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The Expression of Murine Double Minute 2 (MDM2) on *Helicobacter pylori*-Infected Intestinal Metaplasia and Gastric Cancer

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Summary **The overexpression of murine double minute 2 (MDM2) is found in several human tumors, and increased expression of MDM2 inactivates the apoptotic and cell cycle arrest function of p53. Interleukin-16 (IL-16) is a pleiotrophic cytokine and the properties of IL-16 suggest that it involve in the pathophysiological process of chronic inflammatory diseases. In this study, we investigated the expression of MDM2 in intestinal metaplasia and gastric cancer as well as the effect of *H. pylori* infection and IL-16 on epithelial cell proliferation and MDM2 expression in gastric cells *in vitro*. The expression of MDM2 on gastric biopsies was studied immunohistochemistry. AGS cells were incubated with a combination of IL-16 and *Helicobacter pylori (H. pylori)*. Gastric epithelial cell proliferation was studied by BrdU uptake and the expressions of MDM2 were studied by ELISA. There was no significant difference on the expression of MDM2 between with and without *H. pylori* infected chronic gastritis. In *H. pylori* infected gastric mucosa; the MDM2 expression was higher on intestinal metaplasia and gastric cancer than chronic gastritis. IL-16 administration was increased MDM2 expression and cell proliferation on AGS cells, which was decreased by *H. pylori* infection. In conclusion, the expression of MDM2 in long-term *H. pylori* infected gastric mucosa may indicate a risk for carcinogenesis. IL-16 secretion in *H. pylori* infected mucosa is one of the factors for gastric cancer. The expression of MDM2 on mucosa can be a mediator for gastric cancer.**

***Key Words*: *Helicobacter pylori*, gastric cancer, intestinal metaplasia, MDM2**

Introduction

The development of gastric carcinoma involves a multistep process from chronic gastritis to atrophy, intestinal metaplasia and dysplasia. It has been shown that atrophic gastritis and intestinal metaplasia significantly increase the risk of gastric carcinoma. The infection of *Helicobacter pylori* (*H. pylori*) induced chronic gastritis is significantly more pronounced in gastric carcinoma patients [*1*, *2*]. *H. pylori* colonization alters specific host physiology, such as gastrin secretion and gastric epithelial cell cycle events. *H. pylori* colonization also increases apoptosis and proliferation in human gastric tissue [*3–6*]. *H. pylori* infection also induces infiltrations of neutrophils, macrophages, dentric cells, as well as B and T cells [*7*]. This infiltration of inflammatory cells is an important factor for mucosal change from chronic gastritis to intestinal metaplasia, and this infiltration is one of the factors for gastric carcinogenesis.

Interleukin-16 (IL-16) was initially described as a T cell chemoattractant. *In vivo* studies have more fully characterized IL-16 as an immunomodulatory cytokine that contributes to the regulatory process of $CD4^+$ T cell recruitment and activation at sites of inflammation in association with asthma and several autoimmune diseases [*8*]. Although IL-16 can be produced by a variety of cell types, the production

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and timing of release of bioactive IL-16 vary depending on the cell origin. $CD8^+$ T cells contain processed bioactive IL-16 whose release can be triggered within 1–4 h by a variety of stimuli, such as histamine and serotonin. In contrast, $CD4^+$ T cells constitutively generate pro-IL-16 protein that is processed and secreted as bioactive IL-16 approximately 12–24 h after stimulation [*9*]. Lunden *et al.* reported that the increased numbers of $CD4^+$ T cells in the antrum of *H. pylori* infected gastric mucosa, but comparable numbers of $CD8^+$ T cells [*7*]. From these studies, the infiltration of inflammatory cells is important factor for gastric mucosal injury and cell cycle, but the influence of IL-16 expression on gastric mucosa by long-term infection with *H. pylori*, is not clear.

The p53 transcript factor functions as a tumor suppressor by inducing the expression of genes that inhibit cell growth and promote apoptosis [*10*]. The mdm2 gene encodes a 491 amino-acid protein that contains a binding domain for the tumor suppressor of p53 [*11*]. Murine double minute 2 (MDM2) was shown to bind the tumor suppressor p53 and inhibit p53-mediated transsactivation. The overexpression of MDM2 was another mechanism by which the cell could inactivate p53 in the process of transformation. The overexpression of the MDM2 gene is frequent in many human breast carcinomas, soft tissue sarcomas and other cancers, suggesting that MDM2 overexpression may be one of the common causes of oncogenesis [*12*].

In this study, we investigated the expression of MDM2 on chronic gastritis, intestinal metaplasia and gastric cancer in *H. pylori* infected mucosa. *In vitro* study, we also investigated the effect of IL-16 administration and *H. pylori* infection on gastric epithelial cell proliferation and the expression of MDM2 on AGS cells.

Materials and Methods

Gastric samples

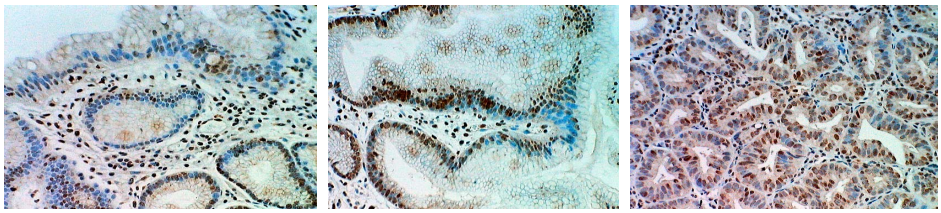
Gastric biopsies were taken through endoscopy, and classified into chronic gastritis, intestinal metaplasia and gastric cancer by histological findings. *H. pylori* infection was studied by taking 2 samples for culture, 2 samples for rapid urea test and 2 samples for histological findings. *H. pylori* infection was diagnosed if anyone of the 6 biopsies tested positive.

Expressions of MDM2 protein on gastric biopsies

The biopsies from chronic gastritis without *H. pylori* infection ($n = 20$), chronic gastritis with *H. pylori* infection ($n = 20$), intestinal metaplasia with *H. pylori* infection ($n = 20$) and gastric cancer with *H. pylori* infection ($n = 20$), were studied. The paraffin-embedded blocks were cut into 5 μm sections. Then they were mounted on slides and dewaxed in xylene and sequentially dehydrated in 100%, 80% and 70% ethanol. DNA in the specimens was degenerated by 4 M HCl for 30 min, and the intrinsic peroxidase was blocked with 0.3% hydrogen peroxide in methanol. Nonspecific binding was blocked with PBS containing 3% skimmed milk (Wako, Tokyo, Japan) for 30 min. To enhance immunostaining, sections were treated with an antigen retrieval solution (10 mM citrate acid monohydrate, pH 6.0, adjusted with 2 N NaOH) and heated 3 times in a microwave oven at high power for 5 min. After the specimens were incubated with anti-MDM2 mouse antibody (Santa Cruz, CA) overnight at 4°C, they were washed with PBS, followed by incubation with biotinylated secondary anti-mouse goat antibody (Dako, Copenhagen, Denmark) diluted 20-fold for 1 h. After washing the tissues with PBS, sections were incubated with avidin-biotin complex (ABC Elite: Vector Laboratoried Inc. Burlingame, CA). Peroxides conjugates were subsequently localized using diaminobenzidine tetrahydrochloride as a chromogen. Sections were counterstained with Haematoxylin. Image Manager Software applied to a light microscope was used for computer analysis of each sample. The degree of immnopositive for MDM2 was evaluated semi-quantitatively. In random fields from representative areas of gastric biopsies, the immunoreactive cells were roughly assessed and expressed as a percentage.

Cell culture and regents

AGS cells were studied because they possess wild-type p53 [*13*], in contrast to KATO-III cells and other cells that have p53 deletions and/or rearrangement, which may affect the response to signals that regulate cell cycle events [*14*]. AGS gastric epithelial cells were grown in F-12 Ham medium (Gibco, Life-Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, MP Biomedicals, Solon, OH) and antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO). All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell culture experiments were carried out using 100 mm dishes (Nunc, Copenhagen, Denmark). *H. pylori* (NCTC 11637) were grown on Skirrow agar (Oxoid, Bashingstone, England) plate with 7% horse blood (Nippon Biotest Lab. Tokyo, Japan) in an atmosphere of 10% CO_2 at 37°C. Colonies were harvested, 10^5 and 10^6 cfu/ml of *H. pylori* were resuspended in antibiotic-free F-12 without FBS. Experiments were performed as 10^4 of AGS cells were incubated with F-12 antibiotic-free media with 0.5% bovine albumin (Trace Biosciences, NZ). Using 6-well polypropylene tissue culture plates, with the combination with/without of 10^5, 10^6 cfu/ml of *H. pylori* and 10^{-10}, 10^{-9} M of recombinant human IL-16 (Pepro Tech, Inc, Rocky hill, NJ) was added into AGS cells. Cells were harvested at 24 h incubation.



Chronic gastritis Intestinal metaplasia Gastric cancer

Fig. 1. The expression of MDM2 on gastric mucosa by ABC staining. A. Chronic gastritis, B. Intestinal metaplasia, C. Gastric cancer MDM2 was stained by DAB as brown area.

ELISA assay

AGS cells were grown on 6 well-plates (Nunc. Copenhagen, Denmark) and maintained in antibiotic-free F-12 medium without serum for 24 h prior to the experiments. The combination of IL-16 and *H. pylori* were added in serum-free medium, and co-cultured for 24 h. Cells were labeled with Bromodeoxyuridine (BrdU: Sigma, St. Louis, MO) for 1 h, and monolayers were washed 3 times with PBS, lysed with 1 ml of lysis buffer (50 mM NaCl, 25 mM Tris-HCl pH 8.0, 0.5% NP-40, 0.5% sodium deoxycholate and 0.02% sodium azide). Cells were scraped, and transferred to eppendolf tubes. Particulate materials were removed by centrifugation. Ten µg/ml of primary anti-BrdU antibody (Dako, Copenhagen, Denmark) and anti-MDM2 antibody (Santa Cruz, CA) were dissolved with 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2 into 10 µl, and coated each well of 96 well-plates, and incubated plates overnight at 4°C. After washing with Buffer B (0.01 M Phosphate buffer, 0.50 M NaCl, 0.1% Tween 20, pH 7.2) 3 times, 100 µl of samples (6 times) were pipetted into wells, and incubated for 2 h at room temperature. After the plates were washed with Buffer B, the plates were then blocked for 30 min with 3% skim milk (Wako, Tokyo, Japan) in buffer B. Secondary antibodies (Dako, Copenhagen, Denmark) were incubated for 1 h. Plates were further incubated with 1:10000 of alkaline phosphatase conjugated streptoavidin (Dako, Copenhagen, Denmark) for 1 h. Plates were washed with 1:20 of AmpliQ washing buffer (Dako, Copenhagen, Denmark) and the amplification reagents A and B were added to all wells. Expressions were determined by a spectrophotometer at 492 nm. The assay was followed by ampliQ detection kit (K6245, Dako, Copenhagen, Denmark). All data were expressed as % AGS cells only.

Statistical analysis

The Turney test was used to evaluate difference. A p value of <0.05 was considered significant.

Results

The expression of MDM2 on gastric mucosa

In chronic gastritis, there was not a significant difference on the expression of MDM2 protein between without *H. pylori* and with the infection (chronic gastritis without *H. pylori* infection vs. chronic gastritis with *H. pylori* infection: 3.60 ± 1.52 vs. 4.77 ± 1.92, $p = 0.31$). But in *H. pylori* infected gastric mucosa, the expression of MDM2 protein was increased in intestinal metaplasia and gastric cancer (chronic gastritis: 4.77 ± 1.92, intestinal metaplasia: 7.12 ± 2.16 $p<0.001$, gastric cancer: 7.76 ± 4.31, $p<0.000$) (Fig. 1, 2).

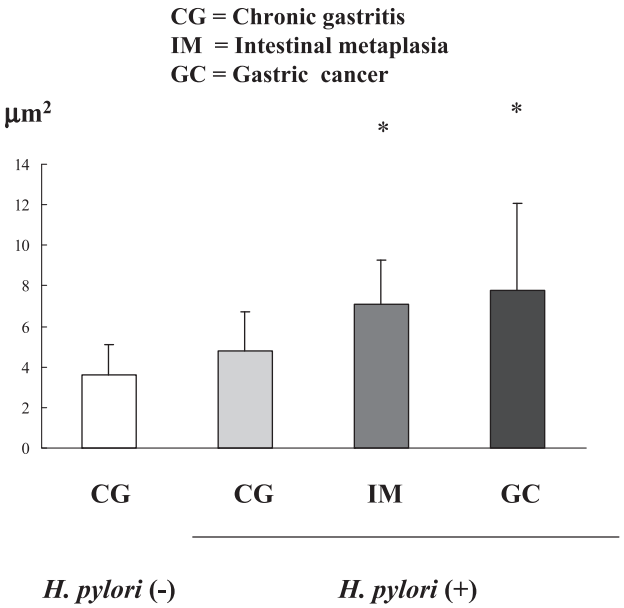


Fig. 2. The Expression of MDM2 on *H. pylori* infected gastric mucosa in chronic gastritis (CG), intestinal metaplasia (IM) and gastric cancer (GC). There was no significant difference on CG between with and without *H. pylori* infection. But in *H. pylori* infected mucosa, the expression of MDM2 increased in IM and GC more than CG. ($p<0.05$)

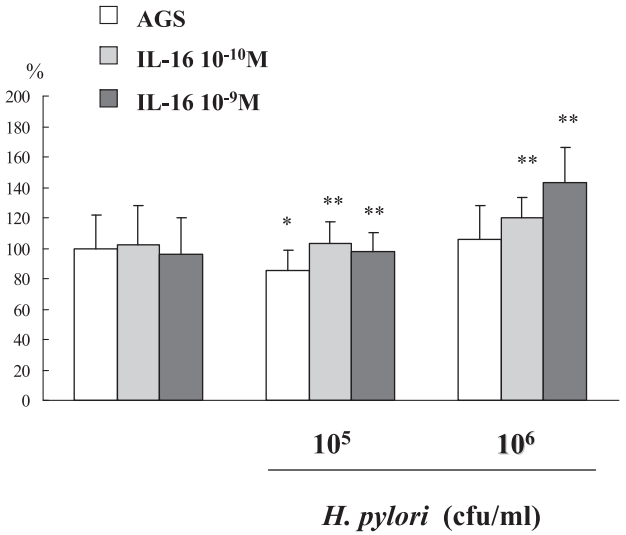


Fig. 3. The effect of IL-16 and *H. pylori* infection on BrdU uptake in AGS cells. The infection of *H. pylori* decreased BrdU uptake on AGS cells. But further administration of IL-16 increased BrdU uptake on AGS cells which had been decreased by *H. pylori* infection.

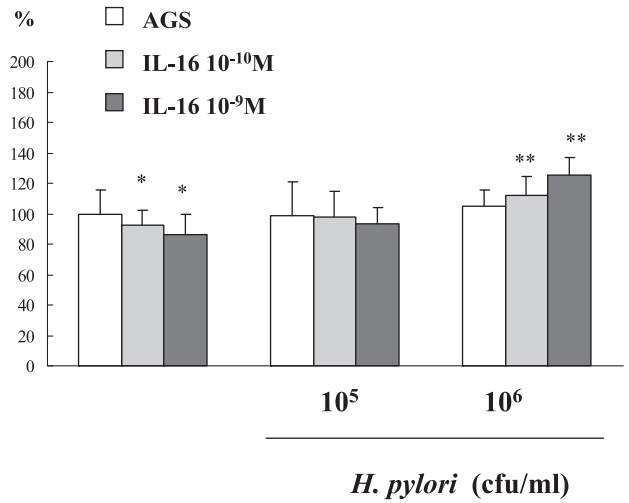


Fig. 4. The effect of IL-16 and *H. pylori* infection on MDM2 expression in AGS cells. The infection of *H. pylori* decreased MDM2 expression on AGS cells, but further administration of IL-16 increased MDM2 expression on AGS cells which had been decreased by *H. pylori* infection.

The effect of H. pylori infection on gastric epithelial cell proliferation

The *in vitro* study showed that the administration of 10^{-10}, 10^{-9} M of IL-16 did not influence the BrdU uptake on AGS cells. (AGS only 100 ± 21.97%, 10^{-10} M IL-16 administration on AGS cells: 95.85 ± 23.90%, 10^{-9} M IL-16: 102.54 ± 25.77%). But the 10^5 cfu/ml of *H. pylori* incubation decreased BrdU uptake on AGS cells (AGS only: 100 ± 21.97% vs. 10^5 cfu/ml of *H. pylori*: 85.19 ± 13.57%, $p<0.001$), 10^6 cfu/ml of *H. pylori* infection did not decrease the BrdU uptake on AGS cells (10^6 cfu/ml of *H. pylori*: 105.78 ± 22.48%). Further 10^{-10}, and 10^{-9} M of IL-16 administration increased BrdU uptake on AGS cells which was decreased by 10^5 cfu/ml of *H. pylori* infection (10^5 cfu/ml *H. pylori*: 85.19 ± 13.57% vs. 10^5 cfu/ml *H.pylori* + 10^{-10} M IL-16: 97.82 ± 15.24%, 10^5 cfu/ml *H.pylori* + 10^{-9} M IL-16: 102.80 ± 14.67%, $p<0.001$). Also the administration of IL-16 increased BrdU uptake on AGS cells with 10^6 cfu/ml of *H. pylori*: (10^6 cfu/ml *H. pylori*: 105.78 ± 22.48% vs. 10^6 cfu/ml *H. pylori* + 10^{-10} M IL-16: 143.36 ± 22.65%, 10^6 cfu/ml *H. pylori* + 10^{-9} M IL-16: 120.38 ± 12.62%, $p<0.001$) (Fig. 3).

The expression of MDM2 protein levels on AGS cells

There was not a significant difference on MDM2 protein levels of AGS cells between with and without 10^6, 10^5 cfu/ml of *H. pylori* infection (AGS cells only: 100 ± 15.83%, 10^5 cfu/ml *H. pylori*: 98.33 ± 22.68%, 10^6 cfu/ml *H. pylori*: 104.65 ± 11.22%). Also administration of 10^{-10}, 10^{-9} M of IL-16 did not have an influence on MDM2 protein levels on AGS cells (AGS cells only: 100 ± 15.83%, 10^{-10} M IL-16: 86.13 ± 13.38%, 10^{-9} M IL-16: 92.28 ± 9.64%). But on 10^6 cfu/ml of *H. pylori* incubation, the further administration of 10^{-10} and 10^{-9} M of IL-16 increased MDM2 protein levels on AGS cells infected with 10^6 cfu/ml of *H. pylori* (10^6 cfu/ml *H. pylori*: 104.65 ± 11.22%, 10^{-10} M IL-16 + *H. pylori*: 111.75 ± 12.30%, 10^{-9} M IL-16 + *H. pylori*: 125.26 ± 11.26%, $p<0.05$) (Fig. 4).

Discussion

Infection with *H. pylori* is a causality of chronic gastritis, gastroduodenal ulceration, and gastric cancer. Intestinal-type gastric carcinogenesis occurs at a later age and progresses through well-defined histological steps initiated by the transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis and intestinal metaplasia and finally to dysplasia and adenocarcinona [*15*]. Correa reported that populations at high risk of developing gastric cancer had a higher prevalence of atrophic gastritis than populations at low risk of developing gastric cancer [*16*]. Gastric epithelial mucosal integrity is controlled by the balance of cell loss and renewal. But epithelial cell hyperproliferation is a factor for gastric carcinogenesis. *H. pylori* infection increased epithelial cell proliferation in gastric mucosa, and for stimulated gastric cell proliferation by *H. pylori*, the infiltration with inflammatory cells is important factor.

The human homologue of the mdm2 gene is frequently overexpressed in many human cancers, suggesting that MDM2 overexpression may be one of the common causes of oncogenesis [*12*, *17*]. Amplification of the mdm2 gene enhances tumorigeneic potential of murine cells. Although human cancer cells frequently overexpress MDM2, and experimental evidences suggest that in normal cells, MDM2 overexpression causes G1 arrest, suggesting that MDM2 overexpression requires genetic alterations. The biochemical activities of MDM2 in cancer cells have been studied intensely because of its association with the tumor suppressor p53 and its implication in human cancer. Cancer cells that overexpress MDM2 may evade these checkpoint regulatory pathways by selecting mutated cells [*18*].

In this study, we demonstrated that on *H. pylori* infected mucosa, the expression of MDM2 was increased in intestinal metaplasia and gastric cancer. But in chronic gastritis, there was no significant difference on the MDM2 expression between with and without *H. pylori* infection. These results indicate that the expression of MDM2 is the trigger for the stimulation and occurrence of gastric cancer, but *H. pylori* infection only did not influence MDM2 expression. Kodama *et al.* reported that in chronic gastritis, *H. pylori* infection increased MDM2 protein levels more than non-infected tissues [*19*]. The difference between their study and ours may be the method of diagnosis for chronic gastritis. They diagnosed chronic gastritis endoscopically, but in this study, we diagnosed chronic gastritis by histological findings. In our study, there was not a significant difference on MDM2 protein levels between with and without *H. pylori* infected chronic gastritis, but the expression of MDM2 seemed to increase. Previous studies demonstrated that the expression of MDM2 increased in gastric cancer [*20*, *21*]. From our study, the expression of MDM2 protein was increased in not only gastric cancer, but also in intestinal metaplasia than chronic gastritis. The histological change of intestinal metaplasia is important for the change to gastric cancer, and the stimulation of MDM2 protein may be one of the factors for the transition to gastric cancer.

To know the mechanisms for the different expression of MDM2, we studied the expression of MDM2 and gastric epithelial cell proliferation on the *H. pylori* infected cells *in vitro*. In this study, we noticed the effect of IL-16 on *H. pylori* infected gastric epithelial cells. Keates *et al.* demonstrated that IL-16 protein levels were increased in Crohn's diseases, and anti-IL-16 antibody reduced TNBS-induces injury [*22*]. IL-16 was originally isolated as lymphocyte chemoattractant factor, and IL-16 is a proinflammatory cytokine that induces migration of $CD4^+$ T lymphocytes as well as activated CD4-bearing monocytes/macrophages and eosinophils. IL-16 is produced predominately by activated $CD8^+$ T lymphocytes and to a lesser extent by $CD4^+$ T lymphocytes and eosinophils [*23*]. In humans, *H. pylori* infection results in active chronic gastritis with continuous infiltration of neutrophils, B cells and T cells. Lundgren *et al.* reported increased numbers of $CD4^+$ T cells in the antrum of *H. pylori*-positive individuals [*7*]. Stimulated infiltration of these inflammatory cells by *H. pylori* infection is known as an important factor for gastric carcinogenesis, and the effect of IL-16 may be one of the important factors for chronic inflammatory diseases including chronic gastritis by long-term infection with *H. pylori*.

In this study, we demonstrated that *H. pylori* incubation decreased BrdU uptake on AGS cells, but further administration of IL-16 increased cell proliferation, which was decreased by *H. pylori* infection. On the other hand, Joe *et al.* demonstrated *H. pylori* increased tritiated thymidine incorporation on KATO III cells [*24*]. The reasons of the differentiation between these studies were the difference of cell lines and concentration of *H. pylori* administration. In most *H. pylori* infected patients, the bacteria infected in childhood and persisted into adulthood. This long-term infection with *H. pylori* causes stimulation of inflammatory cells including monocytes, neutrophils. Especially, the IL-8 is one of the important cytokines for *H. pylori* infected gastric mucosa for the occurrence of gastrointestinal ulceration and gastric cancer. We investigated the expression of IL-16 in gastric mucosa, and there was no significant difference in the expression of IL-16 on gastric mucosa (data not shown). But the administration of IL-16 increased BrdU uptake on AGS cells which was decreased by *H. pylori* infection. *In vivo* studies, we reported that *H. pylori* infection increased cell proliferation on mice gastric mucosa [*25*, *26*]. One of the reasons for the difference between our *in vivo* and *in vitro* study was the presence of inflammatory cell infiltration. In gastric mucosa, infiltration of inflammatory cells and expression of cytokines by *H. pylori* infection, stimulate cell proliferation on gastric mucosa, and the proliferation is increased by not only *H. pylori* infection directly. The expression of IL-16 is one of the important factors for stimulation of cell growth in *H. pylori* infected gastric mucosa. One of the reasons for stimulation of cell growth is, stimulation of oxidative stress by *H. pylori* infection. Inflammation provides several sources of oxidants including leukocytes, cytokines. The infiltration of this reactive oxygen damaged gastric mucosa, and continual damage by long-term infection of *H. pylori* leads to proteome changes related to cell proliferation and carcinogenesis.

In our study, the expression of MDM2 protein was decreased by *H. pylori* infection, but further administration of IL-16 increased MDM2 protein which had been decreased by *H. pylori*. From these results, *H. pylori* infection only is not a trigger for the expression of MDM2, but infiltration of inflammatory cells or the expressions of cytokines including IL-16 are important factors for the expression of MDM2. Ohmiya *et al.* reported that MDM2 SNP309, a single-

mucleotide polymorphism in the MDM2 promoter can impact tumorgenesis especially in the atrophic gastritis. SNP309 elevated the risk for carcinoma in the stomach after establishment of severe atrophic gastritis [*27*]. The presence of SNP309 in the MDM2 gene leads to higher expression of MDM2 protein. From their study, SNP309 can impact tumorigenesis especially in atrophic gastritis. And also SNP309 elevated the risk of carcinoma in the stomach after establishment of atrophic gastritis. The expression of MDM2 and SNP309 can be markers for gastric cancer in atrophic gastritis, but severe inflammatory changes in gastric mucosa are necessary for these expressions.

We conclude, the expression of MDM2 on gastric mucosa may be one of the factors for gastric carcinogenesis by long-term *H. pylori* infection, because MDM2 expresses on gastric mucosa in the early phase of gastric cancer. Also, the infiltration of IL-16 by *H. pylori* infection is a factor for stimulation of MDM2 expression on *H. pylori* infected gastric mucosa. The expression of IL-16 on *H. pylori* infected gastric mucosa can be a trigger for stimulation of epithelial cell proliferation on *H. pylori* infected gastric mucosa.

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