NW, Ota H. The adherent gastric mucous layer is composed of alternating layers of MUC5AC and MUC6 mucin proteins. Dig Dis Sci. 2004; 49:1598–1606. [PubMed: 15573912]
10. 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; 55:2681–2690. [PubMed: 7780985]
11. Weiss AA, Babyatsky MW, Ogata S, Chen A, Itzkowitz SH. Expression of MUC2 and MUC3 mRNA in human normal, malignant, and inflammatory intestinal tissues. J Histochem Cytochem. 1996; 44:1161–1166. [PubMed: 8813081]
12. Sands BE, Podolsky DK. The trefoil peptide family. Annu Rev Physiol. 1996; 58:253–273. [PubMed: 8815795]
13. Wong WM, Poulsom R, Wright NA. Trefoil peptides. Gut. 1999; 44:890–895. [PubMed: 10323896]
14. Hoffmann W. Trefoil factor family (TFF) peptides: regulators of mucosal regeneration and repair, and more. Peptides. 2004; 25:727–730. [PubMed: 15177865]
15. Tomasetto C, Rio MC, Gautier C, Wolf C, Hareuveni M, Chambon P, et al. hSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. Embo J. 1990; 9:407–414. [PubMed: 2303034]

16. Hanby AM, Poulsom R, Singh S, Elia G, Jeffery RE, Wright NA. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. Gastroenterology. 1993; 105:1110–1116. [PubMed: 8405856]
17. Podolsky DK, Lynch-Devaney K, Stow JL, Oates P, Murgue B, DeBeaumont M, et al. Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. J Biol Chem. 1993; 268:6694–6702. [PubMed: 8454642]
18. Kouznetsova I, Peitz U, Vieth M, Meyer F, Vestergaard EM, Malfertheiner P, et al. A gradient of TFF3 (trefoil factor family 3) peptide synthesis within the normal human gastric mucosa. Cell Tissue Res. 2004; 316:155–165. [PubMed: 14968359]
19. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer. 2002; 2:777–785. [PubMed: 12360280]
20. Tsukamoto T, Inada K, Tanaka H, Mizoshita T, Mihara M, Ushijima T, et al. Down-regulation of a gastric transcription factor, Sox2, and ectopic expression of intestinal homeobox genes, Cdx1 and Cdx2: inverse correlation during progression from gastric/intestinal-mixed to complete intestinal metaplasia. J Cancer Res Clin Oncol. 2004; 130:135–145. [PubMed: 14655050]
21. Li XL, Eishi Y, Bai YQ, Sakai H, Akiyama Y, Tani M, et al. Expression of the SRY-related HMG box protein SOX2 in human gastric carcinoma. Int J Oncol. 2004; 24:257–263. [PubMed: 14719100]
22. Tsukamoto T, Mizoshita T, Mihara M, Tanaka H, Takenaka Y, Yamamura Y, et al. Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes. Histopathology. 2005; 46:649–658. [PubMed: 15910596]
23. Bai YQ, Yamamoto H, Akiyama Y, Tanaka H, Takizawa T, Koike M, et al. Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach. Cancer Lett. 2002; 176:47–55. [PubMed: 11790453]
24. Eda A, Osawa H, Yanaka I, Satoh K, Mutoh H, Kihira K, et al. Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. J Gastroenterol. 2002; 37:94–100. [PubMed: 11871772]
25. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965; 64:31–49. [PubMed: 14320675]
26. Nakamura K, Sugano H, Takagi K. Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. Gan. 1968; 59:251–258. [PubMed: 5726267]
27. Sepulveda AR, Wu L, Ota H, Gutierrez O, Kim JG, Genta RM, et al. Molecular identification of main cellular lineages as a tool for the classification of gastric cancer. Hum Pathol. 2000; 31:566–574. [PubMed: 10836295]
28. Shibata N, Watari J, Fujiya M, Tanno S, Saitoh Y, Kohgo Y. Cell kinetics and genetic instabilities in differentiated type early gastric cancers with different mucin phenotype. Hum Pathol. 2003; 34:32–40. [PubMed: 12605364]
29. Tatematsu M, Tsukamoto T, Inada K. Stem cells and gastric cancer: role of gastric and intestinal mixed intestinal metaplasia. Cancer Sci. 2003; 94:135–141. [PubMed: 12708487]
30. Kudo T, Lu H, Wu JY, Graham DY, Casola A, Yamaoka Y. Regulation of RANTES promoter activation in gastric epithelial cells infected with *Helicobacter pylori*. Infect Immun. 2005; 73:7602–7612. [PubMed: 16239564]
31. Yamamoto H, Bai YQ, Yuasa Y. Homeodomain protein CDX2 regulates goblet-specific MUC2 gene expression. Biochem Biophys Res Commun. 2003; 300:813–818. [PubMed: 12559945]
32. Fujimori Y, Akamatsu T, Ota H, Katsuyama T. Proliferative markers in gastric carcinoma and organoid differentiation. Hum Pathol. 1995; 26:725–734. [PubMed: 7628843]
33. Carvalho F, David L, Aubert JP, Lopez-Ferrer A, De Bolos C, Reis CA, et al. Mucins and mucin-associated carbohydrate antigens expression in gastric carcinoma cell lines. Virchows Arch. 1999; 435:479–485. [PubMed: 10592051]
34. Cornberg M, Enss ML, Makkink MK, Beil W, Bock CT, Sobek-Klocke I, et al. Variation of human mucin gene expression in gastric cancer cell lines and gastric mucous cell primary cultures. Eur J Cell Biol. 1999; 78:832–841. [PubMed: 10604660]

35. Motoyama T, Hojo H, Watanabe H. Comparison of seven cell lines derived from human gastric carcinomas. Acta Pathol Jpn. 1986; 36:65–83. [PubMed: 3962675]
36. Sekiguchi M, Sakakibara K, Fujii G. Establishment of cultured cell lines derived from a human gastric carcinoma. Jpn J Exp Med. 1978; 48:61–68. [PubMed: 209229]
37. Byrd JC, Yunker CK, Xu QS, Sternberg LR, Bresalier RS. Inhibition of gastric mucin synthesis by *Helicobacter pylori*. Gastroenterology. 2000; 118:1072–1079. [PubMed: 10833482]
38. 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; 113:455–464. [PubMed: 9247464]
39. Manzo BA, Crabtree JE, Campbell FM, Tweedle D, Potten CS, Bajaj-Elliott M, et al. *Helicobacter pylori* regulates the expression of inhibitors of DNA binding (Id) proteins by gastric epithelial cells. Microbes Infect. 2006; 8:1064–1074. [PubMed: 16473539]
40. Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, Honda S, et al. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of *Helicobacter pylori*. Helicobacter. 2002; 7:192–198. [PubMed: 12047325]
41. Correa P. A human model of gastric carcinogenesis. Cancer Res. 1988; 48:3554–3560. [PubMed: 3288329]
42. Kato S, Matsukura N, Togashi A, Masuda G, Matsuda N, Yamada N, et al. Sex differences in mucosal response to *Helicobacter pylori* infection in the stomach and variations in interleukin-8, COX-2 and trefoil factor family 1 gene expression. Aliment Pharmacol Ther. 2004; 20 Suppl 1:17–24. [PubMed: 15298601]
43. Guillemin K, Salama NR, Tompkins LS, Falkow S. Cag pathogenicity island-specific responses of gastric epithelial cells to *Helicobacter pylori* infection. Proc Natl Acad Sci USA. 2002; 99:15136–15141. [PubMed: 12411577]
44. Shimada T, Koitabashi A, Kuniyoshi T, Hashimoto T, Yoshiura K, Yoneda M, et al. Up-regulation of TFF expression by PPARgamma ligands in gastric epithelial cells. Aliment Pharmacol Ther. 2003; 18 Suppl 1:119–125. [PubMed: 12925149]
45. Michalik L, Desvergne B, Wahli W. Peroxisome-proliferator-activated receptors and cancers: complex stories. Nat Rev Cancer. 2004; 4:61–70. [PubMed: 14708026]
46. Konturek PC, Kania J, Kukharsky V, Raithel M, Ocker M, Rembiasz K, et al. Implication of peroxisome proliferator-activated receptor gamma and proinflammatory cytokines in gastric carcinogenesis: link to *Helicobacter pylori*-infection. J Pharmacol Sci. 2004; 96:134–143. [PubMed: 15492468]
47. Clyne M, Dillon P, Daly S, O'Kennedy R, May FE, Westley BR, et al. *Helicobacter pylori* interacts with the human singledomain trefoil protein TFF1. Proc Natl Acad Sci USA. 2004; 101:7409–7414. [PubMed: 15123808]

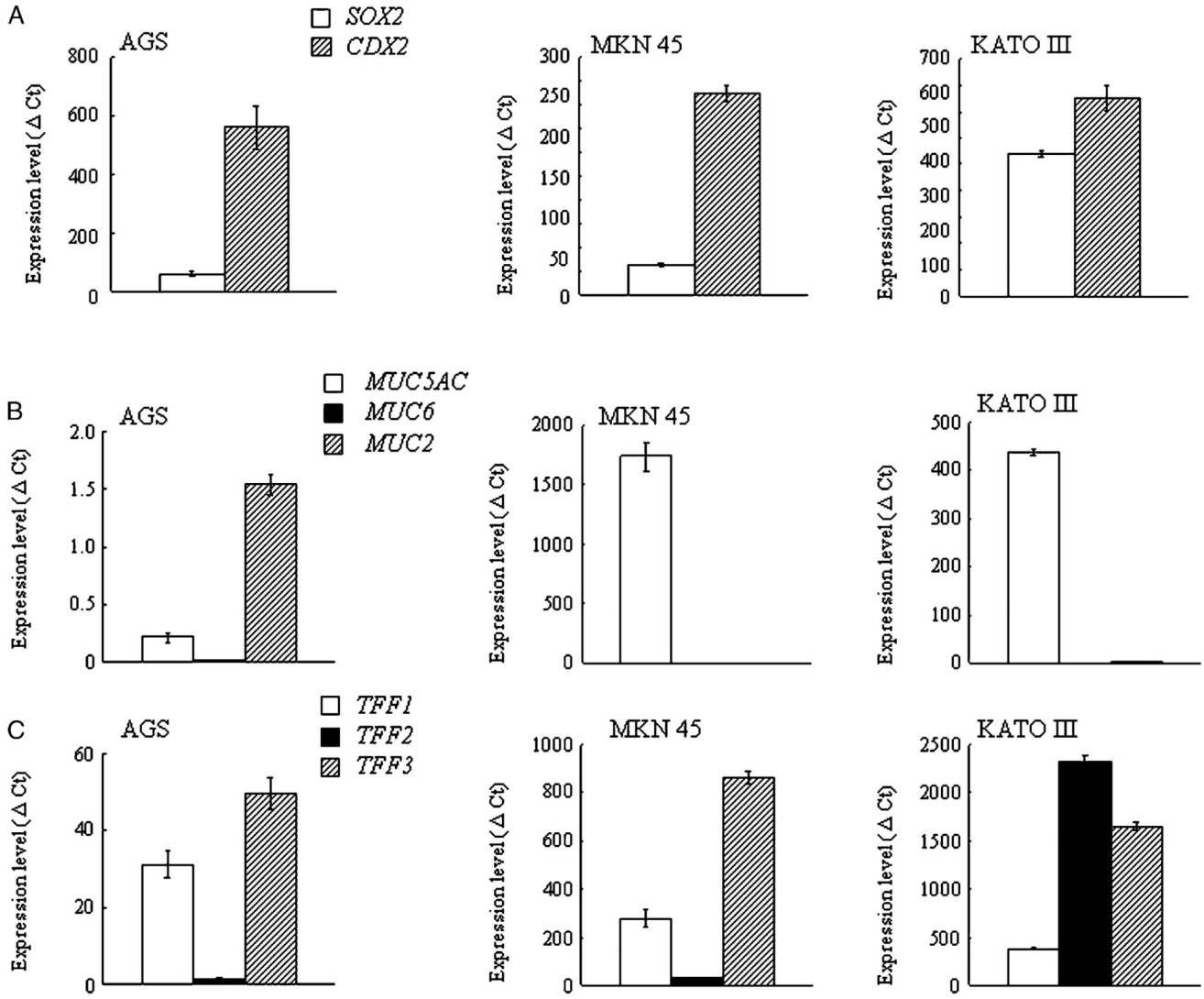
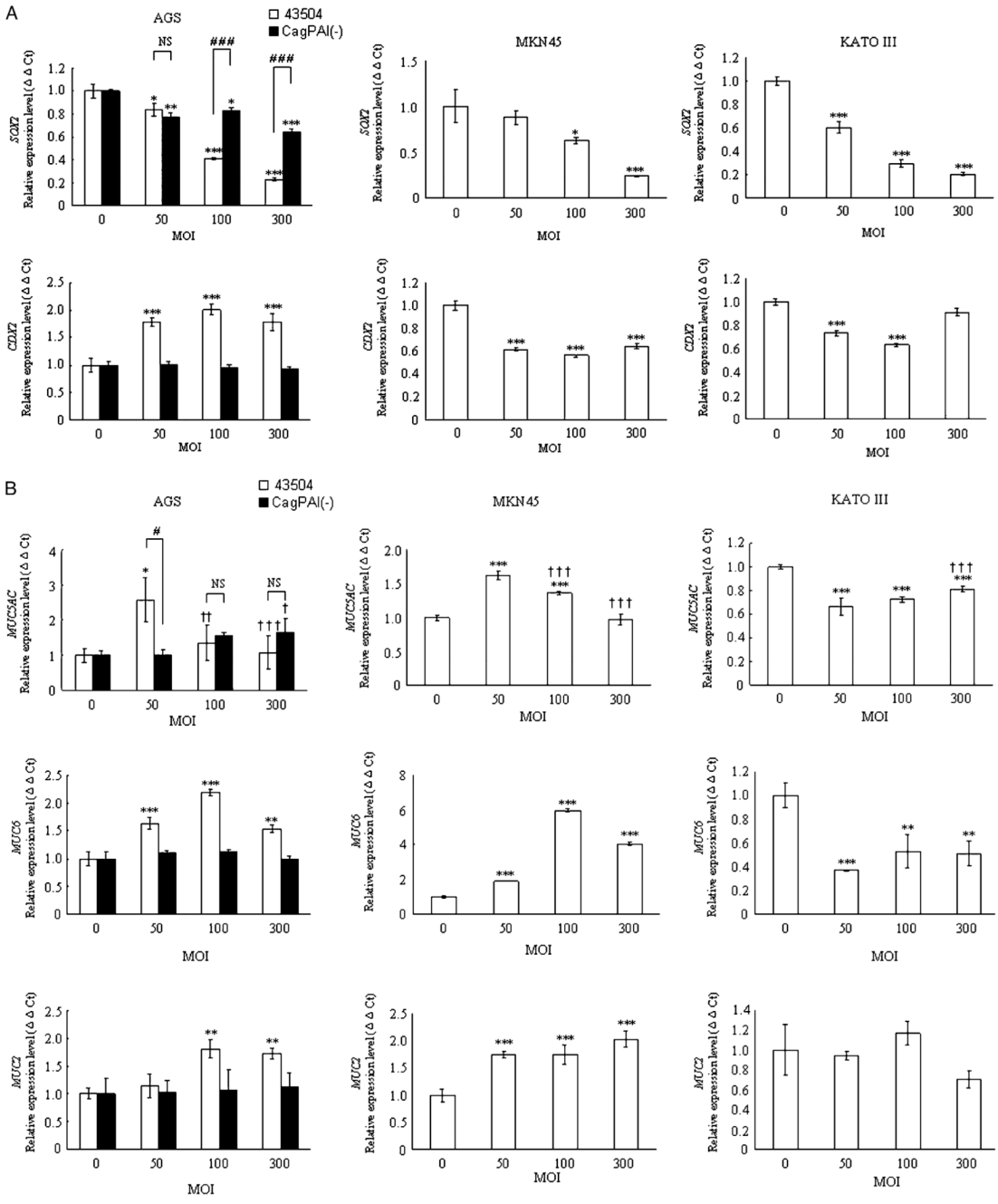


Figure 1.

Unstimulated mRNA levels of transcription factors (*SOX2* and *CDX2*) (A), mucin core proteins (*MUC5AC*, *MUC6*, and *MUC2*) (B), and trefoil factor family peptides (*TFF1*, *TFF2*, *TFF3*) (C) in the AGS, MKN45 and KATO III cells. The change in the comparative threshold cycle (ΔCt) for the target genes was calculated as ΔCt = (Ct of target genes)–(Ct of GAPDH). The ratio of the target genes to GAPDH was calculated as $2^{-\Delta Ct}$ and expressed as $2^{-\Delta Ct} \times 10^5$; this ratio was used to evaluate the expression level within each target gene (*SOX2*, *CDX2*, *MUCs*, and *TFFs*) in the AGS cells, MKN45 cells, and KATO III cells in the unstimulated state. Data are expressed as mean values±SD.



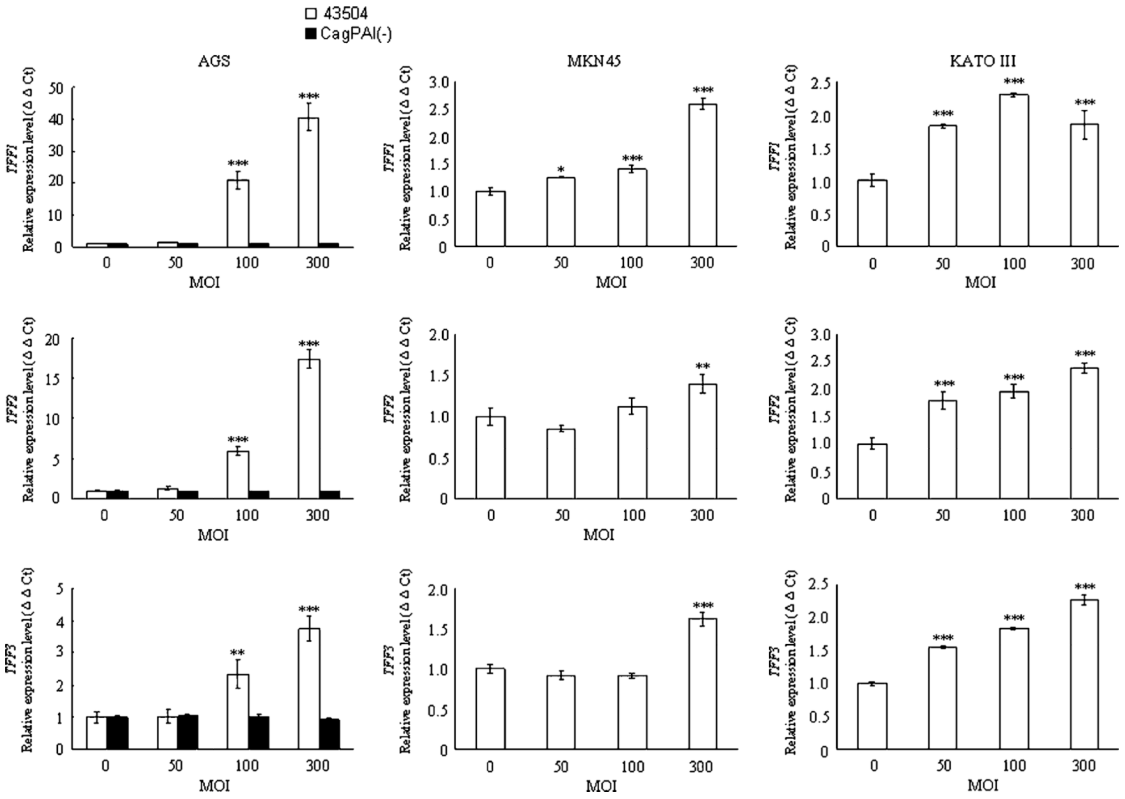


Figure 2.

Effect of treatment with the wild-type *H. pylori* strain and the *cag*-PAI deleted mutant strain on the expression levels of the mRNAs of the transcription factors (*SOX2* and *CDX2*) (A), *MUCs* (*MUC5AC*, *MUC6*, and *MUC2*) (B), and *TFFs* (*TFF1*, *TFF2*, and *TFF3*) (C) in gastric cancer cells at different *H. pylori* multiplicities of infection (MOIs). The abundance of the target genes relative to that of GAPDH was calculated as ΔΔCt = (ΔCt of target genes)–(ΔCt of GAPDH). The ratio was calculated as $2^{-\Delta\Delta Ct}$ to evaluate the alteration of the target genes 12 h after co-culture with the gastric cancer cells and *H. pylori*. MOI. **p* <0.05; ***p* <0.01; ****p* <0.001 in comparison with that of cells cultured at a MOI of 0. †*p* <0.05; ††*p* <0.01; †††*p* <0.001 in comparison with that of cells cultured at a MOI of 50. #*p* < 0.05; ###*p* <0.001 in comparison with that of cells cultured with wild-type *H. pylori*