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Helicobacter pylori infection and administration of non-steroidal anti-inflammatory drugs down-regulate the expression of gastrokine-1 in gastric mucosa

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Background/aims: *Gastrokine-1 is a novel protein that plays an important role in the maintenance of the integrity of the gastric mucosa. However, whether Helicobacter pylori infection and non-steroidal anti-inflammatory drugs, which are known to cause gastric mucosal injuries, affect gastrokine-1 expression in the gastric mucosa is unknown. The aim of the present study was to determine gastric mucosal expression of gastrokine-1 in patients with Helicobacter pylori infection or long-term non-steroidal anti-inflammatory drug administration.* ***Material and Methods:*** *A total of 40 patients with functional dyspepsia (20 with Helicobacter pylori-negative chronic gastritis, and 20 with Helicobacter pylori-positive chronic gastritis), and 37 Helicobacter pylori-negative long-term non-steroidal anti-inflammatory drug users (26 with aspirin, 11 with selective cyclooxygenase-2 inhibitors) were selected. In addition, 20 Helicobacter pylori-negative healthy volunteers were recruited as controls. All subjects underwent endoscopies with biopsies taken from the antrum and the sites with lesions. Gastric mucosal changes were detected endoscopically and histologically, and gastrokine-1 protein expression in the antral mucosa was analyzed by immunohistochemistry.* ***Results:*** *Expression of gastrokine-1 protein was decreased in Helicobacter pylori-positive chronic gastritis compared with Helicobacter pylori-negative subjects and the healthy controls. Similarly, gastrokine-1 expression in non-steroidal anti-inflammatory drug users was also decreased, compared with the healthy controls, but there was no significant difference in gastrokine-1 expression between the aspirin group and selective cyclooxygenase-2 inhibitor group. Moreover, gastrokine-1 expression levels tended to be associated with the severity of chronic gastritis.* ***Conclusions:*** *Both Helicobacter pylori infection and long-term non-steroidal anti-inflammatory drug administration down-regulate gastrokine-1 expression in the gastric mucosa, which may contribute to the gastric mucosal injuries induced by these two factors.*

Key words: *Helicobacter pylori,* non-steroidal anti-inflammatory drugs, gastrokine-1, gastric mucosal injury

Helikobakter pilori enfeksiyonu ve non-streroidal anti enflamatuvar ilaç kullanımı mide mukozasında gastrokin-1 ekspresyonunu baskılamaktadır

Amaç: *Gastrokin-1 gastrik mukozanın bütünlüğünün korunmasında rolü olan yeni tanımlanmış bir moleküldür. Ancak, gastrik mukoza hasarı yaptığı bilinen Helikobakter pilori enfeksiyonunun veya non-streodial antienflamatuvar ilaçların, gastrokin-1 ekspresyonunu etkileyip etkilemediği bilinmemektedir. Bu çalışmanın amacı, Helikobakter pilori enfeksiyonu veya uzun süreli non-streodial antienflamatuvar ilaç kullanımı olan hastalarda gastrik mukozada gastrokin-1 ekspresyonunun araştırılmasıdır.* ***Yöntem:*** *Fonksiyonel dispepsisi olan 40 hasta (20 Helikobakter pilori negatif kronik gastrit ve 20 Helikobakter pilori pozitif kronik gastrit) ve Helikobakter pilori negatif kronik non-streodial antienflamatuvar ilaç kullanımı olan 37 hasta (26 aspirin kullanan ve 11 selektif siklooksigenaz-2 inhibitörü kullanan) çalışmaya alındı. Ayrıca 20 Helikobakter pilori negatif sağlıklı gönüllü de çalışmaya kontrol grubu olarak dahil edildi. Tüm hastalara endoskopi yapıldı ve antrum ve tespit edilen lezyonların biyopsileri alındı. Gastrik mukozadaki değişiklikler endoskopik ve histolojik olarak tespit edildi ve antral mukozadaki gastrokin-1 protein ekspresyonu immunohistokimyasal olarak analiz edildi.* ***Bulgular:*** *Gastrokin-1 ekspresyonu Helikobakter pilori-negatif kronik gastritte, Helikobakter pilori pozitif kronik gastrite ve sağlıklı kontrollere göre azalmış bulundu. Benzer olarak, non-streodial antienflamatuvar ilaçları alan grupta gastrokin-1 ekspresyonu sağlıklı kontrollerinkine göre düşük bulundu ancak aspirin alanlar ile selektif siklooksigenaz-2 inhibitoru alanlar arasında anlamlı farklılık saptanmadı. Bunlara ek olarak, gastrokin-1 ekspresyonu ile kronik gastritin şiddeti arasında ilişki mevcuttu.* ***Sonuç:*** *Helikobakter pilori enfeksiyonu ve uzun süreli non-streodial antienflamatuvar ilaçların kullanımının neden olduğu gastrik mukozadaki gastrokin-1 ekspresyonundaki azalmanın, bu faktörlerin neden olduğu gastrik mukoza hasarına katkısı olabilir.*

Anahtar kelimeler: *Helikobakter pilori,* non-steroidal anti-enflamatuar ilaç, gastrokin-1, gastrik mukozal hasar

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INTRODUCTION

Gastric diseases that are originated from the loss of integrity of the gastric mucosa, such as chronic gastritis and ulceration, have been major problems in the world. Normally, the structural integrity of the gastric mucosa is maintained by the mucosal defense mechanisms (1,2). However, even under physiological conditions, the gastric mucosa is continuously exposed to many factors that may undermine and break the integrity. Once the aggressive factors overwhelm the mucosal defense, the integrity of the gastric mucosa may be impaired, resulting in severe inflammation, erosion, ulceration, bleeding, or even perforation (3).

Among the aggressive factors, *Helicobacter pylori (Hp)* infection is confirmed to be the most important and most common etiological factor for gastric mucosal injuries, such as chronic gastritis and peptic ulceration (4). Multiple and interactive mechanisms, including impairments in gastrin regulation, acid secretion, inflammatory response, and neural pathways, contribute to the development of *Hp*-induced gastric injuries and subsequent consequences (5-7). Use of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective cyclooxygenase (COX)-2 inhibitors, is another risk factor for gastric injuries (8). A variety of changes, such as deficiency in prostaglandins, neutrophil activation, microcirculatory disturbances, oxygen free radicals, and luminal acid, all contribute to the development of NSAID-induced gastric injuries (9,10). Although *Hp* infection and NSAIDs are different etiological factors, and induce gastric injuries via different mechanisms, they may share common pathways involving some key molecular changes during the process of gastric injuries (11-13).

On the other hand, gastric mucosal defense mechanisms withstand the damage factors to maintain the integrity of the mucosa. These mechanisms are multifactorial and many molecular components are involved. Gastrokine-1 (GKN1) is a novel protein that was firstly cloned by a Japanese group in 2000 (14). It is a secreted protein with a molecular weight of approximately 18 KDa and shows high evolutionary conservation across human, mouse, rat, and cow. Previous studies have shown that GKN1 is expressed specifically in the gastric mucosa, but not in other organs such as the lung, liver and pancreas (15,16). These expression patterns of GKN1 indicate that it may play a distinctive role in the stomach. GKN1 has been demonstrated to have mitogenic and motogenic activities and plays an important role in the maintenance of the integrity of the gastric mucosa (15,17). Although GKN1 is abundantly expressed in normal gastric epithelium, its expression is significantly down-regulated or even absent in gastric cancer. Using proteomic techniques, Nardone et al. (18) reported that there were two isoforms for GKN1, and it was the basic isoforms that were down-regulated in *Hp*-positive dyspeptic patients with moderate-severe gastritis. They further investigated GKN1 expression in *Hp*-related precancerous and cancerous gastric lesions and found that GKN1 was significantly decreased in *Hp*-positive patients, compared with *Hp*-negative patients. They also observed a progressive decrease in GKN1 expression from chronic gastritis to precancerous lesions (i.e. gastric atrophy and intestinal metaplasia) and gastric cancer, in which GKN1 expression was undetectable or even absent (19). These findings indicate that down-regulation of GKN1 expression is associated with inflammatory damage to the gastric mucosa, and also the subsequent consequences (19). However, the role of GKN1 in NSAID-induced gastric mucosal injury has not been explored, and the interactive effects of *Hp* infection and NSAIDs on GKN1 expression remain to be elucidated. Therefore, this preliminary study was carried out to address these issues by determining the protein expression of GKN1 in the gastric mucosa in dyspeptic patients with or without *Hp* infection and in long-term NSAID users.

MATERIALS AND METHODS

Patients and Specimens

The study population consisted of a selected 40 patients with functional dyspepsia (20 *Hp*-positive and 20 *Hp*-negative) and 37 long-term NSAIDs users (all *Hp*- negative) selected from outpatients. The functional symptoms were upper abdominal pain or discomfort often exacerbated with eating, early satiety, postprandial abdominal bloating or distention, and nausea, as outlined in the Rome II-I criteria. Of the NSAID users, 26 had received aspirin (acetylsalicylic acid [ASA]) and 11 selective COX-2 inhibitors (celecoxib or meloxicam) for more than six months. These patients were randomly selected from cardiovascular or rheumatismal outpatients. In addition, 20 healthy volunteers who were selected from those individuals attending the hospital for regular physical examinations and confirmed to be negative for *Hp* infection by ^{13}C-urea breath test were recruited (Table 1).

Table 1. Clinical and histological characteristics of the study population

Subject group	Male/ Female	Age (Mean±SD)	Distribution of subjects according to severity of chronic gastritis			
			Absent	Mild	Moderate	Marked
Healthy volunteers (n=20)*	10/10	44.6±12.7	20	0	0	0
Patients with functional dyspepsia (n=40)	23/17	44.5±9.3	0	8	26	6
H. pylori-negative (n=20)	12/8	44.9±10.7	0	6	12	2
H. pylori-positive (n=20)	11/9	43.9±7.9	0	2	14	4
NSAID users, *H. pylori*-negative (n=37)		44.8±12.3	0	7	28	2
Aspirin users (n=21)	15/6	44.7±15.8	0	4	16	1
COX-2 inhibitor users (n=16)	11/5	45.8±6.6	0	3	12	1

All subjects enrolled in this study had no history of gastric ulcer, neoplasia, gastric surgery, liver disease, or severe systemic disease in the past six months, and they did not receive any proton pump inhibitors (PPIs), mucosal protective agents or antibiotics over the past six months. No patient had ever received antibiotic therapy aiming to eradicate *Hp* infection. All subjects were invited to undergo upper gastrointestinal endoscopy. During upper gastrointestinal endoscopy, two biopsies were taken from the gastric antrum for histological examinations including the detection of *Hp* infection with hematoxylin and eosin staining as described below. One additional fresh antral biopsy was obtained for the rapid urease test (RUT), which was performed according to the instructions of the manufacturer (Tianjin Reagent Biotech Company, Tianjin, China). Briefly, after the biopsy was incubated in the test medium for 3 minutes (min) at room temperature, a color change of the medium was observed. An unchanged yellow color was interpreted as *Hp*-negative, while a color change from yellow to pink or red was interpreted as *Hp*-positive. A subject positive for *Hp* as determined by both histology and RUT was defined as having *Hp* infection, and those negative for both tests was defined as having no *Hp* infection.

Written, informed consent was obtained from all patients before the sample collection. The study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University.

Histological Examinations

Gastric biopsy specimens were fixed in 10% neutralized formalin and embedded in paraffin for histological processing. Histological examinations were performed by an experienced pathologist, according to the updated Sydney system (20). Briefly, chronic inflammation, neutrophil polymorph activity, glandular atrophy, and intestinal metaplasia were recorded in all cases of gastritis. Gastritis was graded on a mild, moderate, or marked scale as indicated in the guidelines.

Immunohistochemistry

Paraffin sections (4 μm thick) were cut from the specimens, deparaffinized in xylene and hydrated through a graded series of ethanol concentrations. Antigen retrieval was performed by heating the sections for 10 min at 100°C in 0.01M citrate buffer (pH 6.0), endogenous peroxidase activity was quenched with 3% H_2O_2 for 15 min, and nonspecific staining was reduced by a blocking serum for 10 min. The sections were then incubated with mouse anti-human GKN1 (1:300, Abnova Corporation, China) at room temperature for 2 hours (h). Then, a two-step detection method was used according to the manufacturer's instructions (EnVision™ Detection Kit, Gene Tech Company Limited, China). Briefly, after incubation with the primary antibody, the tissue was incubated with the ChemMate™ EnVision™/HRP for 30 min at room temperature. Then, hematoxylin was used as a counterstain. The reaction was visualized by the CheMate™ DAB plus Chromogen. Negative controls were performed by omitting the first antibodies.

Previous studies had found that GKN1 was expressed in the superficial epithelium and in the upper third of the glandular epithelium, so we calculated the immunoreactive cells in these fields (16,18). A scoring system with two categories was used for evaluation of immunohistochemical results. Category A documented the number of immunoreactive cells as 0 (<5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (>75%). Category B documented the intensity of the immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Finally, values for category A and B were combined to obtain a score. The staining results were measured semi-quantitatively on a

scale of 0 (score less than 2), 1+ (score range from 2 to 3), 2+ (score range from 4 to 5), and 3+ (score range from 6 to 7). Immunostaining was assessed by an experienced pathologist who was blinded to the clinical data of the patients.

Statistical Analysis

All quantitative data were expressed as mean ± standard deviation (SD). The immunoreactive difference in the proportion of subjects with GKN1 expression between the different groups was determined by Mann-Whitney test and Kruskal-Wallis test. All statistical analyses were carried out using the SPSS statistical software package (version 11.0, SPSS Inc. Chicago, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

Expression of GKN1 in Healthy Volunteers, Patients with Functional Dyspepsia and NSAID Users

By definition, *Hp*-positive patients with functional dyspepsia were positive for both histology and RUT, and *Hp*-negative patients with functional dyspepsia and NSAID users were negative for both histology and RUT. All healthy volunteers were negative for histology and RUT.

In normal gastric mucosa without *Hp* infection, strong immunostaining of GKN1 was found in the superficial epithelial cells and in the epithelial cells of the upper part of glands, which was consistent with the results of previous studies (16,18). However, GKN1 expression was gradually decreased and absent in the deeper glands of gastric mucosa (Figure 1A).

The immunopositivity of GKN1 in *Hp*-positive patients with functional dyspepsia was significantly decreased compared with that in *Hp*-negative subjects (Table 2) (Figures 1B, 1C). Similarly, the expression of GKN1 in NSAID users was also decreased; there was no significant difference between ASA and selective COX-2 inhibitor users (Table 2) (Figures 1D, 1E).

Expression of GKN1 in Healthy Volunteers, Patients with Functional Dyspepsia and NSAID Users in Terms of the Presence and Severity of Chronic Gastritis

Chronic gastritis was observed in all patients with functional dyspepsia and NSAID users, but in no-

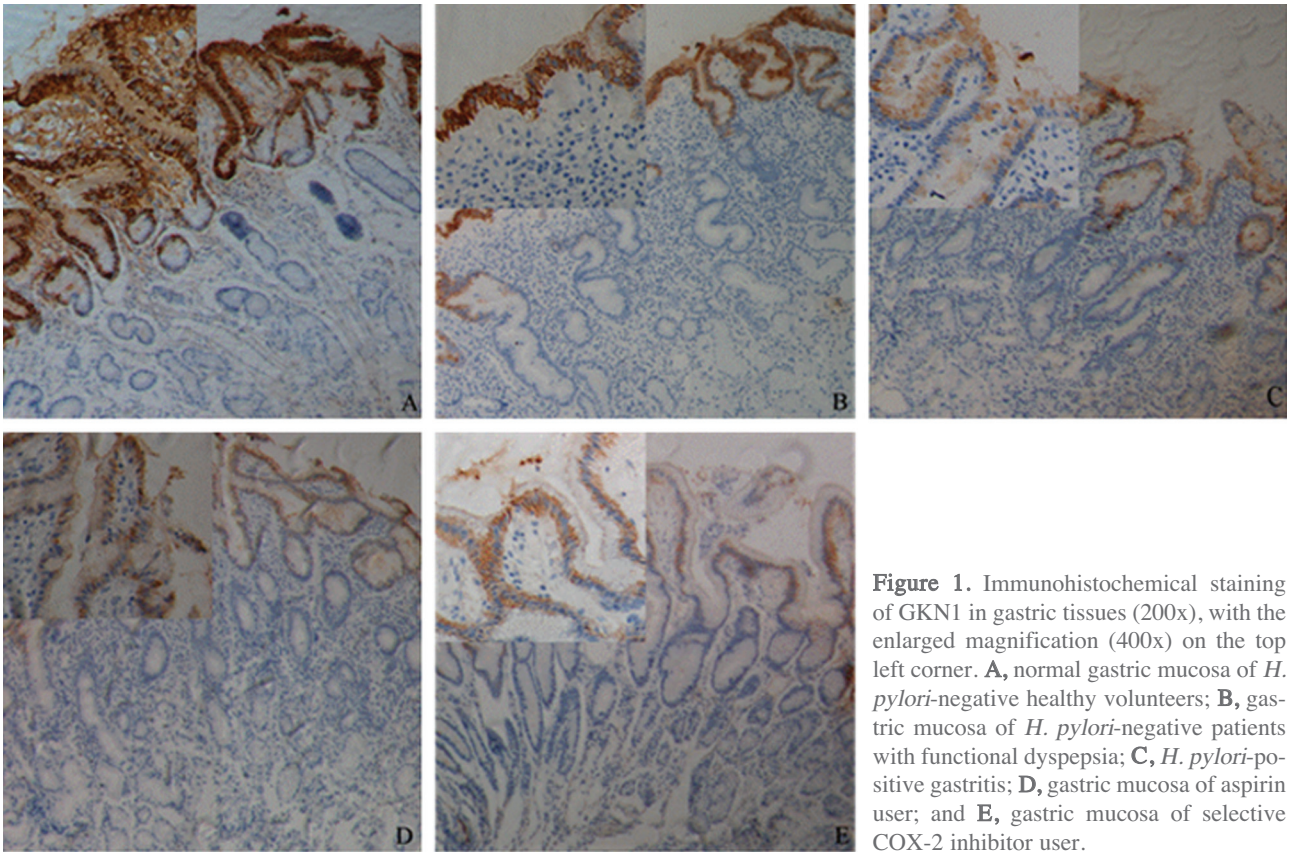

Figure 1. Immunohistochemical staining of GKN1 in gastric tissues (200x), with the enlarged magnification (400x) on the top left corner. **A,** normal gastric mucosa of *H. pylori*-negative healthy volunteers; **B,** gastric mucosa of *H. pylori*-negative patients with functional dyspepsia; **C,** *H. pylori*-positive gastritis; **D,** gastric mucosa of aspirin user; and **E,** gastric mucosa of selective COX-2 inhibitor user.

Table 2. GKN1 expression in healthy volunteers, dyspeptic patients with functional dyspepsia and non-steroidal anti-inflammatory drug (NSAID) users

Subject group	Distribution of subjects according to GKN1 expression levels			
	-	+	++	+++
Healthy volunteers (n=20)*	0	0	0	20
Dyspeptic patients (n=40)	0	5	25	10
H. pylori-negative (n=20) †,	0	0	12	8
H. pylori-positive (n=20)#	0	5	13	2
NSAID users (n=37)*	0	0	30	7
Aspirin users (n=21) †	0	0	18	3
COX-2 inhibitor users (n=16) ##, †	0	0	12	4

*, all were *H. pylori*-negative. †, p<0.001, compared with *H. pylori*-negative healthy volunteers.
#, p=0.014, compared between *H. pylori*-negative and -positive patients. ##, p=0.596, compared between aspirin users and COX-2 inhibitor users.

Table 3. GKN1 expression in healthy volunteers, patients with functional dyspepsia and non-steroidal anti-inflammatory drug (NSAID) users in terms of the severity of histologically confirmed chronic gastritis.

Subject group	Distribution of subjects according to GKN1 expression levels			
	-	+	++	+++
Healthy volunteers (n=20)*	0	0	0	20
Absent (n=20)	0	0	0	20
Mild (n=0)	0	0	0	0
Moderate (n=0)	0	0	0	0
Marked (n=0)	0	0	0	0
Dyspeptic patients (n=40) †#	0	5	25	10
Absent (n=0)	0	0	0	0
Mild (n=8)	0	0	0	8
Moderate (n=26)	0	0	24	2
Marked (n=6)	0	5	1	0
NSAID users (n=37)*†	0	0	30	7
Absent (n=0)	0	0	0	0
Mild (n=7)	0	0	0	7
Moderate (n=28)	0	0	28	0
Marked (n=2)	0	0	2	0

*, all were *H. pylori*-negative. †, p<0.001, compared with *H. pylori*-negative healthy volunteers.
#, p<0.001, compared among patients with different degrees of severity of chronic gastritis.

ne of the healthy volunteers. In normal gastric mucosa of healthy volunteers, the expression of GKN1 was abundantly present. However, in the patients with functional dyspepsia and NSAID users, the expression of GKN1 was progressively decreased from mild to marked chronic gastritis (Table 3).

DISCUSSION

In this study, we first evaluated the expression of GKN1 in normal gastric mucosa. Concordance with previous studies, our data confirmed that GKN1 was abundantly expressed in the surface gastric epithelial cells and the upper part of glands, but absent in the deeper gastric glands (14,16), suggesting that GKN1 may play a role in the defensive barrier of the gastric mucosa. It has been reported that GKN1 is secreted from the gastric epithelial cells as a component of the mucin. On the surface of the gastric epithelium, GKN1 may provide mechanical and chemical protection for the surface epithelial cells, by acting as a lubricant or gel stabilizer and helping to maintain the mucous pH gradient and hydrophobic barrier (15,17). GKN1 possesses mitogenic activities, like epithelial growth factor (EGF), to keep epithelial cell proliferation, migration re-epithelization and reconstruction of gastric glands (21,22). Moreover, previous studies have shown that GKN1 also possesses motogenic activities to promote restitution

in scrape-wounded monolayer cultures and mediate repair after injury to maintain the integrity of the mucosa (15,17). These physiological functions and expression location of GKN1 are similar to trefoil factor 1, which plays an important cytoprotective role in the gastric mucosa (23,24). In addition, GKN1 is involved in gastric maturation in postnatal rats and protection of colonic epithelial cells (25,26). It is noted that another member of the gastrokine family, GKN2, has been viewed as a protective factor in gastric mucosa (27,28).

Hp infection has been established as an etiological cause of gastric inflammation and peptic ulceration. It leads to damage to the gastric mucosa by different sophisticated mechanisms manipulating and regulating various molecular factors and pathways. We detected GKN1 in *Hp*-negative and *Hp*-positive patients with functional dyspepsia, all having different degrees of gastric inflammation, and found that *Hp* infection significantly reduced the expression of GKN1. This observation was consistent with the previous studies that reported a decreased expression of GKN1 in *Hp*-positive patients with chronic gastritis and gastric atrophy (18,19). In addition, it has been reported that the expression of GKN1 is increased after the eradication of *Hp* infection (29). These findings suggest that chronic persistence of *Hp* infection results in the decreased expression of GKN1, which may be one of the mechanisms by which *Hp* infection leads to damage to the gastric mucosa.

NSAIDs are other risk agents that cause damage to the gastric mucosa. In a previous study, indomethacin (a nonselective COX inhibitor) or rofecoxib (a selective COX-2 inhibitor) were gavaged into mice. Whereas indomethacin was associated with a decreased expression of GKN1, followed by gastric mucosal injury, rofecoxib was not associated with the down-regulation of GKN1 in the gastric mucosa and showed no gastric mucosal injury17. This animal experiment suggested that traditional NSAIDs may induce gastric impairment by reduction in the expression of GKN1, but COX-2 selective inhibitors cause gastric mucosal injury without involvement of the down-regulation of GKN1 expression. A recent study demonstrated that GKN1 expression was significantly decreased in the gastric antrum, but slightly elevated in the gastric corpus in 10 *Hp*-negative human volunteers who took ASA for one week (30). The authors postulated that the epithelial cells of the antrum may have lost the ability to express and secrete GKN1 due to the mucosal damage by ASA, whereas epithelial cells of the corpus, less injured by ASA, compensationally expressed and secreted GKN1, at a higher level, by the autocrine and paracrine functions (30). However, this study is limited by its small number of subjects, the use of only ASA, and its short duration. To better understand the effect of long-term usage of NSAIDs on GKN1 expression, we included patients who had used ASA or selective COX-2 inhibitors for more than six months, and detected GKN1 expression in the antral gastric mucosa. Consistent with the previous animal and human volunteer studies, our results showed that GKN1 expression was down-regulated in the patients who had used ASA for more than six months. However, unlike the animal study, we observed that GKN1 expression was also down-regulated in the patients who had used selective COX-2 inhibitors for more than six months. The expression of GKN1 was not significantly different between ASA users and selective COX-2 inhibitor users.

Selective COX-2 inhibitors are claimed to be less toxic to the gastrointestinal tract than ASA because of their smaller effect on the gastric prostaglandin G levels. We suggest that selective COX-2 inhibitors may have no or little effect on GKN1 in the short term. However, long-term administration of selective COX-2 inhibitors may damage the integrity of the gastric epithelium and consequently result in the reduction of GKN1 expression, which depends on the surface integrity of the gastric epithelial cells and upper part of the glands. It was still unknown whether COX-1 or COX-2 could regulate the expression of GKN1.

Based on the findings of the present study, along with findings from previous studies, we hypothesize that *Hp* infection and ASA may directly cause the decrease in GKN1 expression in the gastric antrum, which results in the damage to the gastric mucosa. While selective COX-2 inhibitors may have little effect on the GKN1 expression in the short term, long-term administration may be associated with decreased GKN expression, which in turn contributes to the damage to the gastric mucosa. However, this hypothesis needs to be tested in well-designed clinical studies.

One more important issue that needs to be addressed is whether NSAIDs and *Hp* infection have any additive effects on GKN1 expression, as NSAID usage accompanied by *Hp* infection usually has additive effects and leads to more severe gastric

injury. Unfortunately, we did not include NSAID users with *Hp* infection in our study, and thus further investigation is required to elucidate this critical issue.

In conclusion, both *Hp* infection and NSAID administration down-regulate the expression of GKN1 in the gastric mucosa, which is associated with the severity of gastric inflammation. Further experiments are required to elucidate the detailed molecular mechanisms by which *Hp* infection and NSAIDs regulate the expression of GKN1 and lead to the mucosal damage.

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