Inducible nitric oxide synthase polymorphism is associated with the increased risk of differentiated gastric cancer in a Japanese population

Yasuyuki Goto, Takafumi Ando, Mariko Naito, Hidemi Goto, Nobuyuki Hamajima

Abstract

AIM: To examine the association of inducible nitric oxide synthase (iNOS) C150T polymorphism with gastric cancer, as well as with gastric atrophy and H pylori seropositivity.

METHODS: A single nucleotide polymorphism of iNOS C150T was examined for 454 Japanese health checkup examinees (126 males and 328 females) aged 35 to 85 years without a history of cancer and 202 gastric cancer patients (134 males and 68 females) aged 33 to 94 years with pathologically confirmed diagnosis of gastric adenocarcinoma.

RESULTS: The iNOS C150T polymorphism was not associated with gastric atrophy or with H pylori seropositivity. The odds ratio (OR) of the C/T + T/T genotype for gastric cancer was increased without statistical significance (OR=1.19, 95% confidence interval (CI): 0.68-2.08). In the differentiated subgroup (n = 113), however, the OR of the C/T genotype for gastric cancer was significant (OR = 2.02, 95% CI: 1.04-3.92) relative to the C/C genotype. In addition, considering the location of gastric cancer (n = 105), there were significant differences between the controls and non-cardia group with the OR of 2.13 (95% CI: 1.08-4.18) for C/T and 1.94 (95% CI: 1.00-3.78) for C/T + T/T.

CONCLUSION: The iNOS C150T polymorphism is associated with the risk of H pylori-related gastric cancer in a Japanese population. This polymorphism may play an important role in increasing the risk of gastric cancer in Asian countries with the highest rates of gastric cancer.

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Key words: Gastric cancer; Inducible nitric oxide synthase; Polymorphism; H pylori

INTRODUCTION

Gastric cancer is the second most frequent cancer in the world, accounting for a large proportion of cancer cases in Asia, Latin America, and some countries in Europe\[1\]. H pylori strains carrying the cytotoxin-associated gene A (cagA) are strongly associated with increased risk of gastric adenocarcinoma\[2\]. However, only some of those infected with H pylori developed H pylori-related gastric cancer, even in Asian countries including Japan with high prevalence of cagA-positive H pylori infection. Therefore, it is important to examine any host genetic predisposition to H pylori-related gastric cancers.

Nitric oxide (NO) produced by activated phagocytes has been reported to play a role in the processing of carcino genesis\[3\]-\[5\]. NO is synthesized enzymatically from L-arginine by a family of three distinct nitric oxide synthase (NOS) isoforms\[6\]. Two NOS isoforms, eNOS (expressed in vascular endothelial cells) and nNOS (expressed in neurons of the central and peripheral nervous system), are constitutively expressed and are calcium dependent. The third isoform, inducible NOS (iNOS), is calcium independent, and expressed in response to bacterial endotoxins and cytokines to cause sustained NO release. 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. iNOS can produce larger amounts of NO than either eNOS or nNOS. iNOS expression in the gastric mucosa is higher in H pylori positive gastric cancer patients than in
Several types of polymorphisms have been identified in the promoter region of the iNOS gene: G to C at -954, C to T at -1173, and tandem repeat number polymorphism of (TAAA)n and (CCTTT)n[8,11]. iNOS production is mainly regulated at the transcriptional level[2]. The human iNOS gene comprises 27 exons, with the transcription start site in exon 2 (E2) and the stop codon in E27[3]. Johannesen et al[6] have detected 10 polymorphisms in 8 exons of the iNOS gene, of which one polymorphism (C150T) in exon 16 resulting in an amino acid substitute, Ser608Leu, showed the only transmitted mutation in the transmission disequilibrium test. This polymorphism is associated with cigarette- and alcohol-induced gastric cancer in Chinese population[9], indicating that iNOS Ser608Leu allele may have a dramatic effect on the enzyme activity.

Accordingly, we have investigated the associations of iNOS C150T polymorphism with H pylori seropositivity, gastric atrophy and gastric cancer in Japanese population.

### MATERIALS AND METHODS

#### Study subjects

Detailed information of the characteristics of healthy controls and gastric cancer patients in this study has been published in our previous paper[10]. Briefly, the control group included 454 health checkup examinees (126 males and 328 females) aged 35 to 85 years with no history of cancer who attended a health checkup program supported by the Nagoya Municipal Government in August and September, 2000. The case group consisted of 202 patients (134 males and 68 females) aged 33 to 94 years with a pathologically confirmed diagnosis of gastric cancer who attended a health checkup program before gastric resection. Pepsinogens I and II (PG I and PG II) in serum were measured by radioimmunoassay using a commercially available kit (DINABOT, Tokyo, Japan). Gastric atrophy was defined as PG I < 70 ng/mL and PG I/PG II ratio < 3. These parameters for atrophy are widely used in Japan and have been validated against histological confirmatory studies.

#### Tests for H pylori antibody and pepsinogens

Anti-H pylori IgG antibody tests, high molecular weight campylobacter-associated protein (HM-CAP) ELISA (Enteric Products Inc., Westbury, NY) and HM-CAP with antigens extracted from clinically isolated Japanese H pylori strain (J-HM-CAP) ELISA (Kyowa Medex, Tokyo, Japan), were used for the identification of H pylori-infected participants. An ELISA value of 2.3 or over was regarded as positive for both tests. The infection was confirmed in all gastric cancer cases by culture and bacteriological tests (Gram-negative, oxidase, catalase, and urease test—positive spiral, curved rods) using biopsy specimens before gastric resection. Pepsinogens I and II (PG I and PG II) in serum were measured by radioimmunoassay using a commercially available kit (DINABOT, Tokyo, Japan). Gastric atrophy was defined as PG I < 70 ng/mL and PG I/PG II ratio < 3. These parameters for atrophy are widely used in Japan and have been validated against histological confirmatory studies.

#### Genotyping

DNA was extracted from theuffy coat fraction by QIagen QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, CA). We carried out PCR-RFLP to identify iNOS C150T gene genotype as previously described by Shen et al[13]. However, the way to do PCR was somewhat different. Genomic DNA was used per 25 μL of reaction with 0.12 mmol/L dNTPs, 25 pmol of each primer, 0.5 units of “AmpliTaq Gold”, and 2.5 μL GeneAmp 10 × PCR buffer including 15 mmol/L MgCl2 (Perkin-Elmer Corp., Foster City, CA). Amplification conditions were 10 min of initial denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C, then a final extension at 72°C for 5 min. The PCR product was digested with a restriction enzyme (Tip 509 I) by the same way as previously described by Shen et al[13].

#### Statistical analysis

The strength of associations of H pylori seropositivity, gastric atrophy and gastric cancer with the iNOS C150T polymorphism was measured as odds ratios (ORs). ORs adjusted for sex and age with 95% confidence intervals (CIs) were calculated using logistic regression analysis. Hardy-Weinberg equilibrium was tested for iNOS C150T polymorphisms. The strength of associations of iNOS C150T gene genotype as previously described by Shen et al[13].

#### RESULTS

The characteristics of study subjects have been described elsewhere[16]. Only one case could not be genotyped. The distributions of the iNOS C150T gene was in the Hardy-Weinberg equilibrium ($\chi^2 = 1.37$ and $P = 0.24$). The iNOS C150T polymorphism had no significant effect on H pylori seropositivity. Table 1 shows the sex-age-adjusted ORs and 95% CIs of the iNOS C150T genotypes for gastric atrophy (GA) among H pylori seropositive controls. The iNOS C150T polymorphism was not associated with gastric cancer.

<table>
<thead>
<tr>
<th>iNOS polymorphism</th>
<th>$n$</th>
<th>GA $n$ (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>217</td>
<td>121 (55.8)</td>
<td>1.00</td>
<td>Reference</td>
</tr>
<tr>
<td>C/T</td>
<td>30</td>
<td>16 (53.3)</td>
<td>0.90</td>
<td>0.42-1.94</td>
</tr>
<tr>
<td>T/T</td>
<td>3</td>
<td>0 (0)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>C/T+T/T</td>
<td>33</td>
<td>16 (48.5)</td>
<td>0.75</td>
<td>0.36-1.56</td>
</tr>
</tbody>
</table>

### Table 1  The sex-age-adjusted ORs and 95% CIs of the iNOS C150T genotypes for gastric atrophy (GA) among H pylori seropositive controls

<table>
<thead>
<tr>
<th>iNOS polymorphism</th>
<th>Cases’ $n$ (%)</th>
<th>Controls’ $n$ (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>175 (87.1)</td>
<td>403 (88.8)</td>
<td>1.00</td>
<td>Reference</td>
</tr>
<tr>
<td>C/T</td>
<td>25 (12.4)</td>
<td>48 (10.6)</td>
<td>1.22</td>
<td>0.69-2.17</td>
</tr>
<tr>
<td>T/T</td>
<td>1 (0.50)</td>
<td>3 (0.66)</td>
<td>0.71</td>
<td>0.07-7.54</td>
</tr>
<tr>
<td>C/T+T/T</td>
<td>26 (12.9)</td>
<td>51 (11.2)</td>
<td>1.19</td>
<td>0.68-2.08</td>
</tr>
</tbody>
</table>

*One case subject could not be genotyped.*
Regulation of Nitric oxide (NO) and polyamine pathways.

Our control iNOS gene was differentiated type and located in non-cardia, namely was associated with gastric cancer where the cell type was differentiated type and located in non-cardia, namely H pylori-related gastric cancer. Our control iNOS gene frequency is coincident with the results presented by Shen et al. [3], which represents the genotype frequency in China. We could not evaluate the interaction between iNOS C150T polymorphism and smoking-induced risk of gastric cancer as the study of Shen et al. [3], because we have no information on smoking habit in case group. iNOS is expressed in response to bacterial endotoxins and cytokines to cause sustained NO release. This excess NO would contribute to the development of gastric atrophy, reacting with superoxide produced by H pylori infection [60] to form peroxynitrite which has strong oxidizing properties. We chose the seropositive controls as the comparison group. The iNOS C150T polymorphism, however, was not associated with gastric atrophy as the precursor lesion of gastric cancer in this study.

It was reported that immune dysregulation induced by H pylori in which stimulated spermene synthesis by the arginase-ornithine decarboxylase (ODC) pathway inhibits iNOS translation and NO production, can lead to persistence of the bacterium [20] (Figure 1). We hypothesized that the iNOS C150T polymorphism might affect H pylori infection. But, this gene polymorphism was not associated with H pylori seropositivity in this study, confirming the finding that there is no correlation between H pylori infection and iNOS expression [21]. On the contrary, it has been reported that iNOS detection is significantly associated with H pylori infection [22,23].

There are many reports concerning the high expression of iNOS in gastric cancer, which increases with the stage of cancer and lymph node metastasis. It was reported that the long forms of iNOS promoter region are associated with intestinal gastric cancer in Japanese women [27]. Nam et al. [26] reported that iNOS contributes to H pylori-associated gastric carcinogenesis, and that H pylori is associated with non-cardiac tumor but not with cardiac tumor [29].

### DISCUSSION

This study showed that the iNOS C150T polymorphism was associated with gastric cancer where the cell type was differentiated type and in non-cardia, namely H pylori-related gastric cancer. Our control iNOS gene frequency is coincident with the results presented by Shen et al. [3], which represents the genotype frequency in China. We could not evaluate the interaction between iNOS C150T polymorphism and smoking-induced risk of gastric cancer as the study of Shen et al. [3], because we have no information on smoking habit in case group. iNOS is expressed in response to bacterial endotoxins and cytokines to cause sustained NO release. This excess NO would contribute to the development of gastric atrophy, reacting with superoxide produced by H pylori infection [60] to form peroxynitrite which has strong oxidizing properties. We chose the seropositive controls as the comparison group. The iNOS C150T polymorphism, however, was not associated with gastric atrophy as the precursor lesion of gastric cancer in this study.

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### Table 3  The distribution of the iNOS C150T genotype in case, considering the location of gastric cancer and the phenotype iNOS

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Phenotype</th>
<th>Total cases $^\dagger$ n (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>Non-cardia n (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>Cardia n (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>Differentiated</td>
<td>95 (82.3)</td>
<td>1.00</td>
<td></td>
<td>86 (81.9)</td>
<td>1.00</td>
<td></td>
<td>7 (87.5)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>Differentiated</td>
<td>20 (17.7)</td>
<td>2.02</td>
<td>1.04-3.92$^\ast$</td>
<td>19 (18.1)</td>
<td>2.13</td>
<td>1.08-4.18$^\ast$</td>
<td>1 (12.5)</td>
<td>1.28</td>
<td>0.15-11.0</td>
</tr>
<tr>
<td>T/T</td>
<td>Differentiated</td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>C/T + T/T</td>
<td>Differentiated</td>
<td>20 (17.7)</td>
<td>1.84</td>
<td>0.96-3.54</td>
<td>19 (18.1)</td>
<td>1.94</td>
<td>1.00-3.76$^\ast$</td>
<td>1 (12.5)</td>
<td>1.16</td>
<td>0.14-9.95</td>
</tr>
<tr>
<td>C/C</td>
<td>Undifferentiated</td>
<td>69 (58.8)</td>
<td>1.00</td>
<td></td>
<td>69 (65.8)</td>
<td>1.00</td>
<td></td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>Undifferentiated</td>
<td>2 (17.8)</td>
<td>0.25</td>
<td>0.06-1.06</td>
<td>2 (17.8)</td>
<td>0.25</td>
<td>0.06-1.06</td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>Undifferentiated</td>
<td>1 (1.9)</td>
<td>1.74</td>
<td>0.17-18.1</td>
<td>1 (1.9)</td>
<td>1.74</td>
<td>0.17-18.1</td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>C/T + T/T</td>
<td>Undifferentiated</td>
<td>3 (4.17)</td>
<td>0.35</td>
<td>0.10-1.17</td>
<td>3 (4.17)</td>
<td>0.35</td>
<td>0.10-1.17</td>
<td>0 (0)</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

$^\dagger$P < 0.05 vs control group. $^\ast$Information on gastric cancer phenotype was not available for 16 cases; $^\dagger$One case subject with undifferentiated type could not be genotyped.

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### Figure 1  Regulation of Nitric oxide (NO) and polyamine pathways. H pylori induces inducible nitric oxide synthase (iNOS) expression, arginase II and ornithine decarboxylase (ODC). iNOS competes with arginase for arginine. Gastric cancer relates to the high expression of iNOS. H pylori-induced iNOS is inhibited by spermene produced by the arginase-ODC pathway.
high incidence of \textit{H pylori} infection in patients with gastric cancer including both intestinal type and diffuse type, particularly in those with intestinal type has been confirmed\cite{39}. Our study has confirmed that iNOS C150T polymorphism is related to the risk of \textit{H pylori}-related gastric cancer, by stratification of iNOS genotype frequency among cases according to the location of gastric cancer and histologic phenotype. It may take decades for superficial gastritis to progress to atrophic gastritis. \textit{H pylori} infection is highly associated with each stage of this progression\cite{31}. Loss of serological markers of \textit{H pylori} infection following onset of severe atrophy and intestinal metaplasia is a well described phenomenon, including in Japan\cite{32}. Considering these facts, the comparison with the controls with \textit{H pylori} seropositivity and/or gastric atrophy might be adequate to evaluate the effect of the genotype on \textit{H pylori}-related gastric cancer risk. In fact, the corresponding OR was significantly stable (OR = 2.20 for C/T, 95% CI: 1.08-4.49) in our study.

iNOS differs independently of Ca\textsuperscript{2+} from the constitutive forms of NOS (eNOS and nNOS). One major divergence in the close sequence similarity is a 40-50-amino acid insert in the middle of the FMN-binding domain of eNOS and nNOS. When this insert is removed from nNOS, the deleted mutant retains maximal NO synthesis activity at a lower concentration of free Ca\textsuperscript{2+} than the wild type enzyme\cite{33}. The iNOS C150T polymorphism is located at the position near to this deletion. The amino acid change in E16 may be of functional interest. As in the study of Shen \textit{et al}\cite{34}, our study indicated that those with T allele might increase iNOS expression and the level of NO in gastric mucosa. But specific functional tests of this polymorphism remain to be elucidated.

It is important to discuss our finding that iNOS C150T polymorphism is associated with \textit{H pylori}-related gastric cancer but not with \textit{H pylori} infection or with gastric atrophy (especially the association with the latter two is controversial). iNOS-derived NO is a central effector molecule in the innate immune response to pathogens, with essential antimicrobial functions in host defense. \textit{H pylori} induces both arginine \textit{II} and ODC in macrophages\cite{35} (Figure 1). Arginine converts L-arginine to L-ornithine, which is metabolized by ODC to produce putrescine that is converted to polyamines (spermidine and spermine). Spermine inhibits \textit{H pylori}-stimulated NO production in macrophages by a post-transcriptional effect on \textit{iNOS} translation\cite{36}. The temporal switch of arginine as a substrate for the cytostatic iNOS/NO axis to the pro-growth arginase/ornithine/polyamine and proline axis is regulated by inflammatory cytokines as well as interregulated by the arginine metabolizes themselves. Satriano\cite{51} has proposed that agmatine, converted from arginine by agmatine decarboxylase, coordinates the early and repair phase pathways of arginine in the inflammatory response as a gating mechanism at the transition from the iNOS/NO axis to the arginase/ODC/polyamine axis. Therefore the stage of \textit{H pylori} infection and gastric atrophy might follow the arginase/ODC/polyamine axis. On the other hand, the stage of gastric cancer might follow the iNOS/NO axis. In addition, there is no significant association between cagA positive \textit{H pylori} strains and iNOS expression\cite{38}. CagA might affect the mechanisms that regulate this temporal switch, resulting in the rgnase/ODC/polyamine axis.

In conclusion, iNOS C150T polymorphism is associated with the risk of \textit{H pylori}-related gastric cancer in Japanese and Chinese population. This polymorphism may play an important role in increasing the risk of gastric cancer in Asian countries with the highest rates of gastric cancer. The confirmation of this finding requires much larger studies of different ethnic groups to stimulate the interest in the molecular mechanisms of this polymorphism function.

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