Short Communication

Cyclooxygenase-2 and Inducible Nitric Oxide Synthase Gene Polymorphisms and Risk of Reflux Esophagitis, Barrett’s Esophagus, and Esophageal Adenocarcinoma

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Abstract

The incidence of esophageal adenocarcinoma has increased in recent years, and Barrett’s esophagus is a recognized risk factor. Gastroesophageal reflux of acid and/or bile is linked to these conditions and to reflux esophagitis. Inflammatory disorders can lead to carcinogenesis through activation of “prosurvival genes,” including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Increased expression of these enzymes has been found in esophageal adenocarcinoma, Barrett’s esophagus, and reflux esophagitis. Polymorphic variants in COX-2 and iNOS genes may be modifiers of risk of these conditions. In a population-based case-control study, we examined associations of the COX-2 8473 T>C and iNOS Ser608 Leu (C>T) polymorphisms with risk of esophageal adenocarcinoma, Barrett’s esophagus, and reflux esophagitis. Genomic DNA was extracted from blood samples collected from cases of esophageal adenocarcinoma (n = 210), Barrett’s esophagus (n = 212), and reflux esophagitis (n = 230) and normal population controls frequency matched for age and sex (n = 248). Polymorphisms were genotyped using TaqMan allelic discrimination assays. Odds ratios and 95% confidence intervals were obtained from logistic regression models adjusted for potential confounding factors. The presence of at least one COX-2 8473 C allele was associated with a significantly increased risk of esophageal adenocarcinoma (adjusted odds ratio, 1.58; 95% confidence interval, 1.04-2.40). There was no significant association between this polymorphism and risk of Barrett’s esophagus or reflux esophagitis or between the iNOS Ser608 Leu polymorphism and risk of these esophageal conditions. Our study suggests that the COX-2 8473 C allele is a potential genetic marker for susceptibility to esophageal adenocarcinoma. (Cancer Epidemiol Biomarkers Prev 2008;17(3):727-31)

Introduction

The incidence of esophageal adenocarcinoma has been increasing markedly over the last three decades (1). Barrett’s esophagus increases the risk of esophageal adenocarcinoma by 30- to 125-fold (2).

Traditionally, reflux esophagitis has been seen as an early development on the reflux esophagitis-Barrett’s esophagus adenocarcinoma spectrum (3). However, recent evidence has suggested that reflux esophagitis and Barrett’s esophagus may be distinct clinical entities (4). Although the underlying stimulus in these three conditions is gastroesophageal reflux of acid and/or bile, it is likely that various genetic and environmental factors interact to determine an individual’s response to refluxate in the esophagus.

Inflammatory disorders, which can be caused by chemical agents (e.g., gastric refluxate and cigarette smoke), are recognized risk factors for carcinogenesis. Chronic inflammation leads to activation of “prosurvival genes,” including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS; ref. 5). Increased expression of both these enzymes has been found in esophageal adenocarcinoma (6-8), Barrett’s esophagus (6, 9), and reflux esophagitis (10). It has been shown that downstream mediators on the COX-2 and iNOS pathways lead to tumor promotion by stimulation of proliferation, promotion of angiogenesis, and inhibition of apoptosis (11, 12).

Various single nucleotide polymorphisms have been identified in the COX-2 and iNOS genes.
The COX-2 T>C polymorphism at position 8473 in the 3' untranslated region is in an area shown to contain multiple regulatory elements, which alter COX-2 expression in a murine model (13). The iNOS Ser<sup>608</sup> Leu allele (C>T polymorphism) leads to an amino acid alteration in multiple regulatory elements, which alter COX-2 expression. The iNOS Ser<sup>608</sup> Leu allele has been studied in association with various cancers but not in esophageal adenocarcinoma. Because of their putative functional roles, these polymorphisms have been studied in association with various cancers but not in esophageal adenocarcinoma or its precursor lesion Barrett’s esophagus. We examined the association between COX-2 8473 T>C and iNOS Ser<sup>608</sup> Leu polymorphisms and esophageal adenocarcinoma, Barrett’s esophagus, and reflux esophagitis in a population-based case-control study.

### Materials and Methods

#### Design.
The study methods have been described in detail elsewhere (15). Briefly, the Factors Influencing the Barrett’s Adenocarcinoma Relationship study collected data and samples between March 2002 and July 2005. The study recruited four groups of Caucasian subjects: (a) patients with esophageal adenocarcinoma, (b) patients with long segment Barrett’s esophagus, (c) patients with reflux esophagitis, and (d) normal population controls. Subjects were recruited throughout the island of Ireland with the exception of reflux esophagitis patients who were recruited only in Northern Ireland.

### Genotyping.
DNA was extracted from venous blood samples using the Puregene DNA purification kit.

### Table 1. Characteristics of controls, reflux esophagitis, Barrett’s esophagus, and esophageal adenocarcinoma patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls n (%)</th>
<th>Reflux esophagitis n (%)</th>
<th>Barrett’s esophagus n (%)</th>
<th>Esophageal adenocarcinoma n (%)</th>
<th>P (reflux esophagitis vs controls)</th>
<th>P (Barrett’s esophagus vs controls)</th>
<th>P (esophageal adenocarcinoma vs controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>220 (84.6)</td>
<td>189 (82.2)</td>
<td>185 (82.6)</td>
<td>192 (84.6)</td>
<td>0.468</td>
<td>0.548</td>
<td>0.992</td>
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<tr>
<td>Female</td>
<td>40 (15.4)</td>
<td>41 (17.8)</td>
<td>39 (17.4)</td>
<td>35 (15.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (y)</td>
<td>63.0</td>
<td>61.7</td>
<td>62.4</td>
<td>64.2</td>
<td>0.219</td>
<td>0.567</td>
<td>0.276</td>
</tr>
<tr>
<td>Education (full time)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (y)</td>
<td>12.0</td>
<td>10.8</td>
<td>11.3</td>
<td>10.7</td>
<td>&lt;0.001</td>
<td>0.013</td>
<td>&lt;0.001</td>
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<td>Gastroesophageal reflux symptoms*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>211 (81.2)</td>
<td>140 (60.9)</td>
<td>60 (26.8)</td>
<td>117 (51.5)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>49 (18.8)</td>
<td>90 (39.1)</td>
<td>164 (73.2)</td>
<td>110 (48.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status t</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>102 (40.2)</td>
<td>109 (48.4)</td>
<td>87 (39.2)</td>
<td>45 (20.4)</td>
<td>&lt;0.001</td>
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<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>107 (42.1)</td>
<td>68 (30.2)</td>
<td>85 (38.3)</td>
<td>99 (44.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>45 (17.7)</td>
<td>40 (17.3)</td>
<td>30 (22.5)</td>
<td>77 (34.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (g/d)</td>
<td>26.1</td>
<td>22.0</td>
<td>22.3</td>
<td>19.2</td>
<td>0.151</td>
<td></td>
<td></td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>27.0</td>
<td>27.8</td>
<td>27.0</td>
<td>28.7</td>
<td>0.047</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Symptoms of heartburn or acid reflux experienced at least once a week or more than 50 times per year, more than 5 y before the interview date.

†Never smoker” is defined as those subjects who had never smoked, who had smoked <100 cigarettes in their lifetime, or who had smoked <1 cigarette per day for ≥6 mo. “Ex-smoker” is defined as smokers who had stopped ≥5 y before the interview date. “Current smoker” is defined as smokers who had smoked at least 1 cigarette per day for ≥6 mo, 5 y before the interview date.
Table 2. Frequency of COX-2 and iNOS genotypes and risk of reflux esophagitis, Barrett’s esophagus, and esophageal adenocarcinoma relative to asymptomatic controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n = 248)</th>
<th>Reflux esophagitis (n = 230)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>COX-2 8473 T&gt;C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>111</td>
<td>91</td>
</tr>
<tr>
<td>TC</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>CC</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>TC + CC</td>
<td>137</td>
<td>139</td>
</tr>
<tr>
<td>iNOS Ser&lt;sup&gt;608&lt;/sup&gt; Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>167</td>
<td>155</td>
</tr>
<tr>
<td>CT</td>
<td>72</td>
<td>70</td>
</tr>
<tr>
<td>TT</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>CT + TT</td>
<td>81</td>
<td>75</td>
</tr>
</tbody>
</table>

*One esophageal adenocarcinoma subject was “undetermined” for both polymorphisms and was excluded from analysis.

† Adjusted for age, sex, years of full-time education, body mass index, smoking, alcohol, and gastroesophageal reflux symptoms.

(Gentra Systems). The COX-2 8473 T>C (rs5275) and iNOS Ser<sup>608</sup> Leu (rs2275218) polymorphisms were genotyped using predesigned and validated TaqMan allelic discrimination assays (Applied Biosystems). Reactions were done using the Applied Biosystems Prism 7900HT sequence detection system, with an annealing temperature of 60°C.

Samples were processed without knowledge of their case-control status. Positive and negative control samples were included in each reaction. Genotyping was repeated for a random selection of 10% of samples, with 100% concordance for all assays. Any samples labeled as “undetermined” by the sequence detection system software were excluded from the analysis. One subject was “undetermined” after genotyping for each assay.

Statistical Analysis. Deviation from Hardy-Weinberg equilibrium among cases and controls was tested for using the goodness-of-fit χ<sup>2</sup> test. Genotype frequencies among cases of esophageal adenocarcinoma, Barrett’s esophagus, and reflux esophagitis were compared with controls using the χ<sup>2</sup> test for association while adjusting for potential confounding factors by logistic regression.

Significant differences were observed between the study groups after analysis of baseline characteristics (Table 1). We therefore identified age at interview, sex, body mass index 5 years before interview (kg/m<sup>2</sup>), years of full-time education, smoking status (never, ex-smoker, current smoker), and alcohol intake (g/d) as potential confounding factors. Trend tests were conducted by assigning the ordinal values 1, 2, and 3 to homozygous wild-type, heterozygotes, and homozygous variant genotypes, respectively, and by adding these scores as a continuous variable in the logistic regression model. Statistical significance was set at P < 0.05. All analyses were done using SPSS for Windows version 13.0 (SPSS).

Ethical Approval. The Factors Influencing the Barrett’s Adenocarcinoma Relationship study was approved by the Research Ethics Committee of Queen’s University Belfast, the Clinical Research Ethics Committee of Cork Teaching Hospitals, and the Research Ethics Committee Board of St. James’s Hospital Dublin.

Results

A total of 227 esophageal adenocarcinoma patients, 224 Barrett’s esophagus patients, 230 reflux esophagitis patients, and 260 population controls were recruited as part of the Factors Influencing the Barrett’s Adenocarcinoma Relationship study. The demographic characteristics of each group of subjects are presented in Table 1. The participation rate of eligible, alive esophageal adenocarcinoma patients was 74.2% and the overall esophageal adenocarcinoma response rate was 63.9%. The participation rates of Barrett’s esophagus and reflux esophagitis patients and control subjects were 82.4%, 68.7%, and 41.8%, respectively. DNA samples were available for analysis from 210 (92.5%) esophageal adenocarcinoma patients, 212 (94.6%) Barrett’s esophagus patients, 230 (100%) reflux esophagitis patients, and 248 (95.4%) controls.

The distribution and frequency of genotypes for cases and controls are shown in Table 2. There were no significant departures from Hardy-Weinberg equilibrium for either of the polymorphisms in any of the subject groups. For controls, the frequency of polymorphic variants was 0.32 (COX-2 8473 C) and 0.18 (iNOS Ser<sup>608</sup> Leu). These were similar to frequencies reported in Caucasians in the SNP500 Database. There was no statistically significant association between the iNOS Ser<sup>608</sup> Leu polymorphism and risk of esophageal adenocarcinoma, Barrett’s esophagus, or reflux esophagitis either in the unadjusted analysis or after adjustment for a number of other potential confounding factors (see Table 2). In addition, there was no statistically significant association between the COX-2 8473 T>C polymorphism and risk of Barrett’s esophagus or reflux esophagitis. However, the presence of at least one COX-2 8473 C allele was associated with a significantly increased risk of esophageal adenocarcinoma both before and after adjustment for potential confounding factors (see Table 2). The adjusted odds ratio (OR) of COX-2 8473 TC + CC versus TT was 1.58 [95% confidence interval (95% CI), 1.04-2.40; P = 0.03]. In addition, a significant additive effect across this genotype was shown (P<sub>trend</sub> = 0.04).
will be necessary before a conclusion on the function
analysis of the genetic regulation of COX-2 expression
''wild-type'' 8473 T allele. However, further
adenocarcinoma, may be higher than that of the
associated with an increased risk of esophageal
the expression of the COX-2 8473 C allele, which was
previous studies (6-8). We therefore hypothesize that
in esophageal adenocarcinoma has been well shown in
model (13). Increased expression of the COX-2 enzyme
has been shown to alter COX-2 expression in a murine
has a functional role. However, variation in this region
COX-2 ¶
humans that this polymorphism, located in the 3
reported null findings. There is no direct evidence in
(19), prostate (20), and colorectal (21) cancers have
produced conflicting results. Increased risks of
cancer (22), and similar null findings were reported in a
Korean cervical cancer study (23). These results may in
part be explained by the relatively low variant allele
frequency in these populations (15.2% and 13.8%,
respectively; refs. 22, 23), limiting study power. This
may also be a factor in our study, with a variant allele
frequency of 18% in controls.

A further limitation of our study was that misclassi-
fication bias may have arisen because asymptomatic
control subjects did not have endoscopy done to exclude
Barrett’s esophagus or reflux esophagitis. However,
given that the population prevalence of Barrett’s esoph-
agus has been estimated at 2.6% in a similar Caucasian population
with endoscopically diagnosed reflux esophagitis has
5.7% (24, 25), this is unlikely to have had a major effect on
our findings. In addition, not all patients with reflux
esophagitis diagnosed at endoscopy had biopsies taken
to definitively exclude Barrett’s esophagus, which may
have led to further misclassification bias. However, the
prevalence of histologic Barrett’s esophagus in subjects
with endoscopically diagnosed reflux esophagitis has
been estimated at 2.6% in a similar Caucasian population
(25). If applied to the 230 reflux esophagitis subjects in
our study, only 6 subjects with Barrett’s esophagus may
have been misclassified.

Given that the sample size of our study is relatively
small compared with those in more common cancers,
such as prostate (20) and breast (19), chance findings
cannot be excluded and we did not have adequate power
to detect a modest excess risk associated with the iNOS
Ser608 Leu polymorphism (OR < 2.0) or to examine the
role of possible gene-environment interactions.

In addition, the low participation rate in the control
group may have introduced selection bias, which is a
common problem in all case-control studies. However,
controls in our study were selected at random from the
population via a General Practice database.

In conclusion, the COX-2 8473 C allele may be a risk
factor for esophageal adenocarcinoma, but further larger
population-based studies are required to confirm this
finding.

Discussion
To the best of our knowledge, this is the only case-control
study to date to explore the association between COX-2
and iNOS polymorphisms and risk of esophageal
adenocarcinoma, Barrett’s esophagus, or reflux esoph-
agitis. Our study results indicate that the presence of the
COX-2 8473 C allele may predispose an individual to
esophageal adenocarcinoma. However, this polymor-
phism was not associated with risk of its precursor
conditions Barrett’s esophagus or reflux esophagitis.
Furthermore, the iNOS Ser608 Leu polymorphism did not seem to modulate risk for any of the studied
esophageal conditions.

The strengths of our study include the population-
based design, the relatively large sample size, and the
efforts made to minimize misclassification bias by
characterizing phenotypes clearly. Detailed interview
data allowed us to control for a range of potential
confounding factors, and DNA was available for
analysis in almost all study subjects (>90%). Both polymorphisms were in Hardy-Weinberg equilibrium and had sufficient statistical power (>80%) to detect an
OR of >2.0. We carefully selected two polymorphisms to
evaluate by a review of published data, thus reducing
the likelihood of type I error.

Previous studies in Caucasians investigating associ-
ation between COX-2 8473 T>C and various cancers
have produced conflicting results. Increased risks of
breast (16), lung (17), and basal cell (18) carcinoma
have been described. However, other studies in breast
(19), prostate (20), and colorectal (21) cancers have
reported null findings. There is no direct evidence in
humans that this polymorphism, located in the 3’
untranslated region of exon 10 in the COX-2 gene,
has a functional role. However, variation in this region
has been shown to alter COX-2 expression in a murine
model (13). Increased expression of the COX-2 enzyme
in esophageal adenocarcinoma has been well shown in
previous studies (6-8). We therefore hypothesize that
the expression of the COX-2 8473 C allele, which was
associated with an increased risk of esophageal
adenocarcinoma, may be higher than that of the
“wild-type” 8473 T allele. However, further in vitro
analysis of the genetic regulation of COX-2 expression
will be necessary before a conclusion on the function-
ality of the COX-2 8473 T>C polymorphism can be
drawn.

Only two studies to date have investigated an
association between the iNOS Ser608 Leu polymorphism
and cancer both in Asian populations. From China, Shen
et al. reported no significant association with gastric
cancer (22), and similar null findings were reported in a
Korean cervical cancer study (23). These results may in
part be explained by the relatively low variant allele
frequency in these populations (15.2% and 13.8%,
respectively; refs. 22, 23), limiting study power. This
may also be a factor in our study, with a variant allele
frequency of 18% in controls.

Table 2. Frequency of COX-2 and iNOS genotypes and risk of reflux esophagitis, Barrett’s esophagus, and esophageal adenocarcinoma relative to asymptomatic controls (Cont’d)

<table>
<thead>
<tr>
<th>n</th>
<th>COX-2 genotypes</th>
<th>Unadjusted OR (95% CI)</th>
<th>P_trend</th>
<th>Adjusted OR (95% CI)</th>
<th>P_trend</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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</tr>
<tr>
<td>89</td>
<td>1.00</td>
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<tr>
<td>99</td>
<td>1.09 (0.74-1.61)</td>
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<tr>
<td>24</td>
<td>1.75 (0.66-2.34)</td>
<td>1.19 (0.62-2.34)</td>
<td>1.30 (1.03-3.51)</td>
<td>1.52 (1.04-2.22)</td>
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<td>123</td>
<td>1.12 (0.77-1.62)</td>
<td>1.12 (0.77-1.65)</td>
<td>1.36 (1.04-2.22)</td>
<td>1.58 (1.04-2.40)</td>
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<td>54</td>
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<td>9</td>
<td>1.12 (0.43-2.90)</td>
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<td>63</td>
<td>0.87 (0.59-1.30)</td>
<td>0.92 (0.60-1.42)</td>
<td>0.97 (0.57-1.54)</td>
<td>0.92 (0.61-1.40)</td>
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Cancer Epidemiol Biomarkers Prev 2008;17(3). March 2008
References


