RESEARCH NOTE

Association of specific haplotype of TNFα with *Helicobacter pylori*-mediated duodenal ulcer in eastern Indian population

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Introduction

In the present study, we investigated the association between cytokine gene polymorphisms and *Helicobacter pylori*-mediated gastroduodenal ulcer in eastern Indian population. The analysis of promoter polymorphisms of *TNF-α*, *IL6* and *IL8* revealed no association with *H. pylori*-mediated duodenal ulcer at the genotype level. However, *TNF-α* haplotype GCAT, was present at a significantly higher frequency in *H. pylori* infected individuals with duodenal ulcers, than in those infected individuals without ulceration (odds ratio (OR) = 8.07, with 95% confidence intervals (95%CI) = 1.26–50.4, $P < 0.05$). We also observed difference of expression of these cytokine genes between *H. pylori* infected symptomatic and asymptomatic individuals. However, no correlation was observed between the expression of these cytokine genes and the polymorphic status of the individuals.

H. pylori is the most common gastrointestinal pathogen distributed worldwide and causes a wide spectrum of gastroduodenal diseases. However, even though half of the world's population is infected by *H. pylori*, only 20%–30% actually develop any disease. Bacterial virulence factors, such as the cag pathogenicity island (cag PAI), the vacuolating cytotoxin (Vac A) and Ice A (a designation derived from the phrase 'induced by contact with epithelium') have been associated with enhanced inflammation and gastric cancer development (Blaser *et al*. 1995). However, bacterial factors alone do not explain variable disease manifestation. Thus, the nature of the host immune response might explain these differences in clinical outcome (Gillen and McColl 2005).

A key element in the response to *H. pylori* infection is the immediate production of proinflammatory cytokines in the gastric mucosa. Polymorphisms in *IL-1B, IL6, IL8* and *TNF-α* have been reported to influence cytokine expression, thereby influencing inflammatory processes in response to infectious diseases (Graham 1997; Jordanides *et al*. 2000; El-Omar *et al*. 2001a,b; Hull *et al*. 2001; McColl and El-Omar 2002). Recent studies have described the association of specific variants of the *IL-1B* and *TNF-α* genes with susceptibility to *H. pylori*-related diseases (Kato *et al*. 2001; El-Omar *et al*. 2003; Garcia-Gonzalez *et al*. 2003; Lee *et al*. 2003; Chakravorty *et al*. 2006). Individuals with *IL6*-174 G/G have been shown to produce higher levels of *IL6* than those with the C/C genotype, and the former genotype is associated with the high-mucosal levels of *IL6* in *H. pylori*-associated gastritis (Lobo Gatti *et al*. 2005). Savage *et al*. (2004) reported that the homozygous polymorphic variants of *IL8* (-251 A/A and +396 G/G) increase the risk for gastric cardia carcinogenesis in the high-risk Chinese population.

Since a complex trait disease, such as duodenal ulcer is likely to involve the interaction of alleles at multiple loci, the goal of the current study was to investigate possible associations between specific alleles of *TNF-α* (-308, -857, -863, -1031), *IL6* (-172, -572, -594) and *IL8* (-251) with *H. pylori*-mediated duodenal ulcer, as well as to analyse the expression levels of these cytokines in patients and controls from eastern India as no such reports are available in Indian populations.

Materials and methods

Subjects

The subjects included for this study were 310 unrelated individuals (215 males and 95 females, mean age 38.0 ± 2.0), who underwent enodoscopy at the SSKM Hospital, Kolkata, India, from January 2002 to January 2004. The case (*H.*

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pylori infected with duodenal ulcer, *H. pylori* uninfected with duodenal ulcer) individuals were suffering from gastroenterological problems, from the out-patient department of SSKM Hospital, Kolkata. The controls (*H. pylori* infected without duodenal ulcer, *H. pylori* uninfected without duodenal ulcer) were normal healthy individuals who agreed to participate in this study. The diagnosis of duodenal ulcer was established on the basis of conventional, clinical and endoscopic findings. No subjects had received treatment for *H. pylori* infection. Patients taking nonsteroidal anti-inflammatory drugs, those receiving anti-secretory therapy, or those with gastric carcinoma, were excluded from this study. Duodenal-ulcer patients with gastrointestinal bleeding or suffering from osteoarthritis and cardiovascular diseases were also excluded. Prior to sample collection, written informed consent was obtained from each individual, approved by the ethical committee of the SSKM Hospital.

Diagnosis of H. pylori infection

From each patient, three antral and three corpus biopsy specimens were collected for rapid urease test (RUT), *H. pylori* culture (Yamaoka *et al*. 1999) and histological examination of modified Giemsa stained slides according to the Sydney system (El-Zimaity *et al*. 1996). Diagnosis of *H. pylori* infection was made on the basis of positive results in at least two of the three tests conducted, and absence of *H. pylori* was inferred when all the three tests were negative.

Genotyping of cytokine gene polymorphisms

DNA was extracted from 5-ml blood taken from each individual using standard protocol (Sambrook *et al*. 1989). Cytokine gene polymorphisms were genotyped by PCR, restriction fragment length polymorphism (RFLP) analysis. For genotyping the *TNF-α* (-308 G/A, -857 C/T, -863 C/A, -1031 T/C) polymorphism, the PCR products for each polymorphism was digested with *Nco*I, *HpyCH*IV, *HpyCH*IV, and *Bbs*I, respectively (Skoog *et al*. 1999). *IL6* (-172 G/C, -570 G/C, -594 G/A) polymorphism was genotyped by PCR and subsequent restriction digestion with *Nla*III, *Fnu4H*-I and *Fok*I, respectively (Jordanides *et al*. 2000). Genotyping of *IL8*, -251 A/T polymorphism was performed by digestion of the PCR products with the restriction enzyme *Mfe*I.

Quantitative RT-PCR for TNF-α, IL6 and IL8 expression

Total RNA was isolated from the gastric biopsies of a subset of individuals from different groups using TRIZOL (Invitrogen, LifeTechnologies, Carlsbad, USA). Two μl of DNase-treated RNA was reverse transcribed using Ambion Retroscript kit (Ambion, Austin, USA), in a total volume of 20 μl and stored at -20°C until use. *TNF-α* mRNA expression was determined by RT-PCR in the iCycler (Biorad, Hercules, USA) using the Taqman technology. Quantitative cytokine mRNA expression for *IL6* and *IL8* expression was determined by RT-PCR in the iCycler (Biorad, Hercules, USA) using Sigma jumpstart Syber Green (Sigma, St Louis, USA). The mRNA levels of *TNF-α*, *IL6* and *IL8* were normalized to house keeping gene β-actin.

Statistical analysis

Hardy–Weinberg equilibrium was tested at all SNPs within each group by χ^2 test. Haplotype frequencies were calculated using the software ARLEQUIN version 2.1 (Schneidar *et al*. 2000). Risk of duodenal ulcer upon *H. pylori* infection was calculated as age-adjusted and sex-adjusted OR with 95% CI. for the different genotypes, using the SPSS statistical package (SPSS 2003; SPSS12.0 for windows, Chicago, USA).

Results

Clinical and demographic characteristics of patients and controls

Our sample comprised of 310 unrelated individuals, who were classified on the basis of endoscopic results, as well as bacterial culture reports, into four categories: with both *H. pylori* infection and duodenal ulcer (Hp^+ $Ulcer^+$, n = 91), with *H. pylori* infection but without duodenal ulcer (Hp^+ $Ulcer^-$, n = 62), without *H. pylori* infection but with duodenal ulcer (Hp^- $Ulcer^+$, n = 72) and without either *H. pylori* infection or ulcer (Hp^- $Ulcer^-$, n = 85). The subjects selected for this study were from the same geographical location, belonging to the same caste/ethnic (Bengali–Hindu) background. The mean age and sex ratio were similar among the four groups. We have reported previously that the different caste subpopulations in this geographical area (West Bengal, India) do not show any differences either in allelic or genotypic distributions of various cytokine gene polymorphisms (Chakravorty *et al*. 2004).

TNF-α, IL6 and IL8 gene polymorphisms

Table 1a summarizes the genotype frequencies of all the polymorphisms of *TNF-α*, *IL6* and *IL8* in the four groups of patients and controls according to their *H. pylori* status. Genotype frequencies of any of these polymorphisms did not deviate significantly from Hardy–Weinberg equilibrium in all the four groups. We also did not observe any significant association of allele and genotype frequencies between the *H. pylori* infected individuals with or without duodenal ulcer and uninfected symptomatic and asymptomatic individuals except for the *TNF-α*-1031 locus where the combined C/C and C/T genotypes showed protection over T/T genotype (OR = 0.463, 95%CI = 0.239–0.893, P = 0.032) (table 1a) in *H. pylori* infected with or without duodenal ulcer individuals.

Analysis of the haplotype distribution of *TNF-α* gene in these four groups showed that a particular haplotype GCAT

was present insignificantly in a higher frequency in *H. pylori* infected individuals with duodenal ulcer than in those individuals with infection but without ulceration, OR = 8.07 (95%CI = 1.26–50.4, $P < 0.05$) (table 1b). Pairwise haplotype of *IL6* (-570, -594) showed prevalence of G-G haplotype in all four groups, with frequency of 0.54 in Hp^+ $Ulcer^+$, 0.40 in Hp^+ $Ulcer^-$, 0.58 in Hp^- $Ulcer^+$ and 0.49 in Hp^- $Ulcer^-$ groups, respectively. No significant association between the *IL6* haplotypes and *H. pylori*-mediated duodenal ulcer could be found in our population. The *IL6*-172 locus was not included in the haplotype analysis, as it was almost monomorphic for the G allele.

Combined IL-1B and TNF-α genotypes

We had earlier observed a significant association between *IL-1B*-31 C/C genotype with *H. pylori*-associated duodenal

Table 1a. Distribution of genotypes and estimation of risk of *TNF-α, IL6, IL8* genes in *H. pylori* infected individuals with and without duodenal ulcer.

Polymorphism	Genotype	*Hp^+/$Ulcer^+$ (n = 91)	*Hp^+Ulcer^- (n = 62)	Odds ratio (OR)	*Hp^-/$Ulcer^+$ (n = 72)	*Hp^-/$Ulcer^-$ (n = 85)
TNF-308	G/G	70 (0.76)	47 (0.75)	Referent (G/G) = 1.0	55 (0.76)	60 (0.70)
	G/A	19 (0.20)	14 (0.22)	Combined risk (G/A+A/A)	15 (0.20)	21 (0.24)
	A/A	2 (0.02)	1 (0.01)	OR = 0.94 (95%CI = 0.44–2.00)	2 (0.02)	4 (0.04)
TNF-857	C/C	87 (0.95)	55 (0.88)	Referent (C/C) = 1.0	65 (0.90)	75 (0.88)
	C/T	4 (0.04)	6 (0.09)	Combined risk (C/T+T/T)	7 (0.09)	9 (0.10)
	T/T	0 (0)	1 (0.01)	OR = 0.3612 (95%CI = 0.1–1.29)	0 (0)	1 (0.01)
TNF-863	C/C	54 (0.59)	32 (0.51)	Referent (C/C) = 1.0	54 (0.75)	55 (0.64)
	C/A	31 (0.34)	25 (0.40)	Combined risk (C/A+A/A)	16 (0.22)	26 (0.30)
	A/A	6 (0.06)	5 (0.08)	OR = 0.7309 (95%CI = 0.38–1.40)	2 (0.02)	4 (0.04)
TNF-1031	T/T	54 (0.59)	25 (0.40)	Referent (T/T) = 1.0	35 (0.48)	35 (0.41)
	C/T	28 (0.30)	32 (0.51)	Combined risk (C/T+C/C) OR = 0.463	22 (0.30)	33 (0.38)
	C/C	9 (0.09)	5 (0.08)	(95%CI =0.23–0.89) P = 0.032	13 (0.18)	17 (0.2)
*IL*6-172	G/G	72 (0.79)	54 (0.87)	Referent (G/G) = 1.0	62 (0.86)	75 (0.88)
	G/C	18 (0.19)	7 (0.11)	Combined risk (G/C+C/C)	9 (0.12)	10 (0.11)
	C/C	1 (0.01)	1 (0.01)	OR = 1.7813 (95%CI = 0.72–4.37)	1 (0.01)	0 (0)
*IL*6-570	G/G	57 (0.62)	37 (0.59)	Referent (G/G) = 1.0	59 (0.81)	65 (0.76)
	G/C	27 (0.29)	20 (0.32)	Combined risk (G/C+C/C)	11 (0.15)	18 (0.21)
	C/C	7 (0.07)	5 (0.08)	Odds ratio = 0.8828 (95%CI = 0.45–1.71)	2 (0.02)	2 (0.02)
*IL*6-594	G/G	53 (0.57)	41 (0.66)	Referent (G/G) = 1.0	55 (0.76)	62 (0.72)
	G/A	29 (0.31)	16 (0.25)	Combined risk (G/A+A/A)	15 (0.20)	20 (0.23)
	A/A	10 (0.10)	5 (0.08)	OR = 1.4367 (95%CI = 0.73–2.8)	2 (0.02)	3 (0.03)
*IL*8-251	A/T	46 (0.50)	28 (0.45)	Referent (G/G) = 1.0	29 (0.40)	34 (0.40)
	A/A	25 (0.27)	16 (0.25)	Combined risk (G/A+A/A)	25 (0.34)	26 (0.30)
	T/T	20 (0.21)	18 (0.29)	OR = 0.7885 (95%CI = 0.41–1.50)	18 (0.25)	25 (0.29)

*Hp^+ $Ulcer^+$, *H. pylori* infected doudenal ulcer patients; Hp^+ $Ulcer^-$, *H. pylori* infected nonulcer individuals; Hp^- $Ulcer^+$, *H. pylori* uninfected duodenal ulcer patients; Hp^- $Ulcer^-$, *H. pylori* uninfected nonulcer individuals. n, number of individuals. OR was calculated among Hp^+/$Ulcer^+$ and Hp^+/$Ulcer^-$ groups.

Table 1b. Haplotype frequencies of the *TNF-α* polymorphisms in *H. pylori* infected individuals with and without duodenal ulcer.

	Frequencies			
Haplotypes	Hp^+ $Ulcer^+$ ($n = 91 \times 2 = 182$)	Hp^+ $Ulcer^-$ ($n = 62 \times 2 = 124$)	Hp^- $Ulcer^+$ ($n = 72 \times 2 = 144$)	Hp^- $Ulcer^-$ ($n = 85 \times 2 = 170$)
G-C-C-C	0.145	0.181	0.116	0.251
A-C-C-C	0.00	0.035	0.023	0.00
G-C-C-T	0.395	0.410	0.288	0.271
G-C-A-C	0.217	0.256	0.00	0.024
*G-C-A-T	0.161	0.011	0.291	0.236
G-T-C-T	0.00	0.00	0.021	0.044
A-C-C-T	0.072	0.00	0.203	0.063
A-C-A-T	0.00	0.077	0.00	0.086
A-C-A-C	0.00	0.026	0.034	0.00
A-T-A-T	0.008	0.00	0.00	0.020
G-T-A-T	0.00	0.00	0.021	0.00
A-C-A-C	0.00	0.00	0.034	0.00

Hp^+ $Ulcer^+$, *H. pylori* infected duodenal ulcer patients; Hp^+ $Ulcer^-$, *H. pylori* infected nonulcer individuals; Hp^- $Ulcer^+$, *H. pylori* uninfected duodenal ulcer patients; Hp^- $Ulcer^-$, *H. pylori* uninfected non ulcer individuals. *n*, number of choromosomes. *GCAT haplotype is significantly higher in *H. pylori* infected individuals with duodenal ulcer than in individuals who are infected but asymptomatic. Estimation of risk of the G-C-A-T haplotype was calculated considering the combined other haplotypes as reference.

ulcer in the eastern Indian population (Chakravorty *et al*. 2006). To investigate possible associations between the specific genotype of *IL-1B*-31 and *TNF-α* alleles, the distribution of *TNF-α* haplotypes among individuals bearing the C/C genotype at the *IL-1B*-31 locus was analysed. We grouped individuals who were C/C homozygous at -31 position as well as homozygous for all four *TNF-α* polymorphic sites in one class (these individuals will bear the C at -31 *IL-1B* position and GCAT at *TNF-α* loci on both chromosomes) and individuals who, based on their genotypes at individual locus, could be determined with certainty as noncarriers of the *IL-1B*-31C and *TNF-α* GCAT, in another class. The simultaneous carriage of *IL-1B*-31C and *TNF-α* GCAT haplotype was increased in *H. pylori* positive duodenal ulcer patients, compared to *H. pylori*-infected asymptomatic carriers (70% versus 0%), but no formal statistics could be performed due to the small sample size of the *H. pylori* infected asymptomatic group.

The correlation of TNF-α, IL6 and IL8 polymorphisms with their mucosal expression in the gut

We sought to determine the mucosal expression levels of TNFα, IL8 and IL6 mRNA in *H. pylori* infected individuals with and without duodenal ulcer (figure 1). Although the level of *TNF*α and *IL6* expression was significantly higher (lower ΔC_t value) in *H. pylori* infected individuals with duodenal ulcers compared to *H. pylori* infected asymptomatic individuals, no significant association of *TNF-α* and *IL-6* promoter polymorphisms with expression level was observed (data not shown). No significant difference in the expression of *IL8* between *H. pylori* infected individuals with or without duodenal ulcer was observed. Correlation of *IL8* promoter polymorphism with its expression was also not significant (data not shown).

Discussion

We carried detailed analyses of *IL6, IL8* and *TNF-α* gene polymorphisms in both *H. pylori*-infected and uninfected individuals, with and without duodenal ulcers. No significant association between any of the polymorphic loci with *H. pylori*-mediated duodenal ulcer was observed. However, we did observe an association between the *TNF-α* haplotype GCAT, which was present significantly at a higher frequency in *H. pylori*-infected individuals with duodenal ulcers than in those individuals with infection but without ulceration (OR = 8.07, 95%CI = 1.26–50.4, $P < 0.05$). Mucosal expression levels of *TNF-α* showed a trend of lower expression in *H. pylori*-infected individuals with duodenal ulcers, compared to *H. pylori*-infected asymptomatic individuals. On the other hand, *IL6*, being a proinflammatory cytokine mediating a wide variety of inflammatory response, showed a significantly higher secretion ($P < 0.001$) in *H. pylori*-infected duodenal ulcer patients in comparison to *H. pylori*-infected asymptomatic individuals, although this could not be associated with the promoter-polymorphic alleles.

We had previously reported a strong association between the *IL-1B*-511 T/T and *IL-1B*-31 C/C genotypes and *H. pylori*-mediated duodenal ulcer (OR = 4.2, 95%CI = 1.8–9.4, and OR = 2.16, 95%CI = 1.12–4.15, respectively) both at genotype and at haplotype level (Chakravorty *et al*. 2006). However, in the same population, we failed to observe

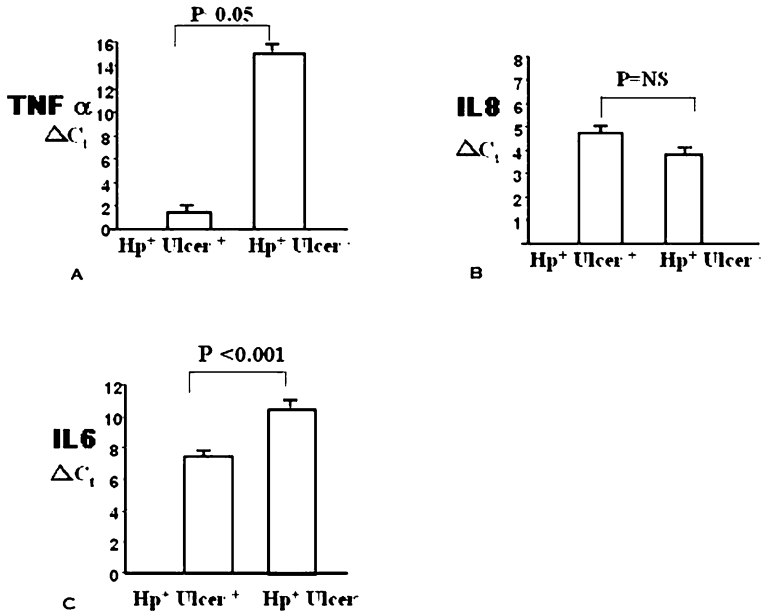

Figure 1. Gastric mucosal lelvels of TNFα, IL6 and IL8 mRNA (expressed as ΔC_t) in Hp^+ $Ulcer^+$ and Hp^+ $Ulcer^-$ individuals. The bars represent the mean ΔC_t values withing each group with ±SD. (A) Gastric mucosal TNFα mRNA (expressed as ΔC_t), in Hp^+ $Ulcer^+$ and Hp^+ Ulcer individuals. (B) Gastric mucosal IL8 mRNA (expressed as ΔC_t) in Hp^+ $Ulcer^+$ individuals. (C) Gastric mucosal IL6 mRNA (expressed as ΔC_t) in Hp^+ $Ulcer^+$ and Hp^+ $Ulcer^+$ individuals.

any significant association between the polymorphic alleles of *TNF-α*, *IL6* and *IL8* with *H. pylori*-mediated duodenal ulcer in the present study. Hence, we conclude that the promoter polymorphisms of the *IL-1B* promoter serve as a better marker for the study of association of cytokine polymorphisms with *H. pylori*-mediated duodenal ulcers.

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