ORIGINAL ARTICLE INFECTIOUS DISEASES

Helicobacter pylori dupA gene is not associated with clinical outcomes in the Japanese population

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Abstract

The *dupA* gene of *Helicobacter pylori* was suggested to be a risk factor for duodenal ulcer but protective against gastric cancer. The present study aimed to re-examine the role of *dupA* in *H. pylori*-infected Japanese patients. We found that *dupA* status was not associated with any gastroduodenal disease, histological score of chronic gastritis or with the extent of interleukin-8 production from gastric cell lines. These results indicate that *dupA* is unlikely to be a virulence factor of *H. pylori* in the Japanese population.

Keywords: Duodenal ulcer, *dupA*, gastric cancer, *Helicobacter pylori*, Japanese
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Introduction

Helicobacter pylori is a spiral bacterium that chronically infects the human stomach and plays a causative role in gastritis, duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) and mucosa-associated lymphoid tissue lymphoma [1,2]. However, only a minority of *H. pylori*-infected patients develop severe disease, suggesting that clinical outcomes are determined by the interaction of bacterial virulence, host genetic susceptibility and environmental factors [3].

Several *H. pylori* virulence factors associated with peptic ulcer and GC have been reported, including *vacA*, *cagA*, *babA* and *oipA* [2,4–7]. Most recently, Lu *et al.* [8] described a novel virulence factor, duodenal ulcer promoting gene (*dupA*), which consists of two open reading frames *jhp0917* and *jhp0918* located in the plasticity region of the *H. pylori* genome. Interestingly, *dupA* is homologous to *virB4,* a gene encoding a component protein of the type IV secretion system (TFSS) in *Agrobacterium tumefaciens*. Lu *et al.* [8] reported that infections with *dupA*-positive strains increased the risk for DU but were protective against gastric atrophy, intestinal metaplasia and GC in the Japanese, Korean and Columbian subjects. Intriguingly, *dupA* is the first genetic factor of *H. pylori* to be associated with differential susceptibility to DU and GC, and thus it could be considered as a disease-specific virulence marker. The pathogenic mechanism of *dupA* appears to involve the induction of high interleukin (IL)-8 production in the antrum, leading to antrum-predominant gastritis, a well-recognized characteristic of DU [8].

However, the role of *dupA* was questioned subsequently when a number of studies were unable to reproduce the observations of Lu *et al.* [8] in other populations [9–12]. Because the role of *dupA* as a virulence marker is still controversial, in the present study, we attempted to re-examine the relationship between *dupA* and gastroduodenal diseases in a Japanese population.

Materials and Methods

Patients

A total of 244 Japanese patients (114 females, 130 males) aged 21–91 years (mean 57.5 years) were recruited (Table 1).

Ethical approval and written informed consent were obtained from all participants before the study. Four biopsy samples (two from the antrum, two from the corpus) were taken endoscopically from each patient and used for *H. pylori*

TABLE 1. Characteristics of the study population

Number of patients	244
Age, mean ± SD (years)	57.5 ± 14.1
Female/male	114/130
Diseases	
Chronic gastritis	84
Duodenal ulcer	63
Gastric ulcer	61
Non-cardia gastric cancer	36
Diffuse type	4
Intestinal type	30
Mixed type	2

culture and histopathologic examination. GU and DU were determined by endoscopic observation, and non-cardia GC and chronic gastritis were diagnosed histologically, as described previously [13–15].

Histology

Biopsy specimens from the antrum and corpus were examined by an experienced pathologist (M.K.) who was unaware of the characteristics of the infecting *H. pylori* strain. For each biopsy specimen, the grade of neutrophil infiltration, mononuclear cell infiltration, atrophy and intestinal metaplasia were scored on the basis of the updated Sydney System (0, none; 1, mild; 2, moderate; and 3, severe) [15].

H. pylori culture and genomic DNA extraction

For *H. pylori* isolation, biopsy specimens were homogenized and inoculated onto Mueller Hinton II Agar medium (Becton Dickinson, Frankin Lakes, NJ, USA) supplemented with 7% horse blood. The culture plates were incubated for 4 days at 37°C under microaerophilic conditions (10% O_2, 5% CO_2 and 85% N_2). Samples from multiple colonies were pooled and stored at −80 °C in Brucella Broth (Difco, Frankin Lakes, NJ, USA) containing 10% dimethylsulphoxide and 10% horse serum.

For DNA extraction, *H. pylori* was subcultured from the stock. Multiple colonies on the agar plates were harvested and genomic DNA was extracted according to a standard phenol–chloroform method.

Detection of *jhp0917*, *jhp0918*, *cagA* and *cagE*

To detect the presence of *jhp0917* and *jhp0918*, we used a PCR method with the two sets of primers designed by Lu *et al.* [8] (Table 2). The amplified DNA fragments were separated electrophoretically on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

To avoid false-negative results on PCR as a result of variations in the primer annealing sites, a dot blot was performed in all cases. For each single dot, 100 ng of sample DNA was mixed with 100 μL of denaturing buffer (0.8 M NaOH, 1.5 M NaCl) and spotted onto a Hybond N^+ membrane (Amersham Biosciences, Little Chalfont, UK) via the 96-well Bio-Dot apparatus (Bio-Rad, Ivry-sur-Seine, France). DNA from the reference strain J99 (GenBank accession number AE001439) and human DNA were used as positive and negative controls, respectively. Human DNA was extracted from peripheral blood cells.

TABLE 2. List of primers used in the present study

Gene	Primer	Primer sequence (5′- to 3′)	PCR product size (bp)
jhp0917[a]	jhp0917-F	TGGTTTCTACTGACAGAGCGC	307
	jhp0917-R	AACACGCTGACAGGACAATCTCCC	
jhp0918[a]	jhp0918-F	CCTATATCGCTAACGCGCGCTC	276
	jhp0918-R	AAGCTGAAGCGTTTGTAACG	
dupA	dupA-F	TAAGCGTGATCAATATGGAT	350
	dupA-R	TGGAACGCCGCATTCTATTA	
cagA[b]	CagA-F1	AACAGGACAAGTAGCTAGCC	701
	CagA-R1	TATTAATGCGTGTGTGGCTG	
	CagA-F2	GATAACAGGCAAGCTTTTGA	349
	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	
cagE[b]	CagE-F	GCGATTGTTATTGTGCTTGTAG	329
	CagE-R	GAAGTGGTTAAAAAATCAATGCCCC	
oipA[c]	OipA-F	CCATGAAAAAAGCTCTCTTAC	516
	OipA-R	GCCCTTTTACCCTTCGTTCAAC	
16S rRNA[d]	16S rRNA-F	TGGCAATCAGCGTCAGGTAATG	520
	16S rRNA-R	GCTAAGAGATCAGCCTATGTCC	

[a]Lu *et al.* [8].
[b]Ikenoue *et al.* [16].
[c]Yamaoka *et al.* [7].
[d]Engstrand *et al.* [17].

Parts of *jhp0917* and *jhp0918* were amplified by PCR using the corresponding primer sets (Table 2). The amplified fragments were purified with Illustra GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences) and used as probes. With the use of ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Biosciences), the probes were labelled with horseradish peroxidase, hybridized to the membrane at 42°C overnight and finally exposed to Hyperfilm ECL.

cagA and *cagE* were detected by a PCR method using the primer sets described previously (Table 2) [16].

Nucleotide sequencing

A DNA fragment covering the intergenic region between *jhp0917* and *jhp0918* was amplified with the primer set *dupA* (Table 2). The amplified fragments were sequenced with a Big Dye Terminator v3.1 Cycle Sequencing kit on a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The signal sequence of *H. pylori oipA* was also sequenced as described previously (Table 2) [7].

RNA extraction and RT-PCR

H. pylori RNA was isolated with ISOGEN (Nippongene, Toyama, Japan) and then treated with DNase I (Takara Biotech-

nology, Shiga, Japan) to remove DNA contamination. The absence of DNA contamination was verified by PCR with a 16S rRNA primer set [17] (Table 2) using RNA as the template.

For RT-PCR, 500 ng of total RNA was reverse transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). The synthesized cDNA was then subject to PCR amplification with the *dupA* primer set and 16S rRNA as a positive control.

Analysis of *H. pylori*-induced IL-8 production in gastric cell lines

The experiments were performed independently on two occasions, each time in duplicate, as described previously [8,18]. Briefly, AGS and MKN45 cells were seeded into 24-well plates and grown overnight at 37°C in RPMI 1640 (Sigma-Aldrich, Ayrshire, UK) medium supplemented with 10% foetal bovine serum.

Randomly selected stock *H. pylori* strains were cultured on the agar, harvested and washed twice with phosphate-buffered saline before being added to the culture wells (bacterium-to-cell ratio, 100:1). The inoculated plates were incubated at 37°C in 5% CO_2 and 95% air for 6 h. The concentration of IL-8 in the cell culture supernatant was measured with a Quantikine Human CXCL8/IL-8 ELISA kit (R&D Systems, Minneapolis, MN, USA).

Results

Prevalence of *jhp0917*, *jhp0918*, *cagA* and *cagE*

Among the 244 *H. pylori* strains, 68 and 78 were positive for *jhp0917*, and 65 and 72 were positive for *jhp0918* by PCR and dot blot, respectively. PCR and dot blot showed inconsistent results (negative PCR but positive dot blot) for *jhp0917* and *jhp0918* in ten and seven cases, respectively. Positive results for PCR but negative for dot blot were not observed. Overall, the agreement between PCR and dot blot results was 95.9% (234/244) for *jhp0917* and 97.1% (237/244) for *jhp0918*.

On the basis of the combined results of PCR and dot blot, 72 strains (29.5%) were judged to have both *jhp0917* and *jhp0918*, whereas 166 (68.0%) possessed neither. The remaining six strains (2.5%) contained only *jhp0917*. These results indicate that *jhp0917* and *jhp0918* are strongly linked ($p < 0.01$). *cagA* and *cagE* were positive in 98.8% (241/244) and 98.4% (240/244) of the examined *H. pylori* strains, respectively.

dupA status in *H. pylori* strains from Japan

In strain J99, *jhp0917* and *jhp0918* are separate genes. *jhp0917* is homologous to the 5′ end of *virB4* but lacks the 3′ end, whereas *jhp0918* is homologous to the 3′ end of *virB4* but lacks the 5′ end. However, in many *H. pylori* strains possessing both *jhp0917* and *jhp0918*, there is a 1-bp insertion (C or T) after position 1385, resulting in a frameshift leading to one continuous gene, named *dupA*, homologous to the complete *virB4* [8]. Therefore, the presence of *jhp0917*, *jhp0918* and the 1-bp insertion after position 1385 are all required for the formation of *dupA*.

To investigate the status of *dupA* in the *H. pylori* strains used in the present study, we analysed the sequence around position 1385 in the 72 strains that had both *jhp0917* and *jhp0918*. We found that all of them contained the 1-bp insertion referred to above. Therefore, we conclude that these 72 strains are all *dupA*-positive, accounting for 29.5% of the

TABLE 3. *dupA* prevalence in different regions

Region/ethnicity	Reference	*dupA* prevalence (%) by diagnosis (*dupA*-positive strains/analysed strains)				
		Overall	CG	DU	GU	GC
Japan	[8]	21.3 (34/160)	14.0 (7/50)	36.7 (11/30)	26.0 (13/50)	10 (3/30)
Korea	[8]	19.4 (34/175)	6.7 (2/30)	36.9 (24/65)	16.7 (5/30)	6.0 (3/50)
Colombia	[8]	33.3 (55/165)	37.5 (15/40)	48.9 (22/45)	40.0 (12/30)	12.0 (6/50)
India[a]	[21]	31.3 (52/166)	22.9 (16/70)	37.5 (36/96)	–	–
South Africa	[9]	84.8 (39/46)	73.3 (11/15)	92.3 (12/13)	–	88.9 (16/18)
Belgium	[9]	43.7 (59/135)	38.2 (29/76)	50.0 (20/40)	–	52.6 (10/19)
USA	[9]	43.5 (20/46)	45.0 (9/20)	42.9 (9/21)	66.7 (2/3)	–
Iran	[10]	49.7 (78/157)	50.0 (34/68)	50.0 (15/30)	39.1 (9/23)	55.6 (20/36)
Brazil	[11]	93.9 (445/474)	96.2 (230/239)	92.3 (144/156)	–	89.9 (71/79)
Australia[a]	[12]	37.8 (17/45)	37.8 (17/45)	–	–	–
Sweden[a]	[12]	63.5 (33/52)	65.0 (13/20)	63.6 (7/11)	–	61.9 (13/21)
Chinese Singaporeans and Malaysians[a]	[12]	41.1 (37/90)	28.9 (15/52)	62.5 (10/16)	–	54.5 (12/22)
China	[22]	35.3 (127/360)	38.3 (51/133)	45.5 (46/101)	23.4 (11/47)	24.1 (19/79)
Japan	This study	28.8 (67/233)	29.5 (23/78)	27.4 (17/62)	28.8 (17/59)	29.4 (10/34)

CG, chronic gastritis; DU, duodenal ulcer, GU, gastric ulcer; GC, gastric cancer.
[a]Functional dyspepsia patients with *H. pylori* infection are considered as CG.

total strains analysed. Table 3 shows a comparison of the prevalence of *dupA* in *H. pylori* isolates in different disease states observed in the present study with those reported in previous studies in a range of populations.

Using BLAST, we collected a total of 89 sequences covering the intergenic region between *jhp0917* and *jhp0918* deposited in GenBank. We found that 95.5% (85/89) of them had the 1-bp insertion after position 1385, whereas 4.5% (4/89) did not. These results, when taken together with those obtained in the present study, indicate that most, but not all, strains possessing both *jhp0917* and *jhp0918* are *dupA*-positive.

dupA expression

Previous studies have shown that several genes in the plasticity region are not expressed and can be regarded as pseudogenes [19]. Because *dupA* is also located in the plasticity region, we aimed to investigate whether it is a true gene. RT-PCR of ten randomly selected *dupA*-positive strains confirmed that *dupA* was always expressed under the conditions tested, and thus is a true gene.

Association between *dupA* and clinical outcomes

To investigate the association between *dupA* and clinical outcomes, five *H. pylori* strains without *cagE* and/or *cagA* and six strains that had only *jhp0917* were excluded from the analysis. Therefore, the analysed population consisted of 233 patients (109 female, 124 male) divided into four disease groups: chronic gastritis (n = 78), DU (n = 62), GU (n = 59) and non-cardia GC (n = 34; diffuse type, four cases; intestinal type, 28 cases; mixed type, two cases). Among these 233 patients, 67 (28.8%) and 166 (71.2%) were infected with *dupA*-positive and *dupA*-negative strains, respectively (Table 4).

dupA-positive strains were distributed almost equally among the four disease groups (DU, 27.4%; gastritis, 29.5%; GU, 28.8%; GC 29.4%) and thus there was no association between *dupA* status and clinical outcomes (p >0.05). In the chronic gastritis group, the histological scores of patients infected with *dupA*-positive and -negative strains were not significantly different (1.00 ± 0.90 vs. 1.29 ± 0.66, 2.30 ± 0.47 vs. 2.49 ± 0.57, 1.39 ± 0.66 vs. 1.33 ± 0.80 and 0.35 ± 0.78 vs. 0.4 ± 0.81, and 1.39 ± 0.66 vs. 1.18 ± 0.80, 2.48 ± 0.80 vs. 2.18 ± 0.75, 0.87 ± 1.18 vs. 0.49 ± 0.77 and 0.09 ± 0.29 vs. 0.09 ± 0.40 for neutrophil infiltration, mononuclear cell infiltration, atrophy and intestinal metaplasia in the antrum and corpus, respectively) (p >0.05), (Fig. 1a, b). The prevalence of *dupA* did not differ significantly in patients with and without atrophy (21/69 vs. 2/9, p >0.05) and in those with and without intestinal metaplasia (6/21 vs. 17/57, p >0.05).

TABLE 4. *dupA* prevalence in different *Helicobacter pylori*-associated diseases

	CG (n = 78)	DU (n = 62)	GU (n = 59)	GC (n = 34)	Total (n = 233)	p value
dupA positive	23 (29.5%)	17 (27.4%)	17 (28.8%)	10 (29.4%)	67 (28.8%)	>0.05
dupA negative	55 (70.5%)	45 (72.6%)	42 (71.2%)	24 (70.6%)	166 (71.2%)	>0.05

CG, chronic gastritis; DU, duodenal ulcer, GU, gastric ulcer; GC, gastric cancer.

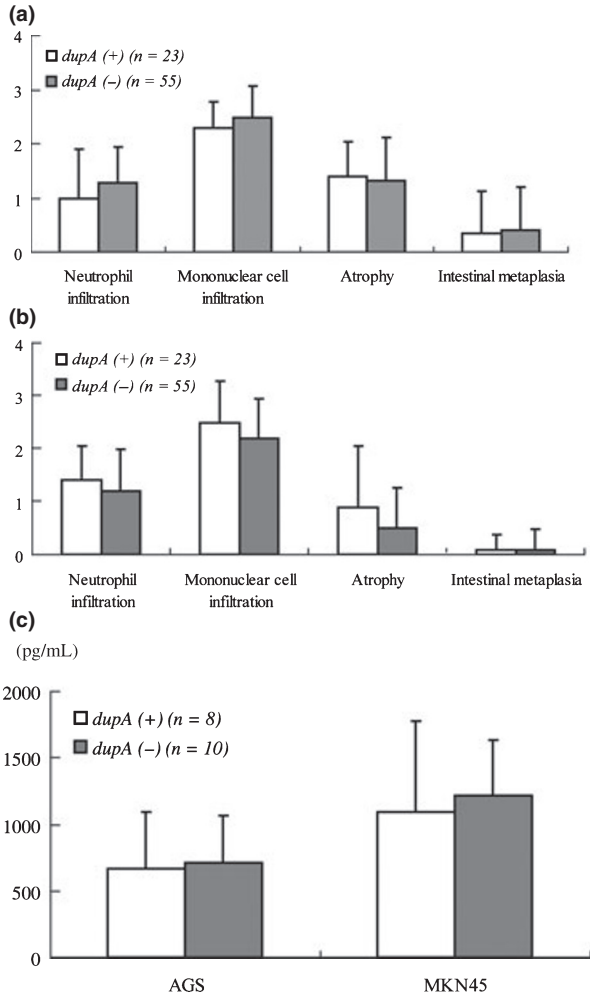

FIG. 1. *dupA* status is not associated with histological scores of chronic gastritis in either the antrum (a) or the corpus (b). *dupA*-positive and -negative strains do not significantly differ in their ability to induce interleukin-8 production from AGS and MKN45 gastric cell lines (c).

Association between IL-8 secretion from gastric cell lines and *dupA* status

We compared the ability of *dupA*-positive and *dupA*-negative strains to induce IL-8 secretion from gastric cell lines. Eight

dupA-positive strains, which had been used for RT-PCR previously, and ten *dupA*-negative strains with both *cagA* and *cagE*, were randomly selected and used for the infection experiments. Because the *H. pylori oipA* gene also affects IL-8 secretion [20], we sequenced the signal sequence of this gene in the 18 strains and confirmed that all had *oipA* in 'on' status. We found that the amounts of IL-8 secreted by gastric cell lines (AGS and MKN45) infected with *dupA*-positive and *dupA*-negative strains were not significantly different (670 ± 419 vs. 707 ± 359 pg/mL for AGS, and 1093 ± 687 vs. 1220 ± 420 pg/ml for MKN45) ($p > 0.05$) (Fig. 1c).

Discussion

Originally, Lu *et al.* [8] reported that the prevalence of *dupA* was significantly higher in DU (36.7%) than in gastritis (14%), GU (26%) or GC (10%) in the Japanese population (Table 3), and proposed that *dupA* was associated with an increased risk for DU but was protective against GC [8].

Table 3 reports these figures from Ref. 7 (not Lu *et al.*); please correct as appropriate.

This is in contrast to our results showing that *dupA*-positive strains are distributed almost equally among those four groups of diseases, with no link between *dupA* status and type of gastroduodenal disease. Moreover, in contrast to the results obtained by Lu *et al.* [8], our data also indicate that *dupA*-positive status is not protective against atrophy and intestinal metaplasia. At present, it is difficult to explain the differences between the results obtained in the present study and those of Lu *et al.* [8] because both studies were conducted on Japanese patients. However, the discrepancy might be a result of geographical variation in the prevalence of *dupA* because our samples and those of Lu *et al.* [8] were obtained from different regions of Japan (Oita and Kyoto, respectively). Alternatively, the discrepancy might result from differences in the proportion of gastric cancer subtypes (diffuse vs. intestinal) and/or the percentage of chronic gastritis patients with atrophy and/or intestinal metaplasia in the two study populations.

From 2007 to early 2009, six studies have examined the relationship between *dupA* status and clinical outcomes but all failed to reproduce fully the results of Lu *et al.* Among them, two studies from India and China supported the role of *dupA* in DU, although its association with GC was not investigated in one [21] and not established in another [22]. Please match the text to each specific reference citation.. Adding to the controversy, two other studies identified *dupA* as a risk factor, and not as a protective factor, for GC [9,12]. The remaining two studies, from Brazil and Iran, showed no association between *dupA* status and gastroduodenal diseases [10,11].

Currently, consensus is likely to be reached on the formation of the *dupA* gene from *jhp0917* and *jhp0918* by a frameshift insertion. Furthermore, Gomez *et al.* [11] have reported that 14/86 (16.3%) of *dupA* sequences from Brazil contained an adenine frameshift insertion after position 1426, creating a premature stop codon that in turn may have considerable effects on protein expression or function. This finding raises questions about the presence of other similar frameshift mutations elsewhere in the *dupA* sequence that cannot be detected by a simple PCR method. Therefore, further studies using immunoblotting with anti-DupA antibody are required to elucidate the true relationship between *dupA* and clinical outcomes.

Using both *dupA* knockout and clinical strains, Lu *et al.* [8] reported that *dupA* plays a role in induction of IL-8 expression. By contrast, and consistent with the study by Schmidt *et al.* [12], the results obtained in the present study indicate that clinical *H. pylori* isolates with or without *dupA* did not differ significantly in their ability to induce IL-8 secretion from gastric cell lines. *dupA* has been reported to be homologous with *virB4*, comprising a gene encoding one component protein of the TFSS. However, it should be noted that the TFSS typically consists of 11 virB proteins (virB1–virB11) and one coupling protein (virD4) [23,24], but other *virB* and *virD4* genes linked with *dupA* have not yet been discovered in the *H. pylori* genome. Therefore, it is still unclear whether the *dupA* gene, together with others, encodes an independent TFSS in addition to *cagPAI* and has a role in the induction of IL-8, or is just a *virB4* homologue by chance.

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Transparency Declaration

The authors declare no conflicting interests.

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