Original Paper

# Ten-second Endoscopic Breath Test using a 20-mg Dose of $^{13}$C-urea to Detect *Helicobacter pylori* Infection

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ABSTRACT

**Background/Aims:** Attempts to improve the $^{13}$C-urea breath test (UBT) have focused on decreasing the amount of substrate used and reducing the duration of the test. To render the test less expensive and more convenient, we designed a more rapid and less expensive endoscopic UBT with a low dose of 20mg and a shortened measurement time.

**Methodology:** A total of 178 patients who underwent diagnostic upper endoscopy were enrolled. At endoscopy, 150mL of intragastric gas sample were collected through a biopsy channel. Following inflation with air, 20mL of water containing 20mg of $^{13}$C-urea were sprayed onto the gastric mucosa using a spraying instrument. After 10 seconds, a gastric gas sample was collected again. The standard UBT was performed after 3-10 days.

**Results:** The $\Delta^{13}CO_2$ values of intragastric samples in *H. pylori*-positive patients and *H. pylori*-negative patients were 76.7±132.9 ‰ and 1.6±1.2 ‰, respectively. With intragastric samples, the maximum sensitivity and specificity of intragastric samples were 83.7% and 100% with cutoff point of 8‰, respectively.

**Conclusions:** Ten-second endoscopic UBT using a 20-mg dose of $^{13}$C-urea is a rapid, inexpensive, and accurate method for the detection of *H. pylori* infection in clinical practice.

**KEY WORDS:** Endoscopic $^{13}$C-urea breath test; 20-mg dose of $^{13}$C-urea; *Helicobacter pylori*

**ABBREVIATIONS:** $^{13}$C-urea Breath Test (UBT); *Helicobacter pylori* (*H.pylori*); per mil (Δ‰)

## INTRODUCTION

$^{13}$C-urea breath test (UBT) has become the most convenient non-invasive method for the diagnosis of the presence of *Helicobacter pylori* (*H. pylori*) infection (1-3). Various modifications have been used to optimize the simplicity and minimize the costs of the test. In a previous study, we reported on the utility of endoscopic UBT in which the $^{13}$C-urea was sprayed with a spraying instrument under endoscopic observation and an intragastric gas sample was collected through a biopsy channel (4). Unlike the standard UBT, $^{13}CO_2$ values of the modified endoscopic UBT, in which intragastric gas samples are collected before $^{13}CO_2$ is absorbed from the digestive tract, are not affected by absorption, kinetics, or exhalation of $^{13}CO_2$, as well as gastric emptying, a great separation between *H. pylori*-positive and *H. pylori*-negative patients was obtained. Based on these results, the urea dose and measurement duration could be reduced in this new endoscopic UBT, while still maintaining diagnostic accuracy. In order to render the test less expensive and more convenient, we designed a more rapid and less expensive protocol for the endoscopic UBT with a low dose of 20mg and a shortened measurement time.

## METHODOLOGY

A total of 178 patients who underwent diagnostic upper endoscopy for gastrointestinal symptoms were enrolled in the present study, including 119 females and 59 males, with a mean age of 58.8 (24-77) years. They all gave their informed consents. None of these patients took proton pump inhibitors, $H_2$-receptor antagonists, antibiotics, or bismuth salts in the previous two months. The study was approved by our local ethical committee.

At endoscopy, after noting the presence and location of abnormal findings and two biopsy specimens were taken from two sites on the greater curvature of the antrum and the midbody of the stomach. The biopsy specimens were placed in 10% buffered formalin fixative for routine processing, sectioning, and staining with hematoxylin and eosin and Giemsa stains. *H. pylori* was determined by Giemsa-staining. After that, 150mL of intragastric gas sample were collected through a biopsy channel and the stomach was inflated with air again. Next, 20mL of water containing 20mg of $^{13}$C-urea were sprayed onto the gastric mucosa using a spraying instrument. Ten seconds after completion of spraying, a gastric gas sample was collected through a biopsy channel using a 50-mL syringe.

Three to seven days after endoscopic UBT, standard UBT was performed in all subjects. After overnight fasting, 100mL of tap water and 100mg $^{13}$C-

Hepato-Gastroenterology 2007; 54:951-954
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**TABLE 1 Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy of Ten-second Endoscopic Breath Test using a 20-mg Dose of $^{13}$C-urea at Each Cutoff Value**

| Cutoff point | TP | FP | TN | FN | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|---|---|---|---|
| 0.5 | 97 | 53 | 12 | 1 | 99.0 | 18.5 | 64.7 | 92.3 | 66.9 |
| 1.0 | 94 | 47 | 18 | 4 | 95.9 | 27.7 | 66.7 | 81.8 | 68.7 |
| 1.5 | 94 | 33 | 32 | 4 | 95.9 | 49.2 | 74.0 | 88.9 | 77.3 |
| 2.0 | 92 | 17 | 48 | 6 | 93.9 | 73.8 | 84.4 | 88.9 | 85.9 |
| 2.5 | 90 | 11 | 54 | 8 | 91.8 | 83.1 | 89.1 | 87.1 | 88.3 |
| 3.0 | 86 | 7 | 58 | 12 | 87.8 | 89.2 | 92.5 | 82.9 | 88.3 |
| 3.5 | 84 | 5 | 60 | 14 | 85.7 | 92.3 | 94.4 | 81.0 | 88.3 |
| 4.0 | 82 | 5 | 60 | 16 | 83.7 | 92.3 | 94.3 | 78.9 | 87.1 |
| 4.5 | 82 | 3 | 62 | 16 | 83.7 | 95.4 | 96.5 | 79.5 | 88.3 |
| 5.0 | 82 | 1 | 64 | 16 | 83.7 | 98.5 | 98.8 | 80.0 | 89.6 |
| 5.5 | 82 | 1 | 64 | 16 | 83.7 | 98.5 | 98.8 | 80.0 | 89.6 |
| 6.0 | 82 | 1 | 64 | 16 | 83.7 | 98.5 | 98.8 | 80.0 | 89.6 |
| 6.5 | 82 | 0 | 65 | 16 | 83.7 | 100 | 100 | 80.2 | 90.2 |
| 7.0 | 82 | 0 | 65 | 16 | 83.7 | 100 | 100 | 80.2 | 90.2 |
| 7.5 | 82 | 0 | 65 | 16 | 83.7 | 100 | 100 | 80.2 | 90.2 |
| 8.0 | 82 | 0 | 65 | 16 | 83.7 | 100 | 100 | 80.2 | 90.2 |
| 8.5 | 80 | 0 | 65 | 18 | 81.6 | 100 | 100 | 78.3 | 89.0 |
| 9.0 | 78 | 0 | 65 | 20 | 80.0 | 100 | 100 | 76.5 | 87.7 |

TP: true positive; FP: false positive; TN: true negative; FN: false negative.

urea solution were ingested. Breath samples were taken at baseline and at 20 minutes after administration. $^{13}$C was measured as the $^{13}CO_2/^{12}CO_2$ ratio and was expressed delta per mil (Δ‰).

The Student's *t* test was used to analyze differences in$\Delta^{13}CO_2$ values between the *H. pylori*-positive and -negative groups. The difference of mean $\Delta^{13}CO_2$ values between the endoscopic UBT and the standard UBT was assessed with Student's paired t test. A p value of $<0.05$ was considered significant. The cut-off values of the UBT at each time point were calculated separately according to the sensitivity, specificity, and accuracy. The optimal cut-off value of excess $\Delta^{13}CO_2$ each protocol was determined by the accuracy.

## RESULTS

Eight patients were excluded from analysis because they had positive serology and negative histology. Of the remaining 170 patients, 98 had *H. pylori* infection, based on histology.

The $\Delta^{13}CO_2$ values of intragastric samples in *H. pylori*-positive patients and *H. pylori*-negative patients were 76.7±132.9 ‰ (0.2-661.9) and 1.6±1.2 ‰ (0-6.3), respectively. As shown in **Figure 2**, the $\Delta^{13}CO_2$ value of intragastric samples in *H. pylori*-positive patients was significantly higher than those of breath samples at 20 minutes ($p<0.001$). The $\Delta^{13}CO_2$ values of breath samples at 20 minutes in *H. pylori*-positive patients and *H. pylori*-negative patients were 16.2±12.6 ‰ (0.4-58.3) and 0.7±0.8‰ (0-3.7), respectively. The $\Delta^{13}CO_2$ value of intragastric samples in *H. pylori*-positive patients was significantly higher than those of breath samples at 20 minutes, whereas there was no significant difference between the two tests in *H. pylori*-negative patients. The great difference between *H. pylori*-positive and *H. pylori*-negative patients was obtained in the values of intragastric samples.

**TABLE 2 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the standard $^{13}$C-urea breath test at each cutoff value.**

| Cutoff point | TP | FP | TN | FN | Sensitivity | Specificity | PPV | NPV | Accuracy | |
|---|---|---|---|---|---|---|---|---|---|---|
| 94.3 | 79.1 | 96.2 | 90.2 | | | | | | | |
| 5 | 8 | 57 | 97 | 53 | 12 | 1 | 9992.0 | 90.5 | 91.4 | |
| .0 | 18.5 | 64.7 | 0 | 18 | 4 | 95.9 | 95.7 | 88.4 | 92.6 | |
| 27.7 | 66.7 | 1.5 | 4 | 95.9 | 49.2 | 795.7 | 85.9 | 91.4 | | |
| 4.0 | 88.9 | 2.0 | 92 | 17 | 48 | 6 | 93.9 | 95.5 | 81.3 | 89.0 |
| 3 | 73.8 | 2.5 | 90 | 11 | 54 | 8 | 9 | 97.6 | 79.7 | 89.0 |
| 1.8 | 83.1 | 3.0 | 58 | 12 | 5 | 84 | 100 | 80.2 | 90.2 | |
| 5 | 60 | 14 | 4.0 | 82 | 5 | 60 | 16 | 100 | 80.2 | 90.2 |
| 4.5 | 82 | 3 | 62 | 16 | 5.0 | 5.5 | 6 | 100 | 80.2 | 90.2 |
| .0 | 6.5 | 7.0 | 7.5 | 83.3 | 100 | 100 | 80.2 | 90.0 | | |
| 6.0 | 78 | 0 | 65 | 20 | 79.6 | 100 | 100 | 76.5 | 87.7 | |
| 6.5 | 76 | 0 | 65 | 22 | 77.6 | 100 | 100 | 74.7 | 86.5 | |
| 7.0 | 76 | 0 | 65 | 22 | 77.6 | 100 | 100 | 74.7 | 86.5 | |

TP: true positive; FP: false positive; TN: true negative; FN: false negative.

**Tables 1 and 2** show that the cut-off $\Delta^{13}CO_2$ values of intragastric samples and breath samples at 20-minute time point were optimal. With intragastric samples, the maximum sensitivity and specificity of intragastric samples were 83.7% and 100% with cutoff point of 8‰, respectively **(Table 1)**. Based on histology, the sensitivity and specificity of breath samples at 20 min were 91.8% and 93.8% with cutoff point of 2‰, respectively **(Table 2)**. The diagnostic accuracy of 10-minute endoscopic UBT was comparable with that of standard UBT.

## DISCUSSION

Various modifications of UBT have been reported, including changes in dosage of urea, type of test meals used, timing of sample collection, and position of patients (5-8). A Japanese standard protocol, using 100mg $^{13}$C-urea, was proposed in 1998 as having high sensitivity and specificity for UBT (1). In this method, the patients who have fasted are given 100mg of $^{13}$C-urea in 100mL of water and breath samples are collected at the baseline and at 20 minutes after ingestion of $^{13}$C-urea. The cut-off value of UBT for the diagnosis of *H. pylori* infection is 2.5‰. However, 75mg (9) and 50mg (10) $^{13}$C-urea have been shown to be equally effective. Isomoto *et al.* validated the endoscopic UBT with 25mg of the test urea (11).

Attempts to improve the UBT have focused on decreasing the amount of substrate used and reducing the duration of the test. The shorter the duration of the test, the more convenient it is for the patient. Moreover, lower dosage of $^{13}$C-urea is also advantageous from an economic point of view. This study showed that it was possible to reduce the amount of the expensive $^{13}$C-urea from 100mg to 20mg and the duration of the test from 20 min to 10 seconds without loss in sensitivity and specificity.

Unlike the standard UBT, the endoscopic UBT, in which $^{13}$C-urea is sprayed directly into the stomach through a biopsy channel, allows diffuse distribution and sufficient contact between the substrate and bacteria. Therefore, high sensitivity and specificity of the endoscopic UBT have been reported (11-13). However, these endoscopic UBT are unable to avoid effects of absorption and exhalation of $^{13}CO_2$ produced in the stomach. In addition, influences of intestinal bacteria on the results of UBT may occur, especially in patients with rapid gastric emptying. In our new endoscopic UBT, since it is possible to collect $^{13}CO_2$ gas produced in the stomach before the urea passes through the stomach, the $^{13}CO_2$ values are not affected by absorption, kinetics, or exhalation of $^{13}CO_2$, and then the greater discriminative values of the test are obtained commonly (7). Also, a test meal need not be used. This advantage is clinically important for detection of *H. pylori* in a Japanese population with high prevalence of atrophic gastritis. Since atrophic gastritis consequently decreases *H. pylori* survival, patients with severe atrophic gastritis whose antibody levels were still elevated may have a negative histology and a positive result of serology. Despite the presence of serum

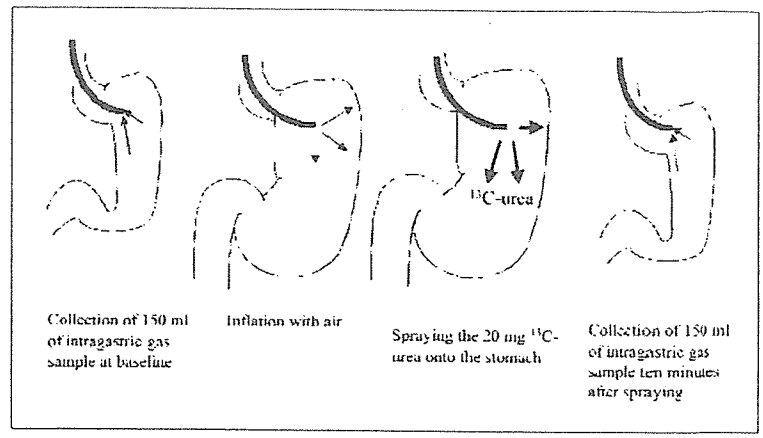


**FIGURE 1** Schematic drawing showing a technique of the new endoscopic $^{13}$C-urea breath test. First, 150mL of intragastric gas sample at baseline was collected through a biopsy channel and the stomach was inflated with air again. Next, 20mL of water containing 20mg of $^{13}$C-urea was sprayed onto the gastric mucosa using a spraying instrument. After completion of spraying, a spraying tube was removed and a gastric gas sample was immediately collected through a biopsy channel using a 50-mL syringe.

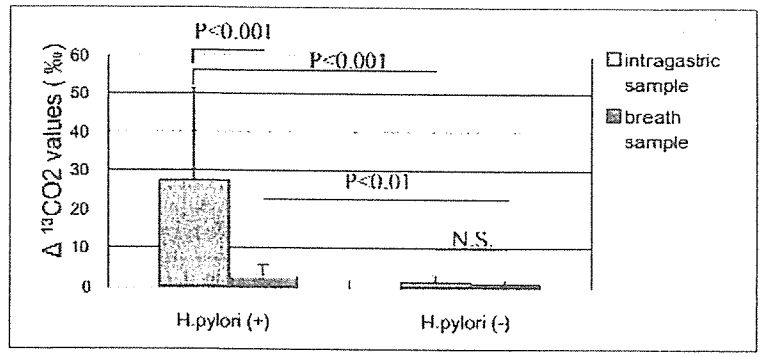


**FIGURE 2** The mean breath and intragastric $\Delta^{13}CO_2$ values for *H. pylori*-positive and -negative patients. The $\Delta^{13}CO_2$ value of intragastric samples in *H. pylori*-positive patients was significantly higher than those of breath samples at 20 minutes, whereas there was no significant difference between the two tests in *H. pylori*-negative patients.

antibodies, failure to detect *H. pylori* in biopsy-based tests could have been due to atrophy or intestinal metaplasia of gastric mucosa (14,15). Similarly, the less sensitive result might be due to low bacterial loads in the stomach of some infected patients after eradication therapy and it might be difficult to interpret the results of UBT to confirm whether infection is cured. In such cases, this new endoscopic UBT is expected to have a higher sensitivity.

Despite the decreased $^{13}$C-urea used from 100mg to 20mg, the optimal cut-off values with intragastric samples was 8‰, higher than those with breath samples, with a sensitivity of 83.7% and a specificity of 100%. With breath samples at 20 minutes using 100mg $^{13}$C-urea, the standard UBT had a sensitivity of 91.8% and a specificity of 93.8%, using 2 ‰ as the best cut-off value. The new endoscopic UBT offered excellent accuracy at earlier sampling time, even at 10 seconds. The duration of 10 seconds is much shorter than that in the rapid urease test. Its speed of diagnosis is very important in clinical practice. Moreover, unlike the RUT, the endoscopic UBT are able to avoid sam-

pling error (16).

In conclusion, our study shows that 10-second endoscopic UBT using a 20-mg dose of $^{13}$C-urea is a rapid, inexpensive, and accurate method for the detection of *H. pylori* infection in clinical practice. It may be a good choice to detect active *H. pylori* infection if endoscopy is indicated for a dyspeptic patient.

## ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Research from the Ministry of Health, Labour and Welfare, Japan (16271201).

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AUTHOR PLEASE CITE FIGURE 1 IN THE TEXT

*Journal of Digestive Diseases* 2007; 8; 8–14 doi: 10.1111/j.1443-9573.2007.00278.x

# Using serum pepsinogens wisely in a clinical practice

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Serum pepsinogen (PG) has been used as biomarkers of gastric inflammation and mucosal status, including atrophic change, before the discovery of *Helicobacter pylori* (*H. pylori*). Serum pepsinogen I (PG I) and pepsinogen II (PG II) levels are known to increase in the presence of *H. pylori*-related nonatrophic chronic gastritis. The measurement of serum PG provides much information on the presence of intestinal metaplasia as well as atrophic gastritis. The eradication of *H. pylori* provokes a significant change in serum PG values: it reduces both PG I and PG II and elevates the PG I to PG II ratio. Recently, the serum PG test method has been the first screening step in Japan, as well as photofluorography. Serum PG tests are used to screen for high risk subjects with atrophic gastritis, rather than as a test for cancer itself. Unlike photofluorography or endoscopy, serum PG screening can identify non-ulcerated differentiated asymptomatic cancer, irrespective of the size and location of the lesion. Most cases detected by the PG method are asymptomatic early gastric cancers and are limited to the mucosa, which are particularly well suited for endoscopic treatment. The PG method can contribute greatly to the patients' quality of life.

KEY WORDS: atrophic gastritis, gastric cancer screening, non-atrophic gastritis, serum pepsinogens I and II.

## INTRODUCTION

Serum PG is classified into two biochemically and immunologically distinct types, namely, PGI and PG II (PG I is also called PGA, and PG II is also called PGC). PG I is produced by chief and mucous neck cells in the fundic glands, while PG II is produced by these cells and also by the cells in the pyloric glands and Brunner's glands.[1–3] It is widely accepted that serum PG levels reflect the functional and morphologic status of stomach mucosa. As the fundic gland mucosa reduces, PG I levels gradually decrease, whereas PG II levels remain fairly constant.[4–6] As a result, a stepwise reduction of the PG I/II ratio is closely correlated with the progression from normal gastric mucosa to extensive atrophic gastritis.

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Serum PG has been used as a biomarker of gastric mucosal status, including atrophic change and inflammation, before the discovery of *Helicobacter pylori* (*H. pylori*). *H. pylori* are now recognized as the main acquired factor in the pathogenesis of peptic ulcer disease and chronic gastritis. The potential mechanisms by which *H. pylori* induces mucosal damage include injury to gastric cells by direct contact of the bacterium through elaboration of enzymes and putative cytotoxins, immune response, and effects of *H. pylori* on the mechanisms that control gastric secretion.[7] Since these changes in gastric secretion affect the serum PG levels, subjects should be divided into two groups according to their *H. pylori* status. Thus, the interpretation of serum PG has changed remarkably since the discovery of *H. pylori*. In this review we reflect on the relevant physiology behind the measurement of serum PG and discuss the relevant literature concerning their use.

## GASTRIC ACID SECRETION

There is a close association between the level of gastric acid secretion and the type of disease affecting the

gastrointestinal tract. Gastro-esophageal reflux disease and duodenal ulcers are likely to occur in patients with high levels of acid secretion,[8,9] whereas gastric ulcers are more likely in patients with moderately reduced secretion.[10] Patients with gastric cancer also have profoundly reduced or absent acid secretion.[11,12]

Patients with duodenal ulcers have increased PG I levels, which were believed to be of genetic origin.[13] Several studies have demonstrated a significant association between serum PG I and gastric secretion,[14–16] whereas a few studies did not show any significant relation.[17] Iijima *et al.*[18] examined the correlation between PG I and maximal acid output and concluded that PG I is influenced not only by parietal cell mass but also by gastric mucosal inflammation induced largely by *H. pylori* infection, which could be responsible for its good correlation with acid secretion in *H. pylori*-infected patients. In using serum PG as a marker of gastric acid secretion, it is necessary to take into account for the *H. pylori* status of the patient.

## *H. PYLORI* INFECTION

*H. pylori* infection is now accepted as the major cause of chronic gastritis and atrophic gastritis is the most common cause of reduced gastric acid secretion.[19,20] Serum PG I and PG II levels are known to increase in the presence of *H. pylori*-related nonatrophic chronic gastritis. In particular, PG II was reported to exhibit a greater rise relative to PG I.[21,22] There was little correlation between PG II and gastric acid secretion because of the wide variety of PG II levels in *H. pylori*-positive subjects.[14] In contrast, in *H. pylori*-negative subjects, PG II is a relatively constant value and correlates with acid secretion, since PG II is derived from both the pyloric gland and the fundic gland.

It is well known that serum PG levels in patients with duodenal ulcers are higher than those observed in *H. pylori* gastritis,[23,24] and increased PG I was believed to be of genetic origin.[13] However, increased PG I levels decrease after *H. pylori* eradication.[23,25,26] Cave *et al.*[27] showed that *H. pylori* sonicate and *H. pylori* lipopolysaccharide stimulate PG release from isolated rabbit gastric glands. This suggests a direct stimulatory effect of *H. pylori* on chief cells. Young *et al.*[28] also showed that purified *H. pylori* lipopolysaccharide increased PG secretion 50-fold while the *E coli* lipopolysaccharide raised this secretion only 12-fold. It was reported that there was no differences in PG secretion between cagA-positive and -negative strains,[29] suggesting that other factors must be involved.

The eradication of *H. pylori* decreases the severity of gastritis and provokes a significant change in serum PG values: it reduces both PG I and PG II and elevates the PG I to PG II ratio.[21,30] To decide whether *H. pylori* has been completely eradicated it is necessary to prove the disappearance of the organisms after eradication therapy. It is sometimes difficult to decide this based on bacterial examinations such as culture tests, histology, and the urease test on endoscopy, because of decreased bacterial density or changes of the organism.[31] Di Mario *et al.*[32] demonstrated that optimal cut-off values to evaluate the success of therapy were: a PG II of 9.47 mg/L, a PG II variation level (the difference between the baseline and after therapy) of 4.54 mg/L, and a PG II delta value (the PG II variation divided by the PG II before therapy of 25% (sensitivity 93%, specificity 91%). Gisbert *et al.*[33] also reported that *H. pylori* eradication was associated with a significant decrease in basal PG II levels that is detected immediately (one month) after completing the treatment. However, the decrease in PG I level occurs progressively for 6 months. They concluded that the measurement of PG I concentration has a limited usefulness in the diagnosis of *H. pylori* reinfection after successful eradication, although PG II determination could be more useful in this situation. Furuta *et al.*[34] determined the optimal cut-off values for percentage changes in serum PG I/PG II ratios. The values were tentatively set as +40%, +25%, and +10% when the serum PG I/PG II ratios before treatment were less than 3.0, not less than 3.0 but less than 5.0, and not less than 5.0, respectively. The serum PG method has an advantage because as no endoscopy is required, repeated examinations will be accepted by patients. Thus, the serological method may be a useful non-invasive method for determining the eradication of *H. pylori*.

## ATROPHIC GASTRITIS AND INTESTINAL METAPLASIA

The clinical importance of atrophic gastritis with intestinal metaplasia is related to the fact that it increases the risk of gastric cancer development.[35–37] In the process of carcinogenesis, at least for intestinal types of gastric carcinomas, it was proposed that the gastric mucosa evolves through the stages of chronic active gastritis, glandular atrophy, intestinal metaplasia, and dysplasia before developing into gastric adenocarcinoma.[17] The risk of gastric neoplasia rises with the increasing grade and extent of atrophic gastritis.[38] Atrophic gastritis is usually diagnosed with endoscopy and biopsies. However, there is a significant potential sampling error in identifying intestinal metaplasia by a random biopsy because the intestinal metaplasia of the gastric mucosa was reported to be patchy.

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It is now clear that intestinal metaplasia is a part of the spectrum of atrophic gastritis with *H. pylori* infection. Xia *et al.*[39] showed that the prevalence of intestinal metaplasia was significantly higher at the gastric antrum of the patients with an *H. pylori* infection compared with uninfected subjects. However, only some of the infected patients go on to develop intestinal metaplasia, suggesting that factors other than *H. pylori*, such as environmental and host genetic factors, may contribute to the progression from atrophic gastritis to intestinal metaplasia. In our previous study,[40] the overall prevalence of intestinal metaplasia was 52% (455/878) and it was higher in subjects with lower PG I/II ratios and lower PG I values. Intestinal metaplasia was found in 252 (82%) of 299 subjects with a PG I/II ratio of less than 2.5 and in 58 (88%) of 66 subjects with a PG I value of less than 25 ng/mL. Thus, it is potentially possible that serum PG is used as a screening test for high risk subjects with atrophic gastritis and intestinal metaplasia. The measurement of serum PG provides much information on the presence of intestinal metaplasia as well as atrophic gastritis.

In Japan, several studies[22,37] have shown that the prevalence of infection is strongly associated with age and this age-related increase in infection falls in the elderly. Thus, the absence of serum antibodies in patients with active or previous infection seems to increase in the elderly.[41,42] It is possible that patients who had a previous infection and do not have serum antibodies are not detected as a high risk group for gastric cancer, despite the presence of severe atrophic gastritis. Measuring serum PG can detect patients with extensive atrophic gastritis, regardless of their *H. pylori* status.

## GASTRIC CANCER SCREENING

Recently, the serum PG method has become the first screening step in Japan, instead of photofluorography,[43–46] because several problems in the latter method have been noted, such as its cost-effectiveness, the risks to those screened of X-ray exposure, and the low sensitivity of photofluorography (less than 40%) in detecting early gastric cancer.[46] The serum PG method has made it possible to screen large populations without the need for endoscopy. Serum PG, especially PG I and PG I/II ratio, have been proven to be markers for atrophic gastritis.[47–49] Therefore, the measurement of serum PG has recently drawn attention as a candidate for a new screening test for gastric cancer in Japan.[43–45]

### Cut-off point of pepsinogen

Although several determinations of a suitable cut-off point for gastric cancer screening have previously been reported, using a serum PG I concentration of less than 70 ng/mL and a PG I/II ratio of less than 3.0 as the cut-off point has been widely accepted in Japan.[43,45]

Dinis-Ribeiro *et al.*[50] demonstrated the validity of the PG test for gastric carcinoma, dysplasia or chronic atrophic gastritis screening. Forty-two data sets were analyzed: 27 population-based screening studies ($n$ = 296 553) and 15 sets of selected individuals ($n$ = 4385). Pooled pairs of sensitivity and false positive rates (Fpr) for PG I ≤70; PG I/II ratio ≤3.0, for PG I ≤50; PG I/II ratio ≤3.0, and for PG I ≤30; PG I/II ratio ≤2.0, were sensitivity 77%/Fpr 27%, sensitivity 68%/Fpr 31%, and sensitivity 52%/Fpr 84%, respectively. Positive predictive values varied between 0.77% and 1.25%, and negative predictive values varied between 99.1% and 99.9%. Kitahara *et al.*[45] report a sensitivity of 84.6% and a positive predictive value of 0.81% with a cut-off point of PG I ≤70 and PG I/II ratio ≤3.0. Miki *et al.*[47] also used the same cut-off point and showed a sensitivity of 96.0% and a positive predictive value of 1.3%. When using a low cut-off point of PG I ≤50, the sensitivity and the positive predictive value were reduced to 68% and 0.7%, respectively. Hattori *et al.*[51] reported a sensitivity of 66.7% and a positive predictive value of 1.3% with a cut-off point of PG I ≤50 and PG I/II ratio ≤3.0.

### Comparison to X-ray method

In Japan, mass-screening programs for gastric cancer by indirect roentogenography are widely used, because gastric cancers are potentially curable if they are diagnosed at early stages, unlike cancers of the lung, liver, and pancreas. In fact, the 5-year survival rate of gastric cancer in Osaka, Japan, where gastric cancer screening is conducted, is 34.1%,[52] a much higher proportion than that in Detroit, where its screening has not been promoted.[53] Since most patients with abdominal symptoms can easily go to the hospital where a further examination by endoscopy or roentogenography can be done, it is likely that patients with the advanced cancer are not included in gastric cancer screening. Consequently, the proportion of early gastric cancer becomes larger in a Japanese screening test. In fact, a number of studies have reported that screen-detected gastric cancers showed an earlier stage distribution and had a lower case fatality rate than symptom-diagnosed cases. The proportion of early gastric cancer among screen-detected cases is 15–30% higher than among symptom-diagnosed cases.[54] Besides, the sensitivity of the X-ray method has been found to be less than 40% in detecting early gastric cancer and greater than 90% in detecting advanced gastric cancer.[16] Thus the

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sensitivity of PG screening for gastric cancer seems superior to that of X-ray method when based on the results of endoscopic examination.

According to the standardized procedure proposed by the Japanese Society of Gastroenterological Mass Survey, seven consecutive photofluorograms, covering the whole area of the stomach, are taken for each screened individual, using roll films that are 100 mm in width. Trained radiographic technicians take the photofluorogram and two gastroenterologists examine the films. A screened individual with a suspected abnormality is referred for further diagnostic examinations, including endoscopy and a full-size radiography. Although the sensitivity (66.1–90.1%) and specificity (77.2–92.0%) of X-ray method showed an acceptable accuracy,[55–57] the identification of false negative cases is a critical part of quantifying these indices. If the photofluorography with direct radiography is used for gastric cancer screening, or if more than seven photofluorograms are taken, false negative cases might decrease.

On the other hand, we have to consider the false-negative rate in evaluation of the screening method. Were there any cases in which the X-ray methods were suggestive but the PG levels were not? I have been very anxious on this point. We previously reported the lower detection rate of gastric cancer in the elderly.[5] In this previous study, the percentage of cancers detected by the PG method was similar (0.15% in subjects less than 40 years of age; 0.14% in those 60 or older), whereas those by the X-ray method were 0.01% in those less than 40-years old and 0.23% in those aged 60 or older. This suggested that some of the older patients with gastric cancer might be missed by the PG method. As suggested by a referee of this article, it is almost certain that there would be some cancer patients with negative results using the PG method who were detected by the X-ray method.

In subjects with mild atrophy, gastric cancer originating in the pyloric gland region is difficult to detect by the PG method. Similarly, in the small type cancer, as the cancer was limited to a small part of the fundic gland area, the serum PG I level and PG I/II ratio were only slightly decreased. The PG method is used as a screening test for high risk subjects with atrophic gastritis, rather than as a test for cancer itself, and thus there is a possibility that the PG method might miss cancer patients without atrophic gastritis.

### Comparison to endoscopy

Endoscopic screening is highly effective for gastric cancer, but it is relatively expensive. However, in the absence of screening, patients present with advanced disease, and their prognosis is poor. There is a nationwide program for the detection of gastric cancer in Chile using screening endoscopy in symptomatic patients.[58] Before these screening programs, only 40% of patients who were found to have gastric cancer could be treated surgically, and there was only a 3% 5-year survival rate. After the induction of endoscopic screening programs, there has been a 75% 5-year survival rate because they have markedly increased the number of early gastric cancers. Dan *et al.*[59] also reported the validity of endoscopic screening for gastric cancer in China. The screening of their cohort of 199 000 subjects prevented 743 gastric cancer deaths and saved 8234 absolute life years. The cost of averting one cancer death is $US 247 600. They conclude that screening of a high risk group of Chinese men from 50- to 70-years old is highly cost effective. Although certain lesions are difficult to detect by the X-ray method; for example, small or flat lesions and even large lesions located in the cardia or on the anterior wall, such cancers can be easily detected by endoscopy. However, who should pay for asymptomatic screening examinations?

On the other hand, the problem of false negatives has been unclear. Hosokawa *et al.*[60] reported a false-negative rate of 22.2% when a new gastric cancer lesion was detected within 3 years by follow-up endoscopy. Nishizawa *et al.*[61] also demonstrated that 6 gastric cancers which were discovered in subjects with a previous definition of normal in a follow-up survey, were advanced. Although endoscopic screening is highly effective, it does not have a sensitivity of 100% and may result in incidental diseases, including endoscopy-related infections such as viral hepatitis or *H. pylori*. It goes without saying that the screening method should avoid such complications during the procedure.

### Advantages and disadvantages of serum pepsinogen method

Although a gastric cancer screening system using a double contrast barium X-ray was introduced in the 1960s throughout Japan, 49 213 people died from gastric cancer in 2002.[62] This suggests that the screening program is unable to cover a small proportion of the high risk population[63] and only a small proportion of people screened positive by serum PG are also given positive results by photofluorography.[64] The test performed for the first time in a population would detect prevalent cases at relatively advanced stages, while the test conducted subsequently in the same population would detect cases at less advanced stages occurring during the screening intervals.[65] Therefore, a test that is

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highly sensitive for the initial prevalent screening may be less so for subsequent incident screenings.[51] Although the serum PG method has been criticized for its relatively low specificity, by using it we would avoid missing gastric cancer in a mass screening.

Serum PG is used as a screening test for high risk subjects with atrophic gastritis rather than as a test for cancer itself. However, unlike photofluorography or endoscopy, serum PG screening can identify non-ulcerated differentiated asymptomatic cancer, irrespective of the size and location of the lesion. Ohata *et al.*[66] reported that cases detected only by the PG method were all asymptomatic early gastric cancers and 89% were limited to the mucosa, and thus are particularly well suited for endoscopic treatment. This suggests that the PG method can contribute greatly to patients' quality of life by detecting cancer in its early stages. On the other hand, in subjects with mild atrophic gastritis, gastric cancer originating in the pyloric gland region, including in an advanced stage, is difficult to detect by the PG method. Therefore, symptomatic subjects or PG method-negative subjects should be screened by a barium X-ray examination instead of endoscopy. The serological examination of *H. pylori* antibody is additional alternative to the X-ray. *H. pylori* are recognized as one of the possible causes of gastric carcinoma. Kikuchi *et al.*[67] reported that among subjects younger than 40 years old, early stage carcinoma has a stronger association with *H. pylori* than advanced carcinoma, and intestinal- and diffuse-type carcinomas have an association with *H. pylori*. It has been demonstrated that the percentages of those with severe serological atrophy increased with age from 10% in those aged 40–49 years to 38% in those aged 70 and more, and the percentages of those with mild serological atrophy were about 30%, independent of age.[68] Although the prevalence of *H. pylori* infection in Japan has fallen in recent years,[22] those who are infected remain at risk of gastric cancer. An *H. pylori* infection was detected in up to 70% of the population by the age of 40 years in Japan.[22] Since early life acquisition of *H. pylori* has been considered to increase the risk of developing gastric cancer,[69] infected individuals aged 40–50 years (belonging to the age group with the largest number of people in Japan), will be at higher risk of gastric cancer in the near future.

Despite the world decline in incidence and mortality, gastric cancer is a leading cause of cancer death in many countries.[70] The high prevalence of intestinal metaplasia among *H. pylori* infected patients suggests that the risk of developing gastric cancer will continue to remain high. Since gastric cancers are potentially curable if they are diagnosed at the early stages, it is insufficient to check the *H. pylori* antibody alone to detect subjects with severe atrophic gastritis. The PG test method is needed for *H. pylori*-positive subjects. Conversely, low-risk subjects who do not have atrophic gastritis or *H. pylori* infection can be detected by the combined screening method using the PG test and *H. pylori* serology. We have to make a further study to determine whether subjects with low-risk for gastric cancer can skip an annual screening.

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Inflammopharmacology 15 (2007) 1–5

DOI 10.1007/s10787-007-0895-z
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Inflammopharmacology

Review

# Salivary gland scintigraphy in gastro-esophageal reflux disease

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Received 12 July 2006; accepted 10 September 2006

**Abstract.** Gastro-esophageal reflux disease (GERD) is associated with a decreased salivary flow as well as gastric acid production. This study therefore aimed to investigate functional disorders of salivary glands in patients with GERD. *Methods:* Thirty-one consecutive patients with GERD underwent salivary gland scintigraphy. *Results:* If the results defined the optimal cutoff point for determining the decreased salivary secretion as 51% in parotid glands and 36% in submandibular glands, a decreased salivary secretion of right parotid gland, left parotid gland, right submandibular gland, and left submandibular gland was found in 39%, 32%, 36%, and 58%, respectively. Overall, salivary function disorder of at least one major salivary gland was found in 24 patients (78%) with GERD. There was no difference in the incidence of impaired salivary function between GERD patients with and without erosive esophagitis. Salivary gland function was more frequently diminished than expected in GERD. We concluded that the presence of impaired salivary gland function was considered to be one of risk factors for developing GERD symptoms.

**Key words:** Salivary scintigraphy; GERD; Washout ratio

## Introduction

The major abnormalities associated with the development of GERD are related to incompetence of the antireflux barrier and impairment of esophageal luminal clearance after reflux [1, 2]. During esophageal acid clearance, salivation plays an important role in defending the esophageal mucosa [3, 4]. Esophageal clearance of regurgitated gastric contents is determined by three mechanisms: gravity, propulsive peristalsis, and salivary secretion [5]. The reduction of the majority of the reflux volume occurs within the first two swallows subsequent to a reflux event, with subsequent swallows producing an acid neutralization of the lining of the esophageal mucosa which eventually returns the mucosa to a pH above 4.0 [6]. Although saliva plays an important role in esophageal acid clearance, facilitation of acid clearance has only been addressed via work on various medications which have been shown to have prokinetic effects on the esophagus [7, 8]. Little attention has been paid to the role of salivation in esophageal clearance. On the other hand, salivary secretion is needed as soon as acidic gastric contents reflux into the esophagus. Immediate salivary response to gastro-esophageal is not evaluated by saliva collection methods. The aim of this study is to evaluate the salivary gland function by means of dynamic salivary scintigraphy and to assess correlation between salivary function disorders and developing GERD.

## Patients and methods

Thirty-one consecutive patients (mean age 55 year old, male/female = 13/18) with GERD underwent salivary gland scintigraphy. GERD was diagnosed by endoscopy and gastro-esophageal reflux self-report questionnaires. As shown in Figure 1, erosive esophagitis was found in 14 of 31 patients with GERD symptoms, whereas the mucosal break of the esophagus was not detected in the remaining 17 patients. Of 14 patients with erosive esophagitis, 11 were classified into grade A according to the Los Angels classification. Three were classified into grade B and severe esophagitis, grade C or D, was not found in the present study. All of the patients were asked to refrain from drugs known to affect salivary secretion, such as anti-depressives, anti-psychotics and anti-hypertensives, which have an anti-cholinergic or anti-adrenergic action [9], for at least one week prior to salivary scintigraphy. Thirteen healthy volunteers (6 men, 7 women, average age 22.6 ± 4.9 years) were also enrolled to define the criteria of salivary dysfunction.

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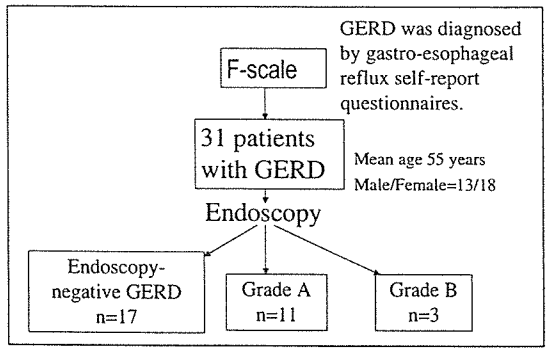


**Fig. 1.** Flow diagram of the study.

*Salivary scintigraphy*

After intravenous administration of 180 to 200 Mbq 99 mTc-pertechnetate, imaging was begun immediately in order not to miss the initial portion of the curve from which parameters were obtained. A single-headed gamma camera was used with a high-resolution collimator and a symmetrical 20% window around a 140 keV photopeak. Anterior sequential imaging was performed every minute for 40 min. At 20 min after injection of radionuclide, a lemon candy was administrated intraorally to stimulate salivary secretion. Regions of Interests (ROI) were selected on the individual submandibular and parotid glands, oral cavity, and thyroid gland (Fig. 2). Time activity curves were drawn for each of these. A background area was selected in shoulder region. The salivary time activity curves were subjected to a two-step background subtraction protocol as follows. Time activity curve of background was normalized to the area of individual salivary gland and subtracted from individual organ curves to yield stage one subtracted curves.

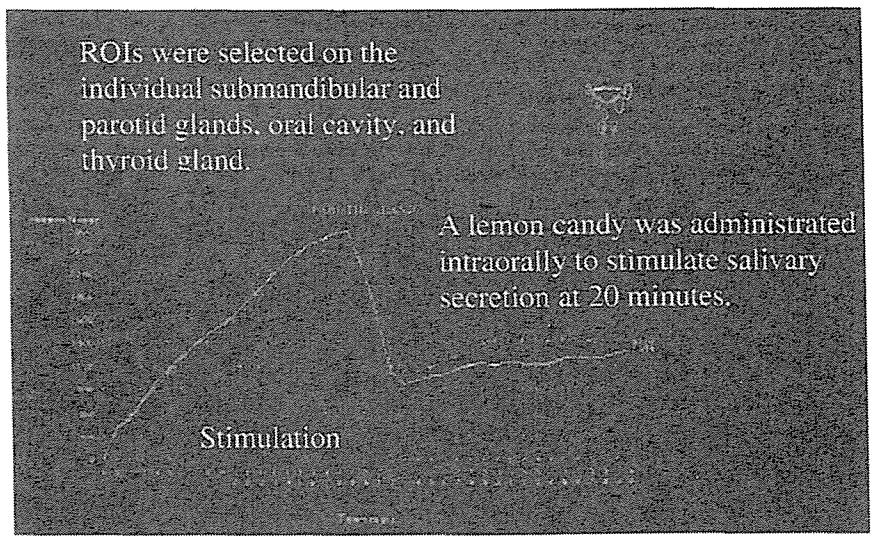


**Fig. 2.** Time activity curves of two parotid glands.

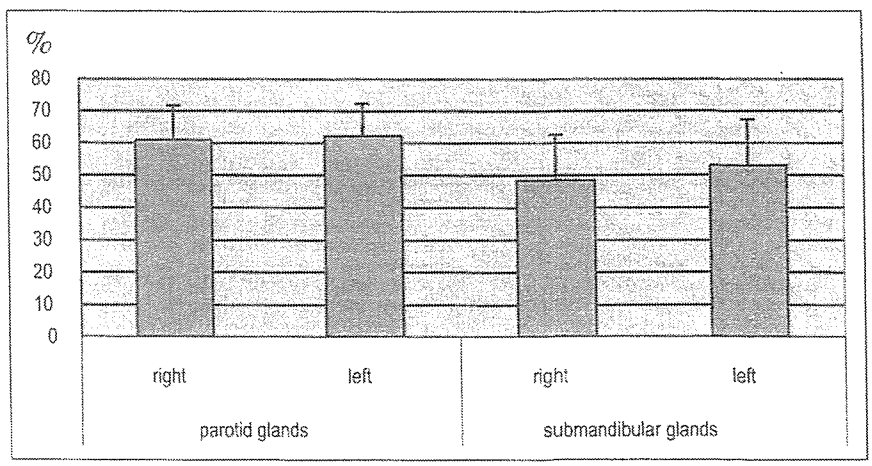


**Fig. 3.** Washout rates of 13 healthy volunteers in each four major salivary gland.

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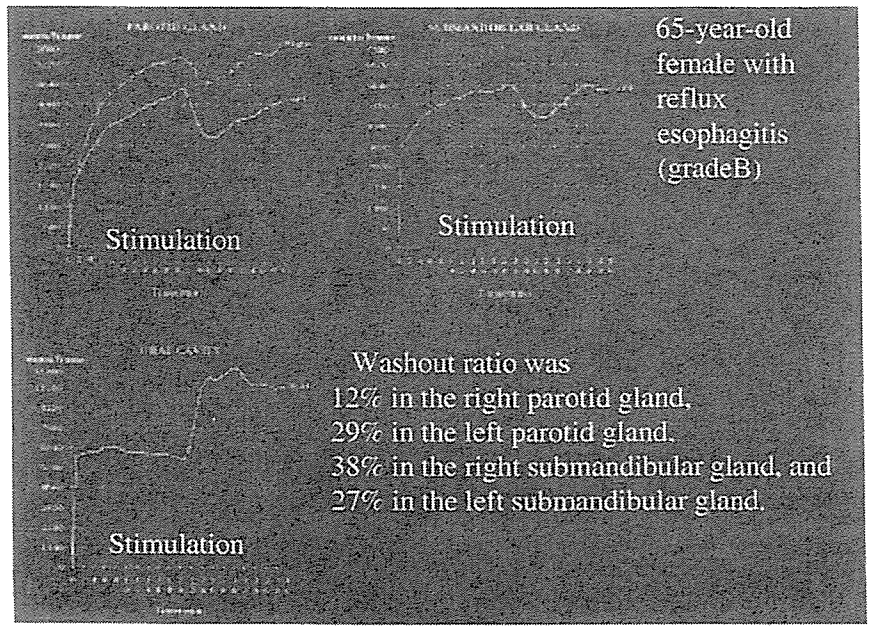


**Fig. 4.** A representative time-activity curve generated from salivary scintigraphy in patients with grade B reflux esophagitis.

Washout ratio was defined as the following formula; peak count before lemon candy administration-lowest count after administration/ peak count before administration. Washout ratios represent the function of saliva excretion of the major salivary glands. They were also asked to abstain from swallowing, chewing, sucking or any other mechanical stimulation of salivary flow.

## Results

As shown in Figure 3, the washout ratios of parotid glands were higher than those of submandibular glands in healthy volunteers. The washout ratio was 60.8 ± 10.7 % in right parotid gland, 62.0 ± 10.1 % in left parotid gland, 48.2 ± 14.0 % in right submandibular gland, and 52.8 ± 14.3 % in left submandibular gland. Since the overall washout ratio was 61.3 ± 10.2 % in parotid glands and 50.5 ± 14.1 % in submandibular glands, the results defined the optimal cutoff point for determining the decreased salivary secretion (mean-SD) as 51 % and 36 %, respectively. Figure 4 demonstrates reduced washout ratios of all major salivary glands in 65-year-old patient with grade B reflux esophagitis.

Results of salivary scintigraphy in 31 GERD patients were demonstrated in Table 1. The mean washout ratio of GERD patients was 55.8 % in the right parotid gland, 57.0 % in the left parotid gland, 53.5 % in the right submandibular gland, and 46.9 % in the left submandibular gland. According to the above-mentioned cutoff points, a decreased salivary secretion was found in 12 (39 %) in the right parotid gland, 10 (32 %) in the left parotid gland, 11 (36 %) in the right submandibular gland, and 18 cases (58 %)in the left submandibular gland.

Overall, salivary function disorder of at least one major salivary gland was found in 24 patients (78 %) with GERD. Ten of 14 patients (71 %) with reflux esophagitis and 14 of 17 patients (82 %) without reflux esophagitis had decreased salivary excretion after stimulation. There was no difference in the incidence of impaired salivary function between GERD patients with and without erosive esophagitis.

## Discussion

Esophageal acid clearance consists of two processes: after reflux, most acid volume is cleared by esophageal peristalsis and gravity leaving only a minimal residue that sustains an acidic pH in the esophageal mucosa until it is neutralized by swallowed saliva 6 [10]. Salivary volume has been reported to be lower in patients with reflux esophagitis than in normal controls [3, 8]. Reduction in salivary function has been considered in the pathogenesis of reflux esophagitis. In the present study, salivary function disorder of at least one major salivary gland was found in 78 % of patients with GERD. There was no difference in the incidence of impaired salivary function between GERD patients with and without erosive esophagitis.

The aim of the present study was to test whether patients with GERD symptoms suffer from impaired salivary secretion. Chronic salivary dysfunction is clinically significant because it may lead to rampant dental destruction, mucosal infection and a variety of speech and digestive disturbances, and in itself may seriously impair the patient's quality of life [11, 12]. It has frequently been shown that salivary secretions decrease with age, the age-related decline being more

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**Table 1.** Results of salivary scintigraphy in 31 GERD patients.

| Age | Gender | diagnosis | grade | washout ratio | | | |
|---|---|---|---|---|---|---|---|
| | | | | right PG | left PG | right SMG | left SMG |
| 26 | M | EE | A | 49 | 50 | 8 | 30 |
| 27 | F | EE | A | 49 | 51 | 100 | 100 |
| 32 | M | EE | A | 20 | 20 | 10 | 10 |
| 33 | F | EE | A | 76 | 76 | 44 | 45 |
| 35 | M | EE | A | 65 | 57 | 77 | 79 |
| 37 | M | EE | A | 62 | 74 | 84 | 89 |
| 48 | M | EE | A | 61 | 65 | 38 | 39 |
| 56 | M | EE | A | 22 | 35 | 35 | 35 |
| 69 | F | EE | A | 49 | 62 | 62 | 61 |
| 70 | F | EE | A | 62 | 51 | 66 | 72 |
| 70 | F | EE | A | 66 | 72 | 70 | 71 |
| 38 | F | EE | B | 50 | 63 | 37 | 31 |
| 28 | M | EE | B | 63 | 67 | 47 | 48 |
| 49 | M | EE | B | 80 | 80 | 70 | 68 |
| 56 | M | NERD | M | 80 | 65 | 50 | 50 |
| 57 | F | NERD | M | 0 | 0 | 5 | 0 |
| 58 | F | NERD | M | 40 | 40 | 35 | 35 |
| 58 | F | NERD | M | 65 | 66 | 63 | 63 |
| 59 | F | NERD | M | 80 | 80 | 67 | 48 |
| 61 | M | NERD | M | 49 | 25 | 60 | 60 |
| 63 | F | NERD | M | 40 | 25 | 26 | 30 |
| 69 | F | NERD | M | 44 | 48 | 54 | 53 |
| 71 | M | NERD | M | 45 | 68 | 65 | 0 |
| 73 | F | NERD | M | 65 | 72 | 86 | 86 |
| 76 | M | NERD | M | 59 | 65 | 65 | 45 |
| 85 | M | NERD | M | 67 | 67 | 51 | 47 |
| 48 | F | NERD | M | 75 | 73 | 55 | 40 |
| 57 | M | NERD | M | 53 | 40 | 10 | 10 |
| 41 | F | NERD | M | 93 | 92 | 43 | 43 |
| 54 | F | NERD | M | 47 | 48 | 39 | 45 |
| 55 | F | NERD | M | 80 | 80 | 70 | 60 |

EE: erosive esophagitis, NERD: non-erosive reflux dosease, PG: parotid gland, SMG: submandibular gland

marked in women and than in men [13]. Since the prevalence of GERD increased with age and was higher than in males in the elderly, the development of reflux esophagitis could be favored by the age related loss of the salivary response to acidic gastroesophageal reflux.

A method for measuring the flow of whole salivary gland in which we ask each participant to spit into a pre-weighed collection tube once each minute for 3 min is unable to evaluate each salivary gland separately [14]. In contrast, scintigraphy has been used to quantify the uptake and the secretion in individual salivary glands. It has been reported that of the total salivary secretion of 1.5 L/day approximately 75% is secreted by the submandibular glands, 20% by the parotid glands and the rest by sublingual and other salivary glands [15]. Therefore, a volume of salivary secretion should differ between patients with impaired salivary glands and those with impaired submandibular glands.

The parotid gland predominantly secretes a protein rich saliva which includes enzymes like amylase while the submandibular secretions are mucin rich which are useful in lubricating the bolus of food [16]. It has been also reported that submandibular glands showed a greater tendency towards profuse unstimulated secretions [17]. These suggest that GERD symptoms caused by impaired salivary secretion may differ widely. It is desirable to evaluate individual salivary gland function separately.

Salivary flow is increased during esophageal acid perfusion, and saliva may act as an endogeneous antacid to protect against symptomatic gastroesophageal reflux [4]. Normal salivary secretion decreases the time that acid is in contact with the esophageal mucosa. The buffering ability of saliva is supplied mainly by bicarbonate [18]. Decreased salivary secretion results in decreased bicarbonate concentration and, therefore, insufficient acid neutralization. Since salivary gland function was frequently diminished in patients with GERD in the present study, decreased saliva seems to have an important role in developing GERD.

Several investigators have demonstrated the impairment of the salivary epidermal growth factor secretory response to mechanical and chemical stimulation of the esophagus in patients with GERD [19–21]. Epidermal growth factor is thought to play an important role in the repair of damaged esophageal mucosa. Furthermore, alterations in the salivary electrolytic composition can influence the protective capacity of the regional mucous membrane [17, 22]. It has been also reported that the pH and volume of saliva can have a clear correlation with some of the symptoms of patients with laryngo-pharyngeal reflux [23]. Costa et al. [24] have also shown the direct correlation between salivary volume and proximal episode of reflux on the esophageal pH-metry. Therefore, it is more desirable that not only volume of saliva but also composition of saliva is evaluated at the same time in GERD patients. Salivary scintigraphy is relatively safe, well tolerated, and easy to perform, and enables an assessment of the function of all major salivary glands individually. Although it can easily assess the salivary secretion in a short time, further studies should be carried out to evaluate the correlation between washout ratios and composition of saliva. We concluded that the presence of impaired salivary gland function was considered to be one of risk factors for developing GERD symptoms.

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1. AK, 10.01.2007 IP 0895

Histol Histopathol (2007) 22: 641-649

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Histology and Histopathology
Cellular and Molecular Biology

# Down regulation of gastric and intestinal phenotypic expression in Epstein-Barr virus-associated stomach cancers

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**Summary.** Aims: We have previously demonstrated the importance of gastric and intestinal phenotypic expression for stomach carcinogenesis. In this study, we focused on Epstein-Barr virus (EBV)-associated stomach cancers, with special attention to Cdx2.

Methods and Results: We evaluated the expression of gastric and intestinal phenotypic markers by immunohistochemistry in 35 EBV-positive [EBV (+)] and 75 EBV-negative [EBV (-)] stomach cancers in Colombia. The lesions were divided phenotypically into gastric (G), gastric-and-intestinal mixed (GI), intestinal (I), and null (N) phenotypes. In the EBV (+) cases, the lesions were divided phenotypically into 9 G (25.7%), 1 GI (2.9%), 3 I (8.6%), and 22 N (62.9%) types. Similarly, the EBV (-) lesions were also classified phenotypically as 15 G (20.0%), 19 GI (25.3%), 24 I (32.0%), and 17 N (22.7%) types. The proportion of N type EBV (+) lesions was higher than for their EBV (-) counterparts ($P<0.0001$). The expression of Cdx2 and MUC2 was also found to be significantly lower in EBV (+) than in EBV (-) stomach cancers ($P=0.0001$; $P<0.0001$). Cdx2 expression in the intestinal metaplastic glands present in non-neoplastic mucosa surrounding EBV (+) lesions was also significantly lower than in EBV (-) tumors ($P=0.016$) despite no evidence of EBV infection.

Conclusions. EBV (+) stomach cancers are characterized by low expression of intestinal phenotype markers, including Cdx2, and only occasional gastric phenotypic expression.

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**Key words:** Stomach cancer, Epstein-Barr virus, N type, Cdx2, MUC2

## Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus implicated in the etiology of many human malignancies, such as Burkitt's lymphoma (zur Hausen et al., 1970), nasopharyngeal carcinoma (Raab-Traub, 1992), Hodgkin's disease (Weiss et al., 1989), lymphoproliferative disorders in immunodeficiency patients (Hanto et al., 1981), and stomach cancer (Fukayama et al., 1998). EBV-associated stomach cancer account for about 10% of all gastric neoplasms (Shibata and Weiss, 1992; Tokunaga et al., 1993), although *Helicobacter pylori* (*H. pylori*) infection is a more important factor for stomach carcinogenesis. There are differences in the proportions of EBV-associated stomach cancers from country to country (Takada, 2000), and the rate in Colombia is significantly higher than in places with heavy gastric cancer burdens, such as Japan, China and Korea (Carrascal et al., 2003). The lesions due to EBV infection resemble nasopharyngeal lymphoepitheliomas and are named lymphoepithelioma-like carcinomas, and specific antigens such as EBV-determined nuclear antigen-1 (EBNA-1) and EBV-encoded small RNA-1 (EBER-1) point to the presence of the virus (Burke et al., 1990; Yanai et al., 1997a,b). Stomach cancers associated with EBV infection were more common in the upper stomach (cardia and fundus), and histologically are most often of undifferentiated type (Yanai et al., 1997). Each EBV-associated stomach cancer appears of monoclonal origin arising from a single EBV-infected cell (Imai et al., 1994). However, there are many obscure points with regard to the

relations between EBV infection and stomach carcinogenesis.

Gastric and intestinal phenotypic expression is important for the histogenesis of stomach cancer (Tatematsu et al., 2003). Several reports have indicated that it is possible to analyze the phenotypic expression of each gastric cancer cell using gastric and intestinal epithelial cell markers (Egashira et al., 1999; Kawachi et al., 2003; Mizoshita et al., 2003; Tsukamoto et al., 2005). Thus, division into gastric (G), gastric-and-intestinal mixed (GI), intestinal (I), and null (N) phenotypes is possible, independent of the histological classification (Tajima et al., 2001; Tatematsu et al., 2003; Inada et al., 2004; Mizoshita et al., 2004a). However, the relation between EBV infection and phenotypic expression has yet to be clarified in detail in stomach cancers associated with the virus. Several authors have demonstrated a correlation between EBV infection and phenotypic marker expression (Lee et al., 2004; Nakamura et al., 2005), but concrete conclusions have yet to be drawn.

In the present study, we therefore evaluated the expression of gastric and intestinal phenotypic markers by immunohistochemistry in 110 stomach cancers in Colombia, along with adjacent non-neoplastic mucosa. The EBV infection status was also evaluated by in situ hybridization in these lesions.

## Materials and methods

### *Samples and tissue collections*

The study subjects were stomach carcinoma patients newly diagnosed during the period between September 2000 and June 2003 in the following four reference hospitals in Colombia: Instituto de los Seguros Sociales "Rafael Uribe Uribe", Hospital Universitario del Valle, Hospital San Juan de Dios in Cali, and Instituto Nacional de Cancerologia in Bogota. We examined EBER-1 expression among formalin-fixed paraffin-embedded blocks of 368 cases with gastric carcinomas, and found that 42 cases were positive (Koriyama et al., manuscript submitted). We selected paraffin-embedded blocks of 35 cases with gastric carcinomas, mainly surgically resected tumors, for the present analysis. Seventy-five EBER-1-negative cases were selected matched for gender, age (5-year category), histology [differentiated (well and moderately differentiated) and undifferentiated (poorly differentiated and signet-ring cell) types in majority area], and area (Bogota or Cali) (Table 1). The Institutional Review Board of the Faculty of Health, Universidad del Valle, Cali, Colombia, approved this study and all subjects gave informed consent.

The patient group comprised 84 men and 26 women, aged 59.0±12.5 years (mean ± standard deviation). All specimens were fixed in 10% buffered formalin. Classification was made according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998) in spite of widely used Lauren's classification (Lauren, 1965), which is inadequate for the studies of histogenesis of stomach cancers and phenotypic expression at the cellular level, because it confuses intestinal phenotypic cancer cells with "diffuse" structure and gastric phenotypes with the "intestinal" (glandular or tubular) morphology. Carcinomas with adjacent non-neoplastic mucosa were serially cut into 5-mm slices in parallel with the lesser curvature and embedded in paraffin, and then sectioned and stained with hematoxylin-eosin (HE) for histological examination.

### *In situ hybridization of EBER-1*

EBER-1 *in situ* hybridization was performed with a kit according to the manufacturer's instructions (Dako, Glostrup, Denmark). Paraffin sections 4 $\mu$m thick were deparaffinized, rehydrated, predigested with proteinase K for 15 min at room temperature and hybridized with a fluorescein-conjugated EBV oligonucleotide probe (EBER PNA Probe/Fluorescein) for 90 min at 55°C. After washing with 0.1M TBS (pH 10) for 25 min at 55°C, hybridization signals were detected by serial incubation with anti-fluorescein isothiocyanate rabbit polyclonal antibody (Anti-FITC/AP), and then with biotinylated Mouse IgG as secondary antibody, followed by the avidin biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% $H_2O_2$ and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. From the results, EBER-positive and EBER-negative lesions were defined as EBV-positive [EBV (+)] and EBV-negative [EBV (-)](Fukayama et al., 2001).

### *Histological and immunohistochemical examination*

Immunohistochemical staining was carried out with monoclonal antibodies against the following antigens:

Table 1. Correlations between clinicopathologic findings and EBV infection in 110 stomach cancers.

| Clinicopathologic findings | EBV (+) (n=35) | EBV (-) (n=75) | P-value |
|---|---|---|---|
| Age | | | |
| Years (mean±SD) | 58.9±13.6 | 59.1±12.0 | P=0.88 |
| Sex | | | |
| Male(n=84) | 28 | 56 | P=0.63 |
| Female(n=26) | 7 | 19 | |
| Histological classification [a] | | | |
| Differentiated type (n=44) | 13 | 31 | P=0.835 |
| Undifferentiated type (n=66) | 22 | 44 | |

SD: standard deviation. [a]: Classified based on structure of elements. "Differentiated type" includes tubular and papillary types, whereas "Undifferentiated type" consists of signet-ring cell and poorly differentiated types.

MUC5AC (CLH2, 1:500; Novocastra Laboratories, Newcastle upon Tyne, UK); MUC6 (CLH5, 1:500; Novocastra Laboratories); MUC2 (Ccp58, 1:500; Novocastra Laboratories); villin (12, 1:20,000; Transduction Laboratories, Lexington, KY, USA); and Cdx2 (Caudal-related homeobox gene 2) (CDX2-88, 1:100; BioGenex, San Ramon, CA, USA).

For gastric and intestinal phenotypic markers, we used normal gastric mucosa and ileum as controls. The precise procedures for immunohistochemical techniques were as previously described (Tatematsu et al., 2003; Mizoshita et al., 2003, 2004b; Tsukamoto et al., 2005). Briefly, 4 $\mu$m-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% $H_2O_2$/methanol solution, antigen retrieval was conducted for detection of binding of the above-mentioned antibodies with 10 mM citrate buffer, pH 6.0, in a microwave oven for 10 min at 98°C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody, followed by the avidin biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% $H_2O_2$ and 0.05% DAB. Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Three independent pathologists (N.H., T.M., and T.T.) judged the histology and immunohistochemical staining for the phenotypic markers and Cdx2. Reactivity for the phenotypic markers and Cdx2 was scored according to the percentage of positively stained tumor cells in the section areas on a 4-point-scale: score 0, <10%; score 1, 10-33%; score 2, 34-66%; score 3, 67-100%. A result was considered positive (+) with a score of 1 or more.

### *Phenotypic classification of cancers*

The phenotypes of stomach cancer cells were determined using two gastric (MUC5AC and MUC6) and two intestinal (villin and MUC2) phenotypic markers. The decisions as to the phenotypes of stomach cancerous areas in which 10% or more of the section area consisted of at least one gastric or intestinal epithelial cell phenotype were classified as gastric (G type) or intestinal (I type) phenotype cancers, respectively. Those which showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype (GI type) cancers, while those showing neither gastric nor intestinal phenotype expression were grouped as unclassified (N type) (Tatematsu et al., 2003; Mizoshita et al., 2003; Tsukamoto et al., 2005).

### *Evaluation of the background gastritis of stomach cancer*

Inflammatory response in non-neoplastic surrounding mucosa [of 26 EBV (+) and 57 EBV (-) stomach cancers] were scored according to the Updated Sydney System (Dixon et al., 1996). The degree of gastric mucosal inflammation including mononuclear cell infiltration, neutrophils infiltration, glandular atrophy, and intestinal metaplasia were classified into four grades as follows: 0 = none, 1 = mild, 2 = moderate and 3 = marked.

### *Expression of gastric and intestinal phenotypic markers and Cdx2 in intestinal metaplastic glands in non-neoplastic surrounding mucosa of EBV (+) and EBV (-) stomach cancers*

Intestinal metaplastic glands were observed in non-neoplastic surrounding mucosa of 9 EBV (+) and 26 EBV (-) stomach cancers. The expression of gastric and intestinal phenotypic markers and Cdx2 was also evaluated in intestinal metaplastic glands of both EBV (+) and EBV (-) cases (Mizoshita et al., 2004b, Tatematsu et al., 2005). Reactivity for the phenotypic markers and Cdx2 was scored according to the percentage of positively stained epithelial cells in the intestinal metaplastic glands on a 4-point-scale: score 0, <10%; score 1, 10-33%; score 2, 34-66%; score 3, 67-100%.

### *Statistical analysis*

The data were analyzed by the Fisher's exact test, c2 test or Mann-Whitney U test for differences between EBV (+) and EBV (-) groups. P-values <0.05 were considered as statistically significant.

## Results

### *Relations between EBV infection and expression of gastric and intestinal phenotypic markers, and Cdx2, in stomach cancers*

Data for comparisons between EBV (+) and EBV (-) lesions for phenotypic marker and Cdx2 expression in cancerous tissues are summarized in Table 2. The average scores for MUC2 and Cdx2 expression were significantly lower in EBV (+) than in EBV (-) cases ($P<0.0001$ and $P=0.0001$, respectively), independently of whether differentiated ($P<0.005$ and $P<0.02$, respectively) or undifferentiated ($P<0.01$ and $P<0.005$, respectively). Regarding the other phenotypic markers, there were no significant differences between the two groups.

### *Comparison of phenotypes between EBV (+) and EBV (-) stomach cancers*

Data for comparisons between EBV (+) and EBV (-) lesions are summarized in Table 3. In the EBV (+) cases, the lesions were divided phenotypically into 9 G (25.7%), 1 GI (2.9%), 3 I (8.6%), and 22 N (62.9%) types. Similarly, the EBV (-) lesions were also classified phenotypically as 15 G (20.0%), 19 GI (25.3%), 24 I

(32.0%), and 17 N (22.7%) types. There was a significant difference in the proportions of each phenotype between EBV (+) and EBV (-) lesions (P<0.0001).

*Comparison of phenotypic markers in differentiated and undifferentiated regions in EBV (+) and EBV (-) stomach cancer cases*

To further analyze the expression of gastric and intestinal phenotypic markers, the phenotypes were compared in mixed structure cases containing differentiated and undifferentiated regions (Table 4). Six EBV (+) cases consisted of 2 adenocarcinomas with differentiated predominance and 4 tumors with larger undifferentiated areas. Among them, 3 cases lacked the phenotypic markers in the undifferentiated regions (3/6=50%). For EBV (-) cases, 2 cases were differentiated region dominant and 7 were undifferentiated predominant, none of them lost the

**Table 2.** Correlations between EBV infection and the expression of the phenotypic markers, and Cdx2 in the stomach cancer cases.

| | The average scores of each marker[a] | | | | |
|---|---|---|---|---|---|
| | MUC5AC | MUC6 | MUC2 | villin | Cdx2 |
| EBV (+) (n=35) | 0.51±0.16 | 0.029±0.029 | 0.057±0.040 | 0.086±0.063 | 0.20±0.099 |
| Differentiated (n=13) | 0.615±0.266 | 0.077±0.077 | 0.077±0.077 | 0.231±0.166 | 0.231±0.166 |
| Undifferentiated (n=22) | 0.455±0.194 | 0±0 | 0.045±0.045 | 0±0 | 0.182±0.125 |
| EBV (-) (n=75) | 1.013±0.15 | 0.16±0.063 | 1.033±0.13 | 0.23±0.070 | 1.060±0.13 |
| Differentiated (n=31) | 1.000±0.2236 | 0.226±0.101 | 0.903±0.169 | 0.484±0.153 | 1.355±0.2 |
| Undifferentiated (n=44) | 1.023±0.191 | 0.114±0.081 | 1.125±0.166 | 0.045±0.032 | 0.852±0.156 |
| P-values between EBV (+) and (-) cases [b] | P= 0.098 | P= 0.58 | P< 0.0001 | P= 0.39 | P= 0.0001 |
| P-values between EBV (+) and (-) differentiated adenocarcinomas | NS | NS | P< 0.005 | NS | P< 0.02 |
| P-values between EBV (+) and (-) undifferentiated adenocarcinomas | NS | NS | P< 0.01 | NS | P< 0.005 |

[a]: Each score is average ± standard error (SE); [b]: Each P-value is analyzed by Mann-Whitney U test. NS, not significant.

**Table 3.** The phenotype classification in EBV (+) and EBV (-) stomach cancers.

| | Phenotypic classification[a] | | | | |
|---|---|---|---|---|---|
| | G type | GI type | I type | N type | total |
| EBV (+) (n=35) | 9 (25.7%) | 1 (2.9%) | 3 (8.6%) | 22 (62.9%) | 35 (100%) |
| Differentiated | 3 | 1 | 2 | 7 | 13 |
| Undifferentiated | 6 | 0 | 1 | 15 | 22 |
| EBV (-) (n=75) | 15 (20.0%) | 19 (25.3%) | 24 (32.0%) | 17 (22.7%) | 75 (100%) |
| Differentiated | 4 | 10 | 11 | 6 | 31 |
| Undifferentiated | 11 | 9 | 13 | 11 | 44 |
| Total | 24 (21.8%) | 20 (18.2%) | 27 (24.5%) | 39 (35.5%) | 110 (100%) |

[a]: P< 0.0001 among G, GI, I , and N types between EBV (+) and (-) cases ($\chi^2$ test).

**Table 4.** Correlation between EBV infection and the expression of the phenotypic markers, and Cdx2 in intestinal metaplasia.

| | The average scores of each marker[a] | | | | |
|---|---|---|---|---|---|
| | MUC5AC | MUC6 | MUC2 | villin | Cdx2 |
| EBV (+) (n=9) | 1.000±0.441 | 0 | 2.333±0.441 | 2.286±0.421 | 0.556±0.377 |
| EBV (-) (n=26) | 1.769±0.256 | 0.231±0.139 | 2.808±0.136 | 2.350±0.244 | 1.654±0.192 |
| P-value[b] | P=0.15 | P=0.61 | P=0.50 | P=0.80 | P=0.016 |

[a]: Each score is average±standard error (SE); [b]: Each P-value is analyzed by Mann-Whitney U test.