Risk Factors, DNA Damage, and Disease Progression in Barrett's Esophagus

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Abstract

Esophageal adenocarcinoma develops on a background of Barrett's esophagus. A number of risk factors have been linked to both conditions, including gastroesophageal reflux and smoking. However, the molecular mechanisms by which these factors influence disease progression remain unclear. One possibility is that risk factors generate promutagenic DNA damage in the esophagus. The comet assay was used to measure DNA damage in esophageal (Barrett's and squamous) and gastric mucosa of Barrett's patients with (n = 24) or without (n = 50) associated adenocarcinoma or high-grade dysplasia in comparison with control patients (squamous mucosa) without Barrett's esophagus (n = 64). Patients completed a questionnaire detailing exposure to some of the known risk factors for Barrett's esophagus and adenocarcinoma. In Barrett's esophagus patients, DNA damage was higher in Barrett's mucosa compared with normal esophageal and gastric mucosa (P < 0.001). In addition, the highest quartile of DNA damage in Barrett's mucosa was associated with an increased risk (odds ratio, 9.4; 95% confidence interval, 1.1-83.4; P = 0.044) of developing adenocarcinoma or high-grade dysplasia compared with *DNA damage levels in the lowest quartile*. Smoking was associated with higher DNA damage in squamous epithelium in all patient groups (P < 0.01) and in Barrett's mucosa (P < 0.05) in Barrett's esophagus patients only. In controls only, current reflux was associated with higher DNA damage, whereas anti-inflammatory drug use resulted in lower levels. Collectively, these data imply a genotoxic insult to the premalignant Barrett's mucosa that may explain the genetic instability in this tissue and the progression to adenocarcinoma. There is an indication for a role for smoking in inducing DNA damage in esophageal mucosa but an understanding of the role of reflux requires further investigation. (Cancer Epidemiol Biomarkers Prev 2005;14(3):620–5)

Introduction

The incidence of esophageal adenocarcinoma is increasing in the western world (1-3). Esophageal adenocarcinoma is thought to develop on a background of Barrett's esophagus, where the normal squamous epithelium is replaced by a metaplastic columnar type epithelium. Although several other types of columnar epithelium can be found in the lower esophagus, it is widely accepted that it is only specialized intestinal metaplasia (SIM), distinguished by the presence of goblet cells, which confers an increased cancer risk. The metaplasia in Barrett's esophagus patients accumulates genetic alterations and can progress through dysplasia to esophageal adenocarcinoma (1).

A number of risk factors have been linked to development of esophageal adenocarcinoma with the strongest evidence for gastroesophageal reflux (4). Other risk factors include obesity, hiatal hernia, and certain medications, all of which may act through predisposing to gastroesophageal reflux and tobacco smoke (3). In addition, diets high in fat, calories, and cholesterol and low in antioxidants and fiber confer an increased risk (5, 6).

Gastroesophageal reflux is common in the western world with as many as 30% of the population experiencing monthly symptoms and ~10% of gastroesophageal reflux patients eventually develop Barrett's esophagus (3). However, it is unclear at a molecular level how gastroesophageal reflux leads to esophageal carcinogenesis in Barrett's esophagus patients. One possibility is that components of reflux either directly or indirectly induce the genetic changes that accompany the disease process. Prolonged acid and bile reflux are common in Barrett's esophagus patients (7, 8) and reflux components can result in mucosal injury (9) and DNA damage either directly or indirectly through an inflammatory process (10-13). The reduced antioxidant capacity in Barrett's mucosa may further contribute to tissue injury and genetic damage (14, 15). Increased free radical–associated cellular damage has also been reported in experimental models of reflux (16).

In the current study, a modified version of the single cell gel electrophoresis assay (comet assay; refs. 17, 18) was used to measure promutagenic DNA damage in biopsies of esophageal squamous, Barrett's, and gastric mucosa from control, Barrett's esophagus, and esophageal adenocarcinoma patients. The comet assay measures strand breaks and alkali-labile sites in DNA; incorporation of the Fapy glycosylase (Fpg) enzyme in to the assay reveals additional damage including 8-hydroxydeoxyguanosine, a common marker of oxidative DNA damage. This study permitted a comparison of DNA damage both with progression of esophageal disease and in relation to exposure to known risk factors for Barrett's esophagus and esophageal adenocarcinoma, including gastroesophageal reflux and smoking.

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Materials and Methods

Materials. DMEM, FCS, and trypsin-EDTA were all from Invitrogen Ltd. (Paisley, United Kingdom). Collagenase (type IV from *Clostridium histolyticum*) and Pronase (from *Streptomyces griseus*) were from Sigma (Dorset, United Kingdom). The A549 cell line was from the European Collection of Cell Cultures (Porton Down, United Kingdom). Fapy glycosylase enzyme was a gift from Professor A. Collins (University of Oslo, Oslo, Norway). Fapy glycosylase was supplied as a crude extract with no reported nonspecific nuclease activity at the concentrations used in this study.

Patient Recruitment. Ethics committee approval was from the Leeds Teaching Hospitals NHS Trust and informed consent was obtained from each participant. Three patient groups were recruited from endoscopy and endoscopic ultrasound lists at Leeds General Infirmary and St. James' University Hospital. The first group comprised 64 patients who were attending endoscopy for a variety of conditions but did not have a diagnosis of Barrett's esophagus; these are called ''control patients''. Indication for endoscopy in this group was primarily dyspepsia and epigastric abdominal pain but also included dysphagia, diarrhea, weight loss, nausea, and suspected celiac disease. Of these, 42 reported a history of reflux symptoms, 21 having current reflux symptoms. The second group initially comprised 62 patients with a histologic diagnosis of Barrett's esophagus with SIM on a previous endoscopy. Histologic analysis was performed on biopsies collected at the time of the current study. This analysis provided confirmation of Barrett's esophagus in 50 patients (i.e., SIM with goblet cells, visualized by alcian blue staining) and this group is referred to as ''Barrett's esophagus patients''. Ten patients with endoscopically visible Barrett's esophagus were found upon histologic analysis not to have SIM. Nine of these were diagnosed as gastric-fundic, junctional, cardia, or gastric type mucosa and were considered in the analysis as a separate group, called ''no SIM patients''; the remaining patient was found to have normal squamous mucosa on histologic examination and was excluded from the analysis. A further two Barrett's esophagus patients were transferred to the esophageal adenocarcinoma group (see below) as they were found to have high-grade dysplasia on this histologic assessment. The third group comprised 24 patients with a histologic diagnosis of Barrett's complicated by high-grade dysplasia (n = 9) or adenocarcinoma (n = 15) and is referred to as ''esophageal cancer patients''. High-grade dysplasia patients were included in this group by virtue of the high frequency of progression and incidental adenocarcinoma reported in such individuals (19). For these patients, histologic confirmation of high-grade dysplasia or adenocarcinoma was obtained from biopsies taken at the most recent endoscopy, normally no more than 2 weeks before the endoscopic ultrasound list at which the biopsies for DNA damage were obtained. None of the esophageal adenocarcinoma patients had received any chemotherapy or radiotherapy before recruitment into the study.

Tissue Collection. Biopsies were obtained from three mucosal areas in Barrett's esophagus and esophageal adenocarcinoma patients: Barrett's esophagus biopsies were collected from the middle of the Barrett's esophagus segment (clear of endoscopically visible tumor in esophageal adenocarcinoma patients), squamous epithelium was sampled clear of the squamocolumnar junction and gastric biopsies were obtained from the corpus of the stomach. In the control patients, biopsies were taken from squamous epithelium above the gastroesophageal junction. Single biopsies were used for comet analysis and each of these was sampled adjacent to biopsies submitted for histology. Biopsies were stored in DMEM and 10% DMSO at 4°C overnight before comet analysis the following day. Storage of esophageal biopsies in this way does not affect the levels of DNA damage (18).

Questionnaire Data. All patients completed a standard questionnaire that detailed their reflux symptoms, medication, alcohol consumption, and smoking status (''never'' versus ''ever'' smokers with the latter group incorporating current and ex-smokers). Patients were asked if they experienced reflux symptoms currently, if they had experienced them previously, and if so for how long, and if they experienced nocturnal reflux. The questionnaire also recorded current prescription of proton pump inhibitors (PPI) for acid suppression, nonsteroidal anti-inflammatory drugs (NSAID), and aspirin. The same investigator (J.R.O.) recruited and interviewed all patients.

Comet Assay. Analysis of esophageal biopsies has been described (17, 18). Biopsies were digested to single cell suspensions with collagenase (0.03%) and pronase (0.05%). Lung epithelial cells (A549) were processed as a negative control with each series of biopsies. Cells were embedded in agarose on microscope slides and subjected to detergent treatment to disrupt cell membranes and remove proteins. After washing, slides were either treated with 50 μl of Fapy glycosylase enzyme (diluted in enzyme buffer) or enzyme buffer alone. Cells were equilibrated in an alkaline buffer before electrophoresis (pH > 13). After neutralization, comets were stained with ethidium bromide viewed using fluorescent microscopy and quantified as a percentage of the total DNA in the comet tail using ''Komet 4.0'' software (Kinetic Imaging Ltd., Nottingham, United Kingdom).

Statistical Analysis. A minimum of 50 randomly selected comet cells was scored for each biopsy and the median percent tail DNA calculated. These data were not normally distributed and were therefore natural log transformed for analysis. For tabulation, data were back transformed and geometric means [±95% confidence interval (95% CI)] presented. In univariable analysis, the χ^2 test was used for categorical data, and t test or ANOVA for continuous data. Variables with P values < 0.2 were included in regression models to determine the risk factors for DNA damage. Significant factors such as age, sex, and smoking status together with DNA damage data were entered into the logistic regression model to determine which of these factors contributed to the presence of adenocarcinoma or high-grade dysplasia among Barrett's esophagus patients. Analyses were performed using STATA 7.0 software (Stata Co., College Station, TX).

Results

The comet assay was used with and without Fapy glycosylase enzyme to measure DNA damage. The latter enzyme can convert additional damage such as 8-hydroxydeoxyguanosine to strand breaks detectable by the comet assay (20). For clarity, comet assay results are presented without Fapy glycosylase inclusion. Where an effect specific to Fapy glycosylase inclusion was identified this is indicated, as it highlights oxidative processes as having particular significance in the DNA-damaging process.

Mucosal Type, Disease Status, and DNA Damage. Demographic data for the patient groups are shown in Table 1. As expected, there were more males among the Barrett's esophagus and esophageal adenocarcinoma patients and these groups had a higher mean age.

Among Barrett's esophagus patients DNA damage was significantly higher in Barrett's epithelium compared with matched squamous (P < 0.001) and gastric mucosa (P < 0.001;

Table 1. Patient details

	Controls	No SIM	Barrett's esophagus	Esophageal adenocarcinoma
No.	64	9	50	24
Mean age (range)	52 (28-83)	53 (32-69)	63 (30-82)	70 (58-86)
Sex (M/F)	36:28*	7:2	34:16	20:4
Current reflux symptoms	21 (33)	2 (22)	15 (30)	5 (21)
Previous reflux symptoms	42 (66)‡	8 (89)	50 (100)	21 (88)
Nocturnal reflux (%)	22 (34)‡	5 (56)	37 (74)	9 (38)
Duration of reflux (median years, range)	3 (1-26)†	5.5 (4-20)	10 (1-50)	10 (1-50)
Hiatus hernia	5 (8)‡	1 (11)	22 (44)	NI
Esophagitis	9 (14)	1 (11)	6 (12)	NI
Ever smoking	32 (50)	2 (22)	25 (50)	13 (54)
Alcohol	48 (75)	7 (78)	38 (76)	16 (67)
NSAID/aspirin	11 (17)	1 (11)	8 (16)	6 (25)
Current PPI use	26 (41)	9 (100)	42 (84)	16 (67)

NOTE: Data shown are no. individuals (%) unless otherwise indicated.
Abbreviation: NI, no information.
*$P < 0.05$ compared to esophageal adenocarcinoma.
†$P < 0.05$ compared to Barrett's esophagus and esophageal adenocarcinoma.
‡$P < 0.001$ compared to Barrett's esophagus.

Fig. 1). Fapy glycosylase enzyme increased the level of detectable DNA damage in all three types of mucosa in all tissues to a similar extent (Fig. 1). The pattern of DNA damage in esophageal adenocarcinoma patients was similar to Barrett's esophagus patients, in that Barrett's mucosa was higher than in matched squamous ($P < 0.001$) and gastric mucosa ($P = 0.116$) and increased following Fapy glycosylase treatment (Fig. 1). Within the esophageal adenocarcinoma group, there was no difference in the DNA damage measured in high-grade dysplasia and adenocarcinoma patients (data not shown).

DNA damage in the Barrett's mucosa of the esophageal adenocarcinoma patients was slightly higher than that in the Barrett's esophagus group but this was not statistically significant (esophageal adenocarcinoma: mean % tail DNA, 25.4; 95% CI, 22.2-29.1 and Barrett's esophagus: 23.3; 95% CI, 21.7-25.0; $P = 0.203$; see Fig. 1). However, when DNA damage in Barrett's epithelium was considered as a categorical variable, the upper quartile of DNA damage was associated with an increased risk of esophageal adenocarcinoma compared with the lowest quartile of damage (odds ratio, 5.3; 95% CI, 1.1-24.9; $P = 0.033$; Table 2). In addition to DNA damage, sex, nocturnal reflux, and PPI medication also showed differences ($P < 0.2$) between Barrett's esophagus and esophageal adenocarcinoma in univariable analysis. These factors, together with smoking status and age were included in a logistic regression model to investigate the contribution of each factor to disease progression. The increased risk of esophageal adenocarcinoma in Barrett's epithelium associated with DNA damage levels in the upper quartile compared with lower quartile remained significant after adjustment for the above factors (odds ratio, 9.4; 95% CI interval, 1.1-83.4; $P = 0.044$). There was no such association with increased esophageal adenocarcinoma risk when DNA damage levels were stratified in squamous or gastric epithelium.

A comparison of the squamous epithelium in control, Barrett's esophagus and esophageal adenocarcinoma patients revealed higher DNA damage in the squamous mucosa of control (17.9%; 95% CI 16.3-19.7) compared with that of Barrett's esophagus patients (15.1%; 95% CI, 13.6-16.8, $P = 0.02$; Fig. 1). The same pattern was present with Fapy glycosylase but the differences were reduced ($P = 0.235$). No difference was seen between the squamous mucosa of control and esophageal adenocarcinoma patients.

We also compared the Barrett's esophagus patients with the patients without histologic confirmation of SIM (''no SIM''). The latter patients had significantly lower levels of DNA damage in ''columnar epithelium'' ($P = 0.029$), squamous ($P = 0.025$), and gastric ($P = 0.007$) mucosa compared with the Barrett's esophagus group (Fig. 2).

Risk Factors and DNA Damage. As expected, hiatus hernia, previous reflux symptoms, and nocturnal reflux were more common in Barrett's esophagus patients than controls (Table 1). In addition, duration of reflux symptoms was significantly longer ($P < 0.05$) in the Barrett's esophagus and esophageal adenocarcinoma patients. However, current reflux symptoms were not associated with the presence of Barrett's esophagus ($P = 0.696$), which may reflect more frequent use of PPI medication in this latter group than in controls (Table 1). All variables from the questionnaire were assessed in relation to DNA damage.

In control patients, the levels of DNA damage were higher with self-reported current and previous reflux symptoms as well as with experiencing nocturnal reflux, although only the association with current reflux symptoms was statistically significant ($P = 0.013$; Table 3). Current PPI use was not correlated with DNA damage (data not shown). No association was found between any measure of reflux and DNA damage in Barrett's esophagus patients (Table 3). There was no significant association in any patient group between reflux and DNA damage when Fapy glycosylase enzyme was included in the assay (data not shown).

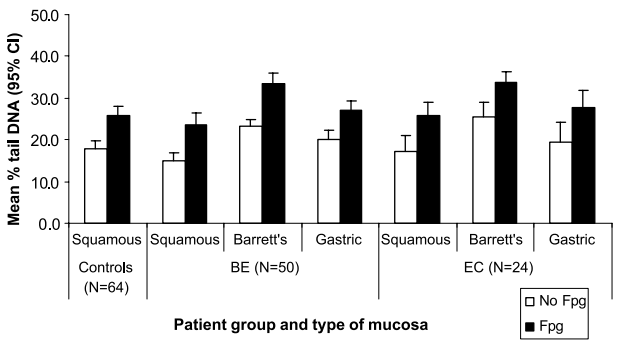

Figure 1. DNA damage levels in control, Barrett's esophagus (*BE*), and esophageal adenocarcinoma (*EC*) patients. DNA damage was measured in the comet assay as described in Materials and Methods. Mean % comet tail DNA with 95% CI for each tissue type examined in each patient group, both with and without incorporation of Fapy glycosylase (*Fpg*) enzyme at a dilution of 1:3,000.

Table 2. DNA damage as a risk factor for development of esophageal adenocarcinoma (adenocarcinoma or high-grade dysplasia)

	DNA damage (% tail) in Barrett's tissue (no Fapy glycosylase)	No. Barrett's esophagus patients	No. esophageal adenocarcinoma patients	Odds ratio, crude (95% CI)	*P* crude	Adjusted odds ratio (95% CI)	*P* adjusted
1st quartile	<20.7	16	3	1.0 (reference)		1.0 (reference)	
2nd quartile	20.7-23.6	11	7	3.4 (0.7-16.1)	0.124	7.2 (0.9-60.1)	0.068
3rd quartile	23.6-28.1	14	5	1.9 (0.4-9.4)	0.430	2.0 (0.2-17.9)	0.538
4th quartile	>28.1	9	9	5.3 (1.1-24.9)	0.033	9.4 (1.1-83.4)	0.044

NOTE: Data adjusted for age, sex, nocturnal reflux, smoking, and PPI medication.

Significant associations were observed between DNA damage and smoking. When considering all patient groups combined DNA damage (mean % tail DNA) was significantly raised in the squamous mucosa of ever smokers (17.9%; 95% CI, 16.3-19.7) compared with never smokers (14.7%, 95% CI, 13.4-16.2; $P < 0.01$); this difference remained after the addition of Fapy glycosylase ($P < 0.01$). Among the individual patient groups, the association with smoking was observed in the squamous epithelium of control patients whereas in Barrett's esophagus patients, DNA damage was higher in both Barrett's and squamous mucosa (Table 4). In addition, current use of anti-inflammatory drugs (NSAID and aspirin) was associated with lower DNA damage levels in control patients particularly after Fapy glycosylase enzyme inclusion (Table 4).

Discussion

The two primary aims of this study were first, to examine whether DNA damage is higher in Barrett's esophagus patients who have developed high-grade dysplasia or adenocarcinoma compared with those who have not, and second, to assess whether the level of DNA damage could be explained by exposure to known risk factors for Barrett's esophagus and esophageal adenocarcinoma.

Mucosal Type, Disease Status, and DNA Damage. DNA damage was higher in Barrett's mucosa compared with both matched squamous esophageal and gastric mucosa in Barrett's esophagus and esophageal adenocarcinoma patients. We did not observe major differences in Fapy glycosylase–sensitive lesions among the three types of mucosa suggesting that this type of damage, which would include some forms of oxidative DNA damage, is not specific to any particular mucosal tissue type. Both acid and bile can induce DNA damage in esophageal cells (10). Other types of DNA damage, including bulky DNA adducts (21) are also found. Further analysis of the chemical nature of the DNA damage in esophageal epithelium in relation to risk factors will be helpful in elucidating the etiopathogenesis of the disease (see below). Overall, these data suggest a particular susceptibility of the metaplastic cells of Barrett's mucosa to DNA damage. Coupled with the increase in cell proliferation observed with exposure to bile salts and acid (22), the presence of increased DNA damage may lead to a cellular environment that is prone to the genetic alterations (e.g., p16 loss of heterozygosity and promoter methylation, *TP53* mutation), seen as metaplasia progresses to dysplasia and adenocarcinoma (1-3).

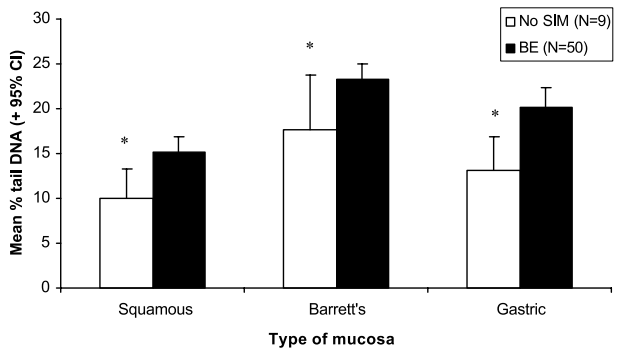

Figure 2. DNA damage levels in Barrett's esophagus (*BE*) and ''no SIM'' patient groups. DNA damage was measured in the comet assay as described in Materials and Methods. Mean % comet tail DNA with 95% CI for Barrett's esophagus patients and patients ascribed to the no SIM group, (i.e., that did not have SIM at the time of the study but had a previous diagnosis of Barrett's esophagus). Data are shown for each tissue type without incorporation of Fapy glycosylase enzyme. *, $P < 0.05$ when compared with mean % tail DNA as measured by the comet assay in the same tissue type in the Barrett's esophagus patient group.

A comparison of Barrett's mucosa in Barrett's esophagus and esophageal adenocarcinoma patients revealed slightly higher DNA damage in the latter group. In a logistic regression model, those Barrett's patients with DNA damage in the highest quartile had a 9.4-fold increased risk of having esophageal adenocarcinoma compared with those in the lowest quartile. There was no association between increased DNA damage and esophageal adenocarcinoma risk when DNA damage was stratified in squamous or gastric epithelium. This suggests that the excess risk of esophageal adenocarcinoma development is specific to elevated DNA damage in Barrett's mucosa, rather than a more general increase in DNA damage levels in cancer patients. Although Barrett's esophagus confers a high relative risk of developing esophageal adenocarcinoma, the majority of Barrett's esophagus patients do not progress to adenocarcinoma. It is therefore possible that a subgroup of Barrett's esophagus patients, more susceptible to DNA damage within Barrett's mucosa, would subsequently be at highest risk of disease progression. Possible causes of increased susceptibility to DNA damage include differential response to reflux in terms of cell proliferation and expression of oxidative stress genes such as *COX-2* (12) or variations in expression of genes coding for carcinogen metabolism (23) or DNA repair (24). We consider it unlikely that the tumor itself was the cause of the higher level of DNA damage in the esophageal adenocarcinoma patients because the Barrett's mucosa biopsies were taken distant from the tumor or any visible nodule within an area of high-grade dysplasia. There is a possibility that the increase in damage in the esophageal adenocarcinoma group may be a consequence of dysplasia in the biopsy analyzed, although unfortunately histology was not available to determine this.

Previously, it has been established that patients with Barrett's mucosa without SIM are not at increased risk of malignancy. In a small group of such patients, we observed a significantly lower level of DNA damage in the columnar mucosa in comparison with the Barrett's mucosa in the Barrett's esophagus group. However, another feature of the ''no SIM'' group is that each of the nine patients had

Table 3. Reflux risk factors and their effect on DNA damage levels in the comet assay (without Fapy glycosylase) in Barrett's esophagus and control patients

Reflux risk factor	Mean % tail DNA (95% CI)			
	Barrett's esophagus group			Controls
	Squamous	Barrett's	Gastric	Squamous
Current reflux symptoms				
No	15.3 (13.6-17.1, n = 35)	23.0 (21.2-24.9)	19.6 (17.4-22.0)	16.5 (14.6-18.7*, n = 43)
Yes	14.8 (11.5-19.1, n = 15)	24.0 (20.6-28.0)	21.3 (16.8-27.0)	21.2 (18.5-24.3, n = 21)
Previous reflux symptoms				
No	NA	NA	NA	16.4 (14.1-19.2, n = 22)
Yes	15.1 (13.6-16.8, n = 50)	23.3 (21.7-25.0)	20.1 (18.1-22.3)	18.8 (16.6-21.2, n = 42)
Nocturnal reflux				
No	14.9 (12.2-18.2, n = 13)	23.6 (20.6-27.1)	21.0 (17.8-24.7)	17.0 (15.2-18.9, n = 42)
Yes	15.2 (13.4-17.3, n = 37)	23.2 (21.3-25.2)	19.8 (17.3-22.6)	20.0 (16.5-24.1, n = 22)
Hiatus hernia				
No	14.2 (12.2-16.5, n = 28)	22.5 (20.3-25.0)	20.4 (17.4-24.0)	17.9 (16.3-19.5, n = 59)
Yes	16.5 (14.2-19.0, n = 22)	24.3 (22.2-26.7)	19.7 (17.1-22.6)	18.8 (7.2-49.2, n = 5)

NOTE: Not applicable (NA) as all Barrett's esophagus patients reported reflux symptoms.
*P = 0.013 (t test for means between no/yes groups).

relatively short segments of endoscopically visible Barrett's esophagus (range, 1-4 cm). Notwithstanding the possibility of sampling error in missing SIM in these patients, it will be of interest to design future studies to specifically test whether low levels of DNA damage are associated with the lower risk of malignancy in patients without SIM or with short-segment Barrett's esophagus.

Risk Factors and DNA Damage. In the squamous epithelium of control patients, there was a positive association between reflux symptoms and DNA damage. However, no such association was found in Barrett's esophagus patients. The majority of Barrett's esophagus patients were prescribed a PPI; therefore, the reflux experienced by this latter patient group may have been less severe than among the control patients. Furthermore, self-reported reflux symptoms have been found inaccurate, with many asymptomatic Barrett's esophagus patients often continuing to experience reflux episodes (25). In addition, if bile contributes to DNA damage then self-reported symptoms after PPI prescription may lead to misclassification of exposure to key reflux components. A more reliable and representative measure of reflux would be valuable, possibly through pH and ''Bilitec'' monitoring, although these techniques also have their limitations.

The strongest risk factor associated with DNA damage in the esophageal mucosa was tobacco smoking. Higher DNA damage was observed in the squamous epithelium across all patient groups and when control and Barrett's esophagus patients were analyzed independently (Table 4). It is unclear why the strength of association varied with and without Fapy glycosylase: control patients with Fapy glycosylase (P = 0.02) and without Fapy glycosylase (P = 0.20); Barrett's esophagus patients with Fapy glycosylase (P = 0.10) without Fapy glycosylase (P = 0.01; adjusted data). This variation may represent the relatively small numbers of subjects rather than differences in types of DNA damage across groups. Despite this, the overall pattern of increased DNA damage in ''ever'' smokers was clear. Higher DNA damage in the squamous mucosa is consistent with the strong increase in risk of squamous cell carcinoma among smokers (26). Smoking is also a moderate risk factor for esophageal adenocarcinoma (3) but is not generally recognized as a significant risk factor for gastric cancer (27) and this is again consistent with the fact that there was an association between DNA damage and smoking in Barrett's mucosa but no such relationship in gastric mucosa.

Use of anti-inflammatory drugs (NSAID/aspirin) was associated with lower levels of DNA damage in squamous mucosa of control patients (Table 4). NSAIDs are associated

Table 4. The effect of smoking and NSAID use on DNA damage levels in Barrett's esophagus and control patients

	Mean % tail DNA (95% CI)				
	Barrett's esophagus group			Control group	
	Squamous (no Fapy glycosylase)	Barrett's (no Fapy glycosylase)	Gastric (no Fapy glycosylase)	Squamous (no Fapy glycosylase)	Squamous (Fapy glycosylase)
Smoke					
Never	13.7 (11.8-15.9, n = 25)*	21.8 (20.2-23.4)*	18.3 (15.9-21.1)	16.8 (14.9-18.8, n = 32)	23.0 (20.5-25.8)†
Ever	16.8 (14.5-19.4, n = 25)	24.9 (22.2-28.0)	22.0 (18.8-25.8)	19.2 (16.4-22.4, n = 32)	28.6 (25.4-32.2)
Adjusted for age, sex and NSAID/aspirin					
Never	20.7 (14.4-30.0)*	21.5 (16.6-28.2)‡	21.1 (14.3-31.5)	13.7 (10.6-18.0)	19.5 (15.6-24.5)*
Ever	26.8 (17.8-40.0)	24.8 (18.5-33.1)	25.8 (16.8-40.0)	15.6 (11.8-20.7)	23.6 (18.4-30.3)
NSAID/aspirin					
No	15.0 (13.3-16.9, n = 42)	23.2 (21.5-25.1)	19.8 (17.5-22.3)	18.6 (16.6-20.7, n = 53)	26.8 (24.3-29.5)*
Yes	15.8 (12.1-20.6, n = 8)	23.6 (19.6-28.5)	21.7 (17.2-27.4)	15.1 (13.0-17.6, n = 11)	21.3 (18.0-25.1)
Adjusted for age, sex, smoking, and PPI use					
No	21.3 (16.8-26.8)	24.5 (20.9-29.1)	22.6 (17.6-29.4)	18.5 (14.9-23.1)	29.1 (23.8-35.5)§
Yes	27.1 (18.0-40.4)	25.3 (19.1-33.8)	26.3 (16.9-40.9)	15.3 (11.4-20.5)	23.8 (18.2-30.9)

*P < 0.05 (t test for means between never and ever smoking or current use or not of NSAID/aspirin).
†P < 0.01 (t test for means between never and ever smoking).
‡P = 0.058 (t test for means between never and ever smoking).
§P = 0.056 (t test for means between current use or not of NSAID/aspirin).

with a reduced esophageal cancer risk (28) possibly through the inhibition of cyclooxygenase-2, an enzyme found overexpressed in both malignant and premalignant tissues in the esophagus (29). These drugs may limit DNA damage indirectly through their anti-inflammatory activity. This concurs with the strongest association between their use and lower DNA damage occurring in the comet assay with Fapy glycosylase (Table 4), which includes measurement of the oxidative lesion 8-hydroxydeoxyguanosine. This observation may prove useful in the management of reflux patients and as such merits further investigation.

Further studies should also consider the role of obesity on levels of DNA damage, as obesity is a risk factor for esophageal adenocarcinoma (3). Unfortunately, body mass index data were not collected in the present study, so we are currently unable to test the association between body mass index, reflux, and DNA damage. In addition, the sample sizes for each of the patient groups, particularly the esophageal adenocarcinoma group were relatively limited in this study. Additional studies should include larger patient groups, to increase confidence in the associations identified in this study between risk factors and DNA damage levels in the esophagus.

In summary, this study shows that preneoplastic Barrett's mucosa contains higher levels of DNA damage than normal squamous or gastric mucosa. The highest levels of DNA damage were measured in Barrett's mucosa of patients who had progressed to adenocarcinoma. These data are consistent with a continuous genotoxic insult in Barrett's mucosa that may, in part, explain the genetic instability of this tissue and have a role in disease progression. DNA damage levels have been shown to be modulated by acid and bile treatments in esophageal cell lines (10) illustrating a possible causal link between levels of DNA damage measured in Barrett's esophagus patients and their risk of progression. Smoking, a known risk factor for esophageal cancer was associated with increased DNA damage, whereas control patients taking anti-inflammatory drugs (NSAID/aspirin), a factor linked to a decreased cancer risk, had lower DNA damage levels. Elevated levels of DNA damage may enhance malignant progression through increasing the likelihood of genetic change, including mutations in key tumor suppressor genes such as *TP53*. Studies into the mechanisms by which reflux contributes to esophageal adenocarcinoma risk requires further investigation with more precise measures of reflux and alternative assays of DNA damage.

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