LETTER TO JMG

Interleukin 12 gene polymorphisms enhance gastric cancer risk in *H pylori* infected individuals

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Bacterial, environmental, population related, and individual host factors are major determinants of the outcome of *H pylori* infection.[1 2] Many bacterial virulence genes—including the pathogenicity island *cagA*, the s1m1 *vacA* alleles, *babA2*, *sabA*, and *oipA*—have been associated with a higher degree of gastric mucosal inflammation, intestinal metaplasia, gastric or duodenal ulcer, gastric adenocarcinoma, and MALToma.[1 3–7] *H pylori* triggers and maintains gastric mucosal inflammation by different mechanisms, which are partly strain dependent and partly strain independent.[1] T and B lymphocyte activation and infiltration of the gastric mucosa depend on *H pylori* antigen processing. The number of infiltrating polymorphonuclear cells varies depending on the virulence of the infecting strain, being much greater when infections are caused by *cagA* positive strains.[3 5 7–9]

The inflammatory cells infiltrating *H pylori* infected gastric mucosa produce a pattern of proinflammatory cytokines.[10 11] High mucosal levels of mononuclear cytokines (IL8, IL6, IL1β, tumour necrosis factor α (TNFα), and interferon γ (IFNγ)) and lymphocytic derived cytokines (IL2, IL2R) have been described in *H pylori* infected patients.[10–13] *H pylori* infection also induces the production of IL12,[14–16] a heterodimeric proinflammatory protein that triggers the production of IFNγ and favours the differentiation of T helper 1 (Th1) cells,[17 18] which, in *H pylori* infected mucosa, prevail over Th2 cells.[15 16 19] The ability of IL12 to induce Th1 is one of the biological bases of the importance of this cytokine in resisting most bacteria, including *H pylori*, and also intracellular protozoa and fungal pathogens.[18 20 21] Cellular sources of IL12 in response to infections are mainly dendritic cells and phagocytes.[16–21] The two subunits of IL12—p35 and p40—are encoded by different genes, named *IL12A* and *IL12B* respectively, which are unrelated and are located on separate chromosomes (3p12–q13.2 and 5q31–33).[18]

Host cytokine gene polymorphisms may be as important as exogenous stimuli in influencing the amount of cytokines produced and consequently the pattern and severity of inflammation.[10 22–26] IL12 gene polymorphisms in particular have been observed to affect autoimmune diabetes[27] and atopic and non-atopic asthma.[28] Both *IL12A* and *IL12B* have a polymorphic dinucleotide repeat region (CT for *IL12A* and TG-TA for *IL12B*) in introns 6 and 4, respectively (Gene bank accession numbers: AF404773 for *IL12A* and AY008847 for *IL12B*), which might affect the amount of the synthesised cytokine. No data have been reported on the possible influence of *IL12A* and *IL12B* polymorphisms on *H pylori* infection and its outcome.

Our aims in the present study were, first, to use denaturating high performance liquid chromatography (DHPLC) to screen the promoters and the coding sequences of *IL12A* and *IL12B* in order to identify any single nucleotide polymorphisms (SNPs); second, to analyse the SNPs identified, together with the number of CT and TG-TA dinucleotide repeats (variable number tandem repeats (VNTR)) of *IL12A* intron 6 and *IL12B* intron 4 in patients with or without *H pylori* infection; and third, to verify the association, if any, between the IL12 gene polymorphisms studied and the outcome of *H pylori* infection (gastric adenocarcinoma in particular).

Key points

- IL12, formed from p35 and p40 subunits encoded by *IL12A* and *IL12B* genes, favours T helper 1 (Th1) differentiation. Th1 lymphocytes prevail over Th2 in *H pylori* associated chronic gastritis, the first step in *H pylori* associated gastric carcinogenesis. In this study, 110 patients with non-cardia gastric cancer were compared with 251 patients with benign gastroduodenal diseases to see whether there was any correlation between IL12 gene polymorphisms and *H pylori* associated gastric adenocarcinoma.
- Two single nucleotide polymorphisms were identified on *IL12A* (−504 T/G and +6686 A/G) and one on *IL12B* (+15485 A/G). Eleven and six alleles were found for CT and TG-TA dinucleotide repeats (VNTR) of *IL12A* intron 6 and *IL12B* intron 4, respectively.
- The frequency of non-cardia gastric cancer was higher in patients with the *IL12A* −504 T/T (odds ratio (OR) = 2.38) or with the *IL12B* VNTR $(TG\text{-}TA)_9/(TG\text{-}TA)_{11}$ genotype (OR = 1.36).
- No IL12 gene polymorphisms were correlated with intestinal metaplasia.
- These findings suggest that *IL12A* and *IL12B* gene polymorphisms may affect the final steps in gastric carcinogenesis in *H pylori* infected subjects.

METHODS

We studied 251 unrelated Italian patients (112 male, 139 female; age range 27 to 88 years) who consecutively underwent upper gastrointestinal endoscopy (EGDS, (o)esophagogastroduodenoscopy) for dyspeptic symptoms. Diagnoses made on the basis of endoscopic findings were: absence of evident endoscopic lesions (10); antral gastritis (94); diffuse gastritis (57); duodenal ulcer (30); gastric ulcer (10); duodenitis (22); reflux gastritis (16); oesophagitis (12).

Three antral and two body biopsies were obtained at endoscopy from each patient for histological evaluation. Two antral and one body biopsy were also obtained for *H pylori*

Abbreviations: DHPLC, denaturating high performance liquid chromatography; EGDS, (o)esophagogastroduodenoscopy; IL, interleukin; SNP, single nucleotide polymorphism; Th, T helper; VNTR, variable number tandem repeats

Table 1 Primer sequences, primer spanning regions, amplified regions, and temperatures of denaturating high performance liquid chromatography analysis

Primer sequences	Primer spanning region	Regions	Temp for DHPLC analysis (°C)
***IL12A* (p35)**			
5′tccccactactctcagcaca3′	−941/−922	Promoter	63; 64
5′cgataggatgaatacacagagtcc3′	−598/−621		
5′ggagcctttgctgactcct3′	−693/−675	Promoter	59.6
5′ggacacccagggagaacag3′	−451/−469		
5′tgtgaacaaaacgccttgag3′	−520/−501	Promoter	61.8
5′gccccgtgtctaagctacc3′	−241/−259		
5′gggaggcactcctctctcta3′	−313/−294	Promoter	65.5; 67
5′cggcgctttcggattaact3′	−71/−89		
5′actgcgaacatttcgctttc3′	−171/−152	Promoter	69.3
5′acatcagcttctcggtgaca3′	−22/−41		
5′cgggagttaatccgaaagc3′	−93/−75	Promoter and exon 1	63.8
5′cgctggatgcagacctgt3′	+129/+112		
5′agtcccgggaaagtcctg3′	+19/+36	Exon 1	65.0; 67.0
5′gagcgagccaaagacctg3′	+213/+196		
5′atccaggctgaagctcctc3′	+1130/+1148	Exon 2	63.2
5′gagggtgcaggtccacag3′	+1357/+1340		
5′ggtgccaggtgcattatcaa3′	+3961/+3980	Exon 3	56.9; 57.9
5′ttttaaccacactgcatgctc3′	+4171/+4151		
5′ttgagacctgaagagtcagaaaga3′	+4385/+4408	Exon 4 and exon 5	56.2; 57.8
5′cccattgcatttgaggaaag3′	+4648/+4629		
5′tggatacttccccatcttgtc3′	+4673/+4693	Exon 6	58.7
5′gcatgaaaaaggtgctctca3′	+4888/+4869		
5′ttggaataccatgtaagtcatgc3′	+6368/+6390	Exon 7 and UTR	56.1; 57.4
5′tgatcagaggtatcatgtggatg3′	+6699/+6677		
5′gaactagggaggggggaaaga3′	+6642/+6661	UTR	55.0
5′gctcagatgctttcatgattacc3′	+6924/+6902		
***IL12B* (p40)**			
5′ggaaagttagcccggaaatc3′	−1246/−1227	Promoter	66.0; 68.0
5′accaggtacacgcagacaga3′	−983/−1002		
5′gtctcgcagcgacctctgt3′	−1045/−1027	Promoter	64.6; 65.6
5′tgctaagaggtatgcaaaggtg3′	−892/−913		
5′ttcctgttcacgtgcagact3′	−965/−946	Promoter	60.7
5′cggatgggagcagaaagtg3′	−707/−725		
5′gccttgagtgtggttgtctg3′	−766/−747	Promoter	60.3; 63.0
5′tttaccacccatcttggctc3′	−521/−540		
5′tcccactcggggaatgtg3′	−572/−555	Promoter	59.3
5′tgagttcatgtcgggatgg3′	−365/−383		
5′ggattttgtttttaagcccttg3′	−419/−398	Promoter	61.2; 63.0
5′aaggaaggagatggagatgct3′	−231/−251		
5′agtgctgtctccaagcacct3′	−298/−279	Promoter and exon 1	57.5
5′agtccaatggccctgaaac3′	+37/+19		
5′tccagtaccagcaacagcag3′	−19/+1	Exon 1	62.0; 64.0
5′agggaggggagggaggtag3′	+96/+78		
5′cttctcttccccctgcttct3′	+3674/+2693	Exon 2	60.0; 61.0
5′ggggcctccacagagaata3′	+3830/+3812		
5′gcttacagcctggttctcca3′	+7124/+7143	Exon 3	57.2; 60.5
5′atgagcgaaagagaaaattgat3′	+7481/+7460		
5′cacaatcaaaacctttctgcaa3′	+7916/+7937	Exon 4	56.1; 59.7
5′aaatgctgagaaaccagagca3′	+8128/+8108		
5′ggaactctctccccaatgtg3′	+9918/+9937	Exon 5	59.0; 62.0
5′tttccttatggagcacatataatca3′	+10221/+10197		
5′tgaactccaaagccttattaggaa3′	+11545/+11568	Exon 6	58.4; 60.0; 61.0
5′gcctccgtaaaactgaccaa3′	+11804/+11785		
5′aggggtcttcacagctttcc3′	+13642/+13661	Exon 7	60.9; 63.2
5′ccaatcatatccctgcatcc3′	+13875/+13856		
5′tagcttccatggcagtcctc3′	+15315/+15334	UTR	59.1
5′gggattccagattttctttgc3′	+15524/+15504		

Primer spanning region relates to DNA sequence numbering, with +1 as the first transcribed nucleotide. Gene bank accession numbers were AF404773 for *IL12A* (p35) and AY008847 for *IL12B* (p40).
DHPLC, denaturating high performance liquid chromatography analysis.

Table 2 Genotype frequencies and Hardy-Weinberg equilibrium analysis of the three single nucleotide polymorphisms studied

Gene SNP	Genotype (frequency)			HWE
***IL12A* −504**	G/G (0.01)	G/T (0.22)	T/T (0.77)	$\chi2 = 2.46$, NS
***IL12A* +6686**	A/A (0.03)	A/G (0.22)	G/G (0.75)	$\chi2 = 3.40$, NS
***IL12B* +15485**	A/A (0.74)	A/C (0.23)	C/C (0.03)	$\chi2 = 3.33$, NS

HWE, Hardy-Weinberg equilibrium; SNP, single nucleotide polymorphism.

culture and genotyping. An EDTA-K_3 treated blood sample was obtained from all patients for host genomic DNA isolation.

A second series of 110 unrelated Italian patients (66 male, 44 female; age range 34 to 90 years) who underwent surgery for non-cardia gastric cancer was also studied. TNM stages were: TNM IA (4); TNM IB (7); TNM II (30); TNM IIIA (18); TNM IIIB (10); and TNM IV (41). According to the Lauren description of gastric cancers, tumours were classified as ''intestinal-type'' in 82 patients and ''diffuse'' in 28. Two tissue samples for *H pylori* genotyping and genomic DNA analysis were obtained from 50 non-cardia gastric cancer patients: one from the neoplastic area and another from the adjacent (but at least 3 cm distant) non-neoplastic mucosa. The tissue samples were stored at −80℃ until DNA extraction was carried out. Whole blood from all patients with non-cardia gastric cancer was used to obtain genomic DNA. Sera were also obtained for measurement of anti-*H pylori* antibodies.

Histological evaluation

In mucosal biopsies from patients who underwent EGDS, *H pylori* colonisation density, chronic inflammation, polymorphonuclear cell infiltration (activity), and intestinal metaplasia were evaluated and graded according to the updated Sydney system.[8] Non-cardia gastric cancer diagnosis was always confirmed histologically on samples taken intraoperatively.

H pylori culture and genotyping

In the series of 251 patients who underwent EGDS, *H pylori* was cultured as described elsewhere.[10] DNA extracted from positive colonies was used to amplify *ureA*, *cagA*, and *vacA* under conditions specified by us elsewhere.[7] In the subgroup of 50 non-cardia gastric cancer patients for whom tissue samples were available, *H pylori* infection and strain virulence gene characterisation were assessed in DNA extracted from tissue. *UreA*, *cagA*, and s1/s2 *vacA* were multiplex polymerase chain reaction (PCR) amplified in a 25 µl final reaction volume containing: 150 ng DNA, 1×PCR gold buffer (Applied Biosystems, Foster City, California, USA), 1.5 mM $MgCl_2$, 200 µM each dNTPs, 500 nM primer URE-A F and URE-A R (5′gacatcactatcaacgaagg3′ and 5′tgaaaaccacgctctttag3′), 160 nM primer Cag-A F and Cag-A R (5′tcaaatacaccaacgcctcc3′ and 5′agcttcttgtggggacaatc3′), 600 nM primer S1/2-F and S1/2-R (5′atggaaatacaacaaacacac3′ and 5′ctgcttgaatgcgccaaac3′), and 2.5 U AmpliTaq gold (Applied Biosystems). The thermocycling conditions were: 95℃ for six minutes, then 42 cycles at 94℃ for 30 seconds, 62℃ for 30 seconds, 72℃ for 30 seconds, and finally 72℃ for seven minutes. The fragments (323 base pairs (bp) for *ureA*, 194 bp for *cagA*, and 259 or 286 bp for s1 and s2 *vacA*, respectively) were separated by electrophoresis on 2% NuSieve 1% Seakem agarose gel (BMA, Rockland, Maine, USA) and stained with ethidium bromide. The PCR amplification conditions used for m1/m2 *vacA* are described by us elsewhere. [7] Past or actual *H pylori* infection in the remaining 60 non-cardia gastric cancer patients was established on the basis of positive findings from serum anti-*H pylori* antibodies (Inova Diagnostics, San Diego, California, USA).

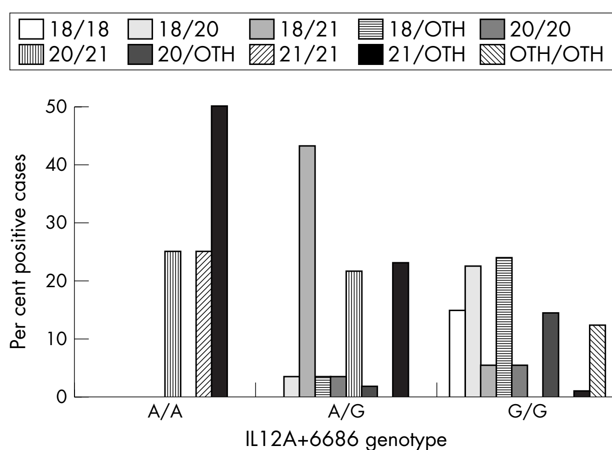

Figure 1 Association between *IL12A* variable number tandem repeats (VNTR) and *IL12A* +6686 genotypes. We genotyped *IL12A* VNTRs by considering separately the most common alleles ($(GT)_{18}$, $(GT)_{20}$, and $(GT)_{21}$) and combining all the remaining alleles as ''others'' (OTH).

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from 3 ml blood samples using the QIAamp DNA blood midi kit (Qiagen, Hilden, Germany). Promoters, coding sequences, splicing sites, and UTR regions of *IL12A* and *IL12B* were PCR amplified using the primers listed in table 1. Briefly, 100 ng of extracted DNA were amplified in a 50 µl final reaction volume under the following conditions: 1×DNA polymerase gold buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 200 µM each dNTPs, 500 nM each primer, and 2.5 U AmpliTaq gold (Applied Biosystems). PCR conditions were: 94℃ for seven minutes, then 40 cycles at 94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 60 seconds, and finally 72℃ for 20 minutes.

DHPLC analyses

PCR amplicons were analysed using a DHPLC screening strategy to detect any sequence polymorphisms (Transgenomic, Omaha, Nebraska, USA). This system uses the principles of reverse phase ion pair high performance liquid chromatography (RP-IP-HPLC) to carry out analytical separations of heteroduplexes. Table 1 reports temperatures for DHPLC analysis. The types of DNA polymorphism and their positions were defined by fluorescence dye terminator cycle sequencing on an ABI PRISM 310 genetic analyser (Applied Biosystems). Data were analysed with Sequencing Analysis 3.3 Software.

Capillary electrophoresis

IL12A intron 6 and *IL12B* intron 4 VNTRs polymorphisms were studied by capillary electrophoresis. Samples (100 ng) of genomic DNA were PCR amplified in a total reaction volume of 20 µl containing 1×PCR buffer with 1.5 mM $MgCl_2$, 200 µM each dNTP, 1.25 U AmpliTaq DNA polymerase (Applied Biosystems), and 500 nM of the following dye labelled primer pairs: HEX-5′tgacgaggacatgggataaa3′ and 5′aagctcattgcctttcaaaca3′ for IL12A, and FAM-5′cagaggg gaaaaggatggtt3′ and 5′cctggccagaactttttcaa3′ for IL12B. The temperature profile was: pre-PCR denaturation of seven minutes at 94℃; 30 seconds at 94℃; 30 seconds at 55℃; and one minute at 72℃ for 30 cycles, and a final extension of 30 minutes at 72℃. Samples for capillary electrophoresis were prepared using 2 µl of amplified products, added to 20 µl deionised formamide and 0.5 µl GeneScan –500 ROX size standard (Applied Biosystems). The mixtures were heated at 95℃ for three minutes and snap cooled at 4℃ for three minutes. The samples were detected by using an ABI PRISM 310 genetic analyser with a 47 cm×50 µm capillary. Results were analysed using GeneScan Analysis software 3.7. The expected size ranges of the two polymorphisms were: *IL12A* VNTR (149–161 bp), *IL12B* VNTR (237–245 bp).

Allelic ladders for each VNTR were constructed by allele sequencing carried out on DNAs from homozygous samples.

Table 3 Variable number tandem repeat (VNTR) allele frequencies of *IL12A* intron 6 and of IL12 B intron 4 found in the present series

IL12A VNTR			*IL12B* VNTR		
Allele	No of $(GT)_n$ repeats	Allele frequency (%)	Allele	No of $(TG\text{-}TA)_n$ repeats	Allele frequency (%)
$(GT)_{11}$	11	0.1	$(TG\text{-}TA)_8$	8	0.8
$(GT)_{12}$	12	0.2	$(TG\text{-}TA)_9$	9	24.1
$(GT)_{15}$	15	0.5	$(TG\text{-}TA)_{10}$	10	3.3
$(GT)_{16}$	16	5.6	$(TG\text{-}TA)_{11}$	11	67.6
$(GT)_{17}$	17	5.9	$(TG\text{-}TA)_{12}$	12	3.9
$(GT)_{18}$	18	37.2	$(TG\text{-}TA)_{13}$	13	0.2
$(GT)_{19}$	19	9.7			
$(GT)_{20}$	20	21.3			
$(GT)_{21}$	21	13.8			
$(GT)_{22}$	22	5.1			
$(GT)_{23}$	23	0.8			

Statistical analysis

Statistical analysis of data involved the χ^2 test, logistic regression analysis, and the non-parametric Mann–Whitney U test.

RESULTS

H pylori infection was diagnosed histologically in 36.4% of patients with no evident endoscopic lesions, 46.3% with antral gastritis, 43.6% with diffuse gastritis, 72.4% with duodenal ulcer, 77.8% with gastric ulcer, 36.4% with duodenitis, 60% with reflux gastritis, and 36.4% with oesophagitis. In patients with non-cardia gastric cancer, *H pylori* infection was diagnosed on the basis of positive *ureA* findings in tissue samples in the subgroup of 50 non-cardia gastric cancer patients for whom tissue samples were available; in the remaining 60 non-cardia gastric cancer patients, *H pylori* infection was established on the basis of positive findings from serum anti-*H pylori* antibodies. Overall *H pylori* infection was established in 69.4% of the non-cardia gastric cancer cases.

IL12A and *IL12B* gene polymorphisms

With DHPLC screening we identified three SNPs: two located in *IL12A* (−504 G/T and +6686 A/G) and one in *IL12B* (+15485 G/T) genes, respectively. The nucleotide substitutions were defined by sequencing. All SNPs were in Hardy Weinberg equilibrium (table 2). The *IL12A* −504 G/G genotype was extremely rare (1% of the cases) and for this reason the four patients with this genotype were excluded from some of the statistical analyses. A significant association was found between *IL12A* −504 and *IL12A* +6686 SNPs ($\chi^2 = 16.52$, $p<0.01$). In particular, the *IL12A* −504 T/G genotype was correlated with the *IL12A* +6686 G/G genotype, while the *IL12A* −504 T/T genotype was correlated with *IL12A* +6686 A/A or A/G genotypes; no association was found between *IL12A* −504 and *IL12B* +15485 ($\chi^2 = 1.18$, NS) or between *IL12A* +6686 and *IL12B* +15485 ($\chi^2 = 4.05$, NS).

The number of GT repeats in intron 6 of *IL12A* (*IL12A* VNTR) and the number of TG-TA repeats in intron 4 of *IL12B* (*IL12B* VNTR) varied widely when patients were considered overall. Table 3 shows the VNTR allele frequencies of *IL12A* and *IL12 B* found in the present series. We genotyped *IL12A* and *IL12B* VNTRs by selecting the most common alleles ($(GT)_{18}$, $(GT)_{20}$, and $(GT)_{21}$ for *IL12A* VNTR; $(TG\text{-}TA)_9$ and $(TG\text{-}TA)_{11}$ for *IL12B* VNTR) and combining them, as reported in table 4. The *IL12A* VNTR genotypes $(GT)_{18}/(GT)_{20}$, $(GT)_{18}/(GT)_{21}$, $(GT)_{20}/(GT)_{20}$, and $(GT)_{20}/(GT)_{21}$ were significantly correlated with *IL12A* −504 T/T, whereas $(GT)_{18}/(GT)_{others}$, $(GT)_{20}/(GT)_{others}$, and $(GT)_{others}/(GT)_{others}$ were correlated with the *IL12A* −504 T/G genotype ($\chi^2 = 68.59$, $p<0.001$). The *IL12A* VNTR genotypes $(GT)_{18}/(GT)_{18}$, $(GT)_{18}/(GT)_{20}$, $(GT)_{18}/(GT)_{others}$, $(GT)_{20}/(GT)_{others}$, and $(GT)_{others}/(GT)_{others}$ were correlated with the *IL12A* +6686 G/G genotype, whereas $(GT)_{18}/(GT)_{21}$, $(GT)_{20}/(GT)_{21}$, and $(GT)_{21}/(GT)_{others}$ were correlated with the *IL12A* +6686 A/G genotype ($\chi^2 = 244.51$, $p<0.001$) (fig 1). The *IL12B* VNTR genotype was correlated

Table 4 Variable number tandem repeat (VNTR) genotype frequencies of *IL12A* intron 6 and of IL12 B intron 4 found in the present series

IL12A VNTR			*IL12B* VNTR		
Genotype	No of $(GT)_n$ repeats	Genotype frequency (%)	Genotype	No of $(TG\text{-}TA)_n$ repeats	Genotype frequency (%)
$(GT)_{18}/(GT)_{18}$	18/18	12.4	$(TG\text{-}TA)_9/(TG\text{-}TA)_9$	9/9	6.1
$(GT)_{18}/(GT)_{20}$	18/20	17.8	$(TG\text{-}TA)_9/(TG\text{-}TA)_{11}$	9/11	31.5
$(GT)_{18}/(GT)_{21}$	18/21	12.7	$(TG\text{-}TA)_9/(TG\text{-}TA)_{others}$	9/others	4.1
$(GT)_{18}/(GT)_{others}$	18/others	19.4	$(TG\text{-}TA)_{11}/(TG\text{-}TA)_{11}$	11/11	50.3
$(GT)_{20}/(GT)_{20}$	20/20	4.1	$(TG\text{-}TA)_{11}/(TG\text{-}TA)_{others}$	11/others	3.8
$(GT)_{20}/(GT)_{21}$	20/21	4.8	$(TG\text{-}TA)_{others}/_{others}$	Others/others	4.1
$(GT)_{20}/(GT)_{others}$	20/others	11.5			
$(GT)_{21}/(GT)_{21}$	21/21	1.3			
$(GT)_{21}/(GT)_{others}$	21/others	7.3			
$(GT)_{others}/(GT)_{others}$	Others/others	8.6			

The most common alleles were considered singly, while the remaining were considered overall and classified as ''others''.

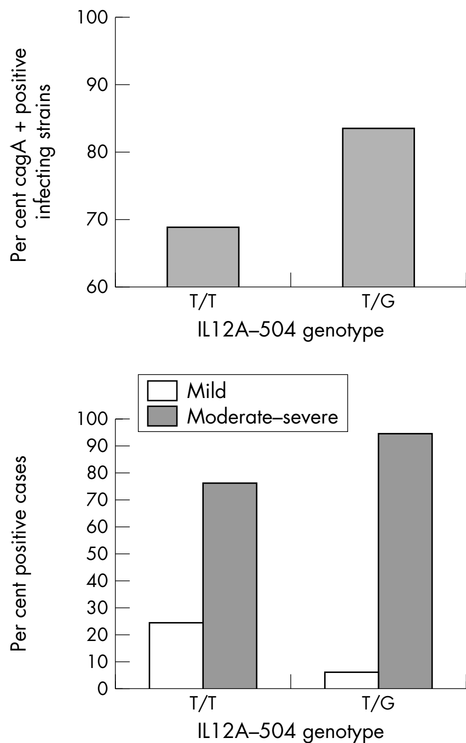

Figure 2 Associations between *IL12A* −504 SNP and *cagA* or antral activity in *H pylori* positive patients.

with *IL12B* +15485 ($\chi^2 = 24.04$, p<0.01); in particular, the genotypes $(TG\text{-}TA)_9/(TG\text{-}TA)_9$, $(TG\text{-}TA)_9/(TG\text{-}TA)_{11}$, and $(TG\text{-}TA)_9/(TG\text{-}TA)_{others}$ were correlated with *IL12B* +15485 A/A, whereas the $(TG\text{-}TA)_{11}/(TG\text{-}TA)_{11}$ genotype was correlated with *IL12B* +15485 A/G and G/G ($\chi^2 = 24.04$, p<0.01).

IL12A and *IL12B* polymorphisms and *H pylori* infection

IL12A and *IL12B* SNPs or VNTRs were not correlated with *H pylori* infection, *cagA*, s or m *vacA*, antral or body inflammation, and antral activity. Body activity was correlated with *IL12B* VNTR: mild to moderate body activity was more commonly recorded in patients with the $(TG\text{-}TA)_{11}/(TG\text{-}TA)_{11}$ genotype ($\chi^2 = 47.75$, p<0.001).

In relation to *H pylori* positive patients, the only associations we found were those between *IL12A* −504 SNP and *cagA* (Fisher's exact test, p<0.05; odds ratio = 2.34, 95% confidence interval (CI), 0.91 to 5.98) or *IL12A* −504 SNP and antral activity ($\chi^2 = 8.83$, p<0.05) (fig 2).

IL12A and *IL12B* polymorphisms and *H pylori* associated diseases

Among the five IL12 genetic polymorphisms studied, and when considering the patients overall, no statistically significant association was found with the disease diagnosis. The patients were then subdivided into two main groups: those with non-cardia gastric cancer and those with benign diseases. *H pylori* infection was more often recorded in non-cardia gastric cancer patients (69.4% *v* 49.0%) (Fisher's exact test: p<0.01). On considering *H pylori* infected patients only, non-cardia gastric cancer was correlated with *cagA* (Fisher's exact test, p<0.05) and s1 *vacA* (Fisher's exact test, p<0.05).

The frequency of non-cardia gastric cancer was higher in patients with the *IL12A* −504 T/T genotype (Fisher's exact test, p<0.05) and in those with *IL12B* VNTR $(TG\text{-}TA)_9/(TG\text{-}TA)_{11}$ genotype ($\chi^2 = 12.40$, p<0.05) (fig 3).

Table 5 shows the association between *IL12B* VNTR polymorphism and the risk of gastric cancer. We selected subjects homozygous for the most common allele $(TG\text{-}TA)_{11}$ as the reference group in the initial odds ratio estimations. The *IL12B* VNTR $(TG\text{-}TA)_9/(TG\text{-}TA)_{11}$ genotype was confirmed to increase the risk of gastric cancer, while $(TG\text{-}TA)_{others/others}$ was associated with a decreased risk of gastric cancer. When performing the logistic regression analysis considering the diagnosis of gastric adenocarcinoma as a dependent variable and the gene polymorphisms of *IL12A* −504, *IL12B* VNTR, and *cagA* as predictors, the odds ratios were 2.38 (95% CI, 0.96 to 5.88), 1.36 (1.05 to 1.76), and 1.68 (0.84 to 3.35), respectively. None of the IL12 gene polymorphisms studied was correlated with Lauren's non-cardia gastric cancer classification.

In relation to patients with benign diseases, we analysed the association between the precancerous intestinal metaplasia and *H pylori* infection, *H pylori* virulence genes, and IL12 gene polymorphisms. Intestinal metaplasia was significantly correlated with *H pylori* infection ($\chi^2 = 10.05$, p<0.01), *cagA* ($\chi^2 = 28.70$, p<0.001), s1 *vacA* ($\chi^2 = 26.18$, p<0.001), and with m1 *vacA* ($\chi^2 = 6.64$, p<0.01), but not with *IL12A* or *IL12B* gene polymorphisms.

DISCUSSION

Using the DHPLC screening strategy followed by direct sequencing we identified the following polymorphisms: *IL12A* −504 T/G, *IL12A* +6686 A/G, and *IL12B* +15485 A/C. The first SNP was located in the promoter region of the *IL12A* gene, while the latter two were localised in the UTR regions of the two genes. These three polymorphisms were in Hardy

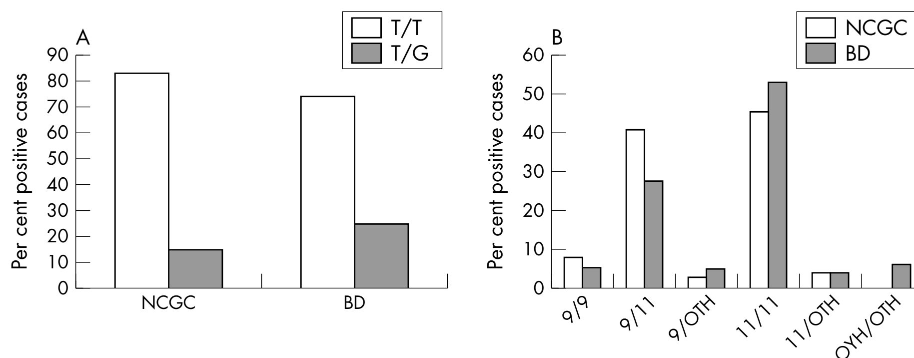

Figure 3 Association between non-cardia gastric cancer and *IL12A* −504 and *IL12B* variable number tandem repeat (VNTR) genotypes. We genotyped *IL12B* VNTRs by considering separately the most common alleles ($(TG\text{-}TA)_9$ and $(TG\text{-}TA)_{11}$) and combining all the remaining alleles as "others" (OTH). BD, benign diseases; NCGC, non-cardia gastric cancer.

Table 5 Association between *IL12B* VNTR polymorphism and gastric cancer risk

Genotype	Case (gastric adenocarcinoma)	Control (benign gastroduodenal diseases)	OR	95% CI
$(TG\text{-}TA)_{11}/(TG\text{-}TA)_{11}$	47	111	1.00	Reference
$(TG\text{-}TA)_{9}/(TG\text{-}TA)_{9}$	8	11	1.42	0.79 to 2.53
$(TG\text{-}TA)_{9}/(TG\text{-}TA)_{11}$	42	57	1.43*	1.02 to 1.99
$(TG\text{-}TA)_{9}/(TG\text{-}TA)_{others}$	3	10	0.77	0.28 to 2.15
$(TG\text{-}TA)_{11}/(TG\text{-}TA)_{others}$	4	8	0.89	0.39 to 2.06
$(TG\text{-}TA)_{others}/_{others}$	0	13	0.70*	0.64 to 0.78

Subjects homozygous for the most common allele $(TG\text{-}TA)_{11}$ formed the reference group in the initial odds ratio estimations.
*$p<0.05$.
CI, confidence interval; OR, odds ratio.

Weinberg equilibrium. The two genes coding for the two IL12 subunits were also analysed for the number of dinucleotide repeats located in intron 6 and intron 4 for *IL12A* and *IL12B*, respectively. These VNTRs were studied bearing in mind that the amount of secreted protein might vary not only in relation to differences in the nucleotide sequence of the promoter or of the coding regions, but also in relation to intron VNTR, as already described for IL1RN,[24] although the exact mechanism linking intron VNTR and protein synthesis has not yet been completely defined. The number of GT repeats of *IL12A* VNTR varied in our patients from a minimum of 11 to a maximum of 23, while TG-TA of *IL12B* ranged from eight to 13.

The wide range of the dinucleotide repeats for both VNTRs implies an excessive data dispersion whenever a statistical analysis is to be carried out. To overcome this limitation other investigators have classified the VNTR alleles of IL1RN on the basis of their length (long/short).[29] This classification is feasible for IL1RN intron 2 VNTR, as each repeat corresponds to an oligonucleotide sequence of 86 bp, but it is difficult to apply to the present VNTRs as the maximum difference between the shorter and the longer alleles corresponds to only 24 bp. Therefore, in agreement with Cai *et al*,[30] who studied the GT dinucleotide repeat polymorphism of the oestrogen receptor α gene, we classified the VNTR genotypes by considering the combinations of the most frequent alleles (three for *IL12A* and two for *IL12B*) and by grouping the less frequent alleles as "others". Interestingly, significant associations were found between the VNTR genotypes and the SNPs of the corresponding gene. These associations might be the expression of linkage or of a selective advantage for some combinations over others.

None of the *IL12A* or *IL12B* polymorphisms and VNTRs studied was correlated with the presence or absence of *H pylori* infection. We therefore suggest that the establishment of *H pylori* infection depends on bacterial characteristics (urease production, expression of adhesins)[1] more than on host cytokine gene polymorphisms, including IL12—although a role has suggested for TNFα –308 G to A transition.[31] By contrast, cytokine gene polymorphisms may correlate with *H pylori* virulence genes in *H pylori* infected subjects. It has already been reported that TNFα polymorphisms are associated with infections from the more virulent *cagA* positive strains.[31] In the present paper, another association was recorded: between *cagA* and the *IL12A* −504 T/G genotype. This finding may be explained on the basis that an association between *cag* PAI and IL12 has already been reported[15 16]: in infections caused by *cagA* positive strains, enhanced gastric mucosal transcription of *IL12B* is recorded, and enhanced release of IL12 by dendritic cells in vitro is found after exposure to *cagE* positive strains. *IL12A* −504 polymorphism might be involved in the regulation of gene expression and, consequently, of IL12 production. This nucleotide is contained within a site possibly recognised by the transcription factor AP2 (GCCT$^{T}/_{G}$GGG), with the penultimate base in our sequence being an A instead of a G.[32] Using the program MATRIX SEARCH 1.0,[33] which allows a search for potential transcription factor binding sites, the sequence with the G allele was recognised for AP2 binding with a match ratio of 0.74, the range which denotes lack of exact match being 0.0 to 1.0. With the T allele, no match was found. The AP2 transcription factor can, in turn, be induced by IL6,[34] potently stimulated by *H pylori*.[11–13] The *IL12A* −504 T/G genotype was also correlated with gastritis activity, which is scored on the basis of the degree of infiltrating polymorphonuclear cells. This finding might either be spurious and consequent on the prevalence of *cagA* positive infecting strains recorded in patients with this genotype, or be the result of an enhanced release of IL12, which is known to stimulate the production by T cells and natural killer cells of GM-CSF.[18]

We also investigated whether there was any association between *IL12A* or *IL12B* polymorphisms and *H pylori* associated diseases. After the patients had been subdivided into two groups—one consisting of those with non-cardia gastric cancer and the other of those with benign gastroduodenal diseases—non-cardia gastric cancer was found to be correlated with the *IL12A* −504 T/T genotype and with the *IL12B* VNTR $(TG\text{-}TA)_{9}/(TG\text{-}TA)_{11}$ genotype, but also with *H pylori* infection and its virulence determinants, *cagA* and s1 *vacA*. For a better definition of the contribution of IL12 gene polymorphisms in enhancing non-cardia gastric cancer risk in *H pylori* infected subjects, logistic regression analysis was undertaken and both *cagA*, *IL12A* −504 and *IL12B* VNTR polymorphisms were confirmed as risk factors for non-cardia gastric cancer. The onset of non-cardia gastric cancer can be considered the result of a process in which the complex interplay between *H pylori* infection, host genetic background, and environmental factors creates conditions favouring or counteracting carcinogenesis.[2 35 36] Taking into account the above hypotheses concerning the involvement of *IL12A*−504 SNP in the regulation of gene transcription, in T/T homozygote subjects the transcription factor AP2 should have a limited transcriptional effect on *IL12A*, and this might lead to reduced IL12 production. It is known that lack of IL12 production results in a reduction in host resistance to infections and tumours[37 38] and that treatment with IL12 has a marked anti-tumour effect on mouse carcinomas.[37] It is more difficult to interpret the association between *IL12B* VNTR $(TG\text{-}TA)_{9}/(TG\text{-}TA)_{11}$ and $(TG\text{-}TA)_{others}/(TG\text{-}TA)_{others}$ genotypes and gastric cancer risk. Increasing numbers of intronic VNTRs have been found to interfere with transcription processes, either by their effect on secondary DNA structure, by their action as protein binding sites, or by their influence on the transcription or stability of mRNA.[24 30] In addition, the VNTR polymorphism may be in linkage disequilibrium with exon alterations that may affect protein function.

Non-cardia gastric cancer, ''intestinal-type'' in particular, is the end stage of a series of lesions which apparently represent a continuum of changes from normal to carcinoma.[39] These include, in order of increasing severity, chronic gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia. In agreement with the multistep process hypothesis, patients with intestinal metaplasia are at increased risk of non-cardia gastric cancer.[40] We therefore looked for an association between the presence or absence of intestinal metaplasia and IL12 gene polymorphisms on the one hand, and *H pylori* infection and its virulent determinants on the other. A strong correlation was found between intestinal metaplasia and *H pylori* infection, *cagA*, and *vacA*. None of the host genetic IL12 gene polymorphisms studied was correlated with intestinal metaplasia. This indicates that they do not play a role in determining this histopathological alteration. The association between IL12 polymorphisms and gastric cancer risk, but not with intestinal metaplasia, suggests that this cytokine probably plays an important role in the progression of intestinal metaplasia to dysplasia and cancer. IL12 gene polymorphisms may act by allowing dysplastic cells, originated from a normal mucosa or from intestinal metaplasia, to escape immune surveillance. This concept is in agreement with the lack of any association between IL12 gene polymorphisms and non-cardia gastric cancer histological subtypes, which are thought to be associated (intestinal type) or not (diffuse type) with the precancerous intestinal metaplasia.[39]

Conclusions

IL12A and *IL12B* gene polymorphisms may affect the latest steps of gastric carcinogenesis in *H pylori* infected subjects. The exact mechanism underlying this phenomenon has yet to be defined, although it is reasonable to suggest that *IL12A* −504 SNP and *IL12B* VNTR may modulate IL12 production in response to *H pylori* infection.

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