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A novel genetic polymorphism of inducible nitric oxide synthase is associated with an increased risk of gastric cancer

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Abstract

AIM: Inducible nitric oxide synthase (iNOS) plays a central role in the pathway of reactive oxygen and nitrogen species metabolism when *Helicobacter pylori* (*H pylori*) infection occurs in humans. iNOS Ser^{608}Leu allele, a novel genetic polymorphism (C/T) occurring within exon 16 of the iNOS reductase domain, may have a dramatic effect on the enzymatic activity. The aim of this study was to determine whether iNOS C/T polymorphism was associated with increased susceptibility to gastric cancer.

METHODS: We conducted a population based case-control study in a high gastric cancer incidence area, Yangzhong, China. Questionnaires from 93 patients with intestinal type gastric cancer (IGC), 50 with gastric cardia cancer (GCC) and 246 healthy controls were obtained between 1997 and 1998, and iNOS genotyping was carried out. Odds ratios (ORs), interaction index (γ), and 95% confidence intervals for the combined effects of iNOS genotype and *H pylori* infection, cigarette smoking or alcohol drinking were estimated.

RESULTS: The frequency of (CT+TT) genotypes was higher in cases than in control group (24.48% *vs* 23.17%), but the difference was not statistically significant. After adjusting for age and gender, past cigarette smokers with (CT+TT) genotypes had a significantly increased risk of IGC (OR = 3.62, 95% CI: 1.23-10.64), while past alcohol drinkers with (CT+TT) genotypes had a significantly increased risk of GCC (OR = 3.33, 95% CI: 1.14-9.67). *H pylori* CagA negative subjects with (CT+TT) genotypes had a significantly increased risk of both IGC and GCC (OR = 2.19 and 3.52, respectively).

CONCLUSION: iNOS Ser^{608}Leu allele may be a potential determinant of susceptibility to cigarette -alcohol induced gastric cancer, but larger studies are needed to confirm the observations.

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INTRODUCTION

On a global scale, gastric cancer remains the world's second most common malignancy. There is a substantial international variation in gastric cancer incidence with the highest rates reported from China, Japan and other Eastern Asian countries[1]. The discovery of *Helicobacter pylori* (*H pylori*) in the early 1980 s has been proven to be a turning point in understanding the pathogenesis of this malignancy. A major advance in this field came with the recognition that chronic *H pylori* infection could induce physiologic and morphologic changes within the gastric milieu, which increase the risk of neoplastic transformation[2]. It has been widely accepted that chronic *H pylori* infection induces hypochlorhydria and gastric atrophy, both of which are precursors of gastric cancer[2]. Epidemiological studies have also indicated that infection with *H pylori* is considered as a risk factor for gastric cancer[3,4] and the WHO IARC has classified this bacterium as a definite biological carcinogen[5]. However, while the majority of infected individuals develop no significant clinical disease, others develop two kinds of divergent clinical outcomes-peptic ulcer disease and gastric cancer[2]. The reasons for developing these two extreme phenotypes, especially important in gastric cancer, have remained poorly understood, and are not explained by bacterial virulence factors alone[2]. This highlights the need to explore potential candidate genes of the host in the pathways involved in the natural history of *H pylori* infection and its interactions with other risk factors in the development of gastric cancer in a high-risk population.

The inducible form of nitric oxide (NO) synthase (iNOS) is one of the most important enzymes involved in the pathway of reactive oxygen and nitrogen species metabolism in the presence of *H pylori* infection in humans. iNOS is a major source of NO production that is produced during inflammation by macrophages[6,7]. Expression of iNOS in response to cytokines is part of the inflammatory response and contributes to tissue damage, suggesting its possible role in the processing of carcinogens[7]. iNOS contains many sites for prosthetic groups and substrate binding[8], which are all potentially important for the function of the enzyme. Furthermore, studies have indicated that even single amino acid changes may have dramatic effects on enzymatic activity[8,9]. The human iNOS gene comprises 27 exons with the transcription start site in exon 2 (E2) and the stop codon in E27[10]. E1-13 code for the oxygenase domain, and E14–27 encode for the reductase domain of the protein. Both of the domains represent different functional parts of the enzyme[9]. Increased iNOS activities have been observed in patients with chronic gastritis caused by *H pylori* infection, and gastric cancer[11,12]. A 13-21 years follow-up study showed that among the *H pylori* positive group, the expression of iNOS and nitro-tyrosine was significantly higher in the group that developed gastric cancer than the one that showed no evidence of gastric cancer, suggesting that *H pylori* positive subjects with high levels of reactive nitrogen species in gastric mucosa may be a high-risk group for gastric cancer[13]. Furthermore, recent studies have revealed that *H pylori* infection may lead to a sustained production of reactive nitrogen species and the formation of nitro-tyrosine contributes to DNA damage and apoptosis in gastric mucosa[12]. Infection with *H pylori* strains

possessing cytotoxin-associated gene (Cag) A, a molecular marker of *H pylori* virulence[14,15], is particularly associated with an increased risk of developing adenocarcinoma of the stomach. It is suggested that iNOS may be a susceptible gene involved in the metabolic pathway of nitrogen and oxygen species of free radicals, and thus may be associated with both gastric cancer risk and *H pylori* infection.

Yangzhong city is one of the areas in China with the highest gastric cancer mortality and incidence rate. The crude mortality rate of gastric cancer was from 96.9 to 110.9/100 000 during 1991 and 1997, and the average adjusted incidence rate in the same period was over 115/100 000 (unadjusted rate was 155.46/100 000), which is over ten times higher than that in the United States[16]. Based on the understanding of the physiology and pathogenesis of gastric cancer, and the genetic pathway related to *H pylori* infection, we hypothesized that higher frequency of iNOS $Ser^{608}Leu$ allele (i.e. C/T polymorphism)[9,17] was responsible for the higher gastric cancer incidence in this area. We were especially interested in knowing whether the association between the polymorphism and gastric cancer was modified by infection with *H pylori* CagA strains and cigarette smoking reflecting high exposure to nitrogen and oxygen species of free radicals. Therefore, the aim of this study was to determine whether iNOS C/T polymorphism was associated with increased susceptibility to gastric cancer, and the effects of *H pylori* infection.

MATERIALS AND METHODS

Study subjects

All gastric cancer patients and "healthy" controls in this study were Han ethnic Chinese living in Yangzhong city for at least 25 years. Gastric cancer was diagnosed according to the International Classification of Diseases for Oncology IX, code = 151, and the criteria of Laurén[18]. Because most diagnosed gastric cancer cases in Yangzhong were intestinal type gastric cancer (IGC) and gastric cardia cancer (GCC), we focused on these two kinds of cancers in the present study. A population based case-control design was used, and 165 gastric cancer cases (108 IGC, 57 GCC) and 295 controls were enrolled. The finally analyzed cases and controls were 143 (93 IGC, 50 GCC) and 246 cases, respectively, because of missing genotype data for some subjects. There were no significant differences comparing the finally analyzed subjects and those with missing data by age and sex. All cases were identified by endoscopic and pathological diagnosis in Yangzhong City Municipal Hospital from January 1997 to December 1998. To reduce misclassification of the histological types, two pathologists reviewed and confirmed all diagnosed cases. Controls were selected from cancer-free subjects living in the same community, who were either cases' siblings or their non-blood relatives (spouses and spouses' siblings with the same gender as cases). Both types of controls differed slightly in demographic features[19]. Their results were combined to increase the sample size and to decrease type I error. This study was approved by the regional ethics committee, and all participants were given an explanation of the nature of the study, and informed consents both written and oral, were obtained. Study subjects completed a questionnaire administered by trained interviewers.

The questionnaire was designed to obtain detailed information on cigarette smoking, alcohol drinking, family history of cancers, and occupational and hazard exposures. Cigarette smokers were defined as subjects who reported ever smoking at least one cigarette per day for 12 mo or more, or whose accumulated cigarette consumption was over 18 packs per year. Past smokers were those who had stopped smoking 1 or more years before the interview. Alcohol drinkers were defined as subjects who reported to have an average of one drink or more per week for one or more yeaes. Past alcohol drinkers were also defined as those who had stopped drinking for 1 or more years before the interview.

Laboratory analysis

Blood was drawn from each participant by the designated coordinator according to the Guidelines of the National Heart, Lung, and Blood Institute Working Group on Blood Drawing, Processing, and Storage for Genetic Studies. Twenty milliliters of forearm venous blood was collected from each subject via venipuncture into two 10-mL vacutainer tubes containing EDTA. Puragene DNA isolation kits (Gentra Systems, Minneapolis MN) were used to isolate genomic DNA for genotyping. All blood samples were separated, and plasma was collected as soon as possible. The plasma was then stored at -20 ℃ in six 1.5-mL tubes for the detection of IgG antibody to *H pylori* CagA.

Denaturing high performance liquid chromatography (DHPLC) was used to scan the potential single nucleotide polymorphisms (SNPs) in all exons of iNOS, and then sequencing was performed to confirm the possible mutations. Finally, a new C/T polymorphism, which changes the coding amino acid from serine (TCG) to leucine (TTG), was identified[17]. PCR-RFLP was carried out to identify the genotype of iNOS according to the features of SNP, which created a restriction enzyme recognition site of *Tsp 509 I*. Genomic DNA was amplified with primers F: 5'-TGTAAACCAACTTCCGTGGTG-3' (Tm = 60.82 ℃) and R: 5'-GTCTCTGCGGGTCTGAGAAG-3' (Tm = 60.14 ℃). PCR was performed in a MJRESEARCH PCR system (PTC-225, USA), and in a 10 μL reaction volume containing 1μL 10×PCR buffer, 1.6 μL dNTPs (1.25 μmol/L), 0.2 μL $MgCl_2$ (25 mmol/L), primers (20 μmol/L, Resgen Corp.) at 0.15 μL each, DMSO 0.5 μL, 6.34 μL dH2O, 50 ng genomic DNA dried on the plate, and Hot Start Taq DNA polymerase 0.06 μL (5 U/μL, Promega Corp.). Touch down PCR procedure was used to amplify the target fragment. After an initial denaturation at 94 ℃ for 15 min, amplification was carried out for 10 cycles at 94 ℃ for 30 s, at 61 ℃ for 45 s, at 72 ℃ for 45 s and decreasing 0.5 ℃ per cycle. Then amplification was again carried out for 35 cycles at 94 ℃ for 30 s, at 56 ℃ for 45 s, and at 72 ℃ for 45 s, followed by a final elongation at 72 ℃ for 7 min. Then, 10 μL PCR products was digested with 0.2 μL *Tsp* 509 I (10 U/μL, NEB Corp.) in a 15 μL volume including 2 μL 10×buffer 1 (NEB Corp.), 0.15 μL BSA (100×) and 2.65 μL dH2O. Digestion was performed for 15 h at 65 ℃. The products were then electrophoresed on a 30 g/L agarose gel to allow unambiguous detection with ethidium bromide staining. Homozygous wide-type individuals (CC) showed 113 bp and 175 bp fragments, heterozygous individuals (CT) showed three bands: 113 bp, 142 bp and 175 bp, and homozygous rare allele individuals (TT) showed two bands: 113 bp and 142 bp[17].

H pylori CagA IgG antibody in plasma was measured by an enzyme-linked immunosorbent assay (ELISA) kit offered by Jingying Biotech Limited Company, Shanghai, China (batch number 0052). The Absorbency at 450 nm was determined after terminating the enzyme reaction. The cutoff value equaled to the average *A* of the negative controls provided by the manufacturer plus 0.3 *A* units. *A* values of samples equaled to or higher than the cutoff point were considered positive.

Statistical analysis

All data were input double blinded into EPI-6 program by two persons separately. After modifying all errors and non-logical data, the differences in the relative associations between cases and controls were assessed by calculating crude odds ratios (OR) from contingency tables. The corresponding chi-square test on the cancer patients and controls was carried out, and 95% confidence intervals (95% CI) were determined using the Fisher exact test. A P-value <0.05 was considered statistically significant. Unconditional logistic regression analysis was performed in both univariate and multivariate models to assess the association between iNOS functional polymorphism and

gastric cancer susceptibility after adjusting for important confounding factors such as age and sex. Test of trend and interaction index (γ) that was determined by coefficient (β) in a multiple logistic regression model were calculated through logistic models based on dummy variables to examine the potential gene-environment interaction[20]. All analyses were performed with the SAS package Genmod (SAS Institute, Cary, NC).

RESULTS

Table 1 compares the characteristics of study subjects. The mean age of cases was significantly greater than the controls (59.36 *vs* 51.89, $P<0.01$). There was no significant difference in the male/female ratio between cases and controls. The proportions of past smokers and alcohol drinkers was significantly greater in the cancer group (36.97% and 30.30%) than in the control group (12.54% and 14.92%). However, there were more current smokers and drinkers in the control (47.12% and 30.85%) than in the case group (26.67% and 10.91%). Compared with controls, cases were significantly less likely to be positive for *H pylori* CagA antibody.

The frequency of iNOS genotypes in gastric cancer and control subjects showed no significant difference, although the frequency of (CT+TT) genotypes was slightly higher in cases than in controls (24.48% versus 23.17%). A gene dose-response effect was not observed, i.e. the effect of heterozygote (CT) genotypes did not lie at or between the homozygotes (CC and TT).

For (CT+TT) the genotype frequency of iNOS in the past smoking subgroup, there were significant differences between the total cases and the controls and between GCC group and controls with an OR of 3.62 (95% CI: 1.23-10.64) and 4.63 (95% CI: 1.15-18.58), respectively. No significant difference was found between IGC cases and controls (Table 2). Although no gene dose-response effect was observed in heterozygote (CT) and homozygote (TT) individuals because of the small number in each cell, there was still a possible interaction between C/T polymorphism and past cigarette smoking in increasing the risk of GCC. In the past alcohol drinkers, there were significant differences in the C/T polymorphism between total cases and controls and between IGC group and controls with an OR of 3.33 (95% CI: 1.14-9.67) and 3.42 (95% CI: 1.03-11.35), respectively. No significant difference between GCC group and control group was found (Table 3). In *H pylori* CagA negative group, subjects with (CT+TT) genotypes had significantly increased risk of both IGC and GCC, with an OR of 2.19 (95% CI: 1.01-4.76) and 3.52 (95% CI: 1.44-8.61), respectively. *H pylori*

Table 1 Covariate distribution among study subjects and ORs for gastric cancer

Characteristics		No Cases (%) (n = 165)	No. Controls (%) (n = 295)	OR (95% CI)
Age (yr)				
	Mean (yr±SD)	59.36±9.29[a]	51.89±10.24	
	Min. (yr)	34.72	30.77	
	Max. (yr)	81.95	78.21	
Gender				
	Male (%)	110 (66.67)	190 (64.41)	
	Female (%)	55 (33.33)	105 (35.59)	
	Ratio	2.00:1	1.81:1	
Smoking habit				
	Never	60 (36.36)	119 (40.34)	1.00
	Current	44 (26.67)	139 (47.12)	0.26 (0.15–0.46)[b]
	Past	61 (36.97)	37 (12.54)	3.15 (1.77–5.61)[b]
Alcohol habits				
	Never	97 (58.79)	160 (54.24)	1.00
	Current	18 (10.91)	91 (30.85)	0.18 (0.10–0.35)[b]
	Past	50 (30.30)	44 (14.92)	1.80 (1.06–3.08)
Plasma *H Pylori* CagA antibody				
	Negative	136 (82.42)	107 (49.31)	1.00
	Positive	29 (17.58)	110 (50.69)	0.18 (0.11–0.31)[b]
iNOS genotyping				
	CC	108 (75.52)	189 (76.83)	1.00
	CT	33 (23.08)	49 (19.92)	1.15 (0.68–1.96)
	TT	2 (1.40)	8 (3.25)	0.42 (0.08–2.16)
	CT+TT	35 (24.48)	57 (23.17)	1.03 (0.59–1.79)

[a]$P<0.05$ *vs* control group after adjusted for age and gender, [b]$P<0.01$ *vs* control.

Table 2 Interaction between C/T polymorphism and past cigarette smoking for the risk of gastric cancer

C/T polymorphism	Past smoking	Total cases (n = 143)	Controls (n = 246)	OR[1]	95%CI	IGC (n = 93)	OR[2]	95%CI	GCC (n = 50)	OR[3]	95%CI
CC	No	67	162	1.00		46	1.00		21	1.00	
CT+TT	No	22	51	0.98	0.55-1.78	12	0.75	0.36-1.54	10	1.44	0.63-3.27
CC	Yes	41	27	2.92	1.53-5.57	27	2.47	1.19-5.09	14	3.65	1.42-9.38
CT+TT	Yes	13	6	3.62	1.23-10.64	8	3.06	0.91-10.35	5	4.63	1.15-18.58

[1]Adjusted for age and gender, $\chi^2_{trend} = 26.26$, df = 1, $P = 0.00$, $\gamma = 1.29/1.07 = 1.21$ [2]Adjusted for age and gender, $\chi^2_{trend} = 18.40$, df = 1, $P = 0.00$, $\gamma = 1.12/0.90 = 1.24$ [3]Adjusted for age and gender, $\chi^2_{trend} = 17.53$, df = 1, $P = 0.00$, $\gamma = 1.53/1.29 = 1.19$.

Table 3 Interaction between C/T polymorphism and past alcohol drinking for the risk of gastric cancer

C/T polymorphism	Past alcohol drinking	Total cases (n = 143)	Controls (n = 246)	OR[1]	95%CI	IGC (n = 93)	OR[2]	95%CI	GCC (n = 50)	OR[3]	95%CI
CC	No	76	157	1.00		53	1.00		23	1.00	
CT+TT	No	24	51	0.83	0.46-1.50	13	0.62	0.30-1.28	11	1.22	0.55-2.70
CC	Yes	32	32	1.34	0.72-2.52	20	1.36	0.67-2.75	12	1.27	0.50-3.19
CT+TT	Yes	11	6	3.33	1.14-9.67	7	3.42	1.03-11.35	4	3.25	0.80-13.13

[1]Adjusted for age and gender, χ^2_{trend} = 10.29, df = 1, P = 0.001, γ = 1.20/0.30 = 4.00 [2]Adjusted for age and gender, χ^2_{trend} = 5.65, df = 1, P = 0.017, γ = 1.23/0.31 = 3.97 [3]Adjusted for age and gender, χ^2_{trend} = 8.95, df = 1, P = 0.003, γ = 1.18/0.24 = 4.9.

Table 4 Interaction between C/T polymorphism and *H pylori* CagA status for the risk of gastric cancer

C/T polymorphism	CagA antibody	Total cases (n = 143)	Controls (n = 178)	OR[1]	95%CI	IGC (n = 93)	OR[2]	95%CI	GCC (n = 50)	OR[3]	95%CI
CC	No	87	64	1.00		61	1.00		26	1.00	
CT+TT	No	28	19	2.53	1.29-4.98	17	2.19	1.01-4.76	11	3.52	1.44–8.61
CC	Yes	21	73	0.45	0.25-0.81	12	0.34	0.17-0.70	9	0.76	0.33-1.73
CT+TT	Yes	7	22	0.43	0.17-1.10	3	0.24	0.07-0.89	4	0.86	0.27-2.79

[1]Adjusted for age and gender, χ^2_{trend} = 33.40, df = 1, P = 0.00, γ = -0.84/-0.79 = 1.06 [2]Adjusted for age and gender, χ^2_{trend} = 28.53, df = 1, P = 0.00, γ = -1.41/-1.08 = 1.31 [3]Adjusted for age and gender, χ^2_{trend} = 12.42, df = 1, P = 0.0004, γ = -0.15/-0.27 = 0.56.

CagA positivity showed significant protective effects in IGC group in both on CC and CT+TT iNOS genotypes, with an OR of 0.24 (95% CI: 0.07-0.89) and 0.34 (95% CI: 0.17-0.70), respectively. However, no significant association was observed between iNOS genotypes and GCC (Table 4).

DISCUSSION

H pylori infection could produce a state of chronic immuno-stimulation in gastric epithelium[21]. It could lead to changes in many factors that are important in the pathogenesis of gastric cancer, including reactive oxygen and nitrogen oxide species [22]. NO, a potentially toxic gas with free radical properties is one of the most important bio-regulatory and signaling molecules produced in the process. It has been recently reported that NO, acting as a messenger molecule mediating various physiological functions[23,24], may also play a role in the process of carcinogenesis.

It has been found that NO is synthesized enzymatically from L-arginine by NO synthase[23,25]. Chronic infection and immuno-stimulation elevate endogenous synthesis of NO. High concentration of NO generated by macrophages after iNOS induction contributed to their cytotoxic and carcinogenic activity[26]. There is now increasing evidence that NO produced by activated phagocytes may play a role in multistage carcinogenesis by mediating DNA damage[27,28]. A to T substitution in the iNOS gene, leads to more activated iNOS expression in the target cells, and finally elevates NO to a high level. Hence, it is reasonable to assume that human iNOS gene may be another important candidate gene for the development of gastric cancer by elevating NO production in target cells when functional polymorphisms occur. Nevertheless its genomic localization at chromosome 17q11.2[29] was not the same as other gastric cancer susceptible genes related to the inflammatory response pathway, such as interleukin 1β and interleukin 1RN, located at 2q14[30]. The key question for gastric cancer agents is how *H pylori* infection could be associated with such totally divergent clinical outcomes as gastric cancer and peptic ulcer disease. A large number of previous studies have focused on the role of the bacterial virulence factors that contribute to the degree of tissue damage in the pathogenesis of these diseases. But these results still could not explain the different outcomes[2,22,31]. With the development of a key concept about the interaction between acid secretion and *H pylori*-induced gastritis during 1990 s, El-Omar proposed the idea for the first time that host genetic factors relevant to pro-inflammatory responses might be relevant to the development of gastric cancer. They explored a candidate IL-1β gene in the context of *H pylori* related disease[32,33]. Because IL-1β can also induce the expression of many other genes, including pro-inflammatory mediator iNOS, by either regulating at the transcriptional level or initiating their mRNA[8,9,34], it is easy to consider that functional polymorphisms occurring in the iNOS gene might also contribute to the increased risk of *H pylori* related gastric cancer.

We have previously reported a newly discovered C/T polymorphism in a Chinese population[17] that had a high mutated allele frequency (24.4%). A report by Johannesen also showed that C/T polymorphism was one of the most frequent SNPs among 10 polymorphisms of human iNOS gene identified in a Danish population. They suggested that the amino acid change in exon 16 might be of functional interest[9]. Our results showed no significant difference in the frequency of (CT+TT) genotypes between cases and controls, and no apparent gene dose-response effect was found. However, in past cigarette smokers and past alcohol drinkers, C/T polymorphism significantly increased the risk of gastric cancer despite the histological subtypes differed, i.e. past cigarette smokers with (CT+TT) genotypes had an increased risk of IGC, while past alcohol drinkers with (CT+TT) genotypes had increased risk of GCC. These findings suggest that C/T polymorphism in iNOS gene alone is not sufficient to show the increasing risk of gastric cancer. The importance of the interaction between C/T polymorphism and cigarette smoking or alcohol drinking varied depending on different histological subtypes of gastric cancer. Similar results were found by Machado for IL-1 genetic markers[35]. Although these findings were not the major hypothesis we proposed, it is biologically plausible that oxidative stress due to carcinogenesis in cigarette might attribute to the increase of gastric cancer risk through interactions with iNOS C/T polymorphism. Larger and independent studies are needed to confirm these findings.

In the *H pylori* CagA positive group, regardless of whether subjects had CC or (CT+TT) genotypes, we always observed a significant protective effect when comparing IGC cases with controls. This suggests that plasma positive for *H pylori* CagA antibody in a highly infected area plays a protective role. This

is in concordance with the finding that *H pylori* density became progressively lower with progression from mild gastritis to severe gastritis, atrophy, intestinal metaplasia and finally gastric cancer[29]. In *H pylori* CagA negative subjects with (CT+TT) genotypes, a high risk was found for gastric cancer group (OR = 2.53, 95% CI: 1.29-4.98) and both subgroups (IGC and GCC). No interaction was found between iNOS genotype and infection with *H pylori* CagA strains.

iNOS protein is a catalytic enzyme with two domains. In terms of functional importance, the deletion mutants retained maximal NO activity at lower concentrations of free Ca^{2+} compared with the wild-type[36]. Identified C/T polymorphism in E16 of iNOS was located at the N-terminal of six amino acids from the deletion reported by Daff *et al*., and the amino acid change in E16 might be of functional interest[9]. To our knowledge, this study was the first one to examine the significance of iNOS polymorphism in gastric cancer. A research on other type of disease might support our observation[9]. Gastric cancer patients having allele T polymorphism could have an increased expression of iNOS, resulting in higher levels of NO in gastric mucosa, mediating many pathological changes and finally leading to carcinogenesis in these patients. But specific functional tests of C/T shift need to be performed to substantiate the putative importance of the $Ser^{608}Leu$ locus in gastric cancer development.

Potential weaknesses in our study include possible recruitment bias in the selection of controls including cases' siblings. This kind of selection might create overmatching. Siblings were more likely to have the same genotypes as the cases than the non-blood related controls, thereby leading to some loss of statistical efficiency, i.e., larger sample sizes were required to attain the same statistical precision[37]. Thus, our data may be more likely to underestimate the true effect of iNOS T alleles on the risk of gastric cancer. But others considered that the use of sibling controls could generally improve efficiency for gene-environment interactions[38,39]. We could not rule out the potential influence of systematic differences between participants and non-participants.

In conclusion, the risk of gastric cancer is increast among past cigarette smoking or alcohol drinking individuals with a C/T polymorphism in E16 of iNOS gene in a Chinese population. But the findings need to be confirmed in other ethnic populations.

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