

Prediction of Malignant Potential in Reflux Disease: Are Cytokine Polymorphisms Important?

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- OBJECTIVES:** Esophageal reflux is common in the Western world and can lead to a number of diseases, such as esophagitis, Barrett's esophagus, and adenocarcinoma. Barrett's predisposes to adenocarcinoma and endoscopic surveillance may lead to earlier detection of adenocarcinoma. However, clinical methods only identify one patient in 15 with Barrett's esophagus. The aim of this study was to find factors that may help identify patients with Barrett's earlier.
- METHODS:** Blood samples and detailed histories were taken from 456 patients with gastroesophageal reflux who were recruited into three study groups: esophagitis, Barrett's esophagus without dysplasia, and Barrett's with dysplasia or adenocarcinoma. PCR was used to determine the frequency of five functional cytokine polymorphisms: interleukin-1 receptor antagonist position +2018 (IL-1 Ra +2018), interleukin-1 β position -511 (IL-1 β -511), tumor necrosis factor- α position -238 (TNF- α -238), interleukin-10 position +1082 (IL-10 +1082), and interleukin-4 receptor position -1902 (IL-4R -1902).
- RESULTS:** IL-1 Ra +2018 genotype 2/2 was associated with Barrett's more commonly than esophagitis (OR-3.7, $p = 0.0345$). The IL-10 +1082 genotype 2/2 was more strongly associated with Barrett's and adenocarcinoma than esophagitis (OR-1.76, $p = 0.056$ and OR 1.96, $p = 0.025$, respectively). There were no differences for the IL-1 β -511, IL-4R -1902, and TNF- α -238 polymorphisms.
- CONCLUSIONS:** Cytokine polymorphisms are more commonly found in patients with Barrett's or adenocarcinoma than those with esophagitis. Together with demographic data, this may help identify those patients with Barrett's who would benefit from surveillance.

(Am J Gastroenterol 2005;100:1012-1018)

INTRODUCTION

Gastroesophageal reflux is a major public health problem in the Western world, with 10% of adults experiencing reflux symptoms daily, sufficient to affect their quality of life (1). Treatment with antacids, H₂ antagonists, and proton pump inhibitors accounts for a significant proportion of the national drug budget (2).

Longstanding reflux leads to a number of inflammatory conditions including esophagitis, Barrett's esophagus, and esophageal adenocarcinoma. The frequency and severity of the reflux is not the only determinant of outcome and many factors remain unknown (3). Reflux disease is increasingly being recognized as a group of distinct disease entities rather than a spectrum of disorders lying on the same continuum (4). There is no compelling evidence to suggest that erosive esophagitis makes the transformation to Barrett's esophagus (a condition that is known to have malignant potential), whereas the known risk of progression of Barrett's to adenocarcinoma is the main reason for follow-up surveillance of Barrett's patients. Romero *et al.* (5) showed a familial aggregation of reflux in relatives of patients with either

Barrett's esophagus or adenocarcinoma, but no such association in patients with esophagitis. Their conclusion was that, after accounting for age and obesity, there is a genetic predisposition for reflux in Barrett's and adenocarcinoma patients.

The interleukin-1 cytokines (IL-1 α , β , and Ra) have been shown to be important in many inflammatory diseases, such as inflammatory bowel disease (6-10), gastric adenocarcinoma (11), periodontitis (12), and rheumatoid arthritis (13). Additionally, IL-4, IL-10, and TNF- α have important roles in both inflammatory and malignant diseases (14-18). Cytokine polymorphisms also appear to affect the inflammatory response to *Helicobacter pylori* infection in the stomach, resulting in atrophic gastritis and an increased risk of adenocarcinoma of the stomach (19). There is evidence that the cytokine profile varies between esophagitis Barrett's and adenocarcinoma, with IL-1 β , IL-10, and IL-4 being the most significant (10, 20).

Our hypothesis is that patients have different inflammatory responses to reflux and that this determines the outcome. These different responses are influenced by genetic variability with the rare alleles of cytokine polymorphisms altering

the biological response to gastroesophageal reflux. This, in turn, renders certain patients more prone to developing Barrett’s esophagus and adenocarcinoma.

We chose to examine five candidate polymorphisms in three groups of prospectively recruited patients, with esophagitis, Barrett’s esophagus, or adenocarcinoma/high-grade dysplasia (HGD), to determine whether differences occur among the esophagitis group (with no malignant potential) and the Barrett’s and adenocarcinoma groups. Both IL-1 Ra +2018 and IL-1 β −511 have been shown to alter the inflammatory response of the stomach, predisposing to atrophic gastritis and adenocarcinoma. When analyzed in combination, they have been shown to be more predictive of the outcome in gastric cancer than individually. IL-4 and IL-10 −1082 have been measured immunohistochemically in tissue from groups of patients with Barrett’s and esophagitis and both were found to be elevated in the Barrett’s tissue. TNF-α −238 has also been shown to be important in inflammation and carcinogenesis (21, 22).

PATIENTS AND METHODS

A total of 456 patients were recruited into the study and allocated prospectively into one of three groups: esophagitis, Barrett’s esophagus without dysplasia, and Barrett’s with dysplasia or esophageal adenocarcinoma. These patients were recruited from the outpatient clinic and endoscopy lists between November 2000 and August 2002. Esophagitis was diagnosed at endoscopy and graded according to the Savary–Millar classification. Barrett’s esophagus and adenocarcinoma were confirmed on histological review by experienced GI pathologists. Barrett’s esophagus was only diagnosed when specialized columnar epithelium (with intestinal metaplasia) was seen and dysplasia was defined as neoplastic epithelium confined within the basement membrane in the absence of inflammation. This study was approved by the South Sheffield Research Ethics Committee (Ref. SS00/225) and each patient gave informed consent. Data collection included patient demographics and lifestyle, past medical history, medication, previous endoscopy results, symptoms, treatment, and surgery. These details were collected at interview and entered into a purpose built database.

A sample of venous blood was taken from each patient and stored in three 7 ml EDTA vacutainers at −19°C until analysis. DNA was extracted using a standard phenol/ethanol method. The polymorphisms examined were interleukin-1Ra + 2018, interleukin-1β −511, interleukin-4R −1902, interleukin-10 −1082, and TNF-α −238. Genotyping of the polymorphisms was performed using a 5’ nuclease PCR method (Applied Biosystems, Warrington, UK), as described by di Giovine *et al.* (23).

Quality control for the genotyping was achieved by using only 72 of the 96 wells in each plate for test samples. Of the remaining 24 wells, 8 were “no sample” controls, 8 were allele 1 controls, and 8 were allele 2 controls. In addition to this, random retesting of samples was carried out.

STATISTICAL ANALYSIS

Power calculations were performed to determine the number of subjects required in each patient group. Assuming a significance level of 5% and a power of at least 80%, it was determined that, if five polymorphisms were to be tested, 150 patients were needed in each of the three groups. These calculations were based on the assumption that the frequency of the rare alleles of a polymorphism in the general population was at least 17% (19) and we chose an OR of 1.8 as the presumed association under the alternative hypothesis.

All data were entered initially into a Microsoft Access database and exported to SPSS, version 10.0 for Windows (SPSS, Inc., Chicago, USA) for statistical analysis. The genotype data obtained in the study were then analyzed by calculating the number of patients in a study group having each of the three possible genotypes 1/1, 1/2, and 2/2. The statistical significance of any difference in the occurrence of the rare allele in our population was assessed using Pearson’s χ² test and by calculating the odds ratios. Mantel-Haenszel and logistic regression tests were used to test for the potential confounding variables of age and sex differences between the groups.

RESULTS

Patient Characteristics

Four hundred and fifty-six patients were recruited into the study. There were 153 patients in the esophagitis group and 153 in the Barrett’s group. Savary–Millar grading of biopsies from the esophagitis group showed that 18.9% were grade 1, 41.8% grade 2, 22.9% grade 3, and 13.7% grade 4 (2.7% unspecified). A further 150 patients were recruited into the dysplasia/adenocarcinoma group, comprising 107 with adenocarcinoma, 14 with HGD, and 29 with low-grade dysplasia (LGD).

General patient group demographics are shown in Table 1. Analysis of variance for these groups showed that there was a statistically significant difference in the median age of the

Table 1. Patient Demographics for the Three Study Groups

Patient Data	Study Group		
	Esophagitis (n = 153)	Barrett’s Esophagus (n = 153)	Dysplasia and Adenocarcinoma (n = 150)
Age	58.0 (20–86)	64.3 (23–95)	71.3 (37–92)
Male: Female	2.4	2.84	5.4
% Smoker/Ex-BMI	60.5	63.0	70.2
BMI	26.2 (14–41)	25.4 (17–38)	26.1 (15–52)
Alcohol intake units/wk	5.0 (0–100)	5.5 (0–100)	3.5 (0–90)
% Patients > 21 U/wk	15.7	15.7	12.6
Median duration of symptoms in year	4.0 (0–57)	10.0 (0–50)	10.0 (0–50)

Numbers represent the median (range).

Table 2. Percentage Distributions for the Alleles of the Cytokine Polymorphisms Tested

Genotype		Percentage with Polymorphism in Study Group		
		Esophagitis	Barrett's Esophagus	Dysplasia and Adenocarcinoma
IL-1 Ra +2018	1/1	55.8	49.0	45.0
	1/2	42.2	43.8	49.0
	2/2	2	7.2	6
IL-1 β -511	1/1	40.4	43.9	38.9
	1/2	48.3	42.6	52.3
	2/2	11.3	13.5	8.7
IL-1RA + IL-1 β	1/1	99.3	94.1	96.0
	1/2 or 2/2	0.7	5.9	4.0
IL-10 -1082	1/1	29.3	24.2	24.7
	1/2	55.1	50.3	48.0
	2/2	15.6	25.5	27.3
IL-4R -1902	1/1	60.7	57.3	67.8
	1/2	37.3	37.3	27.5
	2/2	2.0	5.3	4.7
TNF α -238	1/1	89.1	90.8	91.8
	1/2	0	0.7	0
	2/2	10.9	8.5	8.2

three study groups ($p < 0.001$). The male-to-female ratios were also different between groups ($p = 0.026$). The duration of symptoms was significantly longer in the Barrett's and adenocarcinoma groups ($p = 0.012$). Table 1 also shows the percentage of current or ex-smoker patients, although the differences are not statistically significant. There is no obvious association between increased body mass index (BMI) and a greater risk of Barrett's or adenocarcinoma as compared with esophagitis, with median BMI values similar in the study groups. However, all study groups had a mean BMI of greater than 25, which is categorized as pre-obese. There was little difference in the alcohol intake in the different groups, with each patient drinking a median of 5–6 units per week. The percentage of patients drinking more than the maximum weekly recommended intake of alcohol (21 U/wk for males, 14 U/wk for females) was also similar in all the groups.

Interleukin-1 Receptor Antagonist

There were differences in the frequencies of different genotypes across the study groups for the IL-1 Ra polymorphism at position 2018. The presence of the rare allele 2 in its homozygous form 2/2 is much more common in the Barrett's and adenocarcinoma groups than in the esophagitis group (Table 2). Odds ratios (OR) were calculated comparing the frequencies of the homozygote allele 2 genotype. These calculations gave a significant OR of 3.7 comparing Barrett's patients with those with esophagitis ($p = 0.0345$, Table 3). An OR of 3.04 was calculated comparing adenocarcinoma patients with esophagitis patients, but this was not statistically significant ($p = 0.085$). The distribution was similar

Table 3. Comparison of the Odds Ratios Calculated for the 2/2 Genotype in IL-1 Ra +2018

Comparison of Groups	OR	95% CI	p Value
Barrett's vs esophagitis	3.04	0.81–11.47	0.08
Dysplasia/adenocarcinoma vs esophagitis	3.7	1.02–13.61	0.03*
Dysplasia/adenocarcinoma/Barrett's vs esophagitis	3.4	0.99–11.56	0.04*

*Significant result.

when only males were included in the analysis, but small number of cases limited the interpretation of these data.

Interleukin-1 β

There was no significant difference between the study groups for the IL-1 β -511 polymorphism (Table 2).

Interleukin-1 β and Receptor Antagonist Combined

Those patients homozygous for IL-1 Ra allele 2, with at least one copy of allele 2 in IL-1 β -511, were found almost exclusively in the Barrett's and adenocarcinoma group, with only one patient in the esophagitis group having this combination (Table 2). The ORs comparing the different groups in turn were calculated. Comparing Barrett's with esophagitis gave an OR of 9.5 ($p = 0.01$), while the Barrett's and adenocarcinoma groups compared to the esophagitis group gave an OR of 7.89 ($p = 0.02$) (Table 4). Once again distributions were not significantly different when only males were included because of the small numbers (6 Barrett's, 1 esophagitis, and 0 adenocarcinoma patients).

Interleukin-10

There was a significant difference in the percentages of patients homozygous for allele 2 between the study groups (Table 2). There was an OR of 2.05 comparing cancer patients against those with esophagitis ($p = 0.013$) and 1.84 when comparing Barrett's with esophagitis ($p = 0.035$; Table 5). The results are significant when adjusted for sex and when only males were analyzed. The OR comparing Barrett's and cancer groups to the esophagitis group was 1.9 ($p = 0.010$).

Table 4. Comparison of Odds Ratios for Patients with IL-1 Ra 2/2 Genotype and at least One Copy of Allele 2 in IL-1 β -511

Comparison of Groups	OR	95% CI	p Value
Dysplasia/adenocarcinoma vs esophagitis	6.29	0.75–52.89	0.05*
Barrett's vs esophagitis	9.5	1.19–75.93	0.01*
Dysplasia/adenocarcinoma/Barrett's vs esophagitis	7.89	1.03–60.30	0.02*

*Significant result.

Table 5. Comparisons of the Odds Ratios for Patients with the Rare IL-10 1082 2/2 Genotype

Comparison of Groups	OR	95% CI	<i>p</i> Value
Dysplasia/adenocarcinoma vs esophagitis	2.05	1.15–3.62	0.013*
Barrett's vs esophagitis	1.84	1.04–3.28	0.035*
Dysplasia/adenocarcinoma/ Barrett's vs esophagitis	1.94	1.16–3.25	0.01*

*Significant result.

Interleukin-4 Receptor

The results of IL-4R-1902 polymorphism (Table 2) reveal that the homozygotes for allele 2 are once again more common in the Barrett's and cancer groups, but not statistically significant. Comparing Barrett's to esophagitis, the OR was 3.07 ($p = 0.149$); comparing dysplasia/adenocarcinoma to esophagitis the OR was 3.16 ($p = 0.154$). Adjusting for sex, by including males, again made little difference to the genotype distribution (Table 2). Comparing dysplasia/adenocarcinoma to esophagitis for the 2/2 genotype for males only gave an OR of 2.74 ($p = 0.206$), which is comparable to that for the whole study population.

Tumour Necrosis Factor α

For the TNF- α -238 genotype, there were no differences between any of the study groups (Table 2).

DISCUSSION

Esophagitis may lead to mucosal erosions of differing severity, but does not itself have any known malignant potential. However, Barrett's esophagus is regarded as a premalignant condition, in which the normal squamous mucosa is replaced by columnar epithelium with intestinal metaplasia, arising in response to chronic reflux (24–26). The clinical prevalence of Barrett's is 22.6/100,000, although autopsy studies have demonstrated a higher prevalence of 376/100,000 (27); this suggests that only 1 in 15 patients with Barrett's esophagus is detected. The risk of progression to adenocarcinoma is 30–125 times that of normal population (28–30) and the incidence of esophageal adenocarcinoma is increasing more rapidly than any other cancer in the Western world (31). It usually presents at an advanced stage and the prognosis is poor (32). Surveillance of Barrett's esophagus may lead to earlier detection of adenocarcinoma and could lead to an improved outcome (30, 33, 34). However, surveillance programs have made little impact on the outcome of cancer overall and many clinicians believe that it is not cost effective. On the other hand, it may be that it is because we only detect and survey a small percentage of patients with Barrett's esophagus, that little impact on the overall survival has been achieved.

Our study has extended the findings of Romero (5) by testing the hypothesis that development of Barrett's esophagus

and adenocarcinoma of the esophagus is the result of a different inflammatory response in patients with esophageal reflux. Previous reports (20) have shown that proinflammatory cytokines predominate in inflamed tissue from patients with esophagitis whereas antiinflammatory cytokines predominate in Barrett's tissue. We used a bank of five cytokine polymorphisms, which have been previously shown to have functional significance and all of which have been linked to susceptibility to inflammatory or malignant conditions (35, 36).

The interleukin-1 family are key cytokines in the control of the inflammatory response. There are three members of this family α , β , and Ra, found in most tissues in the body. The interleukin-1 gene cluster is situated on chromosome 2q and contains three related genes within a 430-kilobase (kb) region. They are IL-1A, IL-1B, and IL-1 RN, which code for the IL-1 α , IL-1 β , and IL-1Ra, respectively (37). IL-1 α is a predominantly intracellular molecule that has a regulatory role in cell function, whereas IL-1 β acts more as a systemic hormone (37). There are three diallelic polymorphisms reported in IL-1B, at positions -511, -31, and +3954 base pairs (bp) from the transcriptional start site. The IL1 RN gene has a penta-allelic, 86-bp tandem repeat in intron 2, which is in complete linkage disequilibrium with the IL-1 Ra +2018 gene examined in this study. There have also been many studies showing the effects of IL-1 polymorphisms on disease susceptibility and severity in other conditions including rheumatoid arthritis (38), vulvar vestibulitis (39), multiple sclerosis, alopecia, inflammatory bowel disease (36, 40–43), periodontitis (12, 44–46), type I diabetes mellitus, and gastric adenocarcinoma (19). In this study, the frequency of the IL-1 Ra 2018 2/2 genotype, which has been linked with a high level of IL-1 β *in vivo*, was significantly higher in the adenocarcinoma group than the esophagitis group, with an OR of 3.7 ($p = 0.0345$). The difference between the Barrett's group and the esophagitis group gave an OR of 3.04 ($p = 0.085$). There were no significant differences in the prevalence of the IL-1 β -511 polymorphism between the study groups.

In the study published by El Omar *et al.* (19), the combination of 2 genotypes in IL-1 β and IL-1 Ra resulted in much better discrimination between groups. This was also the case in the present study; when the frequency of genotype 2/2 in IL-1 Ra +2018 combined with genotypes 1/2 or 2/2 in IL-1 β -511 was determined, they were found to occur almost exclusively in the Barrett's and adenocarcinoma groups. Comparing the Barrett's and esophagitis groups gave an OR of 9.5 ($p = 0.01$). When the Barrett's and adenocarcinoma groups were combined, the calculated OR was 7.89 ($p = 0.02$) compared to the esophagitis group.

IL-4 is a Th2 antiinflammatory cytokine selected as a candidate gene because of its importance in the control of inflammation. One of the characteristics of this group of cytokines is that they cause hyperplasia of goblet cells, which are found in Barrett's mucosa but not in normal or inflamed esophageal mucosa (47–49). Additionally, IL-4 levels are

higher in Barrett's mucosa than in normal mucosa or mucosa from areas of esophagitis (20). Our study suggested that the 2/2 genotype was more common, but not significantly so, in the Barrett's and adenocarcinoma groups (OR-3.07, $p = 0.149$ and 3.16 $p = 0.154$, respectively) than the esophagitis group.

The homozygote genotype for allele 2 in the IL-10 -1082 polymorphism also seems to be associated with adenocarcinoma; an OR of 1.96 ($p = 0.025$) was found when the esophagitis and dysplasia/adenocarcinoma groups were compared. This genotype is associated with higher levels of IL-10 and in Barrett's an inflammatory gradient exists with IL-10 levels highest in the distal esophagus (20), where the majority of adenocarcinomas occur (16, 50–53).

TNF has been shown to be important in the pathogenesis of inflammatory, autoimmune, and malignant diseases (54). Originally thought to be anticarcinogenic (55, 56), it has since been shown to be tumorigenic both *in vitro* (14) and *in vivo* (57–59). The -238 polymorphism in the TNF α gene has been shown to be associated with high TNF levels, which is associated with a poor disease outcome in cancer patients (15, 17, 60). However, in this study we found no difference in the distribution of this polymorphism across the study groups.

Although a formal Bonferroni correction was not required for this study, the fact that multiple significance testing was applied has implications for the use of a 5% significance level, raising the possibility of making at least one type I error. However, the results of the association between polymorphisms in inflammatory cytokines and Barrett's esophagus remain noteworthy and warrant a larger, more detailed study.

Power calculations performed before the study assumed that the frequency of the rare alleles in our population would be the same as those of the Scottish and Polish populations previously published (19). In fact, the 2/2 genotype for IL-1 Ra was found in only 5% of our study population compared with the published 17% and could mean that the study was slightly underpowered. However, this variation in frequency of the rare alleles in different populations is consistent with the difference in prevalence of Barrett's esophagus and esophageal adenocarcinoma in diverse populations (61).

In the demographic data, male sex was a significant risk factor for the development of Barrett's and adenocarcinoma, a finding consistent with other studies (62).

Age was another factor that varied across the study groups with the median age of adenocarcinoma patients 6 yr greater than that of Barrett's patients, which was 6 yr greater than that of those with esophagitis. Other studies have reported conflicting findings regarding age as a risk factor (62, 63).

No difference was found between the median BMI for each of the study groups, although all groups fell into the pre-obese range of between 25 and 30, suggesting the possibility that obesity predisposes to reflux, but has no effect on the outcome (5).

There were no significant differences in the number of ex-smokers nor was self-reported alcohol intake significantly different between any of our study groups.

In this study, we used the esophagitis patients as the comparator, since they have an endoscopically verified condition, which has no proven association with either Barrett's or esophageal adenocarcinoma. The results of the dysplasia/adenocarcinoma group were not affected by the inclusion of the LGD patients, who accounted for 32 of the group as a whole. While HGD is recognized as a significant risk factor for malignant progression, the risks for patients with LGD are less predictable.

In conclusion, this study has demonstrated a significant number of genetic and demographic differences between patients with esophagitis, Barrett's esophagus, and esophageal adenocarcinoma. To date, surveillance programs for Barrett's esophagus have had little effect on the overall outcome of cancer in the general population, but they have shown benefits to individuals where the adenocarcinoma was detected at an earlier stage during surveillance endoscopy. Until we identify more of the patients with Barrett's esophagus and detect those patients earlier in the course of the disease, surveillance is unlikely to have any effect on the outcome of adenocarcinoma in the general population. This preliminary work has identified a number of possible genetic markers associated with Barrett's/esophageal adenocarcinoma, which, together with the observed demographic differences, could be used to help identify more patients at risk in the Barrett's population. Larger and more detailed studies may enable the development of more specific and sensitive surveillance programs for detecting malignant change in this group of "at risk" patients.

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Received June 3, 2004; accepted November 11, 2004.

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