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Association of gastric disease with polymorphisms in the inflammatory related genes IL-1B, IL-1RN, IL-10, TNF and TLR4

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Abstract

Objectives—Increasing evidence suggests that polymorphisms in key mediator genes of *Helicobacter pylori* (*H. pylori*) -induced inflammation, influence susceptibility to developing non-cardia gastric cancer. This study aimed to investigate if single nucleotide polymorphisns (SNPs) in a series of inflammatory genes were associated with the development of the most common pathologies thought to precede gastric cancer development namely; *H. pylori* associated gastritis and intestinal metaplasia.

Methods—A total of 250 patients were genotyped for 11 SNPs in the IL-1B, IL-1RN, TNF, TLR4 and IL-10 genes. The study population comprised *H. pylori* uninfected ('normal') control patients (n=96), *H. pylori* positive gastritis (n=91) and intestinal metaplasia patients (n=63). Genotyping was performed using *Taqman* allelic discrimination assays. Odds ratios for gastric disease groups were adjusted for potential confounding factors.

Results—No differences were identified in frequency of carriage, or homozygosity, for any of the 'risk' alleles investigated across the patient groups. There was no evidence to suggest an association with increased risk of developing either chronic gastritis or intestinal metaplasia with SNPs in the IL-1B, IL-1RN, TNF, TLR4 and IL-10 genes.or haplotypes tested.

Conclusion—This study found no evidence of an association with increased risk of developing either chronic gastritis or intestinal metaplasia with the SNPs or haplotypes tested.

Background

Persistent local inflammation is now accepted as a risk factor in the development of a number of cancers. Non-cardia gastric cancer is thought to develop after a long lead-time whereby persistent inflammation, caused by *Helicobacter pylori* (*H. pylori)* colonisation of the gastric mucosa progresses to atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma[1].

Investigations have increasingly considered the role of host/human genetics in *H. pylori* associated gastro-duodenal disease. A number of single nucleotide polymorphisms (SNPs) in genes encoding a variety of inflammatory mediators have apparent functional relevance, in

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that they appear to influence cytokine bio-availability or expression levels. Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine and inhibitor of gastric acid secretion. Polymorphisms in the gene encoding IL-1β (IL-1B) have been associated with an increased risk of hypochlorhydia, gastritis[1] and non-cardia gastric cancer[3] development. Functionally, SNPs at positions -31 and -511 of the IL-1B gene and type 2 of the variable number tandem repeat in the IL-1RN gene (encoding the IL-1 receptor antagonist, IL-1ra) have been putatively associated with increased levels of IL-1β production[4,5]. Expression of IL-1B-511T and IL-1RN*2 together, have also been associated with a significant increase in risk of gastric cancer[6]. A pro-inflammatory profile comprising of SNPs in genes encoding IL-1β, IL-1ra, Tumour Necrosis Factor-α (TNFα) and Interleukin-10 (IL-10) were further reported to confer increased risks of both chronic gastritis and gastric carcinoma development[7,8].

Toll-like receptor 4 (TLR4) is understood to influence the host response to Gram negative infection[9,10] and some studies have reported a role for TLR4 in the host response to *H. pylori* infection[11–13]. Polymorphisms in the coding region of the TLR4 gene at positions +896 and +936 alter the extracellular domain of the receptor at amino acid positions Asp299Gly and Thr399Ile and have been associated with a blunted response to lipopolysaccharide[14]. An association with the Asp299Gly variant with an increased risk of gastric mucosal atrophy and hypochlorhydia has been reported in first degree relatives of patients with gastric cancer [15]. Achyut *et al.*[16], reported an association between the Thr399Ile allele and an elevated risk for gastritis. However, two subsequent studies failed to replicate this association in Mexicans and Europeans[17,18].

Using a cohort of 250 patients we aimed to investigate the role of polymorphisms in genes encoding IL-1β, IL-1ra, IL-10, TNF and TLR-4 in risk of early gastric disease that is thought to occur in the early stages of gastric cancer development. These polymorphisms have been associated with an immune modulatory function, which could be involved in the progression to neoplasia from chronic inflammation. Specifically, our study investigated the frequency of these 'risk' polymorphisms in patients with chronic *H. pylori* associated gastritis and intestinal metaplasia and in a *H. pylori* uninfected control population.

Materials and Methods

Study population

A total of 950 adult patients undergoing endoscopy for clinical reasons were screened through the Gastroenterology Endoscopy Unit at the Adelaide & Meath Hospital, incorporating the National Childrens' Hospital, Dublin. Patients were recruited between 2000 and 2002, and 99% of patients were of 'Irish Ethnicity'. The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St. James's Hospital.

Patients with concurrent inflammatory bowel disease, coeliac disease, rheumatoid arthritis, diabetes and any other chronic inflammatory conditions were excluded from the study. Prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) or gastric pathology believed to be NSAID related also resulted in exclusion. Only patients with sufficient biopsies sets from three sites, namely, the antrum, corpus and duodenum were considered eligible. Gastric mucosal biopsy tissue was reviewed and classified by a Pathologist. Histological status was determined according to the updated Sydney Classification System[19].

Participants were classified into three study groups: gastritis, intestinal metaplasia and normal controls. The gastritis group included patients with histologically confirmed chronic gastritis of either the antrum or corpus or a pan-gastritis and were free from concurrent gastric lesions, (i.e. when ulcers were found in either the stomach or the duodenum, the patient was excluded). All patients in this group were H *pylori* positive. The IM group included all patients presenting

with IM, either 'focal IM' (<25% of epithelium involved) or 'extensive IM' (>25% epithelium involved) who were free from concurrent gastric lesions and were *H. pylori* positive. The normal control group comprised patients with histologically normal gastric mucosa and were *H. pylori* negative.

Assessment of *H. pylori* status

Accurate determination of *H. pylori* status was considered essential to the integrity of the study data. To this end, *H. pylori* status was assessed by a number of methods. Infection was determined according to culture, histological identification, ^{13}C Urea breath test and rapid urease (Clo™) testing. Serological testing for *H. pylori* IgGs was performed for all patients recruited prospectively using an ELISA diagnostic kit against *H. pylori* IgGs (Biohit Plc., Helsinki, Finland).

Genotyping of polymorphisms

DNA was extracted, using a commercially available kit (Qiagen Ltd., West Sussex, UK), from fasting peripheral blood samples taken at the time of endoscopy. Genotyping of the IL-1B-31, -511, +3954, TLR4+896, TNF-238 and -308 assays was carried out using ABI Prism 720 Sequence Detection System, at the Department of Molecular Medicine, Sir Patrick Dunns Laboratory, St. James's Hospital, Dublin. Genotyping of the TLR+936, IL-1RN+2018, IL-10-592, -819 and -1082 SNPs was performed by Kbiosciences (Hertfordshire, UK; www.kbioscience.co.uk). The sequences of the primers and probes used in the assays and the reaction conditions are available upon request from the corresponding author.

Statistical analysis

Distribution of allele and genotype frequencies across patients groups was performed using JMP (JMP IN, Version 4.0.4, SAS Institute Inc.), SPSS (Version 10.0 SPSS Inc.) and Genepop packages (Genepop Online: http://wbiomed.curtin.edu.au/genepop/genepop_op2.html). Testing for Hardy-Weinberg equilibrium was performed using Genepop. Pearsons χ^2 values were calculated using SPSS (v12, SPSS Inc.) and were in agreement with Fisher's χ^2 values reported by Genepop. Haplotype analysis was performed using PHASE 2.1.1 (web-based version, PHASE software for haplotype estimation, Washington, USA[20,21]). Logistic regression was performed using the JMP IN software package and STATA software (STATA v8.2, STATA Press). Odds ratios are reported with 95% confidence intervals (CIs). All odds ratios were adjusted for the effects of age and gender.

Power calculations (Power and Sample Size Calculator, PS Program, v2.1.31: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize) estimated that the sample size of the study conferred between 80 – 90% power to detect significance at the 0.05 level assuming a minor allele frequency of 20%.

Results

Characteristics of the study group

The study group comprised 250 patients classified as gastritis (n=91), intestinal metaplasia (n=63) and normal controls (n=96). Patient characteristics are summarised in Table 1. The groups were similar for gender, presenting complaint and anti-secretary therapy. The IM group was significantly older than the normal controls (Table 1) which was addressed in the statistical analysis.

Based on histopathology, the gastritis group (n=91) were predominantly classified as *H. pylori* positive chronic pangastritis (82%, n = 75) and graded as 'moderately severe'. In the IM group (n=63), most patients (86%, n=54) had focal IM and 14% (n=9) had extensive IM.

Distribution of focal IM by site was as follows: antrum (n=33), corpus (n=4) incisura (n=6) and more than two sites (n=11). In those with IM, 73% (n=46) concurrently displayed a moderate degree of gastritis.

Genotyping analyses

Among controls, all alleles at all loci investigated were in Hardy-Weinberg equilibrium with non-significant χ^2 values. Genotype and allelic frequencies, were in agreement with a number of previous studies[7,8,22,23] and with online databases such as dbSNP (http://www.ncbi.nlm.nih.gov/) and SNP500Cancer (http://snp500cancer.nci.nih.gov/). Preliminary χ^2 testing for differences in allele or genotype frequency across patient groups found no significant differences. Gastric disease groups were combined (gastritis and intestinal metaplasia) and allele number, carriage of 'rare' alleles and homozygosity for 'rare genotypes' were investigated between normal controls and this composite 'gastric disease' group. No significant differences were observed between groups.

Haplotype analysis: Interleukin 10

The frequencies of the four IL-10 haplotypes observed across the patient groups are presented in Table 2. Allelic data for each locus studied in the IL-10 gene was submitted to PHASE v2.1.1 online haplotype analysis software. There were no significant differences found in the haplotype frequencies across the patient groups after analysis with Pearson χ^2 testing.

Logistic regression analysis

Logistic regression analysis was performed using JMP statistical package (detailed earlier). No significant association was noted between carrier status and patient age (means were compared using a two sample t-test, data not shown). Neither did the proportion of carriers differ significantly between men and women (results based on a standard χ^2 testing). As a result, standard odds ratios did not differ significantly from those adjusting for the effect of age and gender. Age and gender adjusted odds ratios relating to carriage of the particular alleles and risk of disease are detailed in Table 3. There was also no significant associations found for any of the variants and 'all gastric disease' when the gastritis and IM patient groups were combined and analyzed together against the normal controls.

'Pro-inflammatory genetic profile' analysis

Combinations of a number of cytokine polymorphisms, which have been reported previously to confer increased risk of disease[7], were also investigated. These combinations included carriage of IL-1B-511T and homozygosity for IL-1RN*2 (≈ IL-1RN -2018C/C) alone and in combination with carriage of TNF-A-308A and homozygosity for IL-10 haplotype ATA. In this study, none of these genotype combinations were associated with a significant increase in the risk of developing gastric disease.

Discussion

Distinct host cytokine responses to *H. pylori*-induced gastric mucosal inflammation appear to play a significant role in clinical outcome, including the development of gastric disease and gastric cancer. However the relationship between gene polymorphisms of these host cytokines and the nature and severity of clinical outcome is not well characterised. The present study examined the significance of several variants in inflammatory cytokine genes in the development of gastric disease. Specifically, we investigated a pro-inflammatory profile of 11 genetic polymorphisms in IL-1B, IL-1RN, IL-10, TNF-A and TLR4 in a patient population of normal controls and *H. pylori* related gastric disease groups. The present study found no

association between carriage of any of the alleles, or combination of alleles, tested and risk of gastritis or intestinal metaplasia development.

Reports of similar studies in the literature yield conflicting results; some support our negative findings while several report associations with one or more SNPs. Regarding IL-10 for example, a study of Venezuelan patients, known to have extremely high *H. Pylori* infection rates, found a 60% increase in risk of intestinal metaplasia and dysplasia combined (OR 1.62, 95% CI: 1.10–2.38) among the carriers of the IL10-1082 low activity allele[1]. In Europeans, an Italian clinical population study reported that patients with low activity IL10-1082 had increased risk of IM (OR=1.53 95%CI=1.07–2.19)[25]. In agreement with the results of the present study, Rad *and* colleagues (2004)[22], studying a German clinical population, found no association between carriage of the rare or 'risk' alleles (or associated haplotype combinations) with the IL-10-592, -819 and -1082 SNPs and intestinal metaplasia or atrophic gastritis.

Carriers of the IL-1B-511 T allele were associated with a modestly greater prevalence of IM among 302 *H. pylori*-infected cancer free subjects from China (OR=2.0, 95% CI = 1.0–3.7). There was no association between IM and polymorphisms in the other inflammatory cytokines tested (IL-1RN, IL-8, IL-10, IL-18, TNF-A, and TGF-B)[26]. Similarly, another Chinese study found no association with the IL-1B-511 T allele or IL-1RN gene polymorphisms in 129 patients with chronic gastritis[27].

A report by Hold *et al.*, in 2007[15] showed that Caucasian carriers of the TLR4+896G allele who were also first degree relatives of patients with gastric cancer, had an increased risk of developing gastric mucosal atrophy and hypochlorhydria, though the confidence intervals are very wide (OR=11, 95%CI=2.5–48 for hypochlorhydria). In 130 patients with non-ulcer dyspepsia and 200 asymptomatic control subjects from Northern India the TLR4+936T (Thr399Ile) allele carriers had a borderline significant elevated risk for gastritis[16]. However, other studies in Mexican and European cohorts have failed to find an association of Thr399Ileu variation with gastritis, duodenal ulcer, and precancerous lesions[17,18].

While the absence of an association between the IL-1B, IL-1RN, TNF and TLR4 polymorphisms and gastric disease reported in this study disagrees with a number of published studies [2,3,5–8,15,16,24–26], other groups have reported a similar lack of association, though these findings often remain as abstracts rather than full papers[17,18,22,27,28–33]. The full reporting of negative studies is important to prevent publication bias and the potential risk of an overestimation of the role these SNPs play in gastritis or IM. The disparity between the results of this study and studies reporting significant risks associated with some of these alleles may be due to different patient groups, different populations, sample size power, differing clinical characteristics, controls drawn from high risk areas for chronic gastritis, multiple comparisons involving several genotypes and several histological endpoints, or confounding factors from other environmental co-factors. Interactions with other genes regulating inflammatory responses may also modify a small affect on risk by any particular variant

This study shows a lack of association between the inflammatory SNPs tested and clinically detected IM, irrespective of extent described on one or more endoscopic visit. The predominant form of IM reported in our study was focal and the prevalence of extensive IM was low. Only 9 patients of the clinical population screened (n=950) presented with extensive IM, suggesting that this is relatively rare in our population. Indeed, regression or progression of IM is also possible in that sites noted to show no metaplasia at a follow up visit, though IM was previously noted at that site, might have it 'reappear' at a subsequent visit. Indeed, follow-up of these patients in the future would be interesting. Long-term follow up in the future, larger sample

sizes combined with well defined patient groups is needed to more thoroughly clarify the relationship between host cytokine genetics and gastric disease.

Rad *et al.*, [22] investigated IL-10 mRNA expression in gastric mucosal biopsy tissue and reported IL-10 expression to correlate with expression of the IL-10 ATA haplotype. In this study, higher levels of IL-10 mRNA were observed in patients with a haplotype other than ATA while carriage of the ATA haplotype was associated with the lowest IL-10 mRNA levels. This lends much credence to the hypothesis that cytokine genotypes influence gastric mucosal inflammatory phenotypes. As IL-10 lowers gastric inflammation[34], the disturbance in the immune response imposed by a 'low-producing' IL-10 genotype could 'tip the scales' in favour of a pro-longed, unimpeded inflammatory response to an immune challenge such as *Helicobacter pylori* infection. A Japanese study by Sugimoto and co-workers[35] assessed the same IL-10 genotypes as those examined in our study in *H. pylori*-positive patients with gastritis only (n = 162), gastric ulcers (n = 110), duodenal ulcer (n = 94), or gastric cancer (n = 105), and *H. pylori*-negative controls (n = 168). They reported that the presence of the ATA/GCC haplotype significantly increased the risk of gastric cancer development (adjusted OR: 2.81, 95%CI: 1.26–6.25) though had no significant effect on the other gastric conditions tested. However due to the rarity of the ATA haplotype a much larger patient cohort will be needed to more fully explore the relationship of this haplotype background to gastric disease susceptibility.

In conclusion, we report that in this Irish patient population, carriage of the 11 variant alleles IL-10-592, -819, -1082, IL-1B-31, -511, +3954, IL-1RN+2018, TLR4+896, +936, TNF-238, -308, confers no significant increase in risk of gastric disease. We believe our study highlights the importance of investigating these polymorphisms in a variety of populations and settings. It remains likely that host polymorphisms in inflammatory mediator genes do influence the course and/or severity of gastric disease but the extent to which they do so varies greatly between individuals and populations. A better understanding of these differences as well as the pathways influenced by pro-inflammatory genetic variants will determine the clinical significance of SNP profiling in the context of *H. pylori* related gastro-duodenal disease.

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Table 1

Characteristics of the study group

	Normal N=96	Gastritis n=91	IM n=63
Description	Normal histology, *H. pylori* negative	Chronic gastritis, *H.pylori* positive	Intestinal metaplasia, *H. pylori* positive
Gender female%	58%	60%	52%
Age mean ± sd (range)	44 ± 17 (18–82)	47± 16 (18–90)	57± 13* (26–84)
Primary presenting complaint			
Epigastric pain	38%	34%	35%
Acid-secretory therapy	29%	34%	29%
Family history of GI cancer	3%	9%	5%

* $p < 0.05$: IM significantly older than normal and gastritis groups

Table 2

IL-10 haplotype frequencies across patient groups.

IL-10 Haplotype	Normal	Gastritis	Intestinal metaplasia
ACC	0.45	0.48	0.46
ATA*	**0.01**	**0.01**	**0.01**
GCC	0.35	0.26	0.32
GTA	0.19	0.25	0.21

* IL-10 ATA haplotype is said to be the low producing haplotype while IL-10 GCC is said to be the high producing haplotype

Table 3

Age and gender adjusted odd ratios (95% confidence intervals) for carriage of individual ‘rare’/’risk’ alleles for normals against gastritis and intestinal metaplasia patient groups.

SNP Locus	Odds ratio (95% confidence interval)	
	Gastritis (n=91)	Intestinal metaplasia (n=63)
TLR 4 +896 G+	1.12 (0.49, 2.52)	1.33 (0.49, 3.59)
TLR 4 +936 T+	0.97 (0.44, 2.11)	0.99 (0.38, 2.63)
TNF -238 A+	1.32 (0.52, 3.4)	0.26 (0.05, 1.34)
TNF -308 A+	1.65 (0.9, 3.02)	1.32 (0.62, 2.83)
IL-1B -31 C+	0.72 (0.4, 1.28)	0.83 (0.41, 1.67)
IL-1B -511 T+	0.75 (0.42, 1.34)	0.88 (0.43, 1.77)
IL-1B +3954 T+	0.74 (0.41, 1.34)	0.97 (0.47, 1.98)
IL-1RN +2018 C+	0.74 (0.41, 1.34)	0.81 (0.39, 1.67)
IL-10 -592 A+	1.58 (0.86, 2.91)	1.42 (0.68, 2.94)
IL-10 -819 T+	1.47 (0.81, 2.68)	1.41 (0.68, 2.91)
IL-10 -1082 A+	0.92 (0.46, 1.84)	1.15 (0.46, 2.83)
IL-10 ATA/ATA haplotype	1.65 (0.38, 7.22)	0.9 (0.09, 9.63)