Jpn. J. Cancer Res. 88, 532–536, June 1997

Cytotoxin Genes of *Helicobacter pylori* in Chronic Gastritis, Gastroduodenal Ulcer and Gastric Cancer: An Age and Gender Matched Case-Control Study

Norio Matsukura,[1,3] Masahiko Onda,[1] Shunji Kato,[1] Hirokazu Hasegawa,[1] Kei-ichi Okawa,[1] Takasi Shirakawa,[1] Akira Tokunaga,[1] Kiyohiko Yamashita[1] and Akio Hayashi[2]

[1]*First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113 and* [2]*Mitsubishi-Kagaku Bio-Clinical Laboratory, 3-30-1 Shimura, Itabashi-ku, Tokyo 174*

***Helicobacter pylori* (*H. pylori*) infection is involved in many gastrointestinal diseases, such as chronic gastritis (CAG), peptic ulcer and gastric cancer (GCA). Both host factors and *H. pylori* strain differences may contribute to differences in the diseases. Thus, we conducted an age and gender matched case-control study of 35 patients each with CAG, gastric ulcer (GUL), duodenal ulcer (DUL) and gastric cancer (GCA) to examine the role of strain differences of the *H. pylori* cytotoxin genes *cagA* and *vacA* in these diseases. We employed polymerase chain reaction to examine the gastric juice for *H. pylori* DNA. The test was positive for 26 (74.3%) CAG, 29 (82.9%) GUL, 28 (80.0%) DUL and 27 (77.1%) GCA patients, showing no statistically significant difference among the diseases ($P=0.84$). *cagA* and *vacA* genes (picked up by using a *vacA*1+*vacA*2 primer pair which detected non-variable regions of the *vacA* gene) were detected by PCR in the *H. pylori* DNA-positive cases as follows: CAG, 92.3% and 76.9%; GUL, 100% and 86.2%; DUL, 89.3% and 89.3%; GCA, 92.6% and 85.2%, respectively. No statistically significant differences were found in the frequencies of these cytotoxin genes in *H. pylori*-positive cases among the various gastric diseases ($P=0.39$ for *cagA* and $P=0.64$ for *vacA*).**

Key words: *Helicobacter pylori* — *cagA* — *vacA* — Gastroduodenal ulcer — Gastric cancer

Helicobacter pylori (*H. pylori*) infection is related to chronic active gastritis, recurrence of gastroduodenal ulcer (GDU) and possibly gastrocarcinogenesis, and the International Agency for Research on Cancer (IARC) has designated *H. pylori* as a definite carcinogen, group 1.[1)] Direct clinical or experimental evidence of *H. pylori*-induced gastric cancer (GCA) has not been reported, but *H. pylori* infection is known to induce chronic atrophic gastritis and atrophic changes of the gastric mucosa, which are high risk factors for gastric carcinogenesis.[2)]

The reason why *H. pylori* contributes to many gastric diseases is not clear, but there are several hypotheses: (1) short-term infection with *H. pylori* may relate to GDU and long, persistent infection may relate to gastrocarcinogenesis,[3)] (2) *H. pylori* strain diversity may be found among gastric diseases, and disease-specific *H. pylori* may exist,[4)] (3) host factors, especially susceptibility to and immunity against *H. pylori*, or interactions between the host and *H. pylori* may specify the disease. To clarify the second possibility, we conducted an age and gender matched case-control study of the *H. pylori* cytotoxin genes *cagA* and *vacA* in chronic gastritis (CAG), gastric ulcer (GUL), duodenal ulcer (DUL) and GCA. The *cagA* gene was isolated from *H. pylori* as a cytotoxin-associated gene[5)] and the *vacA* gene was isolated as a vacuolating toxin gene[6)]; these *H. pylori* virulence factors may be involved in the strain difference in *H. pylori* among gastroduodenal diseases, if it exists.

PATIENTS AND METHODS

Patients All of the patients had undergone endoscopy in our department and were diagnosed endoscopically and histologically as having CAG (superficial gastritis and chronic atrophic gastritis), GUL, DUL or GCA (early and advanced). Thirty-five age and gender matched patients were randomly selected from patients with each of CAG (aged 43–71, average 56), GUL (aged 45–70, average 56), DUL (aged 43–71, average 55) and GCA (aged 45–69, average 58). Twenty-five male and 10 female patients were selected in each disease category and age was matched within 2 years.

Sampling of gastric juice and DNA extraction A sterile suction tube and a new syringe were used for each gastric juice sampling to avoid carry-over of *H. pylori* DNA from previously examined patients. Approximately 1 ml of gastric juice was neutralized with 1 *N* NaOH and centrifuged at 1500 rpm for 10 min. The pellet was resuspended in 0.5 ml of tissue extraction buffer and the DNA was extracted using a previously reported method.[7)]

Detection of *H. pylori* infection by polymerase chain reaction (PCR) The primer pairs, which were homologues of a portion of the 1.9-kb fragment of chromosomal DNA of *H. pylori*, were used as described by

[3] To whom correspondence should be addressed.

Valentine *et al.*[8] Amplified products were analyzed by electrophoresis on 3.0% agarose gels and Southern blot hybridization using an internal probe as described previously.[7]

Detection of cytotoxin genes by PCR *H. pylori* DNA-positive patients with CAG, GUL, DUL or GCA were examined for the cytotoxin genes of *H. pylori*, *cagA* and *vacA*. The *H. pylori* DNA was amplified using a primer pair for *cagA* and *vacA* as described by Tummuru *et al.*[5] and Phadnis *et al.*,[6] respectively (Fig. 1). Nested PCR was used to detect *cagA*: first primer pair, 5′-(GATAACAGGCAAGCTTTTGAGG)-3′ (sense) and 5′-(CTGCAAAAGATTGTTTGGCAGA)-3′ (antisense); second primer pair, 5′-(AGGGAAGAATACTCCAATAAAGCGA)-3′ (sense) and 5′-(CTGCAAAAGATTGTTTGGCAGA)-3′ (antisense). PCR conditions were denaturation at 94°C for 0.5 min, annealing at 55°C for 1.0 min, and extension at 72°C for 1.0 min (40 cycles). The amplified products were analyzed by electrophoresis on a 3.0% agarose gel and stained with ethidium bromide to detect DNA fragments of 349 base pairs (bp) for the first PCR products and 313 bp for the second products. Two primer sets (*vacA*1 and *vacA*2) were used to detect the *vacA* gene, since genetic variation has been reported in the *vacA* gene.[9] Nested PCR was also used to detect *vacA* 1 and *vacA*2 using the following primer pairs: *vacA*1, first primer pair 5′-(ATTAACAGCGCTCAAGATCTCATTA)-3′ (sense) and 5′-(CTTCCACTTGCCCGTTAAGGTAAG)-3′ (antisense); second primer pair 5′-(ATTAACAGCGCTCAAGATCTCATTA)-3′ (sense) and 5′-(TCTCAGTAGGCGTAGAATTGCC)-3′ (antisense), *vacA*2: first primer pair 5′-(GGCCGTTTGCAATACGTGGGAGC)-3′ (sense) and 5′-(TAAAATCCGGTCGCGCTGTCTAT)-3′ (antisense); second primer pair 5′-(ACTAGGTCACTTTTCTCTGGGG)-3′ (sense) and 5′-(TAAAATCCGGTCGCGCTGTCTAT)-3′ (antisense). Both the *vacA*1 and *vacA*2 primer pairs detected non-variable regions of the *vacA* gene. Methods of amplification were the same as for the *cagA* gene. Gel electrophoresis and ethidium bromide staining showed DNA fragments of 1030 bp for the first PCR products and 358 bp for the second products of the *vacA*1 gene, and 1370 bp for the first PCR products and 474 bp for the second products of the *vacA*2 gene.

Statistical analysis Data were analyzed by χ^2 test and $P<0.05$ was taken as the criterion of significance. Relative risk (odds ratio) was determined by using SPSS statistical programs for PC.

RESULTS

Detection of *H. pylori* infection by PCR *H. pylori* DNA was detected by PCR in gastric juice of patients with CGA, GUL, DUL and GCA, as summarized in Table I. *H. pylori* DNA was detected in 26/35 (74.3%) CAG patients, 29/35 (82.9%) GUL patients, 28/35 (80.0%) DUL patients, and 27/35 (77.1%) GCA patients. There were no statistically significant differences in the frequency of *H. pylori* DNA among the diseases ($P=0.84$). Also there were no statistical significant differences in relative risk (odds ratio) between CAG and the other diseases.

Detection of cytotoxin genes in *H. pylori* DNA by PCR The cytotoxin genes, *cagA* and *vacA*, were analyzed by PCR with visualization by agarose gel electrophoresis. Representative examples of agarose gel electrophoretic visualization of PCR amplification products from three patients are shown in Fig. 2. A band of 313 bp was seen for *cagA* and bands of 358 and 474 bp for *vacA*1 and *vacA*2, respectively, in agarose gel electrophoresis (Fig. 2). *cagA* and *vacA* were detected from *H. pylori* DNA-positive patients with CAG, GUL, DUL and GCA as summarized in Table II. The *cagA* gene was detected in 24/26 (92.3%) *H. pylori*-positive CAG patients, 29/29 (100%) GUL patients, 25/28 (89.3%) DUL patients, and 25/27 (92.6%) GCA patients (Fig. 3). The *vacA*1

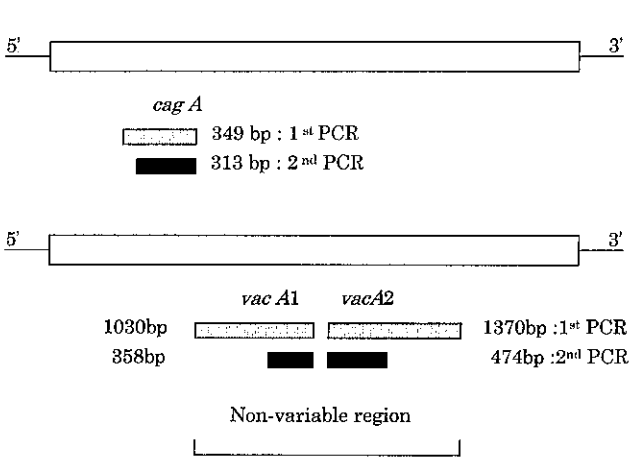

Fig. 1. Schematic representation of the *cagA* and *vacA* primers.

Table I. Positivity of *H. pylori* DNA Detected by PCR in Gastric Juice: An Age and Gender Matched Case-Control Study

Disease	No. of samples tested	No. positive (%)	Odds ratio (95% confidence interval)
Chronic gastritis	35	26 (74.3)	1.00
Gastric ulcer	35	29 (82.9)	1.67 (0.52–5.34)
Duodenal ulcer	35	28 (80.0)	1.38 (0.45–4.26)
Gastric cancer	35	27 (77.1)	1.17 (0.39–3.49)

and *vacA*2 genes were detected in 19/26 (73.1%) and 18/26 (69.2%) *H. pylori*-positive CAG patients, 24/29 (82.8%) and 22/29 (75.9%) GUL patients, 24/28 (85.7%) and 23/28 (82.1%) DUL patients, and 22/27 (81.5%) and 21/27 (77.8%) GCA patients, respectively. There were no statistically significant differences in the frequencies of the *cagA*, *vacA*1 or *vacA*2 gene among the diseases ($P=0.39$ for *cagA*, $P=0.68$ for *vacA*1 and $P=0.73$ for *vacA*2). Also there were no statistical significant differences in relative risk (odds ratio) between CAG and the other diseases. As shown in Fig. 3, *vacA*1 and/or *vacA*2 were detected in 20/26 (76.9%) CAG patients, 25/29 (86.2%) GUL patients, 25/28 (89.3%) DUL patients, and 23/27 (85.2%) GCA patients who were positive for *H. pylori* DNA and *vacA*1 and/or *vacA*2 ($P=0.64$). Cytotoxin genes, *cagA* and/or *vacA* (*vacA*1+*vacA*2) were detected in 24/26 (92.3%) CAG patients, 29/29 (100%) GUL patients, 26/28 (92.9%) DUL patients, and 26/27 (96.3%) GCA patients who were positive for *H. pylori DNA* and a cytotoxin gene ($P=0.48$).

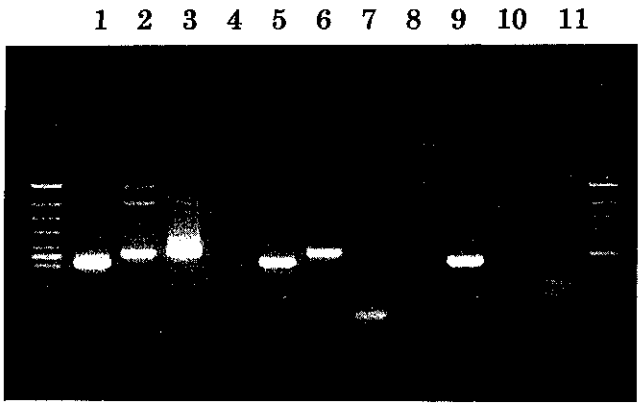

Fig. 2. Agarose gel electrophoresis of amplified PCR products in gastric juice. Lanes 1–3, isolates from a 42-year-old female with GUL; lanes 5–7, isolates from a 68-year-old female with GCA; lanes 9–11, isolates from a 58-year-old male with DUL. Lanes 1, 5 and 9 show *cagA* gene, a band of 313 bp; lanes 2, 6 and 10 show *vacA*1 gene, a band of 358 bp; lanes 3, 7 and 11 show *vacA*2 gene, a band of 474 bp. Lanes 1, 5 and 9 represent *cagA*-positive; lanes 2 and 6 represent *vacA*1-positive, and lane 10 represents *vacA*1-negative; lane 3 represents *vacA*2-positive, and lanes 7 and 11 represent *vacA*2-negative.

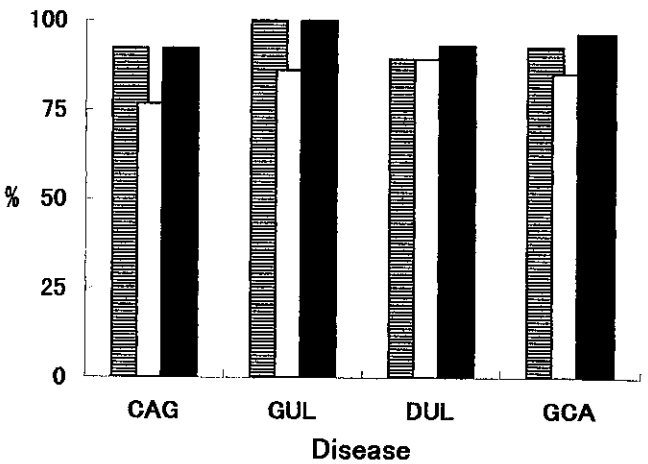

Fig. 3. Positivity (%) of cytotoxin genes by PCR in CAG patients, GUL patients, DUL patients and GCA patients who were positive for *H. pylori* DNA in the gastric juice. Striped columns represent frequency of *cagA*, white columns represent frequency of *vacA*1 and/or *vacA*2, and black columns represent frequency of *cagA* and/or *vacA* (*vacA*1+*vacA*2).

Table II. Positivity of Cytotoxin Genes in *H. pylori* DNA Detected by PCR: An Age and Gender Matched Case-Control Study

Cytotoxin gene	Disease	No. of samples tested	No. positive (%)	Odds ratio (95% confidence interval)
cagA	Chronic gastritis	26	24 (92.3)	1.00
	Gastric ulcer	29	29 (100)	—
	Duodenal ulcer	28	25 (89.3)	0.69 (0.11–4.53)
	Gastric cancer	27	25 (92.6)	1.04 (0.14–8.00)
*vacA*1	Chronic gastritis	26	19 (73.1)	1.00
	Gastric ulcer	29	24 (82.8)	1.77 (0.48–6.46)
	Duodenal ulcer	28	24 (85.7)	2.21 (0.56–8.68)
	Gastric cancer	27	22 (81.5)	1.62 (0.44–5.96)
*vacA*2	Chronic gastritis	26	18 (69.2)	1.00
	Gastric ulcer	29	22 (75.9)	1.40 (0.42–4.59)
	Duodenal ulcer	28	23 (82.1)	2.04 (0.57–7.33)
	Gastric cancer	27	21 (77.8)	1.56 (0.45–5.33)

DISCUSSION

Progress in understanding *H. pylori* infection has shown that this bacterium contributes to almost all the major gastroduodenal diseases, including CAG, GDU, GCA and low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue (MALT lymphoma).[10-12] It is still an open question why this is the case. Blaser and Parsonnet[3] classified *H. pylori* infection as a slow bacterial infection, since it usually persists for a long time. Much research has been done on the strain diversity of *H. pylori*,[13] as well as on host factors, including genetic[14] and environmental factors[15] and variation in acid secretion.[16] Attention has focused on two bacterial factors that may influence the induction of various gastroduodenal diseases, cytotoxin-associated protein (CagA) and vacuolating cytotoxin (VacA), and their genes, *cagA* and *vacA*. The reported frequency of *cagA* and *vacA* genes and their products among CAG, GUL, DUL and GCA patients in Japan have varied widely,[17] though the backgrounds of patients, especially age and sex, with these gastroduodenal diseases have differed among the diseases.[18] Therefore, we conducted an age and gender matched case-control study. Our results showed that the frequency of *H. pylori*, and also those of *cagA* and *vacA*, did not differ significantly among the diseases. Thus, the induction of various gastroduodenal diseases apparently can not be explained in terms of the presence or absence of cytotoxin genes. *cagA* was identified as a gene associated with vacuolating cytotoxin activity[5] and induction of interleukin-8 (IL-8). Recent data showed that *cagA* may have homology to *picA* and *picB* genes and that members of this gene family may induce IL-8 (personal communications). Crabtree and Lindley[19] suggested that IL-8 secretion by gastric epithelial cells may play a key role in *H. pylori*-induced mucosal inflammation. IL-8 attracts neutrophils and stimulates gastrin release. Expression of *cag*A protein (CagA) was associated with an increased risk of developing peptic ulcer and GCA.[19] Moreover, restriction fragment length polymorphism patterns of *cagA* gene were different between peptic ulcer and GCA strains.[17] Recently, molecular cloning and nucleotide sequencing of *cagA* gene were reported.[20] Xiang *et al.*[21] classified *H. pylori* strains into those associated with severe diseases pathology (type I) and those attenuated in virulence (type II), and genetic analysis showed that the *cagA* gene was present only in type I strains, while the *vacA* gene was present in both types. There are reports of a mutated *vacA* gene and different activities may correlate to mutation of the *vacA* gene.[9] Therefore, we used 2 primer pairs, for *vacA*1 and *vacA*2, to detect the *vacA* gene; both the *vacA*1 and *vacA*2 primer pairs detected non-variable regions of the *vacA* gene. *vacA* gene has a mosaic structure consisting of signal sequence types (sla, slb and s2) and mid-region types (m1 and m2). Type s1 strains were associated with DUL, but mid-region types were not. Therefore, there is a possibility that mutant analysis of *vacA* gene may reveal differences among gastroduodenal diseases. Our results show that the frequency of cytotoxin genes does not differ between peptic ulcer and GCA patients. However, further examination is required for CAG. A sequential change from normal gastric mucosa→superficial gastritis →chronic atrophic gastritis→intestinal metaplasia→dysplasia→GCA is considered to represent the course of gastrocarcinogenesis in humans.[22] In this study, we grouped together 20 patients with superficial gastritis and 15 patients with chronic atrophic gastritis/intestinal metaplasia as CAG. Not only positivity of *H. pylori* DNA [13/20 (65%) in superficial gastritis vs. 13/15 (87%) in chronic atrophic gastritis] but also positivity of *cagA* gene [11/13 (85%) in superficial gastritis vs. 13/13 (100%) in chronic atrophic gastritis] and *vacA* gene [7/13 (54%) in superficial gastritis vs. 13/13 (100%) in chronic atrophic gastritis] were lower in superficial gastritis than chronic atrophic gastritis, although this comparison was not an age and gender matched case-control study. A cohort study showed that infection with *cag*A-positive *H. pylori* strains is associated with an increased risk for the development of atrophic gastritis and intestinal metaplasia.[13]

In conclusion, the possible induction of GDU and GCA by *H. pylori* does not appear to be related to the frequency of cytotoxin genes. At present the functions of *cagA* and *vacA* genes of *H. pylori* are not known. There is evidence that virulence factors of *H. pylori* induce neutrophil activation, epithelial cytokines,[23] DNA damage,[24] and free radicals.[25] Interaction of virulence factors of *H. pylori* and host factors such as acid secretion[16] and food intake[21] may be associated with the pathogenesis of the diseases considered here.

(Received February 3, 1997/Accepted April 23, 1997)

REFERENCES

1) International Agency for Research on Cancer. World Health Organization: Schistosomes, liver flukes and *Helicobacter pylori*. *IARC Monogr. Eval. Carcinog. Risks Hum.*, **61**, 218–220 (1994).
2) Ruiz, B., Correa, P., Fontham, E. T. and Ramakrishnan, T. Antral atrophy, *Helicobacter pylori* colonization, and gastric pH. *Am. J. Clin. Pathol.*, **105**, 96–101 (1996).
3) Blaser, M. J. and Parsonnet, J. Parasitism by the "slow"

bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. *J. Clin. Invest.*, **94**, 4–8 (1994).
4) Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., Stemmermann, G. N. and Nomura, A. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res.*, **55**, 2111–2115 (1995).
5) Tummuru, M. K., Cover, T. L. and Blaser, M. J. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infect. Immun.*, **61**, 1799–1809 (1993).
6) Phadnis, S. H., Ilver, D., Janzon, L., Normark, S. and Westblom, T. U. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. *Infect. Immun.*, **62**, 1557–1565 (1994).
7) Matsukura, N., Onda, M., Tokunaga, A., Kato, S., Yamashita, K. and Ohbayashi, M. Detection of *Helicobacter pylori* DNA in gastric juice by the polymerase chain reaction: comparison with findings in bacterial culture and the detection of tissue IgA and serum IgG antibodies against *Helicobacter pylori*. *J. Gastroenterol.*, **30**, 689–695 (1995).
8) Valentine, J. W., Arthur, R. R., Mobley, H. L., Dick, J. D. Detection of *Helicobacter pylori* by using the polymerase chain reaction. *J. Clin. Microbiol.*, **29**, 689–695 (1991).
9) Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K., Blaser, M. J. and Cover, T. L. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J. Biol. Chem.*, **270**, 17771–17777 (1995).
10) NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease. NIH Consensus Conference. *Helicobacter pylori* in peptic ulcer disease. *JAMA*, **272**, 65–69 (1994).
11) Marshall, B. J. *Helicobacter pylori*. *Am. J. Gastroenterol.*, **89**, S116–S128 (1994).
12) Graham, D. Y., Kimura, K., Shimoyama, T. and Takemoto, T. *Helicobacter pylori* infection in Japan: current status and future options. *Eur. J. Gastroenterol. Hepatol.*, **6**, S1–S4 (1994).
13) Kuipers, E. J., Perez-Perez, G. I., Meuwissen, S. G. and Blaser, M. J. *Helicobacter pylori* and atrophic gastritis: importance of the *cagA* status. *J. Natl. Cancer Inst.*, **87**, 1777–1780 (1995).
14) Kato, S., Onda, M., Matsukura, N., Tokunaga, A., Matsuda, N., Yamashita, K. and Shields, P. G. Genetic polymorphisms of the cancer related gene and *Helicobacter pylori* infection in Japanese gastric cancer patients. An age and gender matched case-control study. *Cancer*, **77**, 1654–1661 (1996).
15) Correa, P. and Schmidt, B. A. The relationship between gastric cancer frequency and the ratio of gastric to duodenal ulcer. *Aliment. Pharmacol. Ther.*, **9** (Suppl. 2), 13–19 (1995).
16) Pounder, R. E. *Helicobacter pylori* and gastroduodenal secretory function. *Gastroenterology*, **110**, 947–949 (1996).
17) Awakawa, T., Sugiyama, T., Hisano, K., Karita, M. and Yachi, A. Detection and identification of *cagA* of *Helicobacter pylori* by polymerase chain reaction. *Eur. J. Gastroenterol. Hepatol.*, **7**, S75–S78 (1995).
18) Matsukura, N., Onda, M., Tokunaga, A., Fujita, I., Okuda, T., Mizutani, T., Kyono, S. and Yamashita, T. Significance of serum markers pepsinogen I and II for chronic gastritis, peptic ulcer and gastric cancer. *J. Clin. Gastroenterol.*, **17**, S146–S150 (1993).
19) Crabtree, J. E. and Lindley, I. J. Mucosal interleukin-8 and *Helicobacter pylori*-associated gastroduodenal disease. *Eur. J. Gastroduodenal. Hepatol.*, **6**, S33–S38 (1994).
20) Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. and Covacci, A. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA*, **93**, 14648–14653 (1996).
21) Xiang, Z., Censini, S., Bayeli, P. F., Telford, J. L., Figura, N., Rappuoli, R., Covacci, A. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect. Immun.*, **63**, 94–98 (1995).
22) Correa, P. A human model of gastric carcinogenesis. *Cancer Res.*, **48**, 3554–3560 (1988).
23) Crabtree, J. E., Wyatt, J. I., Trejdosiewicz, L. K., Peichl, P., Nichols, P. H., Ramsay, N., Primrose, J. N. and Lindley, I. J. Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. *J. Clin. Pathol.*, **47**, 61–66 (1994).
24) Kawano, S., Tsuji, M., Fusamoto, H., Sato, N. and Kamada, T. Chronic effect of intragastric ammonia on gastric mucosal structures in rats. *Dig. Dis. Sci.*, **36**, 33–38 (1991).
25) Davies, G. R., Simmonds, N. J., Stevens, T. R., Grandison, A., Blake, D. R. and Rampton, D. S. Mucosal reactive oxygen metabolite production in duodenal ulcer disease. *Gut*, **33**, 1467–1472 (1992).