Interleukin-6 Genetic Polymorphisms are not Related to Helicobacter pylori-Associated Gastroduodenal Diseases

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ABSTRACT

Background. Polymorphisms in the promoter region of the proinflammatory cytokine, interleukin (IL)-6 have been related to several chronic inflammatory diseases. Inter-individual variation in the severity of gastric inflammation may be important in determining the clinical outcome of an Helicobacter pylori infection and relate to polymorphisms in this region.

Materials and Methods. We studied H. pylori-infected patients with duodenal ulcer or gastric cancer. In addition six gastric cancer cell lines, AGS, SNU-668, MKN-1, MKN-7, MKN28 and KATOIII, were cocultured with both cag pathogenicity island-positive and -negative H. pylori. Single nucleotide polymorphisms at positions −174, −572, and −597 in the IL-6 promoter region were identified by PCR-RFLP. The IL-6 production from the cancer cells was determined by ELISA.

Results. Sixty patients with gastric cancer and 60 with duodenal ulcer were studied. The alleles at positions −174 and −597 were closely linked (−174G/−597G or −174C/−597A) regardless of the ethnic group or disease presentation. There was no difference in the allele frequency at any of the sites among patient groups. H. pylori-induced IL-6 production from the gastric cancer cell lines was also independent of the IL-6 polymorphisms or the presence of the cag pathogenicity island.

Conclusions. The genetic polymorphisms in IL-6 can be attributable to ethnicity and appear to be independent of the clinical outcome of an H. pylori infection.

Keywords. Interleukin; polymorphisms; Helicobacter pylori; gastric cancer.

Helicobacter pylori infection induces both chronic and active gastric mucosal inflammation. The outcome of an individual infection is thought to be related to the severity and distribution of H. pylori-related inflammation [1]. The local production of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α are thought to play a central role in the recruitment of inflammatory cells to the gastric mucosa by the presence of H. pylori [2,3]. H. pylori that contain an intact cag pathogenicity island are associated with more severe inflammation and increased mucosal levels of IL-8 [4]. The host’s ability to regulate cytokine production has been shown to be influenced by the presence of polymorphisms in the coding and promoter regions. Genetic polymorphisms in these cytokines may be a host factor that regulates the immune and inflammatory response and is a candidate for the factor that might explain the interindividual variation observed in the severity of gastric inflammation and the clinical outcome of an H. pylori infection. Support for this hypothesis comes from reports suggesting that genetic polymorphisms on IL-1β [5–7] and TNF-α [8] could be related to the risk of H. pylori-associated gastric cancer or peptic ulcer, respectively.

IL-6 is a multifunctional cytokine produced by immune and many nonimmune cells, including monocytes, lymphocytes, macrophages, endothelial cells, intestinal epithelial cells and osteoclasts. IL-6 functions both as an inflammatory mediator and to regulate endocrine and metabolic functions [9]. It also functions as a tumor growth factor for myeloma [9], renal [10],
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prostatic [11] and bladder cancer [12]. IL-6 also enhanced the transformation and growth of nitrosamine-initiated bladder cancer in an in vitro model of inflammation-associated carcinogenesis [12]. The IL-6 gene (IL-6) is located on chromosome 7p21 [13]. Single-nucleotide polymorphisms at the 5′ flanking region of the IL-6 gene promoter (G or C at −174 and at −572, G or A at −597) have been identified [14]. Individu- als with the G allele at position −174 have been shown to produce higher levels of IL-6 [15,16] and are more prone to systemic juvenile onset chronic arthritis [15], lipid abnormalities [15,16] and insulin resistance [17] than those with C/C genotype.

The mucosal levels of IL-6 were reported to be increased in H. pylori-associated gastritis [2,18]. The serum levels of IL-6 were also reported to be greater among patients with gastric cancer compared with patients with benign gastric lesions caused by H. pylori infections [19]. Mucosal IL-6 levels were also reported to be greater in patients with duodenal ulcer compared with patients with simple H. pylori gastritis [20]. Both mucosal IL-6 and IL-8 levels were markedly increased in gastric cancer tissue compared with adjacent noncancerous mucosa [21]. Mucosal IL-6 levels were also greater in early gastric cancer with active H. pylori infection than without H. pylori infection, and the levels decreased dramatically after eradication of infection [21]. Thus, overall, there are data suggesting that IL-6 may play an important role in gastric carcinogenesis in H. pylori-infected patients and the genetic polymorphisms in IL-6 may be a key host factor that increases the predisposition for development of gastric cancer. In the present study, we compared the presence of genetic polymorphisms in IL-6 among H. pylori-infected patients with gastric cancer and duodenal ulcer disease. Duodenal ulcer was chosen as the control as the diseases are mutually exclusive [22].

Materials and Methods

Patients

We studied gastric cancer patients and duodenal ulcer patients from Bogota, Colombia, Seoul, Korea, and Kyoto, Japan. Gastric cancer was confirmed by histology and duodenal ulcer was identified endoscopically. Gastric cancer patients had no other primary malignancies or inflammatory diseases such as rheumatoid arthritis. No subject had received treatment for H. pylori infection. Informed consent was obtained from all patients, and the local ethics committee in each country approved the protocol. H. pylori status was confirmed by culture, histology and/or serology. Gastric biopsies were taken from the greater curvature of the antrum and kept frozen at −20°C until used.

Genetic Analysis

Genomic DNA was extracted from frozen gastric tissue using a commercial DNA extraction kit (QiAmp tissue kit; QIAGEN Inc. Valencia, CA, USA) and stored at −20°C until used. PCR was used for polymorphism screening; negative controls without DNA template were included with each reaction. All analyses were performed blinded with respect to the diagnosis and patient characteristics. A single base pair (bp) polymorphism at −174, −572, −597 in the promoter region of the IL-6 gene was analyzed by the PCR-restriction fragment length polymorphism (RFLP) method.

Position −174 The polymorphic region containing the NlaIII restriction site at position −174 base pairs from the transcription start site (a 198-bp PCR fragment of the IL-6 promoter region) was amplified as previously described [15]. Twenty µL of PCR product was digested with 1 U of NlaIII (New England Biolabs, Inc. Beverly, MA, USA) at 37°C overnight and was run on an ethidium bromide-stained 4% agarose gel. Products either remained intact (allele G; IL-6−174G) or were cut into two fragments of 140 and 58 bp (allele C; IL-6−174C).

Position −572 The polymorphic region containing the BsrBI restriction site at position −572 base pairs from the transcription start site was amplified using the following primers: 5′-AGATTCCAAGGGTCACTTG-3′ and 5′-AGAAGCAGAACCCACTTTC-3′ (a 519-bp PCR fragment of the IL-6 promoter region). PCR was performed for 35 cycles, consisting of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. The final cycle included a 5-minute extension step to ensure full extension of the PCR product. Twenty µL of PCR product was digested with 1 U of BsrBI (New England Biolabs, Inc.) at 37°C overnight and was run on an ethidium bromide-stained 2% agarose gel.

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Products either remained intact (allele C; \( IL-6-572^C \)) or were cut (allele G; \( IL-6-572^G \)) into two fragments of 359 and 160 bp.

**Position \(-597\)** The polymorphic region containing the \( FokI \) restriction site at position \(-597\) base pairs from the transcription start site was amplified using the same primers and same PCR conditions as used for detecting the polymorphism at position \(-572\). Twenty \( \mu \)L of PCR product was digested with 2 U of \( FokI \) (New England Biolabs, Inc.) at 37°C overnight and was run on an ethidium bromide-stained 2% agarose gel.

Products either remained intact (allele G; \( IL-6-597^G \)) or were cut (allele A; \( IL-6-597^A \)) into two fragments of 373 and 146 bp.

**Genetic Polymorphisms of Human Gastric Cancer Cell Lines**

Six human gastric cancer cell lines (AGS, SNU-668, MKN-1, MKN-7, MKN-28 and KATOIII) were studied to detect genetic polymorphisms in \( IL-6 \). AGS and KATOIII were from American Type Culture Collection (ATCC) (Manassas, VA). MKN-1, MKN-7, MKN-28 and SNU-668 were kind gifts from Dr Antonia Sepulveda. AGS cells were of Caucasian origin and the others were of Asian origin. Each cell line was routinely maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 40 \( \mu \)g/ml gentamicin. DNA extraction and PCR-RFLP were performed identically as that described for the patient’s samples.

**IL-6 Polymorphisms in Patient’s Tissues**

The genotypes of the polymorphisms at the three promoter regions of the \( IL-6 \) gene are shown in Table 1. Forty-one (69%) of the 60 Colombian patients had G/G genotype, 17 (28%) had G/C and two (3%) had C/C at the \(-174\) region. In contrast, all 60 Asian patients had the G/G genotype at this region. The Asian
patients had a significantly lower frequency of the C allele at position −174 compared with Colombian patients (0 vs. 17.5%, *p* < 0.0001). Nineteen (63%) of 30 Colombian gastric cancer patients had the G/G genotype, nine (30%) had G/C and two (7%) had C/C at the −174 region. There was no difference in the frequency of genotypes and alleles at position −174 between genetic cancer and duodenal ulcer patients in both ethnic groups (*p* = 0.320).

There was a statistically significant amount of linkage disequilibrium between loci *IL-6*−597 and *IL-6*−174 (*p* < 0.0001). The G allele at position −174 was in linkage disequilibrium with the G allele at position −597 and the C allele at position −174 with the A allele at position −597, irrespective of ethnic group and clinical outcome. Only one (0.8%) Colombian gastric cancer patient was the exception (G/C at −174 and G/G at −597).

At the −572 region, Asian patients had a significantly lower frequency of the G allele at this region as compared to Colombian patients (25.0% vs. 67.5%, *p* < 0.0001). There was also no difference in genotype distribution between duodenal ulcer and gastric cancer patients in both ethnic groups. The −572C allele was associated with the G allele at the −174 and −597 sites except one (0.8%) Colombian gastric cancer patient (G/C at −174, C/C at −572 and G/A at −597). There was no linkage disequilibrium between −572 and −174 or −597 loci, since the lowest permutation-based probability was *p* = 0.13 among Asian patients and *p* = 0.62 among Colombian patients for all pairwise locus tests.

**IL-6 Polymorphisms and IL-6 Production in Human Gastric Cancer Cell Lines**

The six cell lines could be divided into three groups (high, low, and non IL-6 producers) based on the IL-6 levels induced by *H. pylori*

### Table 1  Genotype distribution of single nucleotide polymorphisms of IL-6 in Colombian and Asian patients according to disease by PCR-RFLP analysis

<table>
<thead>
<tr>
<th>Region</th>
<th>Genotype</th>
<th>Total (n = 60)</th>
<th>Total (n = 30)</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GC (n = 30)</td>
<td>DU (n = 30)</td>
<td>GA vs. DU Colombian</td>
<td>GC vs. DU Colombian</td>
<td>GC vs. DU Colombian</td>
</tr>
<tr>
<td>−174</td>
<td>G/G</td>
<td>69%</td>
<td>73%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>G/C</td>
<td>28%</td>
<td>27%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>3%</td>
<td>0%</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>−572</td>
<td>G/G</td>
<td>49%</td>
<td>60%</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>G/C</td>
<td>38%</td>
<td>23%</td>
<td>43%</td>
<td>43%</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>13%</td>
<td>17%</td>
<td>54%</td>
<td>54%</td>
<td>54%</td>
</tr>
<tr>
<td>−597</td>
<td>G/G</td>
<td>70%</td>
<td>73%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>27%</td>
<td>27%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

GC: gastric cancer; DU: duodenal ulcer.
NS: not significant (*p* > 0.01).
coclure (Table 2). Remarkably high levels of IL-6 were induced in MKN-1, MKN-7 and SNU-668 cells by H. pylori compared with the control (cells exposed to medium alone) and these were deemed to be ‘high producers’. Although IL-6 was induced from MKN-28 cells exposed to H. pylori, IL-6 levels were significantly lower compared with in high IL-6 producers and it was deemed to be a ‘low producer’. The other cell lines (AGS and KATOIII) did not produce sufficient IL-6 to be detected by ELISA even when cocultured with H. pylori and were deemed to be ‘nonproducers’.

All the human cell lines had the G/G genotype at the −174 and −597 sites confirming the observation that the G allele at the −174 site was in linkage disequilibrium with the G allele at position −597. Polymorphic diversity in the genotypes was found at the −572 site, however, there was no difference in the frequency of genotypes among groups (high- vs. nonproducers; p = 0.307). We also confirmed the association of the −572C allele with the −174G allele and the −597G allele in cancer cell lines.

Comparison of IL-6 production with cag pathogenicity island-positive H. pylori and cag negative H. pylori showed no difference in the levels of IL-6 induced in any cell line tested showing that IL-6 production was unrelated to the presence of the cag pathogenicity island status in H. pylori.

**Discussion**

We investigated the prevalence of polymorphisms in the IL-6 gene among gastric cancer and duodenal ulcer patients. The rationale for choosing patients with duodenal ulcer as controls was based upon the fact that duodenal ulcer patients have a significantly reduced risk of gastric cancer compared with those with gastritis alone such that duodenal ulcer and gastric cancer are mutually exclusive clinical outcomes of H. pylori infection [22]. Thus, we reasoned that if any host factor predisposed H. pylori-infected patients to the development of gastric cancer, this comparative group should maximize the difference such that a clear demarcation between these two disease outcomes would be discernable. However, no association was found between the IL-6 gene polymorphisms at any of the loci studied and the clinical outcome.

These clinical data support the in vitro data that the polymorphisms at the same sites were unrelated to the levels of IL-6 produced by human gastric cancer cell lines stimulated by H. pylori. These findings suggest that genetic polymorphisms are unlikely to play an important role in determining the capacity of gastric cells to produce IL-6, at least in H. pylori infection. Although human gastric cancer cell lines are not normal, they originated from normal gastric epithelial cells. Of note, gastric cancer cell lines displayed a wide range of IL-6 production when stimulated by coculture with H. pylori, even though these cells all derived from a common disease. These findings may reflect differential cellular characteristics amongst the cell lines tested that are unrelated to genetic polymorphisms in the IL-6 gene. Three cell lines (MKN-1, MKN-7 and SNU-668) produced large quantities of IL-6 without stimulation, which was consistent with previous observations [19,25]. We also found that cocultivation of these cell lines with H. pylori induced high levels of IL-6 production.

The status of the cag pathogenicity island of the H. pylori strain did not influence the IL-6 levels induced in these cell lines. This differs from its effect on IL-8 induction as IL-8 production from gastric cancer cells (e.g. MKN-45, AGS and KATOIII) is related to the presence of the cag pathogenicity island. In fact, the cag
pathogenicity island negative strain in this study (GI2771) produced only 10% to 15% the amount of IL-8 levels compared with the cag pathogenicity island positive strain (ATCC43504 and JK51) (data not shown). The finding that IL-6 production was not related to the cag status was consistent with the previous in vivo observation that gastric mucosal IL-6 levels were not related to the cag status [20].

The individual’s genotype at polymorphic sites in IL-6 (especially −174 site) is thought to determine the IL-6 response to stimuli and predisposes to development of diseases where IL-6 has been implicated. It has been proposed that the C allele at position −174 compared with the G allele was associated with lower levels of plasma IL-6 in normal subjects. Young patients with systemic juvenile rheumatoid arthritis had a significantly lower frequency of the C/C genotype compared with a healthy population [15]. However, this correlation has not been universally observed [16] and the data are still conflicting regarding the functional effects of these polymorphic sites on IL-6 production in vitro. In the initial report, the transcriptional response of IL-6 to stimuli such as endotoxin (lipopolysaccharides) and IL-1 was increased in the −174G construct but not in the −174C construct in HeLa cells [15]. In contrast, other reports indicated that both the −174C and G allele constructs produced the same transcriptional response by IL-1 in HeLa cells [14]. The functional effect of these polymorphic sites on IL-6 production in HeLa cells was different from that in the ECV304 cell line [14]. It has been suggested that the regulation of IL-6 transcription was cell type-specific and not exclusively dependent on single gene polymorphisms. Rather, regulation of IL-6 transcription occurred through complex interactions between several polymorphic sites. Our in vitro observations are consistent with the hypothesis that the level of IL-6 induced by H. pylori is variable amongst cell lines and that IL-6 induction is not related to any single polymorphism in the IL-6 promoter region. All human gastric cell lines that we studied had the G/G genotype at the −174 site and were identified as high, low, or nonIL-6 producers based on the IL-6 levels induced by coculture with H. pylori. Overall, our results do not support the hypothesis that the −174G allele carrier is associated with high levels of IL-6 production.

We confirmed the reported linkage between the −174, −572 and −597 regions of the IL-6 promoter (−174G/−597G, −174C/−597A) [14]. However, the frequency of the −572C allele in our study was higher than that reported previously in study in a Caucasian population (5.2%) [14]. These differences may be explained by differences in the ethnic composition of our study population showing that Asian patients (75%) had a significantly higher frequency of the C allele at this site as compared with Colombian patients (32.5%). These data suggest that the genetic polymorphisms of IL-6 may be primarily related to ethnicity and not to clinical outcome of H. pylori infection. This is consistent with the notion that single nucleotide polymorphisms are indicators of human history because of the low rate of mutation [26], such that these polymorphisms are ethnic characteristics rather than predisposing factors of a common disease. Further studies will be necessary to clarify whether ethnicity influences the allelic frequencies at these polymorphic sites.

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