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RESEARCH ARTICLE

The Effect of Cag A-Positive Helicobacter Pylori Infection on Vascular Risk Factors and the Development of Ischemic Stroke

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Abstract

Background: Chronic *Helicobacter pylori* infections are associated with higher risk of ischemic stroke. Cytotoxin-associated gene-A (*CagA)*-positive *H pylori* strains may enhance the atherosclerotic process by inducing persistent, low-grade inflammatory response in the arterial wall intima. The aim of this study is to investigate the role of *CagA*-positive *H pylori* infection as a risk factor in ischemic stroke and the relation of this infection to vascular risk factors including homocysteine. We also assessed the relation of *CagA*-positive *H pylori* infection to the mean internal carotid artery stenosis.

Materials and Methods: Ninety patients with ischemic stroke and 60 control subjects were included in this study. Sera were examined for *H pylori* antibodies and for *CagA* IgG by ELISA. *CagA* gene was detected in histopathological samples by polymerase chain reaction (PCR). The levels of cholesterol, fibrinogen, homocysteine and carotid artery stenosis were also assessed.

Results: *H pylori* infection was significantly higher in patients versus controls, 77.8% and 53.3% respectively (P = 0.002). *CagA* seropositivity was also significantly higher in patients versus controls (*P* = 0.001). Although *CagA*-positive *H pylori* infection was associated with higher risk of ischemic stroke than *CagA*-negative, no significant difference was detected. However, infection with *CagA*-positive *H pylori* was associated with significantly higher levels of cholesterol, fibrinogen, homocysteine and carotid artery stenosis.

Conclusion: This study revealed that *CagA*-positive *H pylori* infection is a significant risk factor for ischemic stroke and carotid artery stenosis. The association of *CagA*-negative *H pylori* infection with ischemic stroke cannot be excluded.

Introduction

The role of chronic inflammation in atherogenesis has been studied extensively over the past decade. Conceiving atherosclerosis as an inflammatory condition has given rise to a series of hypotheses aimed at finding determinants for the occurrence of cardiovascular and cerebrovascular diseases beyond the traditional risk factors. Among these hypotheses is the contribution of chronic infections such as *Helicobacter pylori*, *Chlamydia pneumoniae*, and cytomegalovirus to the development of atherothrombosis **(Lindsberg and Grau 2003).** Evidences indicate that

inflammation contributes to both initiation and progression of atherosclerosis, and to acute rupture of atherosclerotic plaques, with superimposed thrombus formation increasing the risk of ischemic vascular events **(Ross 1999).**
Infections with *H pylori* are thought to be restricted to the gastric mucosa, but *H pylori* has been found in human atherosclerotic plaques by use of polymerase chain reaction and immunohistochemistry **(Farsak et al. 2000, Ameriso et al. 2001).** Some studies have suggested that specific *H pylori* strains bearing the cytotoxin-associated gene-A *(CagA)* have greater potential for eliciting systemic immune response. The *CagA* virulence factors seem to be involved in the pathogenesis of peptic ulcer disease and gastric cancer and they may lead to enhanced local inflammation, whereas *H pylori CagA*-negative strains provoke a significantly lower inflammatory response **(Blaser et al. 1995, Peek et al. 1995).** Patients with atherothrombotic disease seem to be more likely to be infected by *CagA*-positive *H pylori* strains than healthy subjects **((Pasceri et al. 1998, Pietroiusti et al. 2002, Diomedi et al. 2004).** It has been found that anti-*CagA* antibodies can react with the cytoplasm and the nuclei of smooth muscle cells, with fibroblasts-like cells found in intimal atherosclerotic plaques, and with endothelial cell membrane **(Franceschi et al. 2002).**
CagA-positive *H pylori* strains might enhance the atherosclerotic process by inducing persistent, low-grade inflammatory response in the intima of the arterial wall **(Wick et al. 1997).** These strains have been found to be associated with plaque destabilization, probably caused by the presence of a systemic inflammatory response induced by the organism **(Gabrielli et al. 2004).**
Several risk factors are considered to be important in the pathophysiology of the vascular events. Plasma homocysteine levels are considered to be a vascular risk factor which is implicated in both coronary and cerebrovascular events. Homocysteine significantly differentiates between ischemic stroke and healthy control subjects **(Hasan et al. 2012).** The plasma fibrinogen is another marker that correlates with the parameters of thrombin activation and also correlates positively with hypertension and cholesterol levels **(Haffner 2006).** Further studies are required to assess *CagA*-seropositive *H pylori* infection as a risk factor for ischemic diseases caused by an atherosclerotic mechanism **(Zhang et al. 2008).**
This study is designed to investigate the possible role of *CagA*-positive *H pylori* infection as a risk factor in patients with ischemic stroke and the relation of this infection to vascular risk factors including homocysteine. The relation of *H pylori* infection to mean internal carotid artery stenosis was also assessed.

Subjects and Methods

Subjects

This study was conducted over a period of 12 months on patients attending the Internal Medicine Department of Qassim University Polyclinic as well as King Fahad Specialist Hospital. Ninety patients with ischemic stroke were selected; they included 55 (61%) males and 35 (39%) females with a mean age of 53 ± 8.7 years. All patients had history of peptic ulcer. Patients were excluded from the study if they have hemorrhagic stroke or metronidazole therapy three weeks prior to the study. In addition, sixty normal individuals were included as a control group; thirty five of them (58%) were males and 25 (42%) were females with mean age of 52.6±7.1 years. The absence of atherosclerosis in control subjects was assessed by the following: normal 12-lead ECG, normal echocardiography, stenosis of the carotid tree < 25% by doppler ultrasonography, normal physical examination of the lower limb arteries and absence of clinical history of cardiac disease. The control subjects were recruited from spouses of the same patients to improve controlling of socioeconomic status; since *H pylori* infection may merely be a marker for poor socioeconomic conditions which themselves are vascular risk factors.
The following data were collected for each subject: medical history and complete clinical examination, serum cholesterol, triglycerides and high density lipoproteins (HDL), blood sugar, and findings of electrocardiogram, computed tomography and duplex ultrasound. Homocysteine and plasma fibrinogen were measured in blood of patients and controls. The study was approved by the local clinical research committee and performed according to the ethical procedures. A written informed consent was obtained from each participant.

Methods

Tissue Samples and Histopathology

Gastric biopsies were obtained from patients by endoscope for histopathology and *H pylori* culture. Specimens were immediately placed into sterile, disposable plastic containers. They were fixed in 10% neutral buffered formalin and embedded in paraffin. Several sections of 5µ thickness on microscopic slides were stained with Giemsa stain and evaluated by optical microscope for detection of *H pylori.*

H pylori culture

The biopsy specimens for culture were placed in 300ul of sterile 0.9% saline and transported on ice to the laboratory, where they were processed within two hours. The biopsy specimens were homogenized with a tissue grinder and inoculated onto trypticase soy agar plates supplemented with 7.5% sheep blood. The plates were incubated at 37 ºC under microaerobic conditions up to 10 days. Cultures with typical morphology of *H pylori* by Gram stain were further identified by positive urease, catalase, and oxidase tests.

***CagA* PCR:**

From the primary growth, DNA extraction was performed by Qiagen DNA extraction kit (Hilden, Germany). The PCR mixture contains 10 ul of the extracted DNA, 2.5 U of Taq polymerase, 10 mMTris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.5 mM of each oligonucleotide primer. *CagA* gene of *H pylori* was amplified using the following primers (*CagA*-Forward 5' CCA TGA ATT TTT GAT CCG TTC GG 3', *CagA*-Reverse 5' GAT AAC AGG CAA GCT TTT GAG AGG GA 3') **(Tiwari et al. 2006).** All PCR reagents were purchased from Life Technologies (Gaitherburg, USA).

PCR tubes were subjected to the following cycling conditions: 35 cycles of 94^{o}C for 1 min, 45^{o}C for 1 min, 72^{o}C for 1 min, followed by a final extension step at 72^{o}C for 7 min. A 10 ml sample of PCR products was electrophoresed on a 2% agarose gel stained with ethidium bromide (1 mg/ml), and then examined under UV light for the presence of the 349bp fragment of amplified DNA.

Detection of *H pylori* antibodies and *CagA* IgG

Blood samples were collected from the patients and control subjects in pyrogen-free tubes under aseptic conditions, allowed to be clotted and centrifuged. The sera were stored frozen in aliquots at -70 ºC until used for analysis. The collected serum samples were thawed before starting the assay procedure. ELISA assays were used to test the serum samples for *H pylori* antibodies (Eurospital, Trieste, Italy) and for specific IgG against *CagA* (Radim, Pomezia, Italy).

Statistical analysis:

Data were analyzed with SPSS 16. Values were expressed as mean ± standard deviation. Differences between patient and control groups were assessed by student t-test. Categorical data were compared using Pearson Chi-Square test. Results were considered statistically significant at *P* value ≤ 0.05.

Results

Seventy out of 90 ischemic stroke patients (77.8%) were positive to *H pylori* IgG. Recent active *H pylori* infection was confirmed in 59/70 (84.3%) of *H pylori* seropositive ischemic stroke patients by Giemsa staining of histopathology specimens, and in 55 (78.6%) by culture of *H pylori* from biopsies. *CagA* DNA was detected in 51 out of 55 (92.7%) culture positive samples.

The positivity of *H pylori* IgG was significantly higher in ischemic stroke patients 70/90 (77.8%) compared to 32/60 (53.3%) of controls (P = 0.002). The seropositivity of *CagA* IgG was also significantly higher in patients 60 (66.7%) versus controls 23 (38.3%) with *P* value = 0.001 (Table 1).

A total of 102 subjects were seropositive for *H pylori;* 83 of them were *CagA* positive (60 patients and 23 controls) and 19 were *CagA* negative (10 patients and 9 controls). Among the102 *H pylori* seropositive subjects, the risk of ischemic stroke was higher in *CagA* positive patients 60/83 (72.3%) compared to *CagA* negative patients10/19 (52.6%) but no significant difference was detected (*P* = 0.096).

Smoking, blood sugar, cholesterol, triglycerides, fibrinogen and homocysteine were significantly higher in patients versus controls. While HDL levels were significantly lower in patients versus controls. Data are shown in table (2).

The seropositivity of *H pylori* was associated with significantly higher levels of cholesterol, fibrinogen and homocysteine. *CagA* IgG seropositivity was also associated with significantly higher levels of cholesterol, fibrinogen, homocysteine and blood sugar. Data are shown in table (3) and (4).

The mean percentage carotid artery stenosis was significantly higher in patients infected with *CagA* positive-*H pylori* compared to non-infected patients (33.8% versus 27%; *P* <0.001).

The levels of triglycerides, fibrinogen and homocysteine were significantly higher in ischemic stroke patients with positive *CagA* DNA when compared to those with negative *CagA* DNA (*P* = 0.021, 0.005, 0.013 respectively).

Table (1). Seropositivity of *CagA* IgG in ischemic stroke patients versus controls

		Ischemic stroke patients	Controls	Total
CagA IgG	Positive N (%)	60 (66.7%)	23 (38.3%)	83
	Negative N (%)	30 (33.3%)	37 (61.7%)	67
	Total	90	60	150

P value = 0.001

Table (2): Characteristics of patients with ischemic stroke versus controls

Variable	Patients (n = 90)	Controls (n= 60)	P
Age	53.4 ± 5.1	52.3±5.4	N.S.
Male sex	55(61.1%)	35 (58.3%)	N.S.
Smoking	56 (62.2)	19 (31.7)	<0.001
Blood sugar	144.9 ± 36.3	107.2 ±26.5	<0.001
Cholesterol	245.2 ± 39.6	194.5 ±17.7	<0.001
Triglycerides	160.7 + 33.4	106 ±21.8	<0.001
HDL	37.2+ 6.2	40.9 ±3.6	<0.001
Fibrinogen	3.9+ 0.5	2.5 ±0.5	<0.001
Homocysteine	11.7 ±1	8.1 ±1	<0.001
H pylori IgG	70 (77.8%)	32 (53.3)	0.002
H pylori anti*CagA*	60 (66.7%)	23(38.3%)	0.001

Table (3). Relation of *H pylori* seropositivity to ischemic stroke risk factors among ischemic stroke patients

	H pylori IgG				
	Positive (N=102)		Negative (N=48)		*P* value
	Mean	Std. Deviation	Mean	Std. Deviation	
Triglycerides	141.47	38.05	133.23	42.94	0.237
HDL	38.38	5.59	39.33	5.5	0.330
Fibrinogen	3.54	0.86	2.98	0.62	< 0.001

Homocysteine	10.62	2.04	9.45	1.69	0.001
Blood sugar	133.06	40.61	122.85	29.15	0.121
Cholesterol	231.57	43.64	210.85	30.78	0.001

Table (4). Relation of *CagA* seropositivity to ischemic stroke risk factors among ischemic stroke patients

	CagA IgG Positive (N=83)		*CagA* IgG Negative (N=67)		*P* value
	Mean	Std. Deviation	Mean	Std. Deviation	
Triglycerides	141.87	38.93	135.07	40.67	0.299
HDL	38.43	5.74	39.00	5.36	0.537
Fibrinogen	3.59	0.82	3.07	0.75	0.000
Homocysteine	10.79	1.99	9.57	1.83	0.000
Blood sugar	137.19	41.97	120.63	28.94	0.005
Cholesterol	233.33	43.28	214.55	35.72	0.004

Discussion

In this study, *H pylori* infection was significantly higher in patients versus controls (P = 0.002). *CagA* seropositivity was also found significantly higher in patients versus controls (P = 0.001). A study by **Pietroiusti et al. (2002)** demonstrated higher seroprevalences of *CagA* antibodies in patients with atherosclerotic stroke. A meta-analysis reported little association between stroke and *H pylori* infection which appeared to be stronger for those patients bearing more virulent *CagA* positive *H pylori* **(Cremonini et al. 2004).** In another meta-analysis, a moderate association of ischemic stroke, particularly caused by atherothrombosis with *CagA*-seropositive strains was observed and a statistical significance between the *CagA*-seropositive strains and coronary heart disease was found **(Zhang et al. 2008).**

Diomedi et al. (2008) found a significant correlation between the presence of anti-*CagA* antibodies and the incidence of recurrent stroke. This finding shed light on the interaction of infection with the natural history of atherosclerotic stroke. The increased baseline levels C reactive protein (a sensitive marker of systemic inflammation) in patients with subsequent recurrence suggests that systemic inflammation is probably involved in the process**.** The findings of a longitudinal study, showing a strong predictive role of infection with *CagA*-positive strains for the incidence of first-ever stroke in patients with atherosclerosis, suggested a causal relationship between the two events **(Corrado et al. 2006).**

In the present study, *CagA*-positive *H pylori* infection was associated with higher risk of ischemic stroke than *CagA*-negative but no significant difference was detected. On the other hand, **Mayr et al. (2003)** reported that infections with *CagA* positive but not *CagA* negative *H pylori* strains significantly increase the risk of early atherosclerosis in carotid arteries. The insignificant difference of *CagA* positivity as a risk factor of ischemic stroke may be due to the relatively low number of *CagA*-negative strains in the present study.

Other studies showed no association between *H pylori* infection and increased risk of cardiovascular disease development **(Haider et al. 2002, Markus et al. 2002, Smieja et al. 2003).**

In this study, recent active infection with *CagA*-positive *H pylori* was confirmed by molecular detection of *CagA* DNA in 85% of *CagA* seropositive patients. Moreover, our results showed that infection with *CagA*-positive *H pylori* was associated with significantly higher levels of cholesterol, fibrinogen, homocysteine and carotid artery stenosis.
Interestingly, the levels of triglycerides, fibrinogen and homocystein were significantly higher in ischemic stroke patients with positive *CagA* DNA when compared to those with negative *CagA* DNA (P = 0.021, 0.005, 0.013 respectively). These findings support and explain the association between *CagA*-positive *H pylori* infection and the increased risk for ischemic stroke, as *CagA*-positive *H pylori* infection may have a direct or an indirect effect on ischemic stroke risk factors.

Several mechanisms were suggested to explain this association: (1) infection with *CagA*-positive strains may be linked with plaque instability **(Gabrielli et al. 2004),** some studies report the presence of antigenic material belonging to the organism at the plaque level; the local inflammatory reaction following the presence of the bacterium might induce the instability of the plaque **(Farsak et al. 2000, Ameriso et al. 2001).** (2) Another possible mechanism may be linked to the fact that virulent *H pylori* strains have common antigenic sequences with vascular wall components. The antibodies induced by the infection may cross-react with these antigens, causing an immune-mediated inflammatory reaction, leading to plaque instability. Interestingly, antigenic sequences recognized by anti-*CagA* antibodies are not exposed under normal conditions. However, they may be easily recognized by circulating antibodies when the integrity of the arterial wall is lost, such as in atherosclerotic patients **(Franceschi et al. 2002).** (3) An additional pathogenic mechanism might be linked to the activation of the host inflammatory response to the chronic infection of *H pylori* virulent strains, independently from its presence in the atherosclerotic plaque **(Diomedi et al. 2008).**
The evidence of association between *CagA*-positive *H pylori* infection and increased risk of atherosclerotic stroke may have therapeutic implications; all strains of *H pylori* can be eradicated by a short course of specific antibiotic therapy **(Bytzer and O'Morain 2005),** and the level of antibody response tends to disappear with time after eradication **(Cutler and Vajravel 1996).**

The present study indicated that *CagA*-positive *H pylori* infection plays a significant role in the pathogenesis of ischemic stroke and carotid artery stenosis. However, the association of *CagA*-negative *H pylori* infection and ischemic stroke cannot be excluded. Although, the mechanism is not well understood, it may be mediated by increased homocysteine and fibrinogen as a result of inflammatory reaction to *H pylori*. Confirmation of these results would have important protective and therapeutic implications in patients with ischemic cerebrovascular disease. Therefore, further studies to confirm the impact of eradication of *CagA*-seropositive strains on the prognosis of ischemic vascular diseases are required.

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