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Research Paper

No associations of *Helicobacter pylori* infection and gastric atrophy with plasma total homocysteine in Japanese

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Recent studies have suggested that *Helicobacter pylori* (*H. pylori*) infection might be a risk factor for atherosclerosis. Since the bacterium has not been isolated from atherosclerotic lesions, a direct role in atherogenesis is not plausible. We examined associations of plasma total homocysteine (tHcy) and serum folate, independent risk factors for atherosclerosis, with *H. pylori* infection and subsequent gastric atrophy among 174 patients (78 males and 96 females) aged 20 to 73 years, who visited an *H. pylori* eradication clinic of Nagoya University from July 2004 to October 2005. Polymorphism genotyping was conducted for *methylenetetrahydrofolate reductase* (*MTHFR*) C677T and *thymidylate synthase* (*TS*) 28-bp tandem repeats by PCR with confronting two-pair primers and PCR, respectively. *H. pylori* infection and gastric atrophy were not significantly associated with hyperhomocysteinemia (tHcy ≥ 12 nmol/ml), when adjusted by sex, age, smoking, alcohol, and genotypes of *MTHFR* and *TS*. The adjusted odds ratio of gastric atrophy for low folate level (≤ 4mg/ml) was 0.21 (95% confidence interval = 0.05-0.78). The associations of tHcy with serum folate and *MTHFR* genotype were clearly observed in this dataset. The present study demonstrated that folate and *MTHFR* genotype were the deterministic factors of plasma tHcy, but not *H. pylori* infection and subsequent gastric atrophy, indicating that even if *H. pylori* infection influences the risk of atherosclerosis, the influence may not be through the elevation of homocysteine.

Key words: *Helicobacter pylori*, homocysteine, *methylenetetrahydrofolate reductase (MTHFR)*, *thymidylate synthase (TS)*, gastric atrophy

1. Introduction

Several epidemiologic studies have shown associations between persistent gastric infection with *Helicobacter pylori* and ischemic disorders [1-4]. The ischemic diseases are also associated with plasma total homocysteine (tHcy) level [5-7]. Hyperhomocysteinemia, usually defined as a plasma homocysteine level greater than 15 nmol/ml, has been found in 5% to 10% of general populations [8, 9], and as high as 30% of the population aged 65 and older in the Framingham Heart Study (tHcy > 14 nmol/ml) [10]. Increasing tHcy concentrations accelerate cardiovascular diseases by promoting vascular inflammation, endothelial dysfunction, and hypercoagulability [11].

An important metabolic pathway for homocysteine is the remethylation cycle; in this reaction homocysteine is converted into methionine by methionine synthase. The folic acid–methionine pathway is particularly relevant to the control of genome stability, and involves a number of critical enzymes for which several polymorphisms have been identified. The activity of methylenetetrahydrofolate reductase (MTHFR) can be reduced by polymorphisms of *MTHFR* that alter its affinity for the substrate or cofactor [12]. There were several studies on the association between hyperhomocysteinemia and *H. pylori* infection, but the results were inconsistent [1, 2, 13-16].

Thymidylate synthase (*TS*) gene encodes the enzyme that catalyzes the conversion of deoxyuridylate to thymidylate. The enzyme expression is reportedly affected by a 28bp tandem repeat polymorphism. The *3R3R* homozygotes increased tHcy in a Chinese population, possibly because TS and MTHFR compete for limiting supplies of folate, which is required for the remethylation of homocysteine [17]. However, another study showed that the tHcy concentrations for *3R3R* homozygotes did not differ significantly from those for *2R2R* homozygotes or *2R3R* heterozygotes in healthy young subjects [18].

Several pathways by which *H. pylori* infection could lead to atherosclerosis have been hypothesized

(Figure 1). Among them, the routes from *H. pylori* infection to gastric atrophy and from low folate concentration to hyperhomocysteinemia are well established. But the routes between *H. pylori* infection and hyperhomocysteinemia are not confirmed. The aim of our study was to examine the hypothesized association between *H. pylori* infection and hyperhomocysteinemia adjusted with *MTHFR, TS*, sex, and age. The present study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine (approval number 174).

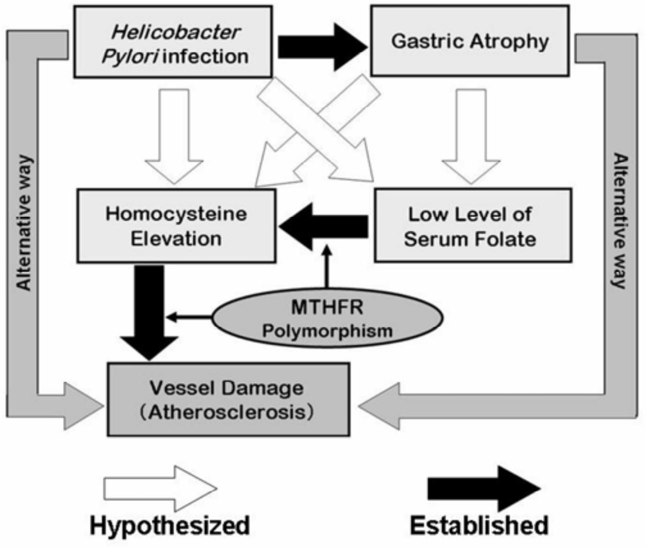

Figure 1. Hypothesized pathways from *Helicobacter pylori* infection to atherosclerosis

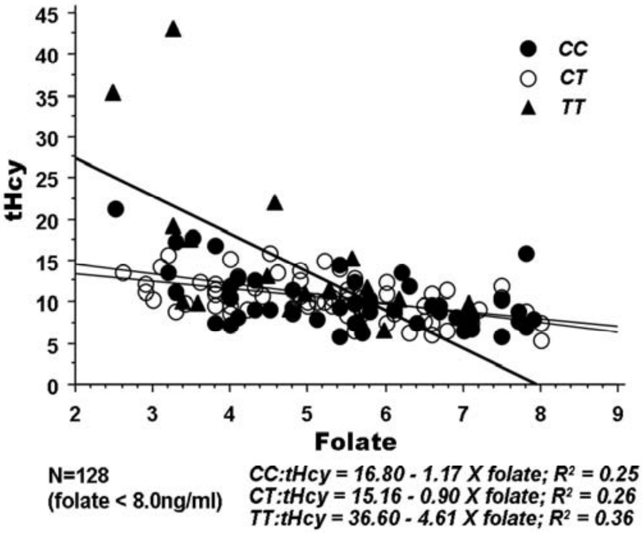

Figure 2. Correlation between serum folate and plasma total homocysteine according to *MTHFR* C677T genotype among those with serum folate < 8.0 ng/ml

2. Patients and Methods

Study Subjects

Subjects were patients who visited Daiko Medical Center of Nagoya University in Nagoya Japan for *H. pylori* infection tests and subsequent eradication treatment, from July 2004 to October 2005. Those aged 20 to 75 years were enrolled after the informed consent on polymorphism genotyping. Participants who had smoked less than 100 cigarettes in their lifetime were categorized as never smokers, and all others were categorized as ever smokers. Participants who had drunk alcoholic beverages at least once a week were categorized as "Yes", and the rest were as "No". When the subjects were positive for serology and/or urea breath test, they were classified into positive for *H. pylori* infection. Gastric atrophy was assessed with serum pepsinogens (PGI < 70ng/dl and PGI/II < 3). Blood samples were collected for measurement of plasma tHcy and serum folate. We defined tHcy levels of 12 nmol/ml and more as hyperhomocysteinemia, and folate levels of 4 mg/dl and lower as lower serum folate based on the frequency distribution (the highest and lowest quartile, respectively).

Genotyping

DNA was extracted from buffy coat conserved at –40℃ using a BioRobot® EZ1 (QIAGEN Group, Tokyo). *MTHFR* C677T polymorphism was genotyped by a polymerase chain reaction with confronting two-pair primers (PCR-CTPP) [19]. Each 25 μl reaction tube contained 50-80ng DNA, 0.12 mM dNTP, 12.5 pmol of each primer, 0.5 U AmpliTaq Gold (Perkin-Elmer, Foster City, CA) and 2.5 μl of 10x PCR buffer including 15 mM $MgCl_2$. The PCR was conducted with initial denaturation at 95°C for 10 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The primers were F1: 5′-AGC CTC TCC TGA CTG TCA TCC-3′, R1: 5′-TGC GTG ATG ATG AAA TCG G-3′, F2: 5′-GAG AAG GTG TCT GCG GGA GT-3′, and R2: 5′-CAT GTC GGT GCA TGC CTT-3′. The amplified DNA fragments were 128-base pairs (bp) for the *C* allele, 93-bp for the *T* allele, and 183-bp for common band. The tandem repeat sequences in the 5′-terminal of the regulatory region of the *TS* gene were detected by PCR assay as previously reported [20].

Statistical analysis

Differences in age, sex, smoking status, alcohol consumption, concentration of serum folate and tHcy, and genotype frequencies between the positives and the negatives of *H. pylori* infection and gastric atrophy were examined by a Mann-Whitney U test or chi-square test. Sex-age-adjusted odds ratio (OR), 95% confidence interval (CI), and interaction term of hyperhomocysteinemia and lower serum folate were estimated with an unconditional logistic model. All statistics were calculated by the computer program StatView Version 5 (SAS Institute Inc.). Hardy-Weinberg equilibrium was also examined.

3. Results

One hundred seventy four patients participated in this study. There were 4 patients whose gastric atrophy was not examined by the pepsinogen test. The *H. pylori* positive perticipants were 115 (66.1%) in all patients, and gastric atrophy was observed in 46/170 (27.1%). The gastric atrophy was in 39.1% among 115 positive individuals. Gastric atrophy without *H. pylori* infection was found for only one patient who had undergone an *H. pylori* eradiation treatment.

The characteristics of patients according to *H. pylori* infection and gastric atrophy are shown in Table 1. Age was significantly associated with *H. pylori* infection and gastric atrophy. There were no significant differences in sex, smoking status and drinking status between the groups compared. The frequencies of *MTHFR* and *TS* genotypes were similar between the positives and the negatives. The observed frequencies of the two polymorphisms did not deviate from Hardy-Weinberg equilibrium (p=0.10 for *MTHFR* and p= 0.59 for *TS*).

Table 1. Characteristics according to *H. pylori* infection and gastric atrophy

Characteristics		*H. pylori* infection			Gastric atrophy		
		Positive	Negative	p value	Positive	Negative	p value
		N=115	N=59		N=46	N=124	
Age (Years)	Mean	53.2	45.6	<0.0001	55.0	49.0	0.0033
	20 - 29	3	10		1	12	
	30 - 39	8	10		3	15	
	40 - 49	20	10		5	25	
	50 - 59	55	19		23	48	
	60 - 69	28	9		13	23	
	70 -	1	1		1	1	
Sex	Female	59	37	N.S	25	68	N.S
	Male	56	22		21	56	
Smoking	Ever	29	10	N.S	11	28	N.S
	Never	85	49		35	96	
Drinking	Yes	28	11	N.S	11	28	N.S
	No	86	47		34	95	
Folate (ng/ml)	Mean	6.6	6.7	N.S	6.6	6.7	N.S
tHcy (nmol/L)	Mean	10.3	10.1	N.S	10.3	10.3	N.S
MTHFR	*CC*	34	26		16	44	
	CT	67	26		23	66	
	TT	14	7	N.S	7	14	N.S
TS	*33*	77	42	N.S	30	87	N.S
	22/23/35	38	16		16	36	

tHcy: total homocysteine *MTHFR: methylenetetrahydrofolate reductase*

TS: thymidylate synthase

There was a strong association between plasma tHcy and the *MTHFR* genotype. Concentration of tHcy was significantly higher for *TT* genotype (13.9± 9.4 nmol/ml) than for *CC/CT* genotype (9.7 ± 2.9 nmol/ml) (p<0.0001). The other factors elevating tHcy level were males and ever smokers. The factors reducing serum folate level were males, ever smokers and drinkers. As the concentration of folate became lower, the difference in elevation of the tHcy concentration due to *MTHFR* polymorphism became larger. The *MTHFR* genotype had the strongest influence on the elevation of tHcy concentration among the factors examined. Figure 2 shows the correlation between folate and tHcy among those with folate < 8.0ng/ml. The slope of the regression line was steepest for those with *TT* genotype (slope = -4.61). Those with folate ≥ 8.0ng/ml had stable tHcy levels.

No significant differences in concentrations of serum folate and tHcy were found between the positives and the negatives of *H. pylori* infection and gastric atrophy according to the genotypes of *MTHFR* and *TS* in any subgroups defined by the genotypes (Table 2).

Table 3 shows the ORs and 95% CIs of hyperhomocysteinemia (≥12 nmol/ml), adjusted for age, sex, and the factors listed in the Table. The OR was above unity for *H. pylori* infection and below unity for gastric atrophy, through not significant. The OR of genotype of *MTHFR* was not significant in the multivariate analysis for hyperhomocysteinemia. Table 4 shows the ORs and 95% CIs for folate ≤ 4 mg/ml, adjusted for the same factors. Gastric atrophy decreased a risk of lower serum folate (adjusted OR=0.21, 95% CI, 0.05-0.78). Smoking status was a significant factor of lower serum folate.

Table 2. Concentrations of serum folate and plasma total homocysteine according to genotypes of *methylenetetrahydrofolate reductase (MTHFR)* and *thymidylate synthase (TS)*, *H. pylori* infection and gastric atrophy

Genotype		*MTHFR*						*TS*					
		CC/CT			*TT*			*33*			*non33*		
		N	Conc.*	p value	N	Conc.	p value	N	Conc.	p value	N	Conc.	p value
Serum folate													
Infection	Positive	98	6.7	0.41	14	6.0	0.63	76	6.8	0.44	36	6.2	0.50
	Negative	51	6.9		7	5.1		41	6.9		16	5.7	
Atrophy	Positive	39	6.5	0.95	7	6.8	0.65	30	7.0	0.68	16	5.7	0.91
	Negative	108	6.8		14	5.2		85	6.8		36	6.1	
Plasma total homocysteine													
Infection	Positive	101	9.9	0.14	14	13.2	0.88	77	9.9	0.77	38	11.1	0.05
	Negative	52	9.4		7	15.2		42	9.7		16	11.2	
Atrophy	Positive	39	9.5	0.32	7	15.2	0.63	30	10.1	0.33	16	10.7	0.88
	Negative	110	9.9		14	13.3		87	9.8		36	11.6	

*Conc.: Consentration: ng/ml for serum folate and nmol/L for plasma total homocysteine

Table 3. Odds ratio (OR) and 95% confidence interval (95% CI) of hyperhomocysteinemia (≥ 12 n mol/ml), adjusted for age, sex, and the factors listed

Factor		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
H. pylori infection	Neg.	1 (Reference)	1 (Reference)	1 (Reference)			
	Pos.	1.14 (0.45-2.84)	1.16(0.45-2.96)	1.29(0.48-3.46)			
Atrophy	Neg.				1 (Reference)	1 (Reference)	1 (Reference)
	Pos.				0.90(0.34-2.34)	083(0.31-2.22)	0.69(0.24-1.98)
Smoking	No		1 (Reference)	1 (Reference)		1 (Reference)	1 (Reference)
	Yes		0.90(0.33-2.42)	0.80(0.29-2.27)		0.90(0.34-2.41)	0.83(0.30-2.30)
Drinking*	No		1 (Reference)	1 (Reference)		1 (Reference)	1 (Reference)
	Yes		0.69(0.26-1.87)	0.95(0.34-2.68)		0.69(0.26-1.86)	0.97(0.34-2.74)
MTHFR C677T	*CC*			1 (Reference)			1 (Reference)
	CT			0.50(0.19-1.34)			0.54(0.21-1.42)
	TT			2.10(0.54.-8.12)			2.30(0.59-8.94)
TS	*33*			1 (Reference)			1 (Reference)
	non33			1.76(0.70-4.40)			1.68(0.67-4.23)

* "No" for < once per week, and "Yes" for ≥ once per week.

Table 4. Odds ratio (OR) and 95% confidence interval (95% CI) of lower serum folate (≤ 4 mg/ml), adjusted for age, sex, and the factors listed

Factor		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
H. pylori	Neg.	1 (Reference)	1 (Reference)	1 (Reference)			
infection	Pos.	0.84 (0.35-2.02)	0.72(0.29-1.78)	0.69 (0.27-1.77)			
Atrophy	Neg.				1 (Reference)	1 (Reference)	1 (Reference)
	Pos.				0.35(0.11-1.09)	0.24 (0.07-0.87)	0.21 (0.05-0.78)
Smoking	No		1 (Reference)	1 (Reference)		1 (Reference)	1 (Reference)
	Yes		3.01(1.09-8.23)	3.05(1.08-8.61)		3.15 (1.11-8.90)	3.18 (1.10-9.20)
Drinking*	No		1 (Reference)	1 (Reference)		1 (Reference)	1 (Reference)
	Yes		0.83 (0.30-2.30)	1.04 (0.36-2.97)		0.83 (0.29-2.36)	1.07 (0.36-3.17)
MTHFR	*CC*			1 (Reference)			1 (Reference)
C677T	*CT*			1.02 (0.39-2.69)			1.04 (0.39-2.74)
	TT			2.99 (0.85-10.54)			3.50 (0.95-12.92)
TS	*33*			1 (Reference)			1 (Reference)
	non33			1.43 (0.59-3.48)			1.41 (0.57-3.51)

* “No” for < once per week, and “Yes” for ≥ once per week.

4. Discussion

Hyperhomocysteinemia is a well-established independent risk factor for the development of atherosclerosis-related diseases due to vascular endothelial damage and hypercoagulability. Some studies demonstrated an association between *H. pylori* infection and hyperhomocysteinemia [1-4], while the other studies did not. [15, 16, 21-23]. Our study taking into the genotypes of folate metabolizing enzymes did not indicate the association.

A number of studies on the possible *H. pylori* involvement for coronary heart disease have been published with conflicting results [24, 25], but a meta-analysis reported no strong association between *H. pylori* infection and ischemic heart disease in 1988 [26]. Recent reports consistently revealed the association between Cag A-positive *H. pylori* and vascular risk [27, 28]. On the other hand, there were many reports of positive associations of stroke and carotid artery disease with *H. pylori* infection. In addition, there was a strong association in subgroup analysis of patients with different etiologies of cerebral ischemia [29-31].

Epidemiologic studies have shown that homocysteine is associated with sex, age, smoking status and drinking status [8]. In Japan, Moriyama *et al* reported that age-adjusted plasma homocysteine levels were higher for both men and women with the *TT* genotype than those with *CC* or *CT* genotype in their cross-sectional study [32]. Casas *et al* searched for all relevant studies on the association between homocysteine concentration and *MTHFR* polymorphism, and reported that homocysteine concentration between *TT* and *CC* homozygotes was 1.93 μmol/L and the odds ratio for stroke was 1.26 (95% CI, 1.14 to 1.40) for *TT* versus *CC* homozygotes [33]. A meta-analysis reported that ischemic stroke risk increased with increasing *MTHFR 677T* allele dose, suggesting an influence of this polymorphism as a genetic stroke risk factor [34]. But another recent meta-analysis reported that no strong evidence existed to support an association heart disease in Europe, North America, or Australia [35]. Devlin *et al* reported that the affection of *MTHFR* polymorphism to hyperhomocysteinemia was significantly strong in lower folate level [36]. Our result supported that the *MTHFR* C677T polymorphism was associated with homocysteine concentration, and persons with *TT* genotype trended to be a hyperhomocysteinemia in lower folate level (Figure 2). It is important to include this factor for evaluation of the association between *H. pylori* infection and homocysteine.

There are three steps in this logical proposition. First step is that *H. pylori* infection causes to gastric atrophy, and second step is that gastric atrophy makes malabsorption to reduce serum folate level. And last step is that lower folate level causes tHcy level elevation.

The first step was well established [37]; our study also showed a very strong association between gastric atrophy and *H. pylori* infection. In the second step, there were few reports demonstrated that gastric atrophy blocked the absorption of folate. Gastric atrophy causes an increase in gastric pH and a decrease in ascorbic acid; the two mechanisms may cause a reduction in folate absorption [38, 39]. In the present study, gastric atrophy decreased a risk of lower folate opposite to what we expected. There were no other factors available for the adjustment, so the reason for this significant association was unknown. Anyway, gastric atrophy may not increase a risk of lower folate. *H. pylori* infection was not significantly associated with lower folate level and hyperhomocysteinemia.

The last step was demonstrated by several studies. Markedly elevated homocysteine concentration have been observed in patients with nutritional deficiencies of the essential cofactor vitamin B_{12} and the cosubstrate folate [40, 41]. But these are negative reports for subjects with high folate levels [1, 10]. Our present study showed that *TT* genotype of *MTHFR* trended to have hyperhomocysteinemia in lower folate level, and tHcy was low among those with high folate level independently on the genotypes.

Because there is no consistent evidence, the hypothesis that *H. pylori* causes hyperhomocysteinemia was still controversial. Recent studies reported that no significant difference in blood homocysteine level between the positives and the negatives [14-16]. Our logistic analysis showed the similar results indicating that there is no link from *H. pylori* infection to hyperhomocysteinemia.

5. Conclusion

This study was the first study that analyzed association between *H. pylori* infection and hyperhomocysteinemia in normal subjects taking into account the polymorphism of *MTHFR*. There was no significant difference in concentration of serum folate and tHcy between those with and without *H. pylori* infection and gastric atrophy. We found a trend to elevate tHcy in *TT* genotype of *MTHFR* as lower serum folate. Our result suggested that there are no pathways from *H. pylori* infection to hyperhomocysteinemia in Japanese and an alternative pathway may exist in association of *H. pylori* infection to atherosclerosis, if exists.

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Conflicts of interest

The authors have declared that no conflict of interest exists.

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