Helicobacter pylori dupA and gastric acid secretion are negatively associated with gastric cancer development

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Few reports have described the cancer prevalence of peptic ulcer patients with long-term follow-up studies. We have conducted a long-term retrospective cohort study of Japanese peptic ulcer patients and evaluated the risk factors for the occurrence of gastric cancer (GCa). A total of 136 patients diagnosed with peptic ulcers from 1975 to 1983 were enrolled. These 136 cases [102 males and 34 females; 69 gastric ulcer (GU) and 67 duodenal ulcer (DU) patients at the time of enrolment; mean follow-up period of 14.4 years (range 1–30 years)] after being matched with a tumour registry database in Hiroshima prefecture were surveyed for GCa. We investigated *Helicobacter pylori* duodenal ulcer promoter gene A (*dupA*) using paraffin-embedded gastric biopsy specimens in 56 cases. Gastric acid secretion and basal acid output (BAO) in 40 cases, and maximal acid output in 68 cases, had been measured at first diagnosis of peptic ulcers. GCa was detected in 24 patients (17 with GU, 7 with DU) during the follow-up. The prevalence of GCa was significantly higher in GU patients than in DU patients (log-rank test $P<0.05$). *dupA*-positive *H. pylori* was detected not only in DU patients (9/20) but also in GU patients (9/36). Gastric acid output was significantly larger in quantity in patients with *dupA*-positive *H. pylori* than in those with *dupA*-negative *H. pylori* ($P<0.05$). The occurrence of GCa was significantly lower in patients with *dupA*-positive *H. pylori* and a high BAO level (log-rank test $P<0.05$). DUs, higher acid output and *dupA*-positive *H. pylori* were negatively associated with GCa.

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INTRODUCTION

Helicobacter pylori plays an important role in the development of gastritis, peptic ulcers and gastric cancer (GCa) (Suzuki *et al.*, 2007). Recent studies have clarified the mechanism of cell injury caused by *H. pylori* (Hatakeyama, 2009; Ohnishi *et al.*, 2008); however, among infected individuals, the status of chronic gastritis, especially the topography of gastritis, and clinical outcome vary. The topography of gastritis in particular contributes to the clinical outcome. Uemura *et al.* (2001) demonstrated that *H. pylori* infection was essential for GCa development and corpus-predominant gastritis favoured GCa in a prospective study covering 7.8 years, while antrum-predominant gastritis was a low-risk factor. We have

previously demonstrated the prevalence of antral-predominant gastritis to be significantly increased in patients with duodenal ulcers (DUs) (Haruma *et al.*, 1995; Imagawa *et al.*, 2008). DU is a representative disease with antrum-predominant gastritis and high gastric acid output (Kamada *et al.*, 1998, 1999). In clinical practice, the simultaneous development of active DU and GCa is very rare. Therefore, patients with DU are regarded as being at low risk of developing GCa. The difference in clinical outcome can be partially explained by the status of a *H. pylori*-derived toxic factor, CagA (Takata *et al.*, 2009). However, we recently clarified that CagA status did not differ between people with DU and those with GCa in young Japanese patients (Ueda *et al.*, 2006).

The duodenal ulcer promoter gene A (*dupA*) was first reported by Lu *et al.* (2005), who found two *virB4* homologues (jhp0917 and jhp0918) in strain J99, and

Abbreviations: BAO, basal acid output; DU, duodenal ulcer; GCa, gastric cancer; GU, gastric ulcer; MAO, maximal acid output; mEq, milliequivalent.

demonstrated that the gene was frequently expressed in *H. pylori* isolated from patients with DU. The true function of the protein encoded by *dupA* is still unclear, but it is regarded as a specific marker for DU. A subsequent study demonstrated that the prevalence of *dupA* differs among species and so the clinical significance of *dupA* polymorphism is still controversial (Yamaoka, 2008).

DU patients are thought to have a low risk of developing GCa; however, most studies to date have been conducted under a cross-sectional study design. In clinical practice, we have found that patients with GCa sometimes have DU scars simultaneously. Indeed, we demonstrated that gastritis may progress from antrum-predominant gastritis to corpus-predominant gastritis in a large-scale cross-sectional study (Imagawa *et al.*, 2008). In the present study, we conducted a long-term retrospective cohort study with cases of active peptic ulcers from our hospital. Then, we tried to confirm the true risk factors for GCa in peptic ulcer patients concerning the location of the ulcer, the status of acid output and the *dupA* status of the *H. pylori*.

METHODS

Patients. A total of 1585 patients with active peptic ulcers diagnosed between 1975 and 1983 were studied retrospectively based on endoscopic records. Periods from the first to the final endoscopy day were confirmed to identify cancer-free patients. In addition, the patients were screened by matching them to a cancer registry database in Hiroshima Prefecture that is considered to be of a high quality, as reported in the World Health Organization/International Agency for Research on Cancer report (IARC, 2002). Finally, a total of 136 cases [102 males and 34 females; 69 gastric ulcer (GU) and 67 DU cases at the time of enrolment; mean age of 48.7 years; mean follow-up period of 14.4 years (range 1–30 years)] were investigated for GCa in a retrospective cohort study. These patients received gastric biopsies upon endoscopic examination; the *H. pylori* infection was confirmed using paraffin-embedded sections. All the patients enrolled were confirmed to be positive for *H. pylori*. Basal acid output (BAO) in 40 cases and maximal acid output (MAO) in 68 cases had been measured at first diagnosis of the peptic ulcers, as previously described (Ohnishi *et al.*, 2008). The study was approved by the Ethical Committee at Hiroshima University School of Medicine (Hiroshima, Japan).

Extraction of DNA from paraffin-embedded sections. In 56 (36 patients with GUs and 20 with DUs) out of 136 cases, biopsy specimens were taken from the gastric corpus and antrum, fixed in buffered formalin, and embedded in paraffin. Tissue sections were placed on glass slides and stained with haematoxylin and eosin. DNA was extracted from sections with 4 µm thickness. The tissue sections were then dehydrated in graded ethanol solutions and dried without a cover glass. Tissues were scraped from the slides with sterile needles. DNA was extracted from the tissues by incubation in 200 µl extraction buffer [100 mM Tris/HCl (pH 8.0), 2 mM EDTA and 400 µg proteinase K µl⁻¹] at 50 °C for 48 h. The tubes were then boiled for 5 min to inactivate the proteinase K, and DNA was collected using the ethanol precipitation method in the presence of Ethachinmate (Nippon Gene). Finally, 1 µl extract was used for PCR.

PCR. To determine the presence or absence of the *dupA* gene in this study, we designed a new set of primers for PCR based on the published sequence of the *dupA* gene (GenBank/EMBL/DBJ accession no. AB196363.1) as follows: forward 5'-CAAAGGAACA-

CAACAAACCT-3' (nt 1361–1380) and reverse 5'-CGATATAGGC-AAACATTATAGGATG-3' (nt 1419–1443). These yielded 82 bp products. Reaction mixtures (20 µl) contained 0.2 µl AccuSure DNA polymerase (BioLine), 2 µl 10 × AccuBuffer, 2 µl dNTP mixture (2.5 mM each), and 1 µM each of the forward and reverse primers (100 µM each). The reaction mixtures were heated to 95 °C for 7 min, then subjected to 60 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 30 s. After the PCR, the products were electrophoresed in 8% polyacrylamide gels containing 1 × TBE buffer (50 mM Tris base, 67 mM borate, 1 mM EDTA). The 23S rRNA gene was analysed as we have described previously (Hiyama *et al.*, 2003).

Sequencing. DNA bands were excised from the gels, and the DNA was eluted and purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified DNA fragments were sequenced using a BigDye terminator version 1.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 genetic analyser (Applied Biosystems) according to the manufacturer's instructions.

Statistics. Statistical differences were evaluated using χ^2 test or Fisher's exact test for 2 × 2 tables, the Wilcoxon test for comparison of mean values and the log-rank test for survival analysis with JMP 5.0.1J software (SAS Institute). A value of $P < 0.05$ was regarded as significant.

RESULTS

Development of GCa in patients with peptic ulcers

Out of 136 patients, 24 (17 with GUs and 7 with DUs) were confirmed to have developed GCa in the follow-up period. The dominant gender was male (19 males and 5 females), but there was no significant difference in gender between the GU and DU groups. Among the 24 patients, there was no statistical difference in age (54.0 ± 10.6 vs 49.0 ± 4.0 for GU vs DU) and the follow-up period until the cancer discovery (13.4 ± 8.1 vs 13.5 ± 4.2 for GU vs DU) between DU and GU. Histologically, these cancers were diagnosed as intestinal type, with only two exceptional cases (one GU and one DU case) diagnosed as diffuse type. A Kaplan–Meier analysis revealed the prevalence of GCa to be significantly higher in the GU patients than in the DU patients (Fig. 1, log-rank test $P < 0.05$). A high risk of GCa was found in the GU patients compared to the DU patients using relative risk (GU vs DU: relative risk = 2.35, 95% confidence interval 1.05–5.32).

dupA genotype in peptic ulcer patients

Since we used shared DNA extracted from paraffin-embedded sections as a template, we designed a PCR primer set for the *dupA* gene producing a product of 82 bp in size for efficient amplification (Fig. 2). PCR products were detected with the expected size by PAGE analysis (Fig. 2a) and the sequence was confirmed by the direct-sequencing method (Fig. 2b). We used the 23S rRNA gene as an internal control to validate the quality of the template DNA in all samples (Fig. 2a). Out of the 56 samples examined, *dupA* was detected in 18 (32%).

Fig. 1. Kaplan–Meier analysis of the development of GCa in peptic ulcer patients. Patients were followed for up to 30 years. DU and GU patients are demonstrated by dotted and solid lines, respectively. The asterisk indicates log-rank test $P < 0.035$.

Characteristics of peptic ulcer patients with *dupA*-positive *H. pylori*

We compared the clinicopathological findings in peptic ulcer patients harbouring *H. pylori* with and without the *dupA* gene. As to age and gender, there was no difference between the *dupA*-positive and *dupA*-negative groups (Table 1). Interestingly, *dupA*-positive *H. pylori* was found not only in DU (9/20, 45%) but also in GU (9/36, 25%) patients, though there was no statistical difference between these groups. Concerning gastric acid output, BAO and MAO were significantly higher in the *dupA*-positive group than in the *dupA*-negative group (Table 1, $P = 0.01$).

Association between *dupA* and GCa development

In 56 out of 136 cases examined, GCa was found in 14 patients (6 with DU and 8 with GU) during the follow-up period. Of the six who had DU and subsequently developed GCa, only one was positive for *dupA*. Only 1 out of the 18 cases (6%) with *dupA*-positive *H. pylori* developed GCa, whereas 13 out of 38 (34%) with *dupA*-negative *H. pylori*

did (Table 1, $P = 0.02$). According to the Kaplan–Meier method, the prevalence of GCa was significantly lower in those with *dupA*-positive *H. pylori* than in those with *dupA*-negative *H. pylori* (Fig. 3; log-rank test $P = 0.04$).

Association between gastric acid secretion levels and GCa development

Since we found that gastric acid secretion (BAO, MAO) was significantly higher in patients with *dupA*-positive *H. pylori* than in subjects without *dupA*-positive *H. pylori* in both the DU and GU groups (Table 1), we analysed the association between gastric acid secretion levels and GCa development (Fig. 4). When patients of $\text{BAO} \geq 0.5 \text{ mEq h}^{-1}$ are considered as displaying a high level of BAO, a high BAO level at the time of enrolment was found to be a negative factor for GCa (Fig. 4, log-rank test, $P = 0.012$).

DISCUSSION

Studies thus far have demonstrated that patients with DU generally have antrum-predominant gastritis, whereas patients with GU almost invariably have corpus-predominant gastritis with some degree of mucosal atrophy, which is considered a precancerous lesion. These findings may reflect that DU is negatively associated with GCa, while the reverse is true for GU. In an 8 year follow-up of Japanese subjects with *H. pylori* infections, none of the patients with DU developed GCa, but 3.4% of the patients with GU did (Uemura *et al.*, 2001).

In our retro-prospective study, we adopted a long-term follow-up period, up to 30 years, and confirmed that GCa had a significantly lower prevalence in DU patients than GU patients. The long-term period was necessary for the discussion of cancer development because it takes a long time for a single cancer cell to grow to a size detectable by endoscopic examination (Haruma *et al.*, 1991; Ito *et al.*, 2009). From our firm results, we concluded that the risk of developing GCa is lower in active-DU patients than in active-GU patients.

However, we have experienced the detection of DU scars in some patients with GCa by endoscopic examination. This was confirmed by this retro-prospective study, by the

Fig. 2. Expression of the *dupA* gene assessed by PCR amplification. *dupA* and 23S rRNA genes were amplified and detected by electrophoresis in polyacrylamide gels. Patient 1 was positive, while patient 2 was negative for a typical *dupA* gene (a). A representative result of direct sequencing of the *dupA* gene PCR product (b).

Table 1. Clinicopathological findings of patients with or without *dupA*-positive *H. pylori*

	<i>dupA</i> positive (n=18)	<i>dupA</i> negative (n=38)	P value
Ulcer location (GU/DU)	9/9	27/11	NS
Gender (male/female)	15/3	31/7	NS
Age (mean $\pm$ SD)	49.2 $\pm$ 10.0	48.6 $\pm$ 11.5	NS
BAO (mean $\pm$ SD)	3.8 $\pm$ 2.1 (n=7)	1.0 $\pm$ 1.2 (n=12)	0.01
MAO (mean $\pm$ SD)	20.3 $\pm$ 2.6 (n=10)	12.4 $\pm$ 1.9 (n=19)	0.01
GCa development (+/-)	1/18	13/38	0.02

NS, Not significant.

finding that one out of seven DU patients subsequently developed GCa. This suggests that in some cases of DU the atrophy may progress, followed by GCa development. Furthermore, the mean age of patients with antrum-predominant gastritis was lower than that of those with corpus-predominant gastritis (Imagawa *et al.*, 2008). Taking this all together, we hypothesize that some cases of DU may have the potential to develop into GCa. Thus, it is clinically important to distinguish such patients from ordinary DU patients who have little risk of developing GCa.

Lu *et al.* (2005) reported that *dupA* is associated with an increased risk for DU, and protection against gastric atrophy and GCa in Japan. In contrast, our results showed that *dupA*-positive *H. pylori* was detected not only in DU patients (9/20) but also in GU patients (9/36), with no significant difference between these groups (Table 1). The reason for this discrepancy is not clear, though it may be due to the limited numbers of subjects examined in this study. Because the number of patients was low, this should be recognized as a preliminary study. However, this study

has provided further support for *dupA* as a negative marker of GCa, consistent with the study of Lu *et al.* (2005). We demonstrated that *dupA* may be a potential prognostic factor for gastric carcinogenesis not only in DU patients but also in GU patients. In our study, the occurrence of GCa was significantly lower in patients with *dupA*-positive *H. pylori*; only one patient developed GCa (Fig. 3, $P=0.04$).

Several studies have demonstrated a relationship between *dupA* and disease outcome after a *H. pylori* infection. Recently, Zhang *et al.* (2008) reported that the prevalence of *dupA* was higher in DU than GU patients, and inversely related to GU and GCa, in a Chinese population. Arachchi *et al.* (2007) obtained similar results in an Indian population. However, contradictory results were reported from South America (Gomes *et al.*, 2008) and East Asia (Douraghi *et al.*, 2008). The prevalence of *dupA*-positive strains differs among species, and so the significance of *dupA* to clinical outcome may differ between countries (Argent *et al.*, 2007; Schmidt *et al.*, 2009; Hussein, 2010; Hussein *et al.*, 2008). Gomes *et al.* (2008) reported

No. at risk:

<i>dupA</i> positive	18	14	10	6	3
<i>dupA</i> negative	38	30	19	13	7

Fig. 3. Kaplan-Meier analysis of the development of GCa in peptic ulcer patients. Patients were followed for up to 30 years. Patients with *dupA*-positive and *dupA*-negative *H. pylori* infections are demonstrated by dotted and solid lines, respectively. The asterisk indicates log-rank test $P < 0.04$.

No. at risk:

Low BAO level	12	12	6	4	2	1
High BAO level	28	27	24	19	14	6

Fig. 4. Kaplan-Meier analysis of the development of GCa in peptic ulcer patients. Patients were followed for up to 30 years. Patients with high and low BAO levels are demonstrated by dotted and solid lines, respectively (low BAO level < 0.5 mEq h^{-1}). The asterisk indicates log-rank test $P < 0.012$.

frame-shift mutations in 14/86 (16%) *dupA*-positive samples. Recently, Hussein *et al.* (2010) analysed a panel of *dupA* polymorphisms and reported that the virulent type of *dupA* (*dupA1*) was linked to the mucosal inflammatory reaction. The analysis of frame-shift-mutations or polymorphisms of the *dupA* gene will be the target for examination in our future studies, when sufficient numbers of subjects are available. In addition, other virulence factors such as CagA or VacA should be examined. As far as CagA is concerned, we had confirmed that most Japanese patients have the same genotype (Ueda *et al.*, 2006).

Gastric acid output is another interesting clinical determinant. Lu *et al.* (2005) described that the absence of the *dupA* gene was associated with increased susceptibility to low pH for *in vitro* experiments and the *dupA* gene was involved in the activation of transcription factors that bind to the interleukin 8 promoter, such as NF- κ B and AP-1. Yamaoka (2008) showed that higher neutrophil infiltration and interleukin 8 production in the antral gastric mucosa of DU patients compared with that in other diseases. The secretion of gastric acid in our study was significantly higher in patients of the GU group as well as the DU group, in case of *dupA*-positive cases (Table 1). The *dupA* gene may have effects on other pH-regulated factors.

As demonstrated by Correa (1988), an increased concentration of nitrite and lower vitamin C concentration stimulated N-nitroso compound formation and increased GCa (Kodama *et al.*, 2003; Tari *et al.*, 2007). In this report, we found that a higher BAO level at the time of enrolment was a negative factor for GCa. Our results indicated that patients with GCa had a lower acid output, and may have additional factors that emphasize these changes, as reported by Correa (1988). Clinical outcome (GU/DU), acid secretion and the *dupA* genotype were all correlated with each other. A larger scale analysis is warranted in the future to determine which factor is the most important clinical predictor.

In conclusion, our study is to the best of our knowledge the first in the world to evaluate the risk factors for the occurrence of GCa in a long-term retro-prospective study. We found that the *dupA* status of *H. pylori* may be a predictor for GCa development in Japanese peptic ulcer patients. The evaluation of *dupA* status may be useful to identify high-risk groups for GCa development and to select patients who need early intervention (eradication) to prevent GCa development. As the next step, a larger-scale study will be necessary.

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