Profile of *Helicobacter pylori* cytotoxin derived from two areas of Japan with different prevalence of atrophic gastritis

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Abstract

Background and Aim—To clarify the roles of *Helicobacter pylori* cytotoxin in gastric atrophy, the cytotoxin positive rate and cytotoxin activity in Fukui and Okinawa, where the prevalence of atrophic gastritis and gastric cancer risk are quite different, were studied.

Materials—Seventy three strains from Fukui and 51 from Okinawa were examined.

Methods—The validation of atrophy was done by endoscopy, being confirmed with histology. The supernatant of liquid *H. pylori* culture media was concentrated 20-fold, serially diluted, using doubling dilutions, and scored from 1 to 8. The semi-quantitated cytotoxin activity was expressed as the maximum dilution score yielding >50% A431 cell vacuolation, being standardised with bacterial density.

Results—The cytotoxin activity of the strains from Fukui was highly diverse compared with that from Okinawa, although the cytotoxin positive rate was not different. In Fukui strains, the grade of atrophy and the cytotoxin activity were correlated ($p < 0.05$). In addition, the cytotoxin activity of the strains from all patients in Okinawa, most of whom showed closed-type/mild atrophy, was significantly lower than that of the strains from the patients with open-type/severe atrophy in Fukui (6.46 (5.53) v 9.76 (8.80), $p < 0.05$), (mean (SEM)).

Conclusion—The difference in profile of the cytotoxin activity in the two areas was related to the difference in the prevalence of atrophic gastritis.

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Keywords: *Helicobacter pylori*, vacuolating cytotoxin, gastric atrophy.

tritis and gastric carcinoma is not known, the epidemiology of gastric cancers suggests that environmental agents are important aetiological factors.⁵

Helicobacter pylori is a spiral shaped Gram negative bacterium that is strongly associated with chronic superficial gastritis.⁶ As has been reported elsewhere,⁷ *H. pylori* infection in Japan has been associated with chronic atrophic gastritis and intestinal metaplasia, the precursor lesions for gastric carcinoma.^{8,9} In addition, there is increasing evidence that persistent infection with *H. pylori* is a risk factor for the development of gastric cancer, especially of the distal stomach.¹⁰⁻¹³

As not all persons infected with *H. pylori* develop gastric cancer, other cofactors must also be important. Such factors could be host related, bacterium related or related to other environmental factors. In terms of bacterium related factors, *H. pylori* strains are highly diverse.^{14,15} Thus, it is appropriate to isolate particular characteristics of *H. pylori* strains that might affect the risk of gastric cancer development. Recent studies have suggested that *cagA*, which is closely associated with vacuolating cytotoxin, may be an important factor for the risk of developing adenocarcinoma of the stomach.¹⁶ Furthermore, the neutralising activity against vacuolating cytotoxin is observed more frequently in serum samples from gastric carcinoma patients than from healthy subjects.¹⁷

In this study, we determined the differences in the presence of vacuolating cytotoxin and its intensity between the strains derived from the patients in Fukui and Okinawa prefectures, in which the prevalence of atrophic gastritis and the gastric cancer risk were quite different, to clarify their roles in the development of gastric atrophy or the increase in gastric cancer risk, or both.

Methods

Patients

The subjects were new patients who were examined for *H. pylori* status by endoscopy in the Second Department of Internal Medicine, Fukui Medical School in Fukui, the middle part of Japan, between February and November 1994 and Okinawa Chubu Hospital in Okinawa, the most south west part of Japan, between September and December 1994. However, nine of 123 examined patients in Fukui and eight of 106 in Okinawa had various diseases other than gastritis or peptic ulcer, that

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is, two with gastric polyp, three with gastric cancer, and four with remnant stomach in Fukui and four with gastric polyp, one with gastric cancer, and three with remnant stomach in Okinawa, and were excluded. The subjects had not been receiving any antisecretory agents or anti-*H. pylori* treatment in the long term before this study. Seventy three of 114 subjects in Fukui (male/female: 45/28) and 51 of 98 subjects in Okinawa (male/female: 24/27) were *H. pylori* positive. In Fukui, 41 patients had mild epigastralgia but no gross findings or diseases in their stomach, that is, had only type B gastritis including erosive or superficial gastritis, or both, six with gastric ulcer, and 26 with duodenal ulcer. Similarly in Okinawa, 33 had gastritis, nine gastric ulcer, and nine duodenal ulcer. The duodenal and gastric ulcer patients also had gastritis. The diagnosis was confirmed endoscopically by two endoscopists. The mean (SD) age of the patients in Fukui and Okinawa was 55.5 (15.4) (range, 16–87) years old and 61.0 (12.1) (range, 35–88) years old, respectively. Although the percentage of duodenal ulcer patients in Fukui seemed to be high, the differences in patients found to have ulcers (43.8% in Fukui *v* 35.3% in Okinawa), the degree of symptoms in the two prefectures was not significantly different.

Validation of atrophy

Firstly, histological atrophy was examined in 84 of 124 biopsied samples of the antrum with haematoxylin and eosin staining from both prefectures (33 from Fukui and 51 from Okinawa) according to the Sydney system,¹⁸ scored 0; none, 1; mild, 2; moderate and 3; severe and compared with endoscopically diagnosed atrophy as described below. Secondly, in all cases, with the location of the endoscopic atrophic border identified by endoscopy, the grade of atrophy was diagnosed according to the criteria of Kimura and Takemoto as reported previously (Fig 1).^{9, 19–21} The endoscopic atrophic border marks the furthest front of the extent of atrophic gastritis from the antrum to the body in the stomach as a result of chronic type B gastritis, although gastritis may be localised to the antrum in Western patients.^{21–23} In brief, by identifying the location of the endoscopic atrophic border in the stomach, the grade of atrophy was divided into two types, closed-type/mild extension of atrophy and open-type/severely extended atrophy. If the border was on the lesser curvature of the stomach, it was defined as closed-type (C-type). They were subdivided into C1, C2, and C3 patterns. If the border was shifted orally and did not exist on the lesser curvature, it was defined as open-type (O-type), and subdivided into O1, O2, and O3 patterns. Therefore, the gastric atrophy was graded as C1, C2, C3, O1, O2, and O3 according to their severity (Fig 1).

Preparation of the strains

Gastric biopsy samples were taken from the lesser curvature of the antrum and large curvature of the body of all patients described

above using a sterilised endoscope, put into Cary-Blair-N transport medium (Nissui Seiyaku Co. Tokyo, Japan), and cultured on Trypticase Soy Agar-II with 5% sheep blood (TSA-II) plate (Nippon Bekton Dickinson, Tokyo, Japan) in the microaerophilic condition (O₂: 5%, CO₂: 15%, N₂: 80%) for five days. The specimens obtained from Okinawa prefecture were sent by air in the same transport medium in an ice box and cultured within 24 hours after biopsy. In addition, a histological study with Giemsa staining was done on the samples from Okinawa prefecture and confirmed by successful isolation of culture regardless of transport by air. *H. pylori* was identified as Gram negative bacilli with urease, catalase, and oxidase activity. A few colonies of the third cultured *H. pylori* isolates (approximately 10⁵ cfu/ml) on a TSA-II plate, which had been well adapted in the in vitro environment, were then cultured in 20 ml of Brucella broth supplemented with 10% fetal calf serum. Broth cultures were incubated with a gyratory shaker at 120 rpm in a microaerophilic condition for three days. The growth of *H. pylori* was assessed by comparison of the optical density at 560 nm of the broth culture medium of each *H. pylori* isolate with that of the culture medium alone.

Figure 1: The extension of endoscopic atrophic border and patterns of endoscopically diagnosed atrophic gastritis by Kimura and Takemoto. The endoscopic atrophic border marks the furthest front of the extent of atrophic gastritis in the stomach as a result of chronic type B gastritis in Japanese patients. Japanese patients tended to have large areas of atrophy and intestinal metaplasia in the stomach. By identifying the location of the endoscopic atrophic border in the stomach, the grade of atrophy was divided into two types, closed-type/mild extension of atrophy and open-type/severely extended atrophy. If the border was on the lesser curvature of the stomach, it was defined as closed-type (C-type). They were subdivided into C1, C2, and C3 patterns. If the border was shifted orally and did not exist on the lesser curvature, it was defined as open-type (O-type), and subdivided into O1, O2, and O3 patterns. Therefore, the gastric atrophy was graded as C1, C2, C3, O1, O2, and O3 according to their severity.

Evaluation of cytotoxin production and quantification of activity

A431 (ATCC CRL 1555) cells were derived from human squamous cell carcinoma and kindly provided by Dr Shimizu (Keio University, Tokyo, Japan). Cells were maintained as described previously.²⁴ A431 cells (0.2×10^4 cells) were inoculated into each well of a 96 well microtitre plate. After a 24 hour incubation period, the medium was replaced with 90 μ l of fresh medium supplemented with 10 mmol/l ammonium chloride to potentiate cytotoxin activity. The supernatant of *H. pylori* culture was concentrated 20-fold by using a 100 kDa ultrafiltration membrane (Ultrafree C3HK, Millipore, Maryland, USA) and then sterilised by passage through a 0.22 μ m filter (Ultrafree C3GV, Millipore, Maryland, USA). For determination of the relative cytotoxin activity, the concentrated culture supernatants were serially diluted, using doubling dilutions of 1, 2, 4, 8, 16, 32, 64, and 128 by the A431 culture medium and scored from 1 to 8. Ten μ l of aliquot was added to 90 μ l of A431 medium in a 96 well plate. After a 24 hour incubation period, cells were examined microscopically for the presence of intracellular vacuolisation. The relative cytotoxin activity was expressed as the maximum dilution score yielding >50% A431 cell vacuolation. The relative activity was defined as 0.5 in a sample of score 1 supernatant that showed vacuolation in less than 50% of the cells. For standardisation by bacterial number, the semi-quantitated cytotoxin activity was expressed by dividing the relative activity with each remainder of optical density between a 3 day old liquid culture of *H. pylori* and culture medium alone.

We compared the positive rate of *H. pylori* cytotoxin and their intensity in all patients and in the patients with gastritis alone between two prefectures. Then, an age matched study was

performed on those from age 40 to 79. The cytotoxin positive rate and the intensity were determined for every decade in Fukui and Okinawa.

Statistics

The cytotoxin positive rate was analysed by the χ^2 test. The relation between atrophy diagnosed histologically and endoscopically and that between the grade of gastric atrophy and the degree of cytotoxin activity were analysed by the Spearman rank correlation test. The difference in cytotoxin activity between Fukui and Okinawa was compared by the Student's *t* test.

Results

There was no significant difference in the cytotoxin positive rate between Fukui and Okinawa for any of the strains isolated (Table I), or between age groups (Table II), although the number in some age groups was too small for comparison.

The histological atrophy of the antrum and the endoscopically diagnosed atrophy were positively correlated according to the Spearman rank correlation test (Fig 2) ($p < 0.01$).

The semi-quantitated cytotoxin activity of the strains from Fukui was highly diverse compared with that of Okinawa (Fig 3). In Fukui strains, there was a positive correlation between the grade of endoscopically diagnosed gastric atrophy and the cytotoxin activity

TABLE I Examined patients in Fukui and Okinawa and their cytotoxin positive rate

	Gastritis	Gastric ulcer	Duodenal ulcer	Total
Examined cases in Fukui	78	8	28	114
<i>H. pylori</i> positive cases	41	6	26	73
Cytotoxin positive cases	31	5	14	50
Cytotoxin positive rate (%)	75.6	83.3	53.8	57.5
Examined cases in Okinawa	77	10	11	98
<i>H. pylori</i> positive cases	33	9	9	51
Cytotoxin positive cases	24	4	6	34
Cytotoxin positive rate (%)	72.7	44.4	66.7	66.7

The cytotoxin positive rate of the strains in Fukui and Okinawa was not different. In each group, the rate was slightly higher in the strains from atrophic gastritis than from duodenal ulcer. Gastritis patients had mild epigastralgia but no gross findings or diseases in their stomach, that is, had only type B gastritis including erosive or superficial gastritis, or both.

TABLE II Cytotoxin positive rate according the decade from age 40 to 79 in Fukui and Okinawa

Age	40-49	50-59	60-69	70-79	Total
Fukui					
<i>H. pylori</i> positive cases	13	18	17	11	59
Cytotoxin positive cases	10	13	11	8	42
Cytotoxin positive rate (%)	76.9	72.2	64.7	72.7	71.2
Okinawa					
<i>H. pylori</i> positive cases	6	10	20	10	46
Cytotoxin positive cases	3	8	13	7	31
Cytotoxin positive rate (%)	50.0	80.0	65.0	70.0	67.4

The cytotoxin positive rate of the strains in Fukui and Okinawa was not affected by age from 40 to 79.

Figure 2: Correlation between histological atrophy of the antrum and endoscopically diagnosed atrophy. They had a positive correlation ($p < 0.01$). The x axis is histological atrophy according to the Sydney system and the y axis is endoscopically diagnosed atrophy according to the criteria of Kimura and Takemoto. The histological atrophy was scored as 0; none, 1; mild, 2; moderate and 3; severe. Endoscopically diagnosed atrophy was graded as C1, C2, C3, O1, O2, and O3 according to their severity.

Figure 3: Correlation between the grade of gastric atrophy and the intensity of cytotoxin activity of isolated *H pylori* (A) in all patients examined and (B) in the patients with gastritis alone in Fukui (●) and Okinawa (○). The cytotoxin activity of isolated *H pylori* was semi-quantitatively described. The grade of atrophy was expressed as C1, C2, C3, O1, O2, and O3 according to its severity. The cytotoxin activity was correlated with the endoscopically diagnosed gastric atrophy in all patients (A) and in gastritis alone (B) in Fukui ($p < 0.05$). Most of the patients in Okinawa had closed-type/mild atrophy. The cytotoxin activity (mean (SEM)) of all the strains from Okinawa was significantly lower than that from the patients with open-type/severe atrophy in Fukui (6.46 (5.53) v 9.76 (8.80), $p < 0.05$).

($p < 0.05$). In the patients with gastritis alone, a positive correlation was also observed ($p < 0.05$). In addition, the cytotoxin activity of *H pylori* from Okinawa was less than 17.24 and the grade of atrophy was likely to be closed-type/mild pattern. The cytotoxin activity (mean (SEM)) of all the strains from Okinawa was significantly lower than that from all the patients with open-type/severe atrophy in Fukui (6.46 (5.53) v 9.76 (8.80), $p < 0.05$). In the comparison of strains from the patients with gastritis alone in both prefectures, the tendency was the same, although it was not statistically significant (7.31 (5.58) v 8.87 (8.75), $p > 0.05$).

When age matched for every decade, a similar finding was clearly observed for those over 60 years old (Figs 4 and 5). The cytotoxin activity was more diverse in Fukui than in Okinawa, correlated with the grade of atrophy. The cytotoxin activity from the patients with open-type/severe atrophy in Fukui was higher than that of all strains in Okinawa.

Discussion

In this study, the cytotoxin activity of *H pylori* strains isolated from Fukui was highly diverse compared with that of the strains from Okinawa. The intensity of the cytotoxin activity in Fukui had a positive correlation with the degree of gastric atrophy when examined in all 73 cases and in 41 cases of gastritis alone ($p < 0.05$), especially in those over 60 years old. More virulent strains on the point of cytotoxin production were associated with the patients in Fukui who had open-type/severe atrophy. In contrast, the virulence of the strains from Okinawa was weak and the degree of atrophy was also mild. In several studies, cytotoxin positive strains have been more associated with peptic ulcers, chronic gastritis with severe inflammation and eventually atrophic gastritis.²⁵⁻²⁷ However, the cytotoxin positive rate was not different between Okinawa and Fukui, regardless of the differences in gastric atrophy. This suggested that the intensity of cytotoxin activity was more closely related to the development of gastric atrophy.

Our study showed that endoscopically diagnosed atrophy was correlated with the histological atrophy of the gastric antrum. In many previous works, the endoscopically diagnosed atrophy is reported to be well correlated with the extension of pyloric gland atrophy and intestinal metaplasia and also negatively correlated with the acid output of the stomach.¹⁹⁻²² As mentioned above, the endoscopic atrophic border marks the furthest front of the extent of atrophic gastritis in the stomach as a result of chronic type B gastritis in Japanese patients. Japanese patients tended to have a large area of atrophy and intestinal metaplasia in the stomach, although gastritis may be localised to the antrum and there is much more multi-focal atrophic gastritis in the West.^{21-23, 28} The Sydney system for the classification of gastritis takes into account the density of *H pylori* infection, the degree of inflammation, the level of gastritis activity, the grade of atrophy and intestinal metaplasia by the histology, and expressed as the antral/corporal/pan-gastritis.¹⁸ It is useful for the expression of closed-type/mild to moderate extension of atrophy (Fig 1). However, a couple of biopsy specimens is not sufficient to clearly subdivide the open-type/severely extended atrophy in the corpus and fornix, as often observed in Japanese patients.²¹ In this study, therefore, the grade of the extension of the atrophy was diagnosed by endoscopy and compared with the cytotoxin activity. In addition to the patients with gastric ulcer, Japanese patients with duodenal ulcer also had gastritis in the antrum and sometimes atrophy or intestinal metaplasia, or both,

Figure 4: Correlation between the grade of gastric atrophy and the cytotoxin activity of patients in their 40s (A) and 50s (B) in Fukui (●) and Okinawa (○). The grade of atrophy was expressed as C1, C2, C3, O1, O2, and O3 according to its severity. Although the cytotoxin activity of the strains from the patients in their 40s showed no difference between Fukui and Okinawa, that from the patients in their 50s in Fukui was diverse and some of them who had open-type/severe atrophy showed high cytotoxin activity.

although the grade of their atrophy was closed-type/mild. Gastric cancer patients also had duodenal ulcers in about 1.2% of the resected specimens in Japan.²⁹ Because, in Japan, almost all patients who had been infected with *H pylori* may have a risk for the development of atrophy, we compared the gastric atrophy in all patients in addition to the patients with gastritis alone. Extension of atrophic gastritis from the antrum to the corpus probably depends on a number of genetic and environmental factors, including diet, alcohol consumption, bile reflux, and infection with *H pylori*. Of them, infection with *H pylori* as well as the intensity of cytotoxin is thought to be a powerful trigger of the extension of gastric atrophy.

Risk factors for atrophic gastritis are also linked to those for gastric cancer,^{4 30} although

more factors and multiple steps are associated with the development of gastric adenocarcinoma.³¹ Recent studies have shown that cytotoxin production does not necessarily require the *cagA* gene.^{32 33} However, cytotoxin production is associated with *cagA* expression.^{26 27} *CagA* positive strains were more virulent and more immunologically strong.²⁷ In addition, by the serological studies, the anti-*CagA* antibody or neutralising antibody to cytotoxin in their sera were more associated with the risk for gastric cancer.^{16 17} As environmental exposure early in life has been implicated in the pathogenesis of gastric cancer,⁵ the exposure to more virulent strains may be correlated with the development of gastric adenocarcinomas. The large difference in the prevalence of atrophic gastritis between Fukui and Okinawa may depend on the difference in the profile of cytotoxin activity of *H pylori* and may eventually reflect the difference in gastric cancer risk to some degree.

Although the simultaneous gastric infection with two *H pylori* strains has been reported at frequencies of 10–13%,¹⁵ the PCR-RFLP patterns using the *ureB* locus showed that almost all patients had the same strain throughout their lifetime.³⁴ Megraud *et al*³⁵ reported by the analysis of molecular fingerprinting of *H pylori* that differences were not due to a change of the strain but to the minor genomic change within the same strain during the long *H pylori* infection. Therefore, the virulence of the strain was continuously conserved for a long period, albeit with some exceptions.³⁶ In addition, divergence of genetic sequences for the vacuolating cytotoxin among *H pylori* strains has been reported and the variation in the length of the *vacA* gene could reflect the phenotype in the strains.^{37 38} Recently, differences in the cytotoxin activity have been also reported and genetically determined.³⁹ In addition, cytotoxin activity is also affected by their secretory function, which has been reported to be similar with the IgA protease type of exoprotein.⁴⁰ Further studies are necessary to clarify the mechanism to induce different cytotoxin activity.

H pylori infection is established in childhood.^{41 42} Several epidemiological studies suggested that the incidence of *H pylori* infection increases in adolescence in Japan and other developed countries.^{43 44} Therefore, patients over 60 years old may have been exposed and affected with *H pylori* infection for more than 20 years. The association between the period of *H pylori* infection and the development of gastric atrophy has been suggested.^{11 12 41 45} In this study, a positive correlation was seen between gastric atrophy and cytotoxin activity especially in those over 60 years old. Therefore, longer exposure to *H pylori* infection as well as the exposure to a more virulent factor may be necessary for the development of severe gastric atrophy. Under 60 years old, the difference between Fukui and Okinawa was not so clear, although the examined number of patients under 50 years old was too small to make a comparison. The other reason may be that the strain from the

Figure 5: Correlation between the grade of gastric atrophy and the cytotoxin activity of patients who were in their 60s (A) and 70s (B) in Fukui (●) and Okinawa (○). The grade of atrophy was expressed as C1, C2, C3, O1, O2, and O3 according to its severity. The cytotoxin activity of the strains from the patients in their 60s in Fukui was highly diverse and correlated significantly with the grade of atrophy ($p < 0.05$). On the contrary, that from Okinawa showed relatively low activity.

younger generation in Okinawa is changing and likely to have a strong cytotoxin activity. A longer chronological study on a younger generation will be needed to clarify this problem.

In Okinawa prefecture, the prevalence of *H pylori* infection has been reported to be lower than that in other prefectures in Japan.³ We also obtained a similar finding in this study, although the difference was not significant (52.0% v 64.0%, $p > 0.05$). The prevalence of atrophic gastritis and the death rate of gastric cancer were also significantly lower than in other prefectures in Japan.²⁻⁴ Many epidemiological studies suggested a correlation between the prevalence of *H pylori* infection and the risk of gastric cancer.^{10-13 46 47} However, several intracountry analyses failed to show any association between gastric cancer risk and *H pylori* infection.^{48 49} Various factors are involved in the development of gastric atrophy or gastric cancer, or both. In terms of *H pylori* infection,

not only the prevalence of *H pylori* infection and the analysis of cytotoxin positive rate but also the analysis of the virulence in cytotoxin activity will be needed to clarify the association between *H pylori* infection and gastric cancer risk.

In contrast, the fact that the strains without cytotoxin activity or with lower cytotoxin activity in Fukui were also found in patients with severe atrophic gastritis showed that factors other than *H pylori* cytotoxin might also be responsible for atrophic gastritis. Many pathogenic factors of *H pylori* strains have been reported.⁶ Differences in the host or in the host strain relation have also been suggested as a cause of the difference in the prevalence of atrophic gastritis and the death rate of gastric cancer between Fukui and Okinawa.^{50 51} Food and life style may also affect the development of gastric carcinoma and the difference in death rate.^{3 31} The HLA-DQA gene is a candidate gene for the host's susceptibility in the establishment of *H pylori* infection and the development of gastric atrophy.^{52 53} The Leb+ antigen is one of the factors for the attachment of *H pylori* infection.⁵⁴ We are now studying these factors in Fukui and Okinawa.

In conclusion, the cytotoxin activity observed in Fukui strains was highly diverse and was correlated with the development of gastric atrophy. The difference in profile of the cytotoxin activity in Fukui and Okinawa was related to the difference in the prevalence of atrophic gastritis, which eventually may reflect the difference in the gastric cancer risk between them.

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