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IL10 Single Nucleotide Polymorphisms are Related to Upregulation of Constitutive IL-10 Production and Susceptibility to *Helicobacter pylori* Infection

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Abstract

Background: *Helicobacter pylori* infection is a strong risk factor for gastric cancer, likely due to the extensive inflammation in the stomach mucosa caused by these bacteria. Many studies have reported an association between *IL10* polymorphisms, the risk of gastric cancer, and IL-10 production. The aim of the study was to evaluate the association between *IL10* genetic variants, *Helicobacter pylori* infection, and IL-10 production by peripheral blood leukocytes in children.

Materials and Methods: We genotyped a total of 12 single nucleotide polymorphisms in *IL10* in 1259 children aged 4–11 years living in a poor urban area in Salvador, Brazil, using TaqMan probe based, 5′ nuclease assay minor groove binder chemistry. Association tests were performed by logistic regression for *Helicobacter pylori* infection and linear regression for IL-10 spontaneous production (whole-blood cultures) including sex, age, and principal components for informative ancestry markers as covariates, using PLINK.

Results: Our results shown that *IL10* single nucleotide polymorphisms rs1800896 (OR = 1.63; 95% CI = 1.11–2.39), rs3024491 (OR = 1.71; 95% CI = 1.14–2.57), rs1878672 (OR = 1.79; 95% CI = 1.19–2.68), and rs3024496 (OR = 1.48; 95% CI = 1.05–2.08) were positively associated with *Helicobacter pylori* infection. Eight single nucleotide polymorphisms were associated with spontaneous production of IL-10 in culture, of which three (rs1800896 and rs1878672, $p = .04$; rs3024491, $p = .01$) were strongly associated with infection by *Helicobacter pylori*.

Conclusions: Our results indicate that *IL10* variants rs1800896, rs3024491, rs1878672, and rs3024496 are more consistently associated with the presence of anti-*H. pylori* IgG by inducing increased production of IL-10. Further studies are underway to elucidate the role of additional genetic variants and to investigate their impact on the occurrence of gastric cancer.

Helicobacter pylori is a gram-negative bacteria that colonizes the gastric mucosa and induces chronic inflammation [1] and is considered the major risk factor for development of gastric cancer, the second leading cause of death from cancer worldwide [2,3]. Studies on the development of gastric cancer suggest that *H. pylori* infection and genetic predisposition are parts of a complex interaction with the host immune system [2,4].

Helicobacter pylori affects about 50% of the world's population and has been associated with poor hygienic conditions [5] with a higher prevalence in developing countries. In Brazil, prevalence of *H. pylori* infection may reach 82% in adults and 78% in individuals among 10–19 years [6].

Helicobacter pylori uses several mechanisms to evade or downregulate both innate and adaptive host

immune responses, including enhancement of Interleukin-10 (IL-10) production [7], which in turn prevents inflammation and favors infection. Levels of IL-10 are critical in immune regulation, controlling the balance between inflammatory and humoral responses and modulation of the balance of T_H1 and T_H2 cells during the infection [1,7]. IL-10 also displays potent abilities to suppress the antigen presentation capacity of antigen presenting cells [3].

Many investigators have reported associations between single nucleotide polymorphisms (SNPs) in the gene encoding *IL10* [8,9], and increasing IL-10 cytokine production [10]; however, genetic studies evaluating the impact of these polymorphisms on the *H. pylori* infection susceptibility have not been available. Considering the impact of *H. pylori* infection and the conflicting data in the literature on the role of *IL10* polymorphisms in IL-10 cytokine production, we sought to evaluate the association between polymorphisms in *IL10* and spontaneous IL-10 production and *H. pylori* infection in a population living in Salvador, Bahia, Brazil, where *H. pylori* seropositivity of 28.7% has been reported [5].

Materials and Methods

Study Population

This study was conducted in Salvador, in Northeastern Brazil, comprised of a population of nearly 2.5 million people. The general study design has been reported elsewhere [11–13]. In summary, the study included 1445 unrelated children between 4 and 11 years old recruited in infancy for a prospective study measuring the impact of a citywide sanitation program on childhood morbidity [14]. These children came from a typical urban poor population characterized by absence of public sewage system in most of their household, and 51.7% of the kids were from families having mensal income equal or less than 147 USD, and only 3.3% had the family income equal or more than 500 USD. Ethical approval for the study was obtained from the Brazilian National Ethical Committee, and written informed consent was obtained from the legal guardian of each child. Data were collected from children born between 1994 and 2001 who lived in sentinel neighborhoods in the city. Standardized questionnaires were applied to the children's guardians between 1997 and 2003 (baseline) to collect data on demographic and social variables as well as on the domestic environment. Children were surveyed again in 2005 to collect the same information and blood samples for DNA extraction, whole-blood culture for IL-10 measurement, and serum for serologic examination.

IL-10 Measurement by ELISA

Spontaneous IL-10 concentration was measured in nonstimulated whole-blood culture supernatants using commercially available antibody pairs and recombinant cytokine standards (BD Pharmingen, San Diego, CA, USA) by sandwich ELISA, according to the manufacturer's instructions. Cytokine concentrations were determined by interpolation of standard curves. Detection limits (low/high) were 31.25/500 pg/mL. A total of 1.356 children were assayed for IL-10 levels.

Serologic Detection of Anti-*Helicobacter pylori* IgG

The presence of *H. pylori* antibodies in serum was determined by ELISA as previously described [5,15] using a commercially available kit (Diamedix, Miami, FL, USA), following the directions provided by the supplier. The cutoff was determined by an index value obtained by the ratio of sample absorbance to the absorbance of a calibrator (a solution containing human serum or defibrinated plasma, with IgG antibodies weakly reactive with *H. pylori* and 0.1% sodium azide). A ratio > 1.1 was considered positive. Borderline subjects were removed from the analysis [5]. A total of 1.259 children were assayed for Anti-*H. pylori* IgG levels.

Genotyping

Twelve *IL10* SNPs were selected for genotyping. A tagging approach suggested five SNPs captured 100% of the genetic variation in a ~ 4.89 kb region on chromosome 1 in the European ancestry CEPH (CEU) and African (Yoruban in Ibadan, Nigeria) YRI populations in HapMap (http://hapmap.ncbi.nlm.nih.gov/). In addition, seven SNPs with prior associations with related phenotypes (rs1800871, rs1800872, rs1800896, rs3024491, rs3024492, rs3024495, and rs3024496) but in high linkage disequilibrium (LD) were included.

DNA was extracted from peripheral blood samples using commercial standard protocols (Gentra® Puregene® Blood Kit; Qiagen, Hilden, Germany). SNPs were typed using the TaqMan™ probe based, 5′ nuclease assay minor groove binder chemistry [16] on the 7900HT Sequence Detection System (Applied Biosystems-ABI, Foster City, CA, USA). TaqMan® validated assays and master mix were manufactured by Applied Biosystems (Applied Biosystems).

PCR was conducted in a 5-μL volume using a universal master mix, predesigned, and validated TaqMan® assays for the SNPs (list of SNPs summarized in Table 1). The thermal cycling conditions were 95 °C for

Table 1 Single nucleotide polymorphisms included in this study

SNP	Chromosome position	Alleles	MA	Gene location
rs1800896	206946897	T/C	C	5′UTR
rs3024491	206945046	C/A	A	Intronic
rs1878672	206943713	G/C	C	Intronic
rs3024496	206941864	T/C	C	Exomic
rs3024495	206942413	G/A	A	Intronic
rs3024492	206944112	T/A	A	Intronic
rs3024505	206939904	C/T	T	Downstream
rs1800872	206946407	A/C	A	5′UTR
rs1518111	206944645	A/G	A	Intronic
rs1554286	206944233	T/C	T	Intronic
rs1800871	206946634	T/C	T	5′UTR
rs3024498	206941529	A/G	G	3′UTR

MA, minor allele.

10 minutes followed by 40 cycles of 95 °C for 15 seconds/60 °C for 1 minutes and an extension step of 60 °C for 5 minutes. Nontemplate negative controls and genotyping-positive controls were included in each genotyping plate. Automatic calling was performed with a quality value above 99%.

Ten percent of the samples were genotyped in duplicate with 100% reproducibility. All 12 SNPs were in Hardy–Weinberg equilibrium.

Statistical Analysis

Tests for genetic association were performed using logistic regression including sex and age as covariates. General, additive, dominant, and recessive models were tested. The recessive model was not performed for SNPs with MAF < 0.1 due to zero cells in analysis. To address the potential effects of population stratification, the first two principal components (PCs) delineated via Eigenstrat on 269 ancestry informative markers (AIMs) were included in the model. For continuous data (i.e., IL-10 levels), analyses were conducted using linear regression adjusted by sex, age, and PCs.

Both analyses were performed considering adaptative permutations (EMP1). Permutation procedures provide a computationally intensive approach to generating significance levels empirically. Such values have desirable properties, for example relaxing assumptions about normality of continuous phenotypes and Hardy–Weinberg equilibrium, dealing with rare alleles and small sample sizes, providing a framework for correction for multiple testing, and controlling for identified substructure or familial relationships by permuting only within cluster. In the case of adaptive permutations, we give up permuting SNPs that are clearly going to be nonsignificant more quickly than SNPs that look interesting [17]. We considered significant associations when the p value was lower or equal to 0.05.

All genetic analyses were performed using PLINK, and the graph for IL-10 production between infected and noninfected subjects was constructed using STATA 8.2 (StataCorp LP, College Station, TX, USA).

Results

Table 2 summarizes clinical and demographic characteristics of the study population included sex, age, and *H. pylori* infection; 55.40% of the children were male and the majority was between 6 and 7 years old (35.52%). The prevalence of anti-*H. pylori* IgG antibodies was nearly identical to previous reports, at 28.04%. Among the original 1445 children, analyses were limited to those for whom both genotype and complete phenotype data were available.

Figure 1 illustrates IL-10 production among all the individuals with available data on IgG anti-*H. pylori,* comparing the IL-10 levels on *H. pylori*-infected and noninfected individuals. Infected individuals showed a tendency for higher IL-10 production, but the difference was not statistically significant.

Table 3 summarizes associations between *IL10* SNPs and spontaneous IL-10 production. Statistically significant negative associations were observed for SNPs rs1518111, rs1800872, rs1800871, and rs1554286 under all models tested. Positive association was observed for SNPs rs3024496, rs3024491, rs1878672, and rs1800896 for at least one genetic model tested.

Table 4 summarizes associations between *IL10* SNPs and *H. pylori* infection. Positive associations were observed for SNPs rs1800896 and rs3024491 under both the additive (OR = 1.25; 95% CI = 1.03–1.51; and

Table 2 Characteristics of the study population

	N (%)
Sex (n = 1.343)	
Female	603 (44.60)
Male	749 (55.40)
Age (n = 1.352)	
≤5 years	472 (35.15)
6–7 years	477 (35.52)
≥8 years	394 (29.34)
IgG anti-*Helicobacter pylori* (n = 1.259)	
Infected	353 (28.04)
Noninfected	906 (71.96)

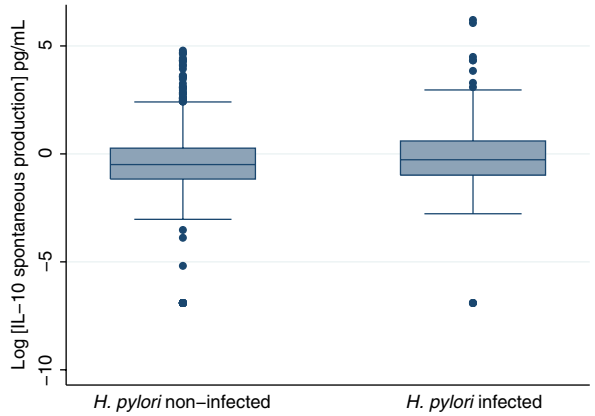

Figure 1 Spontaneous IL-10 production among *Helicobacter pylori* infected or noninfected subjects.

OR = 1.23; 95% CI = 1.01–1.50, respectively) and recessive models (OR = 1.63; 95% CI = 1.11–2.39; and OR = 1.71; 95% CI = 1.14–2.57, respectively). SNPs rs1878672 (OR = 1.79; 95% CI = 1.19–2.68) and rs3024496 (OR = 1.48; 95% CI = 1.05–2.08) were significantly associated with IgG anti-*H. pylori* under the recessive genetic model. Another analysis was carried out taking out children under 5 years of age considering that they can still demonstrate seroconversion; however, the positive association with *H. pylori* persist and some borderline associations became significant suggesting that indeed, polymorphism on *IL10* gene may have an impact on *H. pylori* infection (Supplemental material – Table S1).

Discussion

The differences in IL-10 production were not observed between *H. pylori*-infected and noninfected individuals (Fig. 1). Although the infected group showed a tendency to increased production of IL-10, this difference was not statistically significant. In this study, we measured the spontaneous IL-10 concentration in nonstimulated whole-blood culture supernatants that potentially represent basal IL-10 levels. In contrast, the *H. pylori* antigens-stimulated culture, for example, would allow a better differentiation of IL-10 production between infected and noninfected individuals. In fact, De Vita et al. [18] in a study of cell cultures infected with *H. pylori* demonstrated an increase in IL-10 production among infected individuals. Bodger et al. [19] also reported higher production of IL-10 in *H. pylori*-infected individuals compared with noninfected subjects and those with *Helicobacter*-negative gastritis.

It has previously been shown that polymorphisms in the *IL10* gene may alter the production of IL-10 and its function and/or activity [10]. In the present study, we similarly observed associations between *IL10* SNPs and spontaneous IL-10 production (Table 3); specifically, four markers (rs1518111, rs1800872, rs1800871, and rs1554286) were associated with diminished production of IL-10 ($p < .05$). Marker rs1800872 (−592C/A) was previously described to be associated with lower IL-10 level in patients with food allergy [20] contrasting the data found by Suarez et al. [10].

The IL-10 production was positively associated with the SNPs rs3024496, rs3024491, rs1878672, and

Table 3 Associations between IL10 single nucleotide polymorphisms and spontaneous IL-10 production (n = 1359)*

	Genetic model			
SNP	General β (*p* Value)	Dominant β (*p* Value)	Recessive β (*p* Value)	Additive β (*p* Value)
rs1800896	0.049 (.081)	0.031 (.275)	**0.055 (.047)**	**0.040 (.042)**
rs3024491	**0.060 (.033)**	0.041 (.146)	**0.064 (.023)**	**0.050 (.014)**
rs1878672	0.049 (.080)	0.038 (.175)	0.045 (.112)	**0.043 (.036)**
rs3024496	**0.075 (.008)**	0.049 (.079)	**0.078 (.006)**	**0.058 (.003)**
rs3024495	−0.034 (.225)	−0.035 (.207)	NA	−0.043 (.272)
rs3024492	−0.042 (.140)	−0.046 (.108)	NA	−0.059 (.136)
rs3024505	−0.017 (.588)	−0.018 (.529)	NA	−0.017 (.629)
rs1800872	**−0.112 (<.001)**	**−0.110 (<.001)**	**−0.069 (.014)**	**−0.076 (<.001)**
rs1518111	**−0.115 (<.001)**	**−0.105 (<.001)**	**−0.081 (.004)**	**−0.077 (<.001)**
rs1554286	**−0.097 (.001)**	**−0.094 (.001)**	**−0.060 (.033)**	**−0.066 (<.001)**
rs1800871	**−0.107 (<.001)**	**−0.098 (<.001)**	**−0.076 (.006)**	**−0.071 (<.001)**
rs3024498	−0.049 (.082)	−0.054 (.054)	−0.007 (.807)	−0.041 (.111)

N/A, Not applicable.
*Both *Helicobacter pylori* negative and *H. pylori* positive children were included in the analysis.
Bold values indicates statistical significance.

Table 4 Associations between *IL10* single nucleotide polymorphisms and *Helicobacter pylori* infection (n = 1259)

SNP	Genetic model			
	General (2df) *p* Value	Dominant OR (95% CI)	Recessive OR (95% CI)	Additive OR (95% CI)
rs1800896	**.023**	1.28 (0.99–1.65)	**1.63 (1.11–2.39)**	**1.25 (1.03–1.51)**
rs3024491	**.023**	1.26 (0.97–1.63)	**1.71 (1.14–2.57)**	**1.23 (1.01–1.50)**
rs1878672	**.015**	1.0 (0.77–1.30)	**1.79 (1.19–2.68)**	1.22 (0.99–1.48)
rs3024496	.069	1.17 (0.90–1.53)	**1.48 (1.05–2.08)**	1.17 (0.98–1.42)
rs3024495	.306	1.34 (0.92–1.94)	N/A	1.27 (0.89–1.81)
rs3024492	.506	1.26 (0.86–1.80)	N/A	1.22 (0.84–1.76)
rs3024505	.121	1.16 (0.81–1.67)	N/A	1.14 (0.79–1.63)
rs1800872	.121	1.0 (0.77- 1.30)	0.69 (0.48–1.0)	0.93 (0.78–1.12)
rs1518111	.322	0.95 (0.73–1.23)	0.75 (0.52–1.09)	0.94 (0.78–1.13)
rs1554286	.488	0.99 (0.76–1.28)	0.80 (0.54–1.17)	0.96 (0.80–1.16)
rs1800871	.31	1.02 (0.78–1.32)	0.77 (0.54–1.11)	0.97 (0.81–1.17)
rs3024498	.312	0.92 (0.69–1.22)	1.56 (0.77–3.17)	0.98 (0.76–1.26)

N/A, Not applicable.
Bold values indicates statistical significance.

rs1800896 (Table 3). The SNP rs1800896 was previously associated with higher IL-10 production [7] and has been described as the most important genetic factor in the regulation of constitutive IL-10 mRNA levels [10].

Our data showed that *H. pylori* infection was positively associated with SNPs rs1800896, rs3024491, rs1878672, and rs3024496 (Table 4), suggesting that the presence of these SNPs is associated with susceptibility to *H. pylori* infection. Each of these SNPs were similarly associated with increased production of IL-10 in one or more genetic models tested (Table 3). These findings suggest that elevated levels of certain cytokines might contribute to *H. pylori* infection, as shown in Fig. 2. Previous studies demonstrated that high IL-10 levels could be associated with different types of infection by gram-positive and gram-negative bacteria [21], fungal [22], and dengue infections [23]. The IL-10 SNPs associated with *H. pylori* infection have been also related with others diseases. The rs1800896 is a protective factor to Alzheimer's disease [24]. The rs3024496

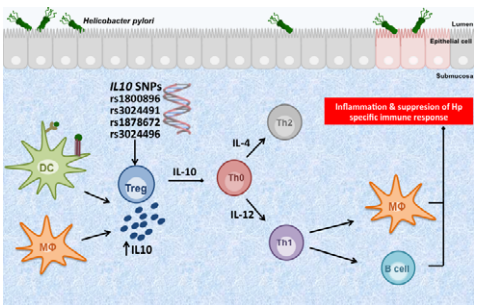

Figure 2 Regulatory single nucleotide polymorphism and *Helicobacter pylori* infection.

and the rs1800896 are associated with increased levels of IgE to allergen and increased risk of asthma [25,26]. Some of our study's limitations were that is not always that positive serology indicates active infection. As such the results of IL-10 expression in the serum may not be reflective of an active infection or may not be even reflect gastric IL-10 levels that could explain our hypothesis. However, considering the strong associations found especially those observed on older children (>5 years of age), we believe our model would be, at least in part, applicable to that.

In summary, we have identified IL10 genetic polymorphisms (rs1800896, rs3024491, rs1878672, and rs3024496) that are consistently associated with the presence of anti-*H. pylori* IgG by inducing increased production of IL-10. IL-10 is an immune regulatory cytokine that can suppress Th1, Th2, and Th17 immune responses, and it is the main immune mechanism to suppress inflammation. Thus, some pathogenic and nonpathogenic organisms can lead to IL-10 upregulation as a way to suppress efficient immune response and then favor infection and parasite survival. In our population, the main organisms related to IL-10 upregulation are helminthes parasites (*Ascaris lumbricoides* and *Trichuris trichiura*) as previously reported [11]. To address whether those organisms could play a role on our results as potential confounders, we run an extra analysis adjusting for helminth infection in addition to age, sex, and AIMs. As can be seen in Table 1 Supplementary, all the associations observed without correction were maintained in this new analysis, suggesting that indeed *H. pylori* can play a role on IL-10 upregulation in those children that have some genetic background (SNPs) on *IL10* gene. In

this sense, the clinical significance of our findings is that people with certain SNPs on IL-10 gene are tend to have more IL-10 when exposed to *H. pylori*, and this could be related to a greater and persistent inflammation that could be related to gastric disease in the future, hypothesis that has to be more clearly elucidated using patients with gastric disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1 Associations between IL10 SNPs and *H. pylori* infection among overall group and children over 5 years old*.