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BRIEF ARTICLE

Inducible nitric oxide synthetase genotype and *Helicobacter pylori* infection affect gastric cancer risk

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Abstract

AIM: To investigate the association of the inducible nitric oxide synthetase (*iNOS*) C150T polymorphism with *Helicobacter pylori* (*H. pylori*) infection and gastric cancer (GC) risk in Iran.

METHODS: In order to determine whether there was a correlation between *iNOS* genotype and GC in Iran, we conducted a case-control study using samples from 329 individuals. For each sample, the C150T *iNOS* polymorphism was genotyped by polymerase chain reaction (PCR) and restriction digestion. Patients were grouped by cancer presence, demographic and behavior characteristics, and *H. pylori* infection status. Statistical tests were conducted to determine whether any behavioral factors or a particular *iNOS* genotype was associated with GC in the study population.

RESULTS: In this population, we found that smoking, hot beverage consumption, a familial history of GC and *H. pylori* infection status were significantly associated with GC development ($P = 0.015$, $P < 0.001$, $P = 0.0034$, and $P < 0.015$, respectively). The distribution of the C150T *iNOS* genotypes among the two study groups was not statistically significant alone, but was impacted by *H. pylori* infection status. When compared to the non-*H. pylori* infected group, cancer patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC [odds ratio (OR) = 2.1, $P = 0.03$] while those with a homozygous TT genotype and infected with *H. pylori* were 5.0 times more at risk of developing GC (OR = 5.0, $P = 0.029$). In contrast, this association was not seen in patients in the control group.

CONCLUSION: A CT or TT polymorphism at position 150 in the *iNOS* gene significantly increases the risk of GC and may be a marker for GC susceptibility.

Key words: Inducible nitric oxide synthetase; Gastric cancer; *Helicobacter pylori*; Heterozygous CT genotype; Homozygous TT genotype

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INTRODUCTION

Gastric cancer (GC) is a major cause of morbidity and mortality throughout the world; it is estimated that GC is responsible for over 700 000 deaths per year[1,2], which makes this disease one of the leading causes of cancer-related death worldwide[3]. One of the most prominent risk factors for development of GC is infection with the bacterium *Helicobacter pylori* (*H. pylori*). Indeed, since its discovery in the early 1980s, *H. pylori* has been recognized as the causative agent of two types of cancer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma[4-8]. The tight association between infection with this pathogen and the development of GC led to the classification of *H. pylori* as a class I carcinogen[9]; it is currently the only bacterium so classified.

Although *H. pylori* infection is relatively common, only a small subset of infected individuals develop GC; some individuals colonized with *H. pylori* carry the organism their entire lives with no overt disease symptoms, while others develop severe disease. Furthermore, recent evidence has begun to suggest that *H. pylori* colonization may be beneficial in some individuals; studies indicate that *H. pylori* protects against the development of esophageal cancer, childhood asthma[10-12] and active tuberculosis[10]. These findings have led researchers to try to define risk factors associated with *H. pylori*-induced disease development. Indeed, years of study have shown that in *H. pylori*-infected individuals, disease development/carcinogenesis is a complex and multifactorial process that involves an intricate interplay between numerous factors; studies indicate that disease etiology is influenced by a combination of bacterial factors, host genetics, and environmental factors such as diet.

Numerous bacterial factors have been identified as being important for *H. pylori*-induced disease development. These include the toxins CagA[13,14] and VacA[15,16], the adhesions BabA[17] and SabA[18], and the outer membrane proteins HomA, HomB, and OipA[19-21]. These and other bacterial factors act in concert to alter host cell signaling pathways, induce epithelial cell proliferation, and disrupt the delicate balance that exists between the bacterium and the host immune system. However, although these bacterial factors play a key role in the development of gastric disease, they represent only one aspect of the story. Indeed, there is a large body of work that suggests environmental factors also affect the risk of GC development. Some of the more prominent environmental risk factors include smoking[22] and high levels of dietary salt intake[23-25].

Besides these exogenous factors, the host immune system also clearly plays a major role in the development of gastric disease. In response to infection with *H. pylori*, the localized gastric environment becomes pro-inflammatory *via* the expression of pro-inflammatory cytokines[26-28]. Because these host factors play an important role in generating and maintaining the pro-inflammatory environment, it is perhaps not surprising that specific mutations in the genes that encode these factors influence gastric disease development. For example, specific interleukin-1 (*IL-1*), *IL-8*, *IL-10*, and tumor necrosis factor-α gene polymorphisms are associated with an increased risk for GC[29,30]. While these cytokines play an important role in the development and maintenance of the pro-inflammatory environment in the gastric mucosa, there are clearly other host factors involved in the disease process.

Nitric oxide (NO) is a molecule that has multiple functions in the gastrointestinal tract. In addition to having anti-microbial properties and playing an active role in vasodilation[31], NO is also a component of the pro-inflammatory immune response that has been linked to carcinogenesis[32]. NO is synthesized from L-arginine by three distinct isoforms of the nitric oxide synthetase (NOS)[31]. Whereas 2 of the 3 isoforms (neuronal NOS and epithelial NOS) are constitutively expressed, the inducible isoform (iNOS) is primarily expressed in response to specific cytokines[31]. Furthermore, expression of iNOS is upregulated during *H. pylori* infection[33]. Several studies have shown a link between increased levels of iNOS and risk of GC development[34,35]. Since one of the end products of NO metabolism is reactive nitrogen species, it is hypothesized that higher levels of iNOS lead to more oxidative DNA damage, thus increasing the risk of cancer development[31]. Interestingly, a single amino acid substitution (Ser 608 Leu) in the iNOS reductase domain has been associated with an increased risk for GC in Asian populations[36,37]. This amino acid change is the result of a single polymorphism (C150T) in exon 16[36,37], which is believed to affect the activity of the iNOS enzyme[36].

Even though the number of GC cases has decreased in many countries in recent years, the incidence of GC in Northern Iran remains high[38]. Several provinces, including Manzadaran, Ardabil, Golestan, Semnan, and the metropolitan area of Tehran, have high rates of GC in both men and women[38]. The age-standardized incidence rate for GC in these areas has been reported as high as 49.1 in men, and 25.4 in women[38]. These incidence rates may be at least partially explained by the fact that a significant proportion of this population is colonized by *H. pylori*. Indeed, the *H. pylori* infection rate for Iranians aged 40 years and over can be as high as nearly 90%[39,40]. A similar prevalence was reported for the Babol city of Iran where the *H. pylori* infection rates are 78% for men and 82% for women[41]. In addition to high rates of *H.*

pylori infection, environmental factors such as consumption of red meat and dairy products, high salt intake, and consuming hot tea have been identified as important risk factors for GC in this region[2].

At present, virtually nothing is known concerning which host genetic factors may influence GC risk in this region. Herein, we describe an epidemiologic study in which we investigate the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among men and women from the Mazandaran region of Iran. We found that in this population, individuals who are infected with *H. pylori* are at a significantly higher risk for development of GC if they also harbor a CT or TT *iNOS* genotype. To date, this report is the first study that links a host-genetic factor to GC risk in this population. Furthermore, it is the first study to investigate the importance of the *iNOS* polymorphism in a non-Asian population. *En masse*, our data indicated that the *iNOS* C150 allele may be a potential marker for susceptibility to *H. pylori*-mediated GC development in multiple populations.

MATERIALS AND METHODS

Study participants

This case-control study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Research Ethics Committee at the Mazandaran University of Medical Sciences. Written informed consent was obtained from each individual, and all study participants were given an explanation of the nature of the study prior to enrollment. Patients were enrolled in the study after seeking treatment at Touba Polyclinics or Imam Hospital between April 2008 and March 2011. Patients with GC were diagnosed by gastric endoscopy and cases were defined using the International Classification of Diseases for Oncology and Lauren criteria[42]. During the course of the study, 159 individuals with GC were enrolled. This group had a mean age of 61.32 ± 12.5 years and was composed of 75 female (approximately 47%) and 84 male (approximately 53%) patients. Patients with inflammatory diseases such as rheumatoid arthritis and/or malignancies other than gastric carcinoma were excluded from the study. Members of the control group were matched by age, sex, geographic location, and ethnic background. This group included 170 individuals with a mean age of 58.86 ± 14.2 years. Ninety-eight were male (approximately 58%) and 72 were female (approximately 42%). For this population, healthy controls who were 40 years of age or greater were recruited from the clinics after undergoing an annual medical screening that included a gastric endoscopy. Healthy controls who were less than 40 years of age were recruited after being referred to the clinic for routine laboratory testing. Demographic information, eating habits, and epidemiologic risk factors were provided by study participants during a face-to-face interview. Risk factors that were evaluated included cigarette smoking, drinking hot beverages, and consumption of salted fish and fast food. A person was considered a smoker if they reported smoking at least one cigarette per day for 12 or more months. Consumption of salted fish or fast food was defined as consuming either item one or more times a week for at least 6 mo. Criteria for drinking hot beverages were essentially as described previously[43]. Patients were considered to have a family history of GC if either of their parents currently had or had been diagnosed with GC in the past, or if any of their siblings had a history of the disease.

Inducible nitric oxide synthetase genotyping

In order to investigate *iNOS* gene polymorphisms, venous blood was collected from study participants and used to isolate genomic DNA by the salting-out method[44]. *iNOS* genotyping was performed using an amplicon-restriction fragment length polymorphism (ARFLP) essentially as previously described[37]. Briefly, for each individual a polymerase chain reaction (PCR) was performed using 2 pmol each of the iNOS-F (5'-TGTAAACCAACTTCCGTGGTG-3') and iNOS-R (5'-174 GTCTCTGCGGGTCTGAAG-3') primers, 200 mmol dNTPs, 10 mmol Tris-HCl, pH 8.3, 50 mmol KCl, 1.5 mmol $MgCl_2$, 0.25 U of Hot start Taq DNA polymerase (Fermentas, Italy) and 50 ng of genomic DNA template in a final reaction volume of 25 μL. Each reaction was denatured at 95 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 60 s, annealing at 58.5 ℃ for 60 s, and extension at 72 ℃ for 40 s. A final extension at 72 ℃ for 5 min was added to ensure complete amplification. Restriction digests were performed by incubating 10 μL of PCR product with 2 U of TSP 509I enzyme (New England Biolabs, Ipswich, MA, United States) in a final volume of 15 μL for 15 h at 65 ℃. Digestion products were separated on a 1.5% SDS-polyacrylamide gel, and visualized after staining with ethidium bromide. Results of the ARFLP analysis allowed us to differentiate between the 3 possible genotypes: (1) digestion of PCR amplicons from homozygous (CC) individuals generated 2 DNA fragments of 113 bp and 175 bp; (2) digestion of amplicons from heterozygous individuals (CT) yielded 3 DNA fragments of 113 bp, 142 bp, and 175 bp; and (3) digestion of amplicons from homozygous (TT) individuals yielded 2 DNA fragments of 113 bp and 142 bp.

H. pylori detection

Infection with *H. pylori* was detected by culturing gastric tissue collected during endoscopy. Identification of clinically isolated *H. pylori* strains was confirmed by Gram stain and urease activity. Additionally, all patients were also tested for infection using an *H. pylori* specific IgG enzyme linked immunosorbent assay (ELISA) (Diagnostic Automation, CA, United States) and by urease breath test (Bahar, Afshan, Iran). Given the difficulties in culturing *H. pylori*, especially from cancerous tissue, individuals were classified as infected with *H. pylori* if they gave a positive result by ELISA.

Table 1 Patient demographics and risk behavior

	Gastric cancer n = 159 (%)	Control n = 170 (%)	P-value
Age (yr)	61.32 ± 12.5	58.86 ± 14.2	0.098
Sex (male/female)	84/75	98/72	0.367
Marital status			
Single	7 (4.4)	3 (1.8)	
Married	149 (93.7)	166 (97.6)	0.002
Divorced	3 (1.9)	1 (0.6)	
Occupation			
Unemployed	4 (2.5)	1 (0.6)	
Employee	24 (15.1)	35 (20.5)	0.001
Housewife	46 (28.9)	74 (43.5)	
Others	85 (53.5)	60 (5.4)	
Smoker	33 (20.8)	22 (12.9)	0.015
Hot beverage	76 (47.8)	38 (22.4)	< 0.0001
Salty fish	15 (9.4)	14 (8.2)	0.09
Fast food	29 (18.2)	39 (22.9)	0.037
Pickles	88 (55.3)	97 (57.1)	0.62
H. pylori positivity	94 (59.1)	77 (45.3)	0.015
Positive family history	34 (21.4)	16 (9.4)	0.0034

H. pylori: *Helicobacter pylori*.

Statistical analysis

Fisher's exact test was used to compare the different *iNOS* allele types, and the frequency of each *iNOS* genotype in the patient population(s) was assessed using a χ^2 test. The difference in age between the 2 study groups was evaluated using a two-tailed Student's *t*-test. Deviation from the Hardy-Weinberg equilibrium was evaluated using a χ^2 test. To evaluate the risk factor(s) in the study populations, unconditional logistic regression models were applied to odds ratios (ORs) and 95% confidence intervals (CIs). For all statistical tests, a P value of less than 0.05 was considered significant.

RESULTS

Demographics and epidemiology of the patient population

Complete epidemiologic and demographic information for the study population is given in Table 1. Across the 329 study participants, ages ranged from 28 years to 86 years. Within the control group the mean patient age was 58.9 ± 14.2 years, whereas within the cancer group the mean patient age was 62.1 ± 12.6 years. There was no significant difference between the ages of patients in the two groups (P = 0.098). The control group was comprised of 98 male and 72 female patients while the cancer group was comprised of 84 male and 75 female patients. There was no significant difference between the distribution of male and female study participants in either group (P = 0.367). Patients were categorized by marital status as single, married, or divorced. The cancer group consisted of 7 single, 149 married, and 3 divorced individuals. In contrast, the control group consisted of 3 single, 166 married, and a single divorced patient. The breakdown of marital status between the case and control groups was significant (P = 0.002). The study participants were also broken down into different groups based on occupation. Patients were considered either unemployed, employed, or worked as a housewife; those participants whose employment status did not fall into one of these 3 categories were grouped into a single category, which was named "other". In the GC group, 4 of the study members were unemployed, 24 were employed, 46 were housewives, and the remaining 85 were in the other classification. Within the control group, 1 participant was unemployed, 35 were employed, 74 were housewives, and 60 were included in the other category. The distribution of patient occupation among the case group and the control group was significantly different (P = 0.001).

Risk factors associated with GC

In addition to gathering demographic information about the study participants, we also analyzed behaviors that are known to be associated with increased GC risk. Of the 159 patients who were diagnosed with GC, 33 were smokers whereas only 22 of the healthy controls reported smoking. Since smoking is an established risk factor for development of GC, perhaps it is not surprising that the increased number of smokers in the cancer group was statistically significant (P = 0.015). Another established risk factor for GC is consumption of hot beverages; drinking hot tea is specifically associated with increased risk of esophageal cancer in Iran[43]. Seventy-six participants in the cancer group reported drinking hot beverages on a regular basis. This number was significantly different than the 38 control patients who regularly drank hot beverages (P < 0.0001). Although consuming high amounts of dietary salt has been linked to GC[23-25], we found no significant difference in the number of patients who regularly ate salted fish among the two groups. Similarly, we found no significant difference in the number of patients who consumed pickles among the groups. However, more of the healthy controls reported regularly eating fast food than did the participants who developed GC. In contrast to the other 2 food criteria, this difference was significant (P = 0.037).

Among the most widely recognized factors that are associated with GC risk are *H. pylori* infection and a family history of cancer. Among the control group, only 18 patients had a family history of GC. In contrast, 34 participants in the cancer group reported having a family history of GC (P = 0.003). As it is the most commonly associated risk factor for GC, we next compared the number of *H. pylori*-positive individuals in both of our study groups. In the cancer group, nearly 60% of the study participants tested positive for *H. pylori*. Compared to this relatively high infection rate, the percentage of healthy controls who tested *H. pylori*-positive (45%) was significantly less (P = 0.015).

Frequency and distribution of C150T inducible nitric oxide synthetase polymorphisms

Having established the relative risk factors of our population, we next sought to determine the distribution of the

Table 2 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in patients with gastric cancer and controls *n* (%)

iNOS C150T	Gastric cancer *n* = 159	Controls *n* = 170	Odds ratio	95%CI	*P*-value
CC	89 (56)	92 (54.1)	1.00[1]		
CT	59 (37.1)	72 (42.4)	0.86	0.54-1.35	0.5
TT	11 (6.9)	6 (3.5)	1.917	0.68-5.41	0.21
C allele	237 (74.5)	256 (75.3)	1.00[1]		
T	81 (25.5)	84 (24.7)	1.05	0.74-1.49	0.78
TT + CT	70 (44)	78 (45.9)	0.94	0.61-1.45	0.77
HWE	0.796	0.07			

[1]The first allele or genotype is the reference for statistical analysis. iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; HWE: Hardy Weinberg Equilibrium; CI: Confidence interval.

possible C150T *iNOS* genotypes among the two study groups (Table 2). Patients were genotyped essentially as described[37]; as reported in Japanese populations[36], the distribution of these specific *iNOS* polymorphisms in the control and cancer patient groups fell within the Hardy-Weinberg Equilibrium ($P = 0.07$ and $P = 0.796$, respectively). Among the 159 members of the GC group, 89 were homozygous CC, 59 were heterozygous CT and 11 were homozygous TT. The distribution of allele types within this group was not significantly different when compared to the study control group, which contained 92 homozygous CC, 72 heterozygous CT, and 6 homozygous TT individuals. There was also no difference in the frequency of each individual allele (C or T) found within the two study populations or the frequency of mutant (either CT or TT) genotypes between the GC and control groups.

CT and TT inducible nitric oxide synthetase polymorphisms contribute to GC risk in H. pylori-infected individuals

Since there was no significant difference in the frequency or distribution of *iNOS* C150T alleles in our two patient populations, we next examined any potential epidemiological interaction between *H. pylori* infection and the particular *iNOS* C150T genotype within the 159 GC patients. As shown in Table 3, the number of GC patients who were infected with *H. pylori* ($n = 94$) was slightly higher than the number of patients who were *H. pylori*-negative ($n = 65$). Among the 94 individuals who were infected with *H. pylori*, 46.8% were homozygous CC, 42.6% were heterozygous CT, and the remaining 10.6% were homozygous TT. In contrast, the *H. pylori*-negative group consisted mostly of the homozygous CC genotype (67.7%), followed by the heterozygous CT genotype (29.2%) and the TT genotype (3.1%). When compared to the non-*H. pylori* infected group, GC patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC (OR = 2.1, $P = 0.03$, χ^2 test). Similarly, having a homozygous TT genotype and being infected with *H. pylori* also led to an increased risk of developing GC; the risk associated with having the homozygous TT genotype was more than twice the risk associated with having only a single T allele (OR = 5.0). This association was also significant ($P = 0.029$, χ^2 test). Similarly, the presence of either mutant allele (CT or TT) increased the risk of GC nearly 2.5-fold (OR = 2.4, $P = 0.01$, χ^2 test). Taken together, these results indicate that for individuals who are infected with *H. pylori*, carrying either a CT or TT *iNOS* polymorphism at position 150 significantly increases the risk of developing GC. Importantly, unlike those patients within the GC group, there was no significant association between *iNOS* genotype, *H. pylori* infection and risk of developing GC in the control group (Table 3).

Table 3 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in *Helicobacter pylori*[3] *n* (%)

	H. pylori+	*H. pylori*-	OR[2]	95%CI	*P*-value
iNOS C150T	*n* = 94	*n* = 65			
GC patients					
CC	44 (46.8)	44 (67.7)	1.00[1]		
CT	40 (42.6)	19 (29.2)	2.1	1.06-4.19	0.03
TT	10 (10.6)	2 (3.1)	5	1.03-24.14	0.029
C	128 (68.1)	107 (82.3)	1.00[1]		
T	60 (31.9)	23 (17.7)	2.2	1.26-3.76	0.004
TT + CT	50 (53.2)	21 (32.3)	2.4	1.23-4.6	0.01
Controls	*n* = 77	*n* = 93			
CC	40 (51.9)	52 (55.9)	1.00[1]		
CT	36 (46.8)	36 (38.7)	1.3	0.7-2.41	0.41
TT	1 (1.3)	5 (5.4)	0.26	0.03-2.31	0.197
C	116 (75.3)	140 (75.3)	1.00[1]		
T	38 (24.7)	46 (24.7)	0.99	0.61-1.64	0.99
TT + CT	37 (48.05)	41 (44.1)	1.2	0.64-2.15	0.6

[1]The first allele or genotype is the reference for statistical analysis; [2]Adjusted for age and gender; [3]Infected or non-infected patients with and without GC. GC: Gastric cancer; iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; C: Number of individual C alleles in the group; T: Number of individual T alleles in the group; HWE: Hardy Weinberg Equilibrium; OR: Odds ratio; CI: Confidence interval; *H. pylori*: *Helicobacter pylori*.

DISCUSSION

The study of *H. pylori*-induced disease is complicated by the fact that the etiology of disease appears to result from the intricate interplay between bacterial virulence factors, host genetics and environmental components. Moreover, an increasing body of literature indicates that colonization by *H. pylori* may even be beneficial to some hosts[10-12]. Because of these facts, there is a pressing need to develop new diagnostics that will clearly identify those individuals most at risk for development of the most severe form of *H. pylori*-induced disease-GC. To this end, numerous studies have looked at the role of virulence factors, environmental risk factors and host genetic polymorphisms in disease development. While these studies have shed an enormous amount of light on development of GC, it is becoming increasingly more evident that vari-

ous factors play different roles based on the population being studied[13-21,23-25,27-30]. Thus, there is a need for larger population-based studies to define the role of particular factors in the disease process. Despite the high prevalence of GC and *H. pylori* infection in Northern Iran, there have been relatively few studies that have examined specific risk factors associated with disease development in this population. Of those that have been conducted, one of the studies that investigated the high rate of cancer development in this region concluded that the classic *H. pylori* virulence factors were not a good predictor of disease outcome[45]. Furthermore, the authors went on to suggest that "...the association between strain virulence markers and disease in Iraq but not Iran suggests that other host and environmental factors may be more important in the disease-prone Iranian population"[45]. Given this suggestion and the deficit in our knowledge, we set out to examine the contribution of particular behavioral factors and genetic polymorphism in the *iNOS* gene to GC development in the Iranian population. Using a case-controlled study design we identified a significant association of several factors with an increased risk for GC. Among the behavioral factors that were important, smoking and consumption of hot beverages were both associated with development of GC. Both of these factors have been previously identified as risk factors for cancer in other populations[36,37]. However, our study is the first to link consumption of hot beverages with GC development in this particular region.

In addition to behavioral factors and similar to other previously reported risk factors[46], we found that a familial history of GC was significantly associated with increased risk of GC development in our study population (Table 1). Also similar to at least one other study in this region[2], we found that infection with the gastric pathogen *H. pylori* significantly increased the risk of developing GC. Finally, we found that carrying either a heterozygous (CT) allele or a homozygous (TT) allele at position 150 of exon 16 of the *iNOS* gene combined with *H. pylori* infection resulted in a significant increase in GC development.

In humans, NO is used as an important signaling molecule. However, in the presence of increased and/or sustained levels of this molecule, the beneficial effects of NO can be overcome by deleterious processes such as DNA damage and chronic inflammation. These pathologic events may be catalyzed by increased iNOS activity, which leads to increased NO production and concomitant increases in the amount of downstream free radicals. As the major source of NO in humans, perhaps it is not surprising that even minor amino acid changes in the iNOS enzyme can affect the levels of NO. The (C150T) polymorphism found in exon 16 of the *iNOS* gene was previously suggested to affect activity of this enzyme, leading to increased production of NO[36]. Interestingly, in our or other populations[36,37], the *iNOS* polymorphism alone was not a risk factor for cancer development. This fact suggests that while the C150T polymorphism may affect NO production, it does not do so to such an extent as to induce cancer development in isolation. Instead, a co-carcinogenic factor is required to synergistically interact in such a way as to promote disease progression. Within the population of Northern Iran, this co-carcinogenic factor appears to be *H. pylori* infection (Table 3). A similar result was also found within the Japanese population[36]. Alternatively, the *iNOS* polymorphism was suggested to act as a co-carcinogenic factor with past cigarette smoking and alcohol consumption within the Chinese population[37]. Thus, in multiple populations, iNOS polymorphism synergistically interacts with various factors to affect GC development.

While the molecular mechanism of synergistic interaction between the *iNOS* polymorphism and *H. pylori* infection is not clear, one plausible hypothesis is that since *H. pylori* infection alone is sufficient to increase NO levels in gastric tissue[47,48], the addition of a polymorphism that also increases iNOS enzyme activity could increase the amount of NO in the local tissue to a further degree. In turn, this combined increase in NO could skew the gastric mucosa toward a pro-inflammatory response and increase the likelihood of carcinogenic cellular damage. It is interesting to note that *H. pylori* infection alone creates a chronic pro-inflammatory environment within the gastric mucosa. Furthermore, it has been well documented that the ability of *H. pylori* to establish a persistent infection depends on maintenance of the proper balance between the host immune system and the bacterium (reviewed in[49]). In patients with either the CT or TT *iNOS* polymorphisms, the increased levels of NO may upset this delicate balance, thus perpetuating pathological and carcinogenic events.

H. pylori-mediated cancer development is a complex and multi-faceted process that involves a combination of bacterial, host, and environmental factors. Moreover, infection with *H. pylori* is inversely correlated with development of esophageal cancer[12], and is thought to provide protection against development of autoimmune disorders such as asthma[11]. Thus, the apparent benefit(s) of asymptomatically harboring this organism brings to bear the question of whether it is best to treat all patients for *H. pylori* infection; if not, the question becomes-how do we identify individuals most at risk for developing serious disease? One possible way to differentiate patients who have a high risk for disease development from those who are low-risk is to specifically examine bacterial, host-genetic and environmental factors that have been shown to be associated with cancer development in *H. pylori*-infected individuals. By screening individual patients for these three types of markers, it may be possible to specifically identify and treat those individuals who are at most risk for developing severe disease while reducing the risk of losing the apparent protective effects of *H. pylori* infection in low-risk individuals.

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COMMENTS

Background

As gastric cancer (GC) remains a serious source of morbidity and mortality worldwide, the identification of individuals most at risk for disease development is a major concern.

Research frontiers

Recent studies have highlighted the importance of polymorphisms in host genes as a risk factor for GC development. However, as many of these studies have been performed on limited patient populations, it remains unclear whether these polymorphisms are important across diverse ethnic groups and geographic areas. In this study, the authors identified an increase in GC risk in *Helicobacter pylori* (*H. pylori*)-infected Iranian patients who harbored a CT or TT polymorphism at position 150 in the inducible nitric oxide synthetase (*iNOS*) gene.

Innovations and breakthroughs

To the best of our knowledge, this study describes the first known link between a host-genetic factor and GC risk in an Iranian population. In addition, this study is the first to define the importance of the *iNOS* C150T polymorphism in a non-Asian population.

Applications

The results of this study suggest that the C150T *iNOS* polymorphism is a potential marker for susceptibility to *H. pylori*-mediated gastric disease.

Peer review

This is a study aimed at investigating the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among patients from a region of Iran. The authors present an interesting study from a region that has not been addressed very often in regard to *H. pylori* and its related pathologies.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108
2 **Pourfarzi F**, Whelan A, Kaldor J, Malekzadeh R. The role of diet and other environmental factors in the causation of gastric cancer in Iran--a population based study. *Int J Cancer* 2009; **125**: 1953-1960
3 **Neugut AI**, Hayek M, Howe G. Epidemiology of gastric cancer. *Semin Oncol* 1996; **23**: 281-291
4 **Blaser MJ**. Helicobacter pylori and gastric diseases. *BMJ* 1998; **316**: 1507-1510
5 **Ernst PB**, Gold BD. The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol* 2000; **54**: 615-640
6 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131
7 **Parsonnet J**, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelman JH, Friedman GD. Helicobacter pylori infection and gastric lymphoma. *N Engl J Med* 1994; **330**: 1267-1271
8 **Talley NJ**, Zinsmeister AR, Weaver A, DiMagno EP, Carpenter HA, Perez-Perez GI, Blaser MJ. Gastric adenocarcinoma and Helicobacter pylori infection. *J Natl Cancer Inst* 1991; **83**: 1734-1739
9 **International Agency for Research on Cancer**. Infection with Helicobacter pylori In Monographs on the evaluation of carcinogenic risks to humans, vol 61. Lyon: International Agency for Research on Cancer, 1994: 177-240
10 **Blaser MJ**, Chen Y, Reibman J. Does Helicobacter pylori protect against asthma and allergy? *Gut* 2008; **57**: 561-567
11 **Chen Y**, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med* 2007; **167**: 821-827
12 **Chow WH**, Blaser MJ, Blot WJ, Gammon MD, Vaughan TL, Risch HA, Perez-Perez GI, Schoenberg JB, Stanford JL, Rotterdam H, West AB, Fraumeni JF. An inverse relation between cagA+ strains of Helicobacter pylori infection and risk of esophageal and gastric cardia adenocarcinoma. *Cancer Res* 1998; **58**: 588-590
13 **Covacci A**, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, Figura N. Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci USA* 1993; **90**: 5791-5795
14 **Higashi H**, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, Hatakeyama M. Biological activity of the Helicobacter pylori virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci USA* 2002; **99**: 14428-14433
15 **Atherton JC**, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* 1995; **270**: 17771-17777
16 **Rhead JL**, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* 2007; **133**: 926-936
17 **Aspholm-Hurtig M**, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, Vikström S, Sjöström R, Lindén S, Bäckström A, Lundberg C, Arnqvist A, Mahdavi J, Nilsson UJ, Velapatiño B, Gilman RH, Gerhard M, Alarcon T, López-Brea M, Nakazawa T, Fox JG, Correa P, Dominguez-Bello MG, Perez-Perez GI, Blaser MJ, Normark S, Carlstedt I, Oscarson S, Teneberg S, Berg DE, Borén T. Functional adaptation of BabA, the H. pylori ABO blood group antigen binding adhesin. *Science* 2004; **305**: 519-522
18 **Mahdavi J**, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science* 2002; **297**: 573-578
19 **Oleastro M**, Cordeiro R, Ménard A, Yamaoka Y, Queiroz D, Mégraud F, Monteiro L. Allelic diversity and phylogeny of homB, a novel co-virulence marker of Helicobacter pylori. *BMC Microbiol* 2009; **9**: 248
20 **Oleastro M**, Cordeiro R, Yamaoka Y, Queiroz D, Mégraud F, Monteiro L, Ménard A. Disease association with two Helicobacter pylori duplicate outer membrane protein genes, homB and homA. *Gut Pathog* 2009; **1**: 12
21 **Yamaoka Y**, Kwon DH, Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proc Natl Acad Sci USA* 2000; **97**: 7533-7538
22 **Sjödahl K**, Lu Y, Nilsen TI, Ye W, Hveem K, Vatten L, Lagergren J. Smoking and alcohol drinking in relation to risk of gastric cancer: a population-based, prospective cohort study. *Int J Cancer* 2007; **120**: 128-132
23 **Joossens JV**, Hill MJ, Elliott P, Stamler R, Lesaffre E, Dyer A, Nichols R, Kesteloot H. Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group. *Int J Epidemiol* 1996; **25**: 494-504
24 **Tsugane S**. Salt, salted food intake, and risk of gastric cancer: epidemiologic evidence. *Cancer Sci* 2005; **96**: 1-6
25 **Tsugane S**, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. *Gastric Cancer* 2007; **10**: 75-83
26 **Noach LA**, Bosma NB, Jansen J, Hoek FJ, van Deventer SJ,

Tytgat GN. Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with Helicobacter pylori infection. *Scand J Gastroenterol* 1994; **29**: 425-429
27 **Peek RM**, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* 2002; **2**: 28-37
28 **Polk DB**, Peek RM. Helicobacter pylori: gastric cancer and beyond. *Nat Rev Cancer* 2010; **10**: 403-414
29 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402
30 **Lu W**, Pan K, Zhang L, Lin D, Miao X, You W. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor {alpha} and risk of gastric cancer in a Chinese population. *Carcinogenesis* 2005; **26**: 631-636
31 **Qidwai T**, Jamal F. Inducible nitric oxide synthase (iNOS) gene polymorphism and disease prevalence. *Scand J Immunol* 2010; **72**: 375-387
32 **Ohshima H**, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 1994; **305**: 253-264
33 **Goto T**, Haruma K, Kitadai Y, Ito M, Yoshihara M, Sumii K, Hayakawa N, Kajiyama G. Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Clin Cancer Res* 1999; **5**: 1411-1415
34 **Koh E**, Noh SH, Lee YD, Lee HY, Han JW, Lee HW, Hong S. Differential expression of nitric oxide synthase in human stomach cancer. *Cancer Lett* 1999; **146**: 173-180
35 **Rajnakova A**, Moochhala S, Goh PM, Ngoi S. Expression of nitric oxide synthase, cyclooxygenase, and p53 in different stages of human gastric cancer. *Cancer Lett* 2001; **172**: 177-185
36 **Goto Y**, Ando T, Naito M, Goto H, Hamajima N. Inducible nitric oxide synthase polymorphism is associated with the increased risk of differentiated gastric cancer in a Japanese population. *World J Gastroenterol* 2006; **12**: 6361-6365
37 **Shen J**, Wang RT, Wang LW, Xu YC, Wang XR. A novel genetic polymorphism of inducible nitric oxide synthase is associated with an increased risk of gastric cancer. *World J Gastroenterol* 2004; **10**: 3278-3283
38 **Malekzadeh R**, Derakhshan MH, Malekzadeh Z. Gastric cancer in Iran: epidemiology and risk factors. *Arch Iran Med* 2009; **12**: 576-583
39 **Malekzadeh R**, Sotoudeh M, Derakhshan MH, Mikaeli J, Yazdanbod A, Merat S, Yoonessi A, Tavangar M, Abedi BA, Sotoudehmanesh R, Pourshams A, Asgari AA, Doulatshahi S, Alizadeh BZ, Arshi S, Madjidpoor A, Mir Moomen S, Fleischer DE. Prevalence of gastric precancerous lesions in Ardabil, a high incidence province for gastric adenocarcinoma in the northwest of Iran. *J Clin Pathol* 2004; **57**: 37-42
40 **Sotoudeh M**, Derakhshan MH, Abedi-Ardakani B, Nouraie M, Yazdanbod A, Tavangar SM, Mikaeli J, Merat S, Malekzadeh R. Critical role of Helicobacter pylori in the pattern of gastritis and carditis in residents of an area with high prevalence of gastric cardia cancer. *Dig Dis Sci* 2008; **53**: 27-33
41 **Ghadimi R**, Taheri H, Suzuki S, Kashifard M, Hosono A, Esfandiary I, Moghadamnia AA, Ghadimi R, Tokudome S. Host and environmental factors for gastric cancer in Babol, the Caspian Sea Coast, Iran. *Eur J Cancer Prev* 2007; **16**: 192-195
42 **Lauren P**. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49
43 **Islami F**, Pourshams A, Nasrollahzadeh D, Kamangar F, Fahimi S, Shakeri R, Abedi-Ardekani B, Merat S, Vahedi H, Semnani S, Abnet CC, Brennan P, Møller H, Saidi F, Dawsey SM, Malekzadeh R, Boffetta P. Tea drinking habits and oesophageal cancer in a high risk area in northern Iran: population based case-control study. *BMJ* 2009; **338**: b929
44 **Miller SA**, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215
45 **Hussein NR**, Mohammadi M, Talebkhan Y, Doraghi M, Letley DP, Muhammad MK, Argent RH, Atherton JC. Differences in virulence markers between Helicobacter pylori strains from Iraq and those from Iran: potential importance of regional differences in H. pylori-associated disease. *J Clin Microbiol* 2008; **46**: 1774-1779
46 **Brenner H**, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. *Methods Mol Biol* 2009; **472**: 467-477
47 **Kaise M**, Miwa J, Iihara K, Suzuki N, Oda Y, Ohta Y. Helicobacter pylori stimulates inducible nitric oxide synthase in diverse topographical patterns in various gastroduodenal disorders. *Dig Dis Sci* 2003; **48**: 636-643
48 **Kim JM**, Kim JS, Jung HC, Song IS, Kim CY. Up-regulation of inducible nitric oxide synthase and nitric oxide in Helicobacter pylori-infected human gastric epithelial cells: possible role of interferon-gamma in polarized nitric oxide secretion. *Helicobacter* 2002; **7**: 116-128
49 **Kusters JG**, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* 2006; **19**: 449-490

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