Effect of Acid Suppression on Molecular Predictors for Esophageal Cancer

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Abstract

Background: Gastroesophageal reflux disease is a risk factor for the development of Barrett’s esophagus and esophageal adenocarcinoma. The effect of antireflux therapy on the incidence of esophageal adenocarcinoma is unknown. Acid exposure in vitro induces hyperproliferation via a cyclooxygenase-2 (COX-2) dependent mechanism. Epidemiologic and animal studies suggest that COX inhibitors decrease the incidence of esophageal adenocarcinoma.

Aim: To study the differential effect of complete compared with incomplete acid suppression on proliferation, apoptosis, and COX-2.

Patients and Methods: Fifty-one patients with Barrett’s esophagus who underwent pH monitoring were divided into two groups according to their DeMeester score: 32 acid-suppressed patients (group 1) and 19 patients with abnormally high acid exposure (group 2). Slides from biopsies taken 3 months before and 4 and 12 months after pH monitoring were stained for Mcm2, COX-2, c-myc, and cleaved caspase-3 (marker of apoptosis).

Results: There was no evidence of a difference between the two groups in terms of age, gender ratio, medication, dysplasia status, and the expression levels of any marker before pH monitoring. In group 1, Mcm2 expression decreased in the luminal surface and throughout the tissue 12 months after monitoring when compared with the two previous time points (P < 0.05). The levels of COX-2 increased overtime (P < 0.01 in group 1, not significant in group 2). There was no correlation between Mcm2 and COX-2 expression. Acid suppression had no effect on c-myc or apoptosis.

Conclusion: Long-term acid suppression reduces proliferation in Barrett’s esophagus samples but has no advantageous effect on c-myc, apoptosis, or COX-2.

Key words: Barrett’s esophagus; esophageal adenocarcinoma; acid suppression; proliferation; apoptosis; COX-2.

Introduction

The incidence of esophageal adenocarcinoma has increased dramatically in the west in the last 30 years (1). Esophageal adenocarcinoma generally arises from a premalignant metaplastic columnar epithelium which replaces the distal native squamous epithelium, called Barrett’s esophagus (2). A long history of gastroesophageal reflux disease has been shown to be an independent risk factor for the development of esophageal adenocarcinoma (3) and Barrett’s esophagus (4) and the duration of the reflux episodes correlated with the length of the Barrett’s esophagus segment (5). The apparent role for gastroesophageal reflux disease in the development of esophageal adenocarcinoma and Barrett’s esophagus has triggered the search for chemopreventive strategies, which involve the complete eradication of gastroesophageal reflux.

Both surgical and antireflux therapies lead to a partial endoscopic regression of Barrett’s esophagus, the appearance of squamous islands (6, 7), and a decrease in the incidence of dysplasia (7-9). Despite the evidence for some endoscopic and histopathologic improvement, these therapies rarely result in the complete eradiation of the Barrett’s segment (10). Some individual studies have shown a small reduction in cancer incidence in patients treated with surgical, compared with medical, antireflux therapies (11-13). However, a meta-analysis by Corey et al. (14) did not show any statistical significance between the two treatments. It should be noted that these studies lacked a control group because all patients had some form of acid suppression treatment.

This apparently contradictory evidence may result from the nonstandardization of the types and doses of acid suppressants used, as well as the variation in surgical procedures. Furthermore, in these studies, the efficacy of the antireflux treatment was generally inferred from the self-reported reflux symptomatology. However, it is clear from several independent investigations that symptom control does not always concord with complete intrasophageal pH normalization (7, 15, 16). Normalization of acid reflux may be important in view of laboratory evidence, which suggests that pulsatile reflux exposure might alter the cell kinetics in favor of malignant progression. Specifically, pulsatile acid exposure of Barrett’s esophagus using ex vivo and in vitro model systems has been shown to increase cell proliferation and reduce apoptosis via activation of mitogen-activated protein kinase pathways (17-19). In a subsequent clinical study, which attempted to control for the amount of reflux exposure, Ouattu-Lascar et al. (16) showed that complete acid normalization with lansoprazole treatment decreased the proliferative index in Barrett’s esophagus patients, as measured by proliferating cell nuclear antigen expression. In the same year, an independent group showed that although complete normalization with omeprazole had no effect on proliferation, incomplete normalization with ranitidine led to an increased proliferation (6). These results were further supported by a surgical series in which incompletely acid-suppressed patients had an increased epithelial proliferation rate (20).

As well as acid containing refluxate, bile reflux has also been shown to occur frequently in Barrett’s esophagus patients (21). In vitro and ex vivo data have shown that acidified bile induced...
c-myc expression (22) and caused hyperproliferation via cyclooxygenase-2 (COX-2) and protein kinase C (23). On the other hand, inhibition of COX-2 or protein kinase C led to a decrease in proliferation in \textit{ex vivo} cultures of Barrett’s explants and \textit{in vitro} adenocarcinoma cell cultures (18, 24). Furthermore, in retrospective, epidemiologic studies of non-steroidal anti-inflammatory drugs were shown to reduce the risk of progression to esophageal adenocarcinoma (25, 26). Animal models of duodenogastresophageal reflux have also suggested that COX-2 inhibitors might decrease the risk of esophageal adenocarcinoma arising within Barrett’s esophagus (27-29). Further to this work, a chemoprevention trial, called Aspirin Esomeprazole Chemoprevention Trial, is in progress to study the effect of high-dose versus low-dose proton pump inhibitor therapy with or without aspirin on the risk of cancer progression in a large prospective male cohort over 8 years (http://www.digestivediseases.org/news.shtml).

Using esophageal adenocarcinoma as the end point for chemoprevention trials is ideal; however, there are a number of practical difficulties with this approach in view of the large study size and the long follow-up period required. Therefore, the use of surrogate markers for cancer progression is a useful adjunct to the definitive studies. In addition, biomarker studies may also provide mechanistic information about the molecular basis for cancer progression which may enable a more targeted approach to chemopreventive therapies in the future (for review, see ref. 30). In the hunt for markers of progression to esophageal adenocarcinoma, a number of targets have been identified. Many of these, including alterations in the expression of p16, p53, and cyclin D1 and surface expression of minichromosome maintenance protein (Mcm) 2, can be linked to cell cycle progression (31-33). Until recently, assessment of cell proliferation on fixed tissue has relied on markers with disadvantages, such as Ki-67 and proliferating cell nuclear antigen (34, 35). The cell biological role of Ki-67 is still not worked out and its expression profile is subject to controversy, and proliferating cell nuclear antigen is involved in DNA repair as well as proliferation. Mcm2 is a more sensitive and specific proliferation marker (36, 37).

The markers for this study, COX-2, c-myc, cleaved caspase-3 (marker of apoptosis), and Mcm2, have been selected on the basis of existing evidence discussed above, which suggests that their expression may be affected by reflux exposure. Hence, the aim of this study was to examine the effect of incomplete and complete acid suppression, as defined by rigorous 24-hour pH monitoring criteria, using surrogate markers as the end point.

Materials and Methods

Patient and Tissue Collection. Following approval from the Cambridge Local Research Ethics Committee, we recruited a retrospective cohort of 51 patients who had been part of the Barrett’s surveillance program at Addenbrooke’s Hospital for a minimum of 2 years and who had all had esophageal pH monitoring. Patients had been referred for pH monitoring because they had low-grade dysplasia with ongoing inflammation or had persistent symptoms despite proton pump inhibitors. The diagnosis of low-grade dysplasia was made as part of the normal hospital diagnostic program. The samples with low-grade dysplasia were independently double-reported by two specialist upper gastrointestinal histopathologists.

Patient demographics, symptoms at the time of pH monitoring, medication before and after pH monitoring, as well as histopathologic diagnoses of dysplasia were recorded. Patients were treated using various acid-suppressive drug regimens according to their clinician preferences and we therefore categorized them into four groups according to the British National Formulary guidelines for each drug: no medication, less than (low), equal to (normal), or more than (high) the recommended therapeutic dose of proton pump inhibitor. The ‘high’ group also included patients taking a combination of a therapeutic dose of a proton pump inhibitor in addition to an H2 receptor antagonist. Paraffin-embedded sections were obtained from biopsies taken in the middle part of the Barrett’s esophagus segment as part of the surveillance program. Biopsies were collected from three time points: 2.9 ± 0.6 months before pH monitoring and 4.1 ± 0.7 and 12.4 ± 1.0 months after the pH monitoring at subsequent surveillance endoscopies.

Stationary Manometry and pH Monitoring. Twenty-four-hour ambulatory pH monitoring was undertaken using a portable data recorder (Orion pH monitor, Medical Measurement Service, Dover, NH) connected to single channel glass pH catheter with internal reference (Mediplus). The catheter was passed transnasally and located 5 cm above the lower esophageal sphincter (determined manometrically). During the recording period, the patients recorded any symptoms, their time, upright and supine periods, and were encouraged to pursue their normal daily activities and diet. The data from the recorder were analyzed to determine standard variables, which were also used to calculate the DeMeester score for each patient. A score of >14.72 was considered to be abnormal (38).

Immunostaining. Paraffin-embedded sections were stained with Mcm2 (gift from Steve Dilworth, Division of Investigative Science, Imperial College London, London United Kingdom and Ron Laskey, Hutchison-MRC Research Centre, Cambridge, United Kingdom), COX-2 (Cayman Chemicals, Ann Arbor, MI), c-myc (1:400 dilution; Oncogene Research Products, Nottingham, United Kingdom), or cleaved caspase-3 (1 in 1/500 dilution; Cell Signaling Technology, Beverly, MA) antibodies. The staining procedure for Mcm2 and COX-2 has previously been described (31, 39). Cleaved caspase-3 and c-myc were stained similarly to COX-2. Briefly, tissue sections were deparaffinized and rehydrated. Antigen retrieval was done by pressure cooking. Blocking steps for endogenous peroxidase activity and nonspecific binding were done before incubation with the primary antibody. Biotinylated secondary antibody (Vector Labs, Peterborough, United Kingdom) was then applied followed by Avidin Biotin Complex. Visualization was done using the 3,3’-diaminobenzidine method (Vector). Sections were counterstained with hematoxylin and a negative control was done by omission of the primary antibody.

Scoring of Immunostaining. Mcm2, cleaved caspase-3, and COX-2 immunostainings were quantified according to previously published methods from our group (36, 39) and c-myc quantification was modified from Yuen et al. (40). Briefly, Mcm2 and cleaved caspase-3 expression in the epithelial surface, defined as the most superficial layer of columnar cells and through out the tissue, was quantified as a percentage of total cells (31). Up to 1000 cells were counted and as many well-orientated crypts as possible. The overall distribution and strength of COX-2 and c-myc staining were examined under low-power magnification (×100). Further scoring was done in at least three high-power fields (×400) in each available section to determine the percentage of the positive cells, especially in the lamina propria. Intensity of staining (1, weak staining; 2, moderate staining; 3, strong staining; and 4, very strong staining) and distribution (1, 25% of cells stained; 2, 25-50% of cells stained; 3, 50-75% of cells stained; and 4, >75% of cells stained) were added together to generate a mean score for COX-2 and c-myc.

Statistical Analysis. The frequency of Mcm2 and COX-2 staining was expressed as mean ± SE. Statistical significance between the groups for medication, symptoms, and manometry...
was analyzed by $\chi^2$ test. The Marginal Homogeneity test was used to test for differences in the degree of dysplasia between the groups and over time within the groups. The Friedman's test was used for comparing continuous variables between the two groups and looking at the values of the markers overtime. Analysis of correlation of expression of markers was done with a Spearman’s correlation test. In all cases, $P < 0.05$ was required for significance. Analysis was carried out with SPSS V12.0 (SPSS, Inc., Chicago, IL) and Stat-Xact V4.0 (Cytel Corporation, Cambridge, MA).

**Results**

**Patient Demographics.** The patients were divided into two groups according to their intraoesophageal acid exposure as measured by the DeMeester score (Table 1). Group 1 was composed of 33 patients with a normal acid exposure and group 2 was composed of 19 patients with an abnormally high acid exposure. The two groups did not differ in terms of age, gender ratio, length of Barrett’s esophagus segment, medication levels, and degree of dysplasia before pH monitoring (Table 1). There was also no evidence of a difference in the symptomatology between the two groups although, as expected, the group with abnormal acid exposure had a greater preponderance of reflux symptoms (25% of group 1 versus 47% of group 2). The manometric characteristics of the two groups were not statistically different but group 2 had a higher percentage of patients with a hypocontracting esophagus than group 1 (79% versus 41%, respectively). This manometric abnormality would be in keeping with the higher proportion of group 2 patients with reflux symptoms. Over the follow-up period, the degree of dysplasia showed no evidence of varying over time in either of the two groups.

**Expression of Biomarkers**

**Mcm2 Expression.** Mcm2 expression was confined to the nuclei of epithelial cells (Fig. 1). As expected from previously published data (36, 41), the levels of Mcm2 correlated with the degree of dysplasia ($P < 0.005$; data not shown). In group 1, the overall Mcm2 expression levels decreased 12 months post monitoring compared with the expression levels observed at the start of the study ($P < 0.02$; Fig. 2A). The decrease in Mcm2 levels was also seen at the luminal surface by 12 months after monitoring compared with the samples taken before and 4 months after monitoring ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2B). This is particularly relevant because we have previously shown that increased expression levels of Mcm2 at the luminal surface were a marker of progression to esophageal adenocarcinoma (31). In group 2, Mcm2 expression levels showed no evidence of a change over time.

**COX-2 Expression.** COX-2 expression was localized to the cell membrane and cytoplasm of epithelial cells and to the cytoplasm of lamina propria cells. The deeper levels of the samples (the lower crypts and the glands) usually displayed a stronger intensity of COX-2 staining than the surface epithelium and upper crypts (Fig. 1). The levels of COX-2 before pH monitoring were similar in the two groups (Fig. 3).

The levels of COX-2 increased after 4 and 12 months when compared with 3 months before pH monitoring in the two groups although statistical significance was only found in group 1 ($P < 0.005$ and $P < 0.05$, respectively, for both time points versus before). There was no difference between groups 1 and 2. There was no correlation between the expression of Mcm2 and COX-2 within a given biopsy (Fig. 4).

**c-Myc and Cleaved Caspase-3 Expression.** For both c-myc (Fig. 1) and cleaved caspase-3 (Fig. 1), similar expression levels were observed in the two groups before pH monitoring. Furthermore, no differences in expression were seen over time in any group (data not shown). The expression of c-myc was higher in dysplastic Barrett’s esophagus than in nondysplastic samples in the surface, upper crypt, and throughout the tissue (Fig. 5).

**Discussion**

In this retrospective study, we have analyzed the link between acid suppression, proliferation (c-myc and Mcm2), COX-2, and apoptosis (cleaved caspase-3). Acid-suppressed patients (group 1) were shown to have a decrease in Mcm2 expression over time whereas COX-2 protein levels increased over the same time scale (Figs. 1 and 2). These statistically significant changes in expression were not seen in patients with abnormal pH monitoring. The expression levels of cleaved caspase-3, a marker of apoptosis, and c-myc were not affected by acid suppression.

In this patient cohort, 37% of patients in group 2 were asymptomatic but not acid suppressed and 25% of group 1 patients had reflux symptoms despite adequate acid suppression. This is in agreement with previous work which has shown that symptom control does not equate with adequate acid suppression (16, 42). Eighteen percent (9 of 51) of patients in our cohort were not adequately acid suppressed despite being on supraphysiologic doses of proton pump inhibitors. This is in keeping with 20% patients reported to be unresponsive to therapeutic doses of proton pump inhibitor in the literature (43). This phenomenon may be explained by an individual variation

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**Table 1. Patient demographics, medication, symptoms, manometry, and degree of dysplasia from the retrospective cohort**

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>DeMeester score</td>
<td>4.4 ± 0.6</td>
<td>59.8 ± 7.8</td>
</tr>
<tr>
<td>Age at pH monitoring</td>
<td>61.0 ± 1.5</td>
<td>62.2 ± 2.8</td>
</tr>
<tr>
<td>Gender ratio (male/female)</td>
<td>3.16:1</td>
<td>1.7:1</td>
</tr>
<tr>
<td>Length of Barrett's esophagus segment (cm)</td>
<td>4.1 ± 0.4</td>
<td>4.7 ± 0.7</td>
</tr>
</tbody>
</table>

**Medication**
- None: 33 (3%) vs 19 (5.3%)
- Low: 17 (21%) vs 3 (16%)
- Standard: 13 (39%) vs 6 (32%)
- High: 12 (36%) vs 9 (47%)

**Symptoms**
- Asymptomatic: 19 (58%) vs 7 (37%)
- Reflux: 8 (27%) vs 10 (53%)
- Other: 5 (15%) vs 2 (11%)

**Manometry**
- Normal: 9 (27%) vs 1 (5%)
- IOM: 10 (30%) vs 8 (42%)
- LP LOS: 4 (17%) vs 7 (37%)
- Other: 1 (3%) vs 0
- N/A: 8 (27%) vs 3 (16%)

**Dysplasia**
- No dysplasia: 8 (24%) vs 6 (32%)
- Indefinite for dysplasia: 4 (12%) vs 1 (5%)
- Low-grade dysplasia: 19 (58%) vs 12 (63%)
- High-grade dysplasia/AC: 0 vs 0
- N/A: 2 (6%) vs 0

**NOTE:** The medication, symptoms and manometry were all recorded at the time of pH monitoring. The dysplasia levels were recorded at the surveillance endoscopy before pH monitoring. In the symptoms sections, “Other” includes symptoms that were not specific to gastroesophageal reflux disease. Abbreviations: IOM, intraoesophageal manometry; LP LOS, low pressure at the lower esophageal sphincter; AC, esophageal adenocarcinoma; N/A, no data for these patients.
in pharmacokinetics, mainly dependent on a cytochrome \( P4502C19 \) polymorphism (reviewed in ref. 44). Furthermore, a recent study showed that in patients with persistent acid-reflux despite proton pump inhibitor therapy, altering their medica-

tion did not significantly alter intraesophageal or intragastric pH exposure (45). This may explain why the proliferation index, as measured by Mcm2, was unaltered over time for group 2 even after their medication was changed.

**Figure 1.** Representative sections from a negative control and immunohistochemistry for COX-2, Mcm2, cleaved caspase-3, and c-myc antibodies. Magnification, \( \times 100 \) (left); \( \times 400 \) (right). **Square**, magnified area.
The reduction in cell proliferation in group 1 patients who remained acid suppressed over a 12-month period is in keeping with a previous prospective study which evaluated the effect of complete versus incomplete acid suppression on proliferating cell nuclear antigen expression over a 6-month period (46). The number of patient years in this study is too small to draw any meaningful conclusions about cancer incidence; however, three patients from group 1 developed high-grade dysplasia or esophageal adenocarcinoma over the course of the study. The significant reduction in Mcm2 observed in group 1 over time is more striking when one considers that these three high-grade dysplasia/esophageal adenocarcinoma patients, which were included in the analysis, had a higher proliferation index throughout the study and were thus likely to mask changes resulting from acid-suppression. Interestingly, the expression levels of Mcm2 at the luminal surface were statistically higher for the three progressors than for the nonprogressors even before the histopathologic diagnosis of dysplasia ($P = 0.01$; data not shown). These data are in keeping with previous work published by our laboratory where high levels of Mcm2 at the luminal surface correlated with later progression to cancer (31).

The expression of COX-2 increased over time in both groups although this only reached statistical significance in group 1. Previous work from our laboratory showed a similar increase of COX-2 over time in patients undergoing surveillance. The explanation may be related to induction of COX-2 by gastrin in patients taking proton pump inhibitors because gastrin increases COX-2 expression through stimulation of the CCK receptor (39, 47).

Amplification of the c-myc gene has been reported in high-grade dysplasia and esophageal adenocarcinoma (48, 49) but, to date, this is the first report of increased c-myc expression with the development of dysplasia. In vitro and ex vivo studies suggested that exposure to a pulse of acidified bile increased c-myc (22) and induced hyperproliferation in a COX-2-dependent fashion (23). In addition, pulsatile acid exposure has been shown to decrease apoptosis (18, 19). However, in this study, no differences were seen in any of the groups for both c-myc and cleaved caspase-3 and there was no correlation between COX-2 expression and proliferation as measured by Mcm2 expression. It is possible that the concentration of bile acid in acid reflux was insufficient to trigger a modulation of c-myc expression. In addition, results from an in vitro system cannot always be directly applied in vivo.

Acid suppression by proton pump inhibitor was shown to decrease the degree of dysplasia (6-8) and we have shown in this study that it decreases proliferation at the luminal surface. These data suggest a possible chemopreventive role for proton pump inhibitors in the context of Barrett’s esophagus-associated cancers. Furthermore, our data suggest that acid suppression may increase, rather than decrease, COX-2 expression and, hence, if inhibiting the COX enzyme system is important for cancer prevention, then a dual chemopreventive strategy may be required. Only a randomized control trial with a large number of patient years, such as the ongoing Aspirin Esomeprazole Chemoprevention Trial chemoprevention trial, will determine the role of these drugs in reducing cancer incidence.

In conclusion, we showed that long-term acid suppression reduces proliferation in Barrett’s esophagus samples but has no advantageous effect on c-myc, apoptosis, or COX-2. The results from prospective studies to test the clinical validity of proton pump inhibitors as part of a chemopreventive strategy are eagerly awaited.

**Figure 2.** Mcm2 expression as a percentage of total cells throughout the whole tissue section which included the surface and the glands (Overall) and at the luminal surface over time ($*, P < 0.05$; $**, P < 0.005$). The time in months corresponds to the time before and after pH monitoring. Group 1 are completely acid suppressed and group 2 have persistent acid reflux despite proton pump inhibitor (PPI) therapy. The graph represents each individual point; bars, mean.

**Figure 3.** COX-2 expression over time in group 1 (acid suppressed) and group 2 (abnormal intraesophageal pH group). $*, P < 0.05$; $**, P < 0.005$. The time in months corresponds to the time before and after pH monitoring. Columns, mean; bars, SE.

**Figure 4.** Correlation of COX-2 and Mcm2 expression in all the samples at the three time points.

**Figure 5.** Expression of c-myc in dysplastic and nondysplastic Barrett’s esophagus in the luminal surface, upper crypts (Upper), proliferative compartment (Prolif), and throughout the tissue (Overall). $**, P < 0.01$; $* P < 0.05$. Columns, mean; bars, SE.
Acknowledgments
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References