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Interleukin-1 polymorphisms associated with increased risk of gastric cancer

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***Helicobacter pylori* infection is associated with a variety of clinical outcomes including gastric cancer and duodenal ulcer disease¹. The reasons for this variation are not clear, but the gastric physiological response is influenced by the severity and anatomical distribution of gastritis induced by *H. pylori*. Thus, individuals**

with gastritis predominantly localized to the antrum retain normal (or even high) acid secretion², whereas individuals with extensive corpus gastritis develop hypochlorhydria and gastric atrophy³, which are presumptive precursors of gastric cancer⁴. Here we report that interleukin-1 gene cluster polymorphisms suspected of enhancing production of interleukin-1-beta are associated with an increased risk of both hypochlorhydria induced by *H. pylori* and gastric cancer. Two of these polymorphisms are in near-complete linkage disequilibrium and one is a TATA-box polymorphism that markedly affects DNA-protein interactions *in vitro*. The association with disease may be explained by the biological properties of interleukin-1-beta, which is an important pro-inflammatory cytokine⁵ and a powerful inhibitor of gastric acid secretion^{6,7}. Host genetic factors that affect interleukin-1-beta may determine why some individuals infected with *H. pylori* develop gastric cancer while others do not.

H. pylori infects half of the world's population and has been implicated in the pathogenesis of gastric cancer¹, the second-most common malignancy worldwide⁸. The mechanism of *H. pylori*-induced carcinogenesis is not clear. The infection almost always causes inflammation of the gastric mucosa, the distribution and severity of which varies widely and affects the clinical outcome. Gastritis that is confined to the antral region is associated with excessive acid production and a high risk of duodenal ulcer disease². In contrast, gastritis involving the acid-secreting corpus region leads to hypochlorhydria, progressive gastric atrophy³ and an increased risk of gastric cancer^{4,9}. These pre-cancerous changes are unfavourable to *H. pylori* growth and some gastric cancers arise long after the infection has disappeared. Duodenal ulceration and gastric cancer seem to be mutually exclusive outcomes of *H. pylori* infection that cannot be explained by differences in bacterial virulence factors alone, as virulent strains seem to be equally associated with both conditions^{10,11}. An alternative explanation is that host genetic factors (in conjunction with bacterial and/or environmental factors) determine the immune and inflammatory responses to *H. pylori* infection. A critical factor in *H. pylori*-induced gastric carcinogenesis is gastric acid secretion, which both influences and is influenced by *H. pylori*-induced gastritis. If inflammation of the corpus mucosa is severe, acid secretion is inhibited and eventually lost through the destruction of gastric glands³. Furthermore, pharmacological inhibition of acid secretion leads to the re-distribution of *H. pylori*-induced gastritis with a reduced intensity of antral inflammation

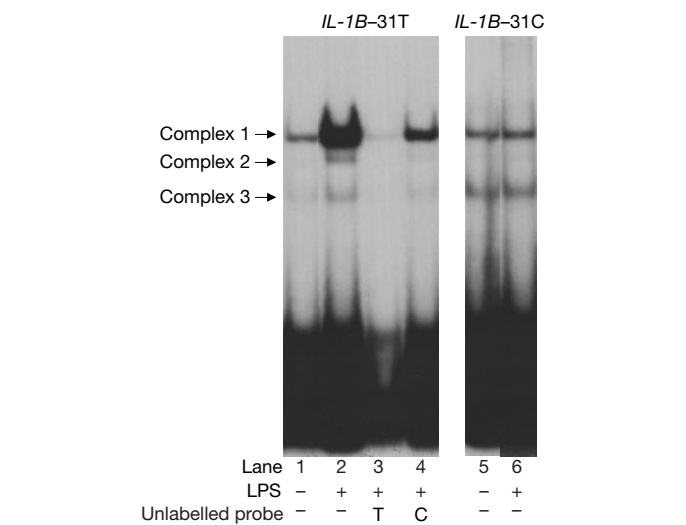


Figure 1 Differential binding patterns between the T- and C-bearing alleles of the *IL-1B* promoter. Nuclear extracts from fresh human monocytes stimulated for 30 min with lipopolysaccharide (LPS) (+) show evidence of induction of DNA-protein complexes 1 and 2 on the *IL-1B* - 31T oligonucleotide. Complex 3 and free probe were equivalent across all lanes. Cold competition with a 100-fold excess of unlabelled probe (T) blocked the formation of complex 1 (lane 3), whereas cross-competition with unlabelled *IL-1B* - 31C (C) only partially blocked complex 1 (lane 4). Results were similar with monocyte extracts from three different donors.

and an increased intensity in the corpus, and may progress to gastric atrophy in the long term¹². Endogenous factors that suppress acid secretion could also contribute to corpus gastritis and atrophy.

The interleukin-1 (*IL-1*) gene cluster on chromosome 2q contains 3 related genes within a 430-kilobase (kb) region, *IL-1A*, *IL-1B* and *IL-1RN*, which encode the pro-inflammatory cytokines IL-1 α and IL-1 β as well as their endogenous receptor antagonist IL-1ra respectively⁵. IL-1 β is upregulated in the presence of *H. pylori* and is important in initiating and amplifying the inflammatory response to this infection¹³⁻¹⁵. IL-1 β is also a potent inhibitor of gastric acid secretion^{6,7}; on a molar basis it is estimated to be 100-fold more potent than proton pump inhibitors and 6,000-fold more potent

Table 1 Estimated haplotype frequencies and linkage disequilibrium coefficients

Population	Loci	Haplotype*						Disequilibrium†		
		1-1	1-2	2-1	2-2	1-3,4,5	2-3,4,5	D'	χ^2	ρ
GCR controls (n = 100)	<i>IL-1B</i> - 31/ <i>IL-1B</i> - 511	0.610	0.010	0.005	0.375			0.98	95.2	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1B</i> + 3954	0.420	0.200	0.380	0.000			-0.53	27.8	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1RN</i>	0.519	0.096	0.131	0.244	0.005	0.005	0.52	26.6	0.0001
	<i>IL-1B</i> + 3954/ <i>IL-1RN</i>	0.495	0.295	0.155	0.045	0.010	0.000	-0.20	3.8	0.05
Low acid GCR (n = 45)	<i>IL-1B</i> - 31/ <i>IL-1B</i> - 511	0.422	0.000	0.022	0.556			0.96	41.4	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1B</i> + 3954	0.273	0.149	0.560	0.018			-0.27	3.3	0.07
	<i>IL-1B</i> - 31/ <i>IL-1RN</i>	0.330	0.092	0.203	0.375	0.000	0.000	0.48	10.5	0.001
	<i>IL-1B</i> + 3954/ <i>IL-1RN</i>	0.408	0.425	0.125	0.042	0.000	0.000	-0.17	1.4	0.24
Normal acid GCR (n = 58)	<i>IL-1B</i> - 31/ <i>IL-1B</i> - 511	0.681	0.017	0.000	0.302			0.95	52.1	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1B</i> + 3954	0.527	0.171	0.275	0.027			-0.20	2.3	0.13
	<i>IL-1B</i> - 31/ <i>IL-1RN</i>	0.611	0.087	0.130	0.155	0.000	0.017	0.47	12.6	0.0004
	<i>IL-1B</i> + 3954/ <i>IL-1RN</i>	0.561	0.223	0.180	0.018	0.017	0.000	-0.25	3.6	0.06
Gastric cancer controls (n = 429)	<i>IL-1B</i> - 31/ <i>IL-1B</i> - 511	0.699	0.002	0.000	0.298			1.00	426.6	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1B</i> + 3954	0.498	0.203	0.250	0.048			-0.16	11.2	0.0008
	<i>IL-1B</i> - 31/ <i>IL-1RN</i>	0.612	0.084	0.109	0.185	0.005	0.004	0.51	112.6	0.0001
	<i>IL-1B</i> + 3954/ <i>IL-1RN</i>	0.525	0.214	0.196	0.056	0.009	0.000	-0.07	2.1	0.14
Gastric cancer cases (n = 366)	<i>IL-1B</i> - 31/ <i>IL-1B</i> - 511	0.576	0.008	0.003	0.413			0.98	352.4	0.0001
	<i>IL-1B</i> - 31/ <i>IL-1B</i> + 3954	0.418	0.166	0.352	0.063			-0.19	12.8	0.0003
	<i>IL-1B</i> - 31/ <i>IL-1RN</i>	0.423	0.155	0.152	0.259	0.007	0.004	0.39	54.7	0.0001
	<i>IL-1B</i> + 3954/ <i>IL-1RN</i>	0.413	0.350	0.162	0.064	0.007	0.004	-0.17	11.0	0.0009

Data given for pairs of *IL-1* loci in gastric cancer relatives (GCR), gastric cancer cases and respective control populations.
* For all *IL-1B* loci, C is denoted by allele 1 and T is denoted by allele 2.
† D' = D/D_{max} for association of most common alleles at each locus.

than H₂ antagonists¹⁶. Three diallelic polymorphisms in *IL-1B* have been reported, all representing C–T base transitions, at positions –511, –31 and +3954 base pairs (bp) from the transcriptional start site¹⁷. There are conflicting data regarding the functional effects of these polymorphisms on IL-1β production^{18,19}. The *IL-1RN* gene has a penta-allelic 86-bp tandem repeat (VNTR) in intron 2, of which the less common allele 2 (*IL-1RN**2) is associated with a wide range of chronic inflammatory and autoimmune conditions¹⁷. *IL-1RN**2 is associated with enhanced IL-1β production *in vitro*¹⁹, but data regarding its effects on IL-1ra production are contradictory^{20–22}.

To determine whether these polymorphisms are important with respect to the different outcomes of *H. pylori* infections, we studied their effects on gastric physiology in healthy subjects. We had previously shown that a cohort of 149 first-degree relatives of gastric cancer patients from the West of Scotland had a high prevalence of hypochlorhydria (defined as a pentagastrin-stimulated peak acid output of less than 15 mmol h⁻¹) in association with *H. pylori* infection²³. Of the 103 (69%) gastric cancer relatives (GCR) infected with the *H. pylori*, 45 had hypochlorhydria and gastric atrophy and 58 had normal or high acid secretion. One hundred unselected newborns from the West of Scotland were available as population controls. We also had available a case-control study of gastric cancer, based on a population from Warsaw, Poland, in which there were 393 gastric cancer cases and 430 controls with DNA samples²⁴. We were thus able to determine whether genotypes that modify IL-1β are associated with low acid secretion and gastric atrophy, and whether these same genotypes increase the risk of gastric cancer. These studies were reviewed and approved by the Institutional Review Boards and Ethics Committees of the US National Cancer Institute, the University of Glasgow Hospitals NHS Trust, and the M. Sklodowska-Curie Memorial Cancer Centre (Warsaw).

In both the Scottish and Polish control populations, the alleles at the individual loci of *IL-1B* and *IL-1RN* were in Hardy–Weinberg equilibrium, with non-significant χ² values. There was marked linkage disequilibrium among the loci within the gene cluster (see also ref. 25). Linkage disequilibrium between *IL-1B* – 31 and *IL-1B* – 511 was almost total, with 99.5% of the inferred haplotypes (*IL-1B* – 31/*IL-1B* – 511) in the combined control groups consisting of either T–T or C–C (Table 1). There was also strong linkage disequilibrium (*D'* ≈ 0.5) between *IL-1B* – 31 and *IL-1RN* in both populations. However, the two groups differed with respect to linkage disequilibrium between *IL-1B* – 31 and *IL-1B* + 3954, which was strong in the Scottish population controls but relatively weak (*D'* = –0.16) in the Polish controls. There was no significant linkage disequilibrium between *IL-1B* + 3954 and *IL-1RN* in either control group.

There were no significant differences in genotype frequency for any *IL-1* marker between the GCR and controls or between the total

H. pylori-infected and uninfected GCR (Table 2). Nevertheless, among the infected GCR, those with low acid secretion had a significantly higher frequency of the pro-inflammatory *IL-1RN**2 allele and the T–T haplotype of *IL-1B* – 31 and *IL-1B* – 511 (*IL-1B* – 31T/*IL-1B* – 511T), as compared with the GCR with normal or high acid secretion. Carriers of the T allele of *IL-1B* – 31 (*IL-1B* – 31T+) had an age-adjusted odds ratio of 9.1 (95% confidence interval (CI), 2.2–37), and there was little difference between the homozygous and heterozygous carriers (Table 2). *IL-1RN**2 homozygotes (*IL-1RN**2/*2) were also at increased risk of hypochlorhydria, although risk among the *IL-1RN**2 heterozygotes was not significantly increased (Table 2). In a logistic regression model including both factors, the estimated age-adjusted odds ratios for *IL-1B* – 31T+ and *IL-1RN**2/*2 were 7.5 (95% CI, 1.8–31) and 2.1 (95% CI, 0.7–6.3), respectively. The *IL-1B* + 3954 genotype was not associated with the risk of hypochlorhydria.

There were similar associations between these alleles and the risk of gastric cancer. Carriers of *IL-1B* – 31T had an increased gastric cancer risk at an odds ratio of 1.9 (95% CI, 1.5–2.6), with no significant difference between homozygotes and heterozygotes (Table 3). Moreover, *IL-1RN**2 was associated with an increased risk in homozygotes but not in heterozygotes (Table 3). In a logistic regression model including both of these genotypes, the estimated odds ratios for *IL-1B* – 31T+ and *IL-1RN**2/*2 were 1.6 (95% CI, 1.2–2.2) and 2.9 (95% CI, 1.9–4.4), respectively. *IL-1B* + 3954T homozygotes seemed to be protected against gastric cancer, although the effect did not reach statistical significance (Table 3).

The estimated effects of *IL-1B* – 31T+ and *IL-1RN**2/*2 were similar in subgroups of gastric cancer cases defined by age, sex, histological type and anatomical site (data not shown). Furthermore, adjustment for other reported risk factors for gastric cancer, including tobacco and alcohol use, ABO blood group and family history of gastric cancer, did not substantially alter the estimates (data not shown).

The *IL-1B* – 31T/*IL-1RN**2 haplotype imparted a greatly increased risk of gastric cancer, as compared with having no copy of *IL-1B* – 31T and at least one copy of the other *IL-1RN* alleles. Twenty-two per cent of cases (compared with eight per cent of controls) had this haplotype, either in its homozygous form or with *IL-1B* – 31C/*IL-1RN**2, with an odds ratio of 4.4 (95% CI, 2.8–6.9). In the absence of *IL-1B* – 31T, homozygous *IL-1RN**2 was associated with a similarly elevated odds ratio of 5.3 (95% CI, 1.9–14), although this genotype combination was uncommon because of linkage disequilibrium between the two loci. In contrast, the odds ratio for *IL-1B* – 31T+ with no more than one copy of *IL-1RN**2 was only 1.7 (95% CI, 1.2–2.3). Nonetheless, because its effect is observed in both homozygotes and heterozygotes, the *IL-1B* – 31T allele accounts for a greater proportion of excess gastric cancer cases than the *IL-1RN**2 allele, despite their comparable frequencies. The

Table 2 *IL-1* genotype frequencies in gastric cancer relatives (GCR) and controls

Locus	Genotype	<i>H. pylori</i> -infected GCR		Odds ratio (95% CI)*	Uninfected GCR (n = 46)	Population controls (n = 100)
		Low acid (n = 45)	Normal acid (n = 58)			
<i>IL-1B</i> – 31	C/C	5	30	1.0	23	37
	C/T	28	21	8.1 (2.0–33)	18	50
	T/T	12	7	13.6 (2.6–71)	5	13
<i>IL-1B</i> – 511	C/C	5	29	1.0	23	36
	C/T	30	21	8.3 (2.0–34)	18	51
	T/T	10	8	11.4 (2.2–58)	5	13
<i>IL-1B</i> + 3954	C/C	30	35	1.0	28	67
	C/T	15	23	0.8 (0.3–1.9)	15	26
	T/T	0	0		3	7
<i>IL-1RN</i>	1/1	17	35	1.0	24	42
	1/2	14	14	2.4 (0.9–6.2)	15	44
	1/3, 4, 5	0	2	0	0	2
	2/2	14	7	5.6 (1.8–17)	7	12

* Odds ratio for low acid versus normal/high acid, adjusted for age and within-family sampling. CI, confidence interval.

fraction of gastric cancer in the population that is attributable to *IL-1B* - 31T is estimated to be 31%, compared with 18% for *IL-1RN**2. Their combined population attributable fraction is estimated to be 38%, which represents the fraction of gastric cancer cases that are caused by these *IL-1* alleles.

The *IL-1B* - 31 polymorphism involves a TATA sequence in the *IL-1B* promoter. To investigate the effect of *IL-1B* - 31 variants on the induction of IL-1 β , we used electrophoretic mobility-shift analysis to assess their DNA-binding activity *in vitro*. Synthetic allele-specific oligonucleotides representing the polymorphic *IL-1B* - 31 sites were incubated with nuclear protein extracts from non-stimulated human monocytes and monocytes activated by lipopolysaccharide (LPS). LPS stimulation induced a fivefold increase in DNA binding (complex 1) on the *IL-1B* - 31T oligonucleotide (Fig. 1, lanes 1 and 2). In contrast, LPS failed to induce complex 1 formation on the *IL-1B* - 31C oligonucleotide (Fig. 1, lanes 5 and 6). Furthermore, complex 1 formation on radiolabelled *IL-1B* - 31T was specifically blocked by competition with the unlabelled *IL-1B* - 31T, but not the *IL-1B* - 31C, oligonucleotide (Fig. 1, lanes 3 and 4). These results indicate that one or more proteins (presumably transcription factors) in complex 1 may be unable to interact with the C-bearing *IL-1B* - 31 allele to form the transcription initiation complex. In parallel experiments assessing allele-specific oligonucleotides for *IL-1B* - 511, there were no differences in binding activity (data not shown), indicating that the effect of *IL-1B* - 511 may be mediated by linkage disequilibrium with the TATA box polymorphism. On the basis of these results and previously published functional data for *IL-1RN*, hypochlorhydria and gastric cancer may be associated with alleles of *IL-1B* - 31 and *IL-1RN* that enhance IL-1 β production.

Here we demonstrate that pro-inflammatory genotypes of the *IL-1* loci (*IL-1B* - 31T+ and *IL-1RN**2/*2) increase both the likelihood of a chronic hypochlorhydric response to *H. pylori* infection and the risk of gastric cancer, presumably by altering IL-1 β levels in the stomach. While the pro-inflammatory effects of high IL-1 β concentrations may facilitate the clearance of *H. pylori* from the gastric mucosa, the concomitant inhibition of acid secretion allows the spread of *H. pylori*-induced inflammation from the antrum to the corpus. This functional inhibition is initially reversible but the progressive destruction of parietal cells eventually leads to irreversible hypochlorhydria. A decreased flow of gastric secretions may therefore heighten mucosal damage by allowing the accumulation of bacterial toxins and by-products of inflammation that would normally be diluted and flushed out. The reactive oxygen and nitrogen oxide species that are derived from inflammation are known mutagens, while hypochlorhydria permits superinfection by other bacteria that enhance the production of highly carcinogenic *N*-nitroso compounds²⁶.

H. pylori-induced hypochlorhydria also markedly reduces the levels of vitamin C in gastric juice²⁷, further facilitating the forma-

tion of *N*-nitroso compounds. The opportunities for DNA damage caused by this cascade of genotoxic factors are amplified by the increased rate of cell turnover in inflamed mucosa. Therefore, genotypes that enhance IL-1 β production may favour the initiation of a set of responses to *H. pylori* that result in hypochlorhydria, corpus atrophy and an increased risk of gastric cancer.

Our findings complement the most widely accepted multi-stage model of gastric carcinogenesis⁴, and provide insights into the etiological role of *H. pylori*. We have shown at least two susceptibility loci in the *IL-1* gene cluster for gastric cancer and its precursors. The effects of these loci influence an early stage of the disease process and require the presence of *H. pylori* infection. Progression towards cancer is probably influenced by other components of the host genetic constitution acting epistatically, as well as by dietary and other factors in the environment. *IL-1B* - 31T/*IL-1RN**2 constitutes a pro-inflammatory haplotype that is in strong linkage disequilibrium, at least in Caucasian populations. We speculate that this linkage disequilibrium reflects past selective pressures for or against enhancement of the IL-1 β response to environmental challenges. Whereas pro-inflammatory genotypes may be advantageous for the host response to some infections, the vigorous IL-1 β production associated with *H. pylori* gastric infection may exacerbate mucosal damage and increase the risk of eventual neoplasia. □

Methods

IL-1 genotyping

IL-1B polymorphisms were distinguished by two separate methods, polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) and 5' nuclease PCR assays (TaqMan). For PCR-SSCP, 50 ng DNA was amplified in a GeneAmp PCR System 9700 (PE Applied Biosystems), using the primer pairs listed in the Supplementary Information. Amplification was performed in a volume of 20 μ l, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each of dATP, dTTP and dGTP, 100 μ M dCTP, 1 μ Ci of [α -³²P]dCTP (3,000 Ci mol⁻¹), 80 ng of each primer and 1 unit of Taq polymerase. The thermocycling conditions were as follows: 94 °C for 10 min; then 5 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s; then 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; then 5 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. SSCP analysis of the radiolabelled amplification products was performed as described²⁸.

For TaqMan assays, primers (Operon Technologies Inc.) and probes (PE Applied Biosystems) were designed using Primer Express software (PE Applied Biosystems). Probes for the T or C allele were 5'-labelled with either FAM (6-carboxyfluorescein) or VIC fluorogenic dyes, and 3'-labelled with TAMRA (6-carboxytetramethylrhodamine) quencher. PCR amplification was performed in a volume of 25 μ l containing 50 ng genomic DNA, 1 \times TaqMan Universal Master Mix (PE Applied Biosystems), 200 nM for each probe and 900 nM for primers. Cycling conditions were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 62 °C for 1 min, as recommended by the manufacturer. Thermal cycling of optical plates was performed in GeneAmp PCR System 9700 and endpoint analysis was performed in the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Sequences of primers and probes for *IL-1B* TaqMan assays are available from the authors.

For *IL-1RN*, genomic DNA was amplified using PCR under the conditions described above for *IL-1B*, using forward primer 5'-CCCCCTAGCAACTCC-3' and reverse primer 5'-GGTCAGAAGGGCAGAGA-3'. The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Alleles were sized relative to a 1-kb DNA ladder and coded conventionally as follows: allele 1 = 4 repeats, allele 2 = 2 repeats, allele 3 = 5 repeats, allele 4 = 3 repeats, allele 5 = 6 repeats; the rarer alleles 3, 4 and 5 were grouped in the statistical analysis.

A total of 93% (366/393) of the gastric cancer cases, 99% (429/430) of their population controls, and all GCR subjects and their newborn population controls were successfully genotyped for all four loci.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from freshly isolated human monocytes as described²⁹, after no stimulation or addition of 1 μ g ml⁻¹ LPS derived from *E. coli* (Sigma) for 30 min. Complementary single-stranded oligonucleotides were synthesized (Life Technologies) as follows (variant nucleotides in bold):



Complementary strands were annealed by combining 2 μ g of each oligonucleotide and 6 μ l of 10 \times annealing buffer (500 mM Tris, 100 mM MgCl₂ and 50 mM dithiothreitol) in a 60- μ l reaction, placing in a boiling water bath for 5 min and allowing to cool to room temperature. The DNA-protein binding reaction was conducted in a 20- μ l volume containing 7 μ g of nuclear protein extract, 1 μ g poly (dI-dC) (Sigma), 4 μ l of 5 \times binding

Table 3 *IL-1* genotype frequencies in gastric cancer cases and controls

Locus	Genotype	Cases (n = 366)	Controls (n = 429)	Odds ratio (95% CI)
<i>IL-1B</i> - 31	C/C	128	219	1.0
	C/T	172	164	1.8 (1.3-2.4)
	T/T	66	46	2.5 (1.6-3.8)
<i>IL-1B</i> - 511	C/C	127	217	1.0
	C/T	170	166	1.8 (1.3-2.4)
	T/T	69	46	2.6 (1.7-3.9)
<i>IL-1B</i> + 3954	C/C	212	242	1.0
	C/T	140	158	1.0 (0.8-1.4)
	T/T	14	29	0.6 (0.3-1.1)
<i>IL-1RN</i>	1/1	148	230	1.0
	1/2	117	152	1.2 (0.9-1.6)
	1/3, 4, 5	8	7	1.8 (0.7-4.8)
	2/2	93	39	3.7 (2.4-5.7)
	2/5	0	1	0

buffer (60 mM HEPES, 7.5 mM MgCl₂, 300 mM KCl, 1 mM ethylenediamine-tetraacetic acid, 2.5 mM dithiothreitol, 50% glycerol and 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride) and 2.5 × 10⁴ c.p.m. of ³²P-labelled oligonucleotide probe. UN-SCAN-IT 5.1 software (Silk Scientific) was used for densitometric analysis of the autoradiographs.

Statistical analysis

Hardy–Weinberg equilibrium of alleles at individual loci was assessed by χ^2 statistics. Haplotype frequencies for pairs of alleles were estimated using the Estimating Haplotype-frequencies (EH) software program (<ftp://linkage.rockefeller.edu/software/eh>). Linkage disequilibrium coefficients $D' = D/D_{\max}$ and χ^2 values were calculated for pairs of the most common alleles at each locus using the LINKDOS software program (distributed with GENEPOP, <ftp://ftp.cfe.cnrs-mop.fr/pub/PC/MSDOS/GENEPOP/>). Odds ratios with Cornfield 95% confidence intervals and logistic regression models controlling for the effects of possible confounders were computed using STATA version 5.0 software (STATA Press). The odds ratios for hypochlorhydria were age-adjusted (categorized as ≤35, 36–45, 46–55 and >55 years) because of its age-dependence²⁵ and their confidence intervals were based on robust variance estimates³⁰ which adjust for within-family correlation, to account for sampling of several members of a given family.

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