Infection with *Helicobacter pylori* Strains Possessing *cagA* Is Associated with an Increased Risk of Developing Adenocarcinoma of the Stomach

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ABSTRACT

To determine whether infection with a *Helicobacter pylori* strain possessing *cagA* is associated with an increased risk of development of adenocarcinoma of the stomach, we used a nested case-control study based on a cohort of 5443 Japanese-American men in Oahu, Hawaii, who had a physical examination and a phlebotomy during 1967 to 1970. We matched 103 *H. pylori*-infected men who developed gastric cancer during a 21-year surveillance period with 103 *H. pylori*-infected men who did not develop gastric cancer and tested stored serum specimens from patients and controls for the presence of serum IgG to the *cagA* product of *H. pylori* using an ELISA. The serum IgG assay using a recombinant *CagA* fragment had a sensitivity of 94.4% and a specificity of 92.5% when used in a clinically defined population; serological results were stable for more than 7 years. For men with antibodies to *CagA*, the odds ratio of developing gastric cancer was 1.9 (95% confidence interval, 0.9-4.0); for intestinal type cancer of the distal stomach, the odds ratio was 2.3 (95% confidence interval, 1.0-5.2). Age <72 years and advanced tumor stage at diagnosis were significantly associated with *CagA* seropositivity. We conclude that infection with a *cagA*-positive *H. pylori* strain in comparison with a *cagA*-negative strain somewhat increases the risk for development of gastric cancer, especially intestinal type affecting the distal stomach.

INTRODUCTION

There is increasing evidence that persistent infection with *Helicobacter pylori* is a risk factor for the development of gastric adenocarcinoma (1, 2), especially of the distal stomach (3-5). The evidence comes mainly from epidemiological investigations (6-9), including nested case-control studies (4, 5, 10), and molecular and pathological studies support its biological plausibility (11).

However, although *H. pylori* infection is highly prevalent in patients with gastric cancer, most *H. pylori*-infected persons never develop these neoplasms (12). A logical next step is to identify other factors that more precisely determine risk among *H. pylori*-infected persons. *H. pylori* strains are highly diverse (13-15), and individuals may harbor more than one strain (14, 15). Thus, it is appropriate to isolate particular characteristics of *H. pylori* strains that might affect risk of gastric cancer development.

Most presently known phenotypic characteristics of *H. pylori* are conserved among virtually all strains. However, approximately 60% of isolates possess a gene, *cagA*, which encodes a high molecular weight protein (CagA) of variable size (M, 120,000-140,000) (16, 17). Immunoblot studies suggest that persons infected with *cagA*+ strains have higher degrees of gastric inflammation and epithelial cell damage than do persons from whom *cagA*− strains have been isolated (18). Persons infected with *cagA*+ *H. pylori* strains have enhanced expression of IL-1α, IL-1β, and IL-8 in gastric biopsies compared to uninfected persons or patients infected with *cagA*− strains (19). Since both intensity of inflammation and epithelial damage may be involved in the pathogenesis of gastric cancer (1), it is reasonable to examine the importance of *cagA* in this context. The presence of serum antibodies to the *cagA* product is strongly associated with peptic ulceration (18, 20, 21), as well as with inflammation.

We benefit from our previous prospective study to determine whether *H. pylori* infection was a risk factor for the development of gastric cancer among American men of Japanese ancestry in Hawaii (5). In this study, 111 patients who developed gastric cancer over a 21-year period were identified. Serum specimens from these patients (and from matched controls) had been obtained an average of 13 years prior to the diagnosis of gastric cancer.

We now report the development and validation of a new serological assay based on a recombinant fragment of *cagA*, and we explore the role of *cagA* as an additional risk factor for gastric cancer among persons from this cohort who were infected with *H. pylori* on entry into the study.

MATERIALS AND METHODS

Selection of Patients for Validation Study. To determine the utility of the *CagA* serological assay, we studied sera from 181 persons in Nashville, TN; Los Angeles, CA; and Syracuse, NY, whose *h pylori* status had been defined previously (19, 22-24). Uninfected persons (*n* = 115) were those who underwent endoscopy and had biopsies that did not reveal *H. pylori* infection by rapid urease test or by histological examination; all patients also had negative serology (in a standardized ELISA) for serum IgG directed to *H. pylori* (25). The 115 uninfected patients were divided arbitrarily into a reference (*n* = 35) and a test (*n* = 80) group. Infected persons were those from whom *H. pylori* was isolated from culture of gastric biopsies; these 66 patients included those with duodenal ulceration (*n* = 14), gastric ulceration (*n* = 6), gastritis alone (*n* = 36), or other diagnoses (*n* = 10). For each infected patient, a single *H. pylori* isolate was evaluated to determine *cagA* status by colony hybridization with a gene-specific probe, as described previously (16, 24). On the basis of the hybridization assay, 36 patients were defined as being infected by a *cagA*+ strain and 30 patients by a *cagA*− strain. All sera had been stored at 20°C until used.

Selection of Patients for Cancer Study. All the patients in this study were part of the Japan-Hawaii Study cohort as described previously (5). During the 21-year period from 1968 to 1989, 109 cases of pathologically confirmed gastric carcinoma had been identified, and previous serological testing indicated that 103 of these patients had been infected with *H. pylori* at the time of their original serum submission in the 1960s (5). Of the 103 matched controls for these cases, 83 had been infected with *H. pylori* at the time of original serum submission. For each of the remaining 20 *H. pylori*-infected cases, 3 additional controls matched on age at examination and date of serum collection were identified following the previous criteria (5). Serology was done on coded samples to determine whether *H. pylori* infection was present. Each of the 20 control sets had at least 1 potential control with *H. pylori* IgG antibody positivity. If more than 1 of the 3 controls for each case was positive, then a suitable control was selected randomly. Thus, in total, the study consisted of...
103 \textit{H. pylori}-infected men who developed gastric cancer and their 103 (83 + 20) matched controls, who also were infected with \textit{H. pylori} but did not develop gastric cancer during the study period.

**Preparation of the Recombinant CagA Antigen.** A 1.7-kilobase BamHI fragment containing base pairs 1921–3648 of \textit{cagA} cloned in pMC3 (16) was subcloned into the BamHI site of pET15b (Novagen, Madison WI) downstream of the T7 promoter. This plasmid (pORV220) was used to transform \textit{Escherichia coli} host strain BL21, a \textit{DE3} lysogen containing the T7 RNA polymerase gene under control of the lacUV5 promoter (26). Addition of isopropyl-\textbeta-D-thiogalactopyranoside to a growing culture of the lysogen induces T7 polymerase to transcribe the target DNA on the recombinant plasmid. This vector allows transcriptionally regulated expression of a fusion protein consisting of the \textit{CagA} fragment with a NH\textsubscript{2}-terminal histidine tag. The fusion protein consists of 600 residues (24 vector + 576 insert) and has a predicted molecular mass of 66.4 kilodaltons. The protein was purified from lysates of induced broth cultures with the use of a nickel-chelating resin and eluted with imidazole as described (27).

**Serological Methods.** The presence of serum IgG antibodies to \textit{H. pylori} was determined by ELISA with the Pyloristat kit (Biowhittaker, Walkerven, MD) as described previously (5). For the \textit{CagA} ELISAs, optimal concentrations of antigen, patient serum, and anti-human IgG conjugate were determined by checkerboard titrations. For orv220, the optimal antigen concentration was 5 \textmu g/ml, and 100–1\textmu l aliquots were loaded into wells in a 96-well microtiter plate. The optimal dilution of human serum was 1:100, and horseradish peroxidase-conjugated goat anti-human IgG was used at a dilution of 1:4000. Other details of the serological methods were exactly as described in similar assays (23, 25).

**Assessment of Stability of Antibody Levels.** To determine whether serum antibodies to the \textit{cagA} product persist over the course of chronic \textit{H. pylori} infection, we evaluated paired serum specimens from 36 healthy epidemiologists who were part of a cohort studied previously for clinical and epidemiological features associated with \textit{H. pylori} infection (28). On average, the specimens were obtained 7.59 years apart, and knowledge of \textit{H. pylori} status was available from serological studies using a conserved \textit{H. pylori} antigen (28). These sera were also examined for antibodies to the \textit{cagA} product with the use of the methods developed in the previous sections.

**Statistical Analysis.** The \textit{t} test for paired samples was used for the comparison of means, and McNemar’s \textit{t} test was used for the comparison of the distribution of various characteristics between patients and control subjects. The OR for stomach cancer, based on the results of the \textit{CagA} assay, was derived from conditional logistic regression methods (29). Tests for linear trend in the logit of risk were derived from conditional logistic regression models through the use of grouped \textit{cagA} test results (coded by quartile as 1, 2, 3, or 4). All models of conditional logistic regression were fitted by the use of grouped data analysis methods and a special application of the proportional hazards regression model (30). The estimate of the attributable risk of gastric carcinoma related to a \textit{cagA}+ strain was based on the method of Walter (31). A receiver-operator-characteristic curve was constructed to summarize the sensitivity and specificity estimates (32).

**RESULTS**

**Concordance of Findings with \textit{pEM3} and orv220.** Previous studies of \textit{CagA} serology used a recombinant antigen that was purified from \textit{E. coli} cell lysates by sequential column chromatography (21). Because purification of this antigen was tedious and prolonged, overlapping \textit{cagA} gene fragments were expressed as fusion proteins in an alternate prokaryotic expression system. Preliminary studies indicated that orv220 had the greatest utility of several of the candidate antigens based on \textit{CagA} fragments (data not shown). We first asked how this truncated protein compared with the antigen purified from \textit{E. coli}. We next asked whether \textit{CagA} serology based on serum IgG could accurately reflect the \textit{cagA} status of \textit{H. pylori} strains with which patients were infected. To establish the assay, sera from 35 persons known not to be infected with \textit{H. pylori} were used for reference, and after multiple runs, thresholds based on mean values plus intervals of SD were established. Concurrently, sera were tested from a second group of 80 uninfected persons, 36 persons known to be infected with a \textit{cagA}+ strain and 30 persons from whom the only \textit{H. pylori} isolate obtained was \textit{cagA}−. Not surprisingly, absorbance ratios for the uninfected persons were nearly identical to those for the reference group (Fig. 1). In contrast, the values for the persons infected with \textit{cagA}+ strains were significantly higher (Student’s \textit{t} test, \textit{P} < 0.001; one-tailed test). Among the 30 sera obtained from persons from whom the only isolate was a \textit{cagA}− strain, a bimodal distribution was observed. For 27 of the sera, the values were similar to those in the 2 groups of uninfected persons, but for 3 sera the values were much higher and similar to those for patients infected with \textit{cagA}+ strains.

To establish a threshold for use in diagnostic assays, we examined the accuracy of several absorbance ratio cutoffs comparing uninfected persons and those known to be infected with \textit{cagA}+ strains (Table 1). Overall, the highest accuracy was obtained when the mean value for the 35 uninfected (referent) patients + 3 SD was used, with sensitivity of 94.4% and specificity of 92.5%. We used this type of threshold in all future studies. On the basis of receiver-operator-characteristic curves, there was a high level of discrimination of \textit{cagA} status with the use of orv220 (Fig. 2).

**Stability of Anti-\textit{CagA} Response.** Having paired sera obtained 7.6 years apart on the average from patients in previous studies (28), we examined the stability of the anti-\textit{CagA} response. Among 36 participants, 27 showed evidence of \textit{H. pylori} infection on initial (mean absorbance, 3.70 ± 0.26) and follow-up (mean absorbance, 4.14 ± 0.24) periods with the use of the ELISA based on conserved \textit{H. pylori} antigens (25, 28). In contrast, among 9 uninfected persons, initial (mean absorbance, 0.21 ± 0.04) and follow-up (mean absorb-
ance, 0.17 ± 0.03) values were much lower. CagA antibody levels were stably low in persons uninfected with *H. pylori*, as expected. In general, mean anti-CagA levels changed little among these patients (Table 2); however, a single patient showed a specific absorbance ratio rise from 0.06 to 0.98 during the 15-year interval between serum specimens, indicating the acquisition of a cagA + strain after prior infection with a cagA − strain. In total, these data indicate that the cagA products induce a persistent and specific immune response as long as *H. pylori* infection persists.

### Association of cagA Positivity with Gastric Cancer

The characteristics of the 103 *H. pylori*-infected men who developed gastric cancer during the 21-year observation period and their age-matched controls are shown in Table 3. The two groups of men were similar with respect to demographic characteristics and laboratory values. We then asked whether initial CagA seropositivity was a risk factor for development of gastric cancer over the 21-year observation period. For this population, presence of serum antibodies to CagA was associated with increased risk of cancer development (OR = 1.9; Table 4), but the association was not statistically significant (P = 0.08). An analysis confined to the 101 cases of cancer of the distal stomach and their controls showed highly similar values, as expected (OR = 1.8; P = 0.11). When the cases with distal cancers were stratified by the histological type of tumor, the strongest association was with the intestinal type (OR = 2.3; P = 0.056) but not the diffuse type (OR = 1.0; P = 1.0); 3 men had an indeterminate histological pattern.

Positivity in the cagA ELISA was not associated with the interval between when serum was obtained and the diagnosis of cancer (OR = 2.5 and 95% CI = 0.5–12.9 for interval <10 years; OR = 1.7 and 95% CI = 0.7–3.8 for interval ≥10 years). In the population of *H. pylori*-infected persons, the presence of CagA antibodies was associated with an attributable risk of 28% (95% CI = 0–57) for gastric cancer, but the height of the CagA antibody response was not associated with an attributable risk of 28% (95% CI = 0–57) for gastric cancer development (Table 5). To assess whether men infected with cagA + strains were more likely to have high antibody levels to conserved *H. pylori* antigens, we compared results from the previous IgG ELISA to detect responses to those antigens (5) and from the present anti-CagA assay. The age-adjusted mean IgG values (2.26 ± 0.06) for 170 subjects (cases and
controls) infected with cagA+ strains were nearly identical with those for the 36 men infected with cagA- strains (2.31 ± 0.13; P = 0.73). Independent analyses restricted to either cases or controls alone showed no significant differences in IgG values to the conserved antigens by cagA status (data not shown).

Next we stratified the analysis by the age of the patients at which gastric cancer was diagnosed. CagA seropositivity was associated with a 3-fold (95% CI = 1.0-9.3; P = 0.057) increase in gastric cancer risk for 52 men diagnosed under the age of 72 years; in contrast, for 51 men who were ≥72 years at the time of diagnosis, the association was not significant (OR = 1.3; 95% CI = 0.5-3.5). CagA seropositivity was associated with risk of presenting with an advanced stage tumor (3 or 4) at time of cancer diagnosis in 81 men (OR = 2.6; 95% CI = 1.1-6.2; P = 0.03) but not an earlier stage tumor (1 or 2) in 21 men (OR = 1.0). The risk of developing gastric cancer associated with CagA seropositivity was not increased when subjects were stratified according to birth order or sibship size.

DISCUSSION

A study of a total of 115 persons known not to be infected with H. pylori showed that there was little background serological reactivity with the recombinant CagA fusion protein encoded by orv220 and therefore confirms the specificity of this diagnostic test. Among persons known to be infected with cagA+ strains, there was a broad range in serological reactivity but without substantial overlap with the reactivity of uninfected persons. The stability of the results over years in the absence of antimicrobial therapy further indicates the utility of the assay.

Simultaneous gastric infection with two H. pylori strains has been reported with frequencies of 10-13% (14, 15), and simultaneous infection with 3 different strains also has been observed (33). In our initial validation study, only a single H. pylori isolate from each patient had been examined so there was no opportunity to detect multiple infection. However, the fact that 3 of 30 (10%) persons from whom the isolate was a cagA- strain had high level serological responses to CagA suggests that these persons were coinfected with a cagA+ strain. Since only the conjunction of simultaneous infection with a cagA- index strain and a second cagA+ strain [and not simultaneous cagA+/cagA+, cagA-/cagA-, and cagA+ (index)/cagA-] could show dichotomous results between colony testing and serological assay, our data suggest that the frequency of multiple infection may be substantially higher than what was reported previously, which was based on a small number of biopsies (14, 15). The present work further supports the notion that noninvasive serological assays for H. pylori infections are desirable because they are global assays that in essence sample the entire stomach. In contrast, biopsy-based techniques only sample a tiny fraction (<1%) of the gastric mucosa. Because CagA is an immunodominant antigen (17), serological assays potentially have the power to detect infections with cagA+ organisms even if numbers are low in relation to cagA- isolates.

Having an accurate assay, we thus were able to test the hypothesis that infection with a cagA+ strain was a risk factor for the development of gastric cancer. The study design of the Japan-Hawaii cohort (34) and our previous investigations of the association (OR = 6.0; 95% CI = 2.1-17.3) of H. pylori infection with the development of gastric cancer (5) facilitated the present study. Infection with a cagA+ strain nearly doubled the risk of developing gastric cancer over the ensuing 21 years, compared with infection with a cagA- strain, and the effect was more marked for persons who developed intestinal type neoplasms.

In these studies, the increase in risk associated with a cagA+ strain compared with a cagA- strain ranged from 1.8 to 2.3, and some of the differences between strata in cagA status were not statistically significant. Undetected differences in risk between the two groups, lack of perfect accuracy of the assay, and variation in host response to this infection could help account for the lack of significance. Furthermore, we used the conservative two-tailed analysis of significance. It may be argued that the one-tailed analysis is more appropriate since the prior literature suggests an association of enhanced inflammation and gastric cancer risk (5, 8, 11); thus, we did not expect an inverse association between cagA positivity and cancer risk. Use of one-tailed analysis indicates that the associations with all cancers and intestinal type cancers reach statistical significance (P = 0.04 and 0.028, respectively). Similarly, Crabtree et al. (35) showed that CagA seropositivity among H. pylori-infected patients, as determined by immunoblotting, was significantly greater in gastric cancer patients at the time of diagnosis (91%) than in infected controls with non-ulcer dyspepsia (72%; P = 0.028).

The associations with the subset of more aggressive tumors (younger age and higher stage when diagnosed) and the consistency of the data with our a priori hypothesis suggest that the effect is real. This positive effect is biologically plausible for several reasons: (a) infection with cagA+ strains has been associated with enhanced epithelial cell injury (18, 19), and injury to surface gastric epithelial cells may promote or possibly initiate oncogenesis; (b) infection with cagA+ strains is associated with higher degrees of gastric inflammation (18, 19) and with enhanced expression of proinflammatory cytokines such as IL-1 and IL-8 (19, 36). These may contribute to epithelial injury; (c) most cagA+ strains also express vacuolating cytotoxin activity (16, 17), Infection with cytotoxin-producing strains, as assessed by presence of serum neutralizing antibodies, may be associated with the presence of gastric cancer (37); and (d) intestinal type gastric cancer (associated with CagA seropositivity in this study) is more highly associated with increased inflammation and advanced atrophic gastritis than is the diffuse type (38, 39). We hypothesize that the enhanced intensity of inflammation induced by the cagA+ strain results in accelerated mucosal damage with loss of epithelial structures and subsequent atrophy and eventually metaplasia, consistent with the model proposed by Correa (11). Considering the small number of cases of diffuse cancer studied, the lack of significance does not preclude an association of cagA positivity and these tumors.

For the original group of 109 patients with gastric cancer and their matched controls, those who were infected with a cagA- strain of H. pylori still had an increased risk of developing cancer (data not shown); thus, cagA status did not account entirely for the association of H. pylori with gastric cancer. In our previous study (5) and in a more recent Japanese study (8), high titered antibody responses to conserved H. pylori antigens were associated with gastric cancer risk. One interpretation of this phenomenon is that high antibody levels are markers for the degree of inflammation, and active inflammation is considered a precursor of oncogenic events (1). The lack of correlation of anti-CagA antibody levels and gastric cancer risk (Table 5) may reflect that this antibody response does not mirror inflammatory phenomena. In any event, the significance of these findings must be tempered by the observation that CagA seropositivity was present in 78% of the H. pylori-infected controls who did not develop cancer [and 72% of the non-ulcer dyspepsia patients in the study by Crabtree et al. (35)]. Thus, infection with a cagA+ strain is neither necessary nor sufficient for oncogenesis but is one of several factors that may be involved in this process.

Finally, the function of cagA to H. pylori is unknown, as is its particular role in induction of gastric inflammation. Presence of cagA in a strain may only be a marker for adjacent genes or for a particular phenotype that itself is relevant to inflammation or to the oncogenic process. Nevertheless, the results of this study provide for the first...
time an indication that particular *H. pylori* strains may be associated with differential risk of gastric cancer. Further work in this direction is warranted.

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