The development of new treatment strategies for oesophageal cancer

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Cyclooxygenase-2 (COX-2) expression has been demonstrated to be upregulated in oesophageal cancer, but its clinical significance remains unclear. Prognostic significance of elevated cyclooxygenase-2 expression in patients with selective COX-2 inhibitors as a novel chemotherapeutic treatment for adenocarcinoma of the oesophagus was analysed in this study.
INTRODUCTION

Adenocarcinoma of the oesophagus is a highly lethal disease, which incidence has markedly increased during the last few decades. The pathogenesis of oesophageal adenocarcinoma follows a sequence of events during which the normal squamous epithelium is replaced by metaplastic specialised columnar epithelium (Barrett oesophagus) in response to (duodeno-)gastro-oesophageal reflux disease. Barrett oesophagus can subsequently progress to low-grade and high-grade dysplasia and eventually to invasive cancer. Surgical resection is currently the only curative treatment. However, postoperative mortality and morbidity are substantial and even after intentionally curative resection, five-year survival rates rarely exceed 25%.

Preoperative knowledge of prognostic factors (i.e. depth of invasion, lymph node involvement and distant metastases) is necessary to identify patients eligible for surgery, but clearly new prognostic markers and novel adjuvant treatment strategies are needed. Epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of cancer especially in the digestive tract. The best known target of NSAIDs is the cyclooxygenase (COX) enzyme. Two COX enzymes are known, but it is the inducible COX-2 isoenzyme that has been linked to diseases, such as chronic inflammation and carcinogenesis. Recently, a selective COX-2 inhibitor was shown to reduce polyp burden in patients with familial adenomatous polyposis. It is, however, not known whether these drugs reduce the incidence of cancer in humans. Recent studies suggest that expression of COX-2 mRNA and protein is elevated in approximately 70-80% of oesophageal, gastric and colorectal carcinomas, but its clinical significance in oesophageal adenocarcinoma has remained unclear. The aim of this study was to assess whether expression of COX-2 protein is associated with clinicopathological parameters and survival in patients undergoing intentionally curative resection for oesophageal adenocarcinoma.

METHODS

Patients

Between January 1st 1993 and December 31st 2000, 306 patients underwent oesophageal resection for adenocarcinoma of the oesophagus or gastro-oesophageal junction with curative intent (i.e. locally resectable disease without distant metastases), at the Department of Surgery of the Academic Medical Center, Amsterdam, the Netherlands. Preoperative work-up consisted
of endoscopy with biopsy, external ultrasonography of abdomen and neck, chest X-ray, oesophageal endosonography and indirect laryngoscopy. Lymph node metastases at the celiac trunk, which are considered distant metastatic disease for intrathoracic oesophageal carcinoma (M1a) according to the ’97 Union International Contre le Cancer (UICC) TNM classification, were only a contraindication for resection when considered irresectable and confirmed by sonographically guided transcutaneous cytological puncture. The data from these original 306 patients were prospectively collected in a database. All pathology reports were reviewed in order to identify those patients who had been operated upon for adenocarcinoma developed in a histologically proven Barrett’s oesophagus (defined by the presence of goblet cells). Patients with an adenocarcinoma of the cardia or gastro-oesophageal junction without a Barrett’s segment were excluded (n=155). Archival material of the remaining 151 patients was re-evaluated by two of the investigators (CJB and BvR) to obtain the sample with deepest invasion of each tumour. Another 6 specimens were excluded during the immunohistochemical analyses (three samples with no definitive invasive cancer, two samples with adenosquamous carcinoma, and one sample that repeatedly detached from the slide), so that the remaining 145 patients were included into this study. All patients were treated with subtotal oesophagectomy and resection of the lesser curvature of the stomach. In 95 patients (65.5%) resection was performed by a transhiatal approach without thoracotomy. Lymphadenectomy comprised en bloc removal of all lymphatic tissue in the lower posterior mediastinum, along the cardia and the lesser curvature of the stomach. Fifty patients (34.5%) underwent oesophagectomy through a right-sided thoracotomy followed by a laparotomy in combination with two-fields lymph node dissection. This procedure included an abdominal lymphadenectomy as described above, plus the removal of lymph nodes along the common hepatic artery, the splenic artery and the celiac trunk and an extended lymph node dissection in the chest (i.e. including the right paratracheal, infra-aortic arch and subcarinal lymph nodes). Between April 1994 and February 2000, 96 patients (66%) were randomly assigned to either transhiatal or transthoracic oesophagectomy as part of a randomized trial comparing both techniques. In the remaining 49 patients, a standard transhiatal procedure was performed. Patients were followed until death or June 30th 2001, ensuring a minimal follow-up of 6 months (median 38.2 months; range 8 days to 7.3 years). They were seen at a regular basis for five years in the outpatient clinic. In the first two years patients were seen at three to four-month intervals, afterwards at six-month intervals. For the present study, patients and/or their family practitioners were contacted by phone to assess their current status when they
had been discharged by the surgeon after five years. No patients were lost to follow-up. None of the patients received chemo- and/or radiotherapy preoperatively, and no adjuvant treatment was administered postoperatively. The study was done in accordance with the guidelines of the local ethics committee.

**COX-2 immunohistochemical staining**

Formalin-fixed and paraffin-embedded specimens were sectioned (5 μm), deparaffinized, and microwaved for 4 x 5 min in 700 W in 0.01 M Na-citrate buffer (pH 6.0) for antigen retrieval. The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and in blocking solution (1.5:100 normal horse serum in PBS) for 15 minutes to block unspecific binding sites. Immunostaining was performed with a COX-2 specific mouse anti-human monoclonal antibody (160112, Cayman Chemical Co., Ann Arbor, MI, USA) in a dilution of 1:200 (2.5 μg/ml) in PBS containing 0.1% sodium azide and 0.5% bovine serum albumin at room temperature overnight. Then the sections were treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (Vectastain ABCComplex, Vector Laboratories). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO, USA), and the sections were counterstained with Mayer’s hematoxylin. Every 20th sample of the trial series was a known colon adenocarcinoma specimen, in which stromal cells at an area of ulceration were scored 3+, cancer cells from 2+ to 3+, and adjacent nonneoplastic epithelium 1+ (for scoring criteria see below). This procedure confirmed that there was no significant intra- and interassay variability of the staining intensity, and helped us to score the trial specimens. Recently we evaluated several COX-2 antibody preparations, and concluded that the monoclonal antibody used in this study provided us with the most specific and reproducible immunoreactivity. Specificity of the antibody was confirmed in this trial by re-staining a randomly selected subset of specimens (every 10th sample, n=15) with and without preadsorption of the primary antibody with a human COX-2 control peptide (10 μg/ml, Cayman Chemical) for one hour at room temperature prior to the staining procedure.

**COX-2 scoring**

COX-2 immunohistochemical staining was scored independently and in a blinded manner by an experienced gastrointestinal pathologist (GJAO) and by another investigator (AR). The following scoring criteria of the tumour cells were agreed upon before the analysis: 0, no staining; 1+, weak diffuse
cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); 2+, moderate to strong granular cytoplasmic staining in 10-90% of the cancer cells; 3+, over 90% of the tumor cells stained with strong intensity. Scores 0 and 1 were categorized as 'COX-2 low' and scores 2 and 3 as 'COX-2 high' for the statistical analyses (see below). The allocation of tumours to the 'COX-2 low' versus the 'COX-2 high' category by the two investigators was similar (>95% of the specimens were categorized identically). In cases of disagreement (n=6) the slides were re-evaluated by a group of investigators (CJB, BvR, GJAO and AR) using a multiheaded microscope. In addition to the tumour cell staining, positivity of nonneoplastic squamous epithelium, metaplastic Barrett's mucosa, and stromal cell staining were noted.

**Statistical analysis**

The association between demographic and clinicopathological features and COX-2 expression was analysed using Student's t-test (continuous data) or Chi-squared test (categorical data). Overall survival was estimated according to the Kaplan-Meier method and compared using the log-rank test. We included all deaths since the time of surgery, including two perioperative deaths.

The Cox proportional hazard model was used to examine the effect of COX-2 expression in relation to other prognostic factors. Age, gender, operation type, and the variables related to tumour characteristics as differentiation grade, tumour stage, and radicality of the resection were included in this model. The limited number of events (from a statistical point of view) meant that only a restricted number of possible confounders could be examined. Therefore, variables with multiple categories were recoded into dichotomous variables by combining categories with comparable prognosis (differentiation grade: well versus moderate and poor; radicality of resection: microscopically radical (R0) versus microscopically nonradical (R1) and macroscopically nonradical (R2); tumour stage: stage I and IIa versus IIb, III and IV). P-values of 0.05 or below were considered statistically significant. All statistical analyses were performed using the Statistical Software Package version 9.0 (SPSS INC., Chicago, USA).
RESULTS

Formation and demographic characteristics of the cohort
Of the original 306 oesophageal resections for an adenocarcinoma, 151 patients had a Barrett’s carcinoma. Six specimens were excluded during the immunohistochemical analyses, so that 145 patients were included into this study (see Methods for details). There were 120 males (82.8%) and 25 females (17.2%) with a mean age of 65.7 years (range 34.8 - 85.2 years).

Immunohistochemical expression of COX-2
COX-2 immunoreactivity was detected in 143 of the 145 (98.6%) oesophageal adenocarcinoma specimens. Moderate to strong staining with a granular cytoplasmic pattern (as opposed to weak and diffuse cytoplasmic positivity;

FIGURE 1
Representative examples of COX-2 immunohistochemistry. A: Strong (3+) immunoreactivity in tumour cells (Tu). Note that the nonneoplastic stroma (Str) including a blood vessel (BV) is negative. This tumour was categorized as ‘COX-2 High' (200x). B: Weak (1+) immunoreactivity in tumour cells (Tu). This tumour was categorized as ‘COX-2 Low’ (200x). C: Intestinal metaplasia (IM) showing weak (1+) immunoreactivity (200x). D: Strong (3+) immunoreactivity in the stromal cells (Str) at a site of an ulceration E: Oesophageal squamous epithelium (Sq) was consistently negative (200x). Blocking controls for A-D are shown in a-d, respectively (see Methods for details).
see Methods for details) was observed in 115/145 (79.3%) cases of which 13 were scored as strong and 102 as moderate. COX-2 expression was mainly localized in the neoplastic cells (Figure 1A and B). Weak epithelial positivity was observed in 50% of the Barrett's metaplasias while the remaining of them were negative (Figure 1C). Similarly, only weak or no staining was observed in stromal cells (connective tissue cells, smooth muscle cells and blood vessels), except at sites of erosions and ulcerations (n=29) and around necrosis (n=4). In fact, a significant proportion (13/30, 43.3%) of the specimens in the ‘COX-2 low’ category expressed high levels of COX-2 in the stromal cells at a site of mucosal injury (Figure 1D). Squamous epithelium of the oesophagus was consistently negative or only weakly positive (Figure 1E). Both intra- and interassay variation of distribution of the immunoreactivity and intensity of the staining were minimal as assessed by using an internal control sample with known staining intensities (see Methods for details). Interobserver variation was 4.1 %, and all specimens which were discrepant (n=6) were re-evaluated as described in the Methods section, and the consensus score was used for further analysis. Furthermore, 15 randomly selected specimens were restained with and without the blocking procedure using the antigenic peptide (Figure 1A-D). All tumour cell signal was blocked by this control procedure in all specimens, and only one sample was scored to a different category, when compared to the original evaluation.

**Correlation between COX-2 expression and clinicopathological parameters**

COX-2 expression was not statistically significant correlated with clinicopathological parameters at the time of operation, although possibly a trend was seen towards a positive association with the presence of lymph node metastases (p=0.09) (Table 1A). A significant association was found between elevated COX-2 expression and the development of distant metastases (p=0.02) and locoregional recurrences (p=0.05) (Table 1B).

**Association of COX-2 expression with overall survival**

Kaplan-Meier curves for patient survival are depicted in Figure 2. A significant difference in survival was observed between patients in the ‘COX-2 low’ category when compared to the ‘COX-2 high’ category (p=0.002; log-rank test). After 2 years the probability for survival was 81.8% (95% CI 67.3 - 96.3) in the COX-2 low group, and 51.7% (95% CI 41.9 - 61.5) in the COX-2 high group which declined after 5 years to 71.6% (95% CI 53.2 - 89.9) and 35.1% (95% CI 23.2 - 47.1) respectively.
### TABLE 1A
Correlation of clinicopathological findings and COX-2 expression.

<table>
<thead>
<tr>
<th>Correlation of clinicopathological findings and COX-2 expression.</th>
<th>COX-2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=30)</td>
</tr>
<tr>
<td><strong>Patient characteristics</strong></td>
<td>No. (%)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>66±9</td>
</tr>
<tr>
<td>Gender</td>
<td>Male (120)</td>
</tr>
<tr>
<td></td>
<td>Female (25)</td>
</tr>
<tr>
<td><strong>Tumour characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Depth of invasion(^a)</td>
<td>T1 (45)</td>
</tr>
<tr>
<td></td>
<td>T2 (17)</td>
</tr>
<tr>
<td></td>
<td>T3 (83)</td>
</tr>
<tr>
<td>Lymph node involvement(^b)</td>
<td>N0 (67)</td>
</tr>
<tr>
<td></td>
<td>N1 (78)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>M0 (117)</td>
</tr>
<tr>
<td></td>
<td>M1 (28)</td>
</tr>
<tr>
<td>Tumour stage(^c)</td>
<td>I (32)</td>
</tr>
<tr>
<td></td>
<td>IIa (33)</td>
</tr>
<tr>
<td></td>
<td>IIb (16)</td>
</tr>
<tr>
<td></td>
<td>III (36)</td>
</tr>
<tr>
<td></td>
<td>IV (28)</td>
</tr>
<tr>
<td>Differentiation grade</td>
<td>Well (11)</td>
</tr>
<tr>
<td></td>
<td>Moderate (56)</td>
</tr>
<tr>
<td></td>
<td>Poor (78)</td>
</tr>
<tr>
<td><strong>Operation</strong></td>
<td></td>
</tr>
<tr>
<td>Operation type(^d)</td>
<td>THE (95)</td>
</tr>
<tr>
<td></td>
<td>TTE (50)</td>
</tr>
<tr>
<td>Radicality of resection(^e)</td>
<td>R0 (112)</td>
</tr>
<tr>
<td></td>
<td>R1 (28)</td>
</tr>
<tr>
<td></td>
<td>R2 (5)</td>
</tr>
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</table>

\(^a\) T1: tumour limited to the submucosa, T2: tumour infiltrates muscularis propria, but not adventitia, T3: tumour infiltrates adventitia; \(^b\) N0: no regional lymph node metastasis, N1: regional lymph node metastasis present; \(^c\) I: T1N0M0, IIa: T2-3N0M0, IIb: T1-2N1M0, III: T3N1M0, IV anyT anyNM0; \(^d\) THE: transhiatal resection, TTE: transthoracic resection; \(^e\) R0: microscopically radical, R1: microscopically nonradical, R2: macroscopically nonradical.
TABLE 1B
Correlation of clinical outcome parameters and COX-2 expression.

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>Low (n=30)</th>
<th>High (n=115)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NO (n)</td>
<td>NO (%)</td>
<td>NO (%)</td>
</tr>
<tr>
<td>Locoregional recurrence</td>
<td>No (83)</td>
<td>22 (73.3)</td>
<td>61 (53.0)</td>
</tr>
<tr>
<td></td>
<td>Yes (62)</td>
<td>8 (26.7)</td>
<td>54 (47.0)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>No (94)</td>
<td>25 (83.3)</td>
<td>69 (60.0)</td>
</tr>
<tr>
<td></td>
<td>Yes (51)</td>
<td>5 (16.7)</td>
<td>46 (40.0)</td>
</tr>
</tbody>
</table>

FIGURE 2
Kaplan-Meier curves of 145 patients with oesophageal Barrett carcinoma. There were 30 patients with low COX-2 expression and 115 with high COX-2 expression. A statistically significant difference was observed between the two groups (p=0.002; log-rank test).
Under the graph, the numbers of patients still at risk at different time points (in months) during the follow-up are shown.
Univariate and multivariate analysis
To evaluate the role of COX-2 as an independent prognostic factor,
a multivariate analysis was performed including seven possible confounding
variables as described in Methods.
In the univariate Cox regression analysis a significant effect was found for
COX-2 expression, tumour stage, differentiation grade, and radicality of the
resection (Table 2). The impact of high COX-2 expression on mortality did not
change after adjustment for age, gender, operation type, tumour stage,
differentiation grade and radicality of resection.

TABLE 2
Results of univariate and multivariate analyses of clinicopathological parameters related to survival by the Cox
proportional hazards model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95% CI</td>
<td>RR</td>
<td>95% CI</td>
</tr>
<tr>
<td>10-year age increase</td>
<td>1.1</td>
<td>0.9-1.5</td>
<td>1.2</td>
<td>0.9-1.5</td>
</tr>
<tr>
<td>Female gender</td>
<td>0.8</td>
<td>0.4-1.5</td>
<td>0.7</td>
<td>0.4-1.5</td>
</tr>
<tr>
<td>TTE Operation type</td>
<td>0.8</td>
<td>0.5-1.3</td>
<td>0.8</td>
<td>0.5-1.4</td>
</tr>
<tr>
<td>High TNM classification</td>
<td>2.2</td>
<td>1.6-3.1</td>
<td>4.2</td>
<td>2.3-7.7</td>
</tr>
<tr>
<td>Poor differentiation grade</td>
<td>1.3</td>
<td>1.1-1.5</td>
<td>2.5</td>
<td>0.3-19</td>
</tr>
<tr>
<td>Non-radical resection</td>
<td>1.2</td>
<td>1.1-1.5</td>
<td>2.5</td>
<td>1.5-4.1</td>
</tr>
<tr>
<td>High COX-2 expression</td>
<td>3.2</td>
<td>1.5-7.1</td>
<td>3.5</td>
<td>1.6-7.9</td>
</tr>
</tbody>
</table>

a Relative Risk; b 95% Confidence Interval

DISCUSSION
Our data indicate that elevated expression of COX-2 is associated with
reduced survival in patients operated upon for adenocarcinoma of the
oesophagus. Patients with tumours with high COX-2 expression had a more
aggressive course of their disease, as they were more likely to develop distant
metastases and local recurrences leading to higher mortality rates. This higher
mortality could not be explained by a more advanced disease stage at the
time of operation. Multivariate analyses suggest that elevated COX-2
expression is an independent prognostic factor for patient survival together
with radicality of the resection and tumour stage, which are the well
established prognostic markers for Barrett’s carcinoma. However, since short-
term mortality and long-term survival occur in both COX-2 groups, COX-2 is
obviously not the only meaningful factor for patient survival and for the
individual patient, COX-2 expression per se will not be able to act as a
guideline for eligibility for surgery.
In a recent study on squamous cell carcinoma of the oesophagus, elevated
COX-2 expression did not correlate with various clinicopathological
parameters including prognosis.15 In gastric adenocarcinoma, elevated
expression of COX-2 has been associated with lymph node metastasis but not
with distant metastasis.15,16 Moreover, no statistically significant correlation
between expression of COX-2 and survival was found in this tumour type.17,18 In
colorectal adenocarcinoma, elevated expression of COX-2 correlates with
several clinicopathological parameters, such as tumour size and tumour
differentiation, and with poorer overall survival, but it remains unclear whether
COX-2 is an independent prognostic factor in this disease.19-21 These
differences may depend on number of patients included in the trials, distinct
pathogenesis of oesophageal versus gastric and colorectal adenocarcinomas,
or variations in the techniques, reagents and scoring criteria used. The
strength of our study is a large number of consecutive patients with an
identified Barrett’s adenocarcinoma combined with the prospectively
collected database with an extensive and complete follow-up of survival. We
chose immunohistochemistry for evaluation of COX-2 expression in the
tumour samples, since this method is virtually the only one that can be used to
analyse protein expression from formalin fixed and paraffin embedded
archival material, the signal can be precisely localized at the cellular level and
large numbers of specimens can be analysed in a reproducible manner. It was
also helpful that the performance of the monoclonal antibody (in comparison
to several other COX-2 antibodies) was recently characterized.10 Our data
suggest that the immunohistochemical evaluation of COX-2 protein
expression was specific, sensitive and reproducible. However, quantitation is a
challenge when using immunohistochemistry. To this end, we were able to
design a relatively simple scoring method that produced highly concordant
results between two independent evaluators.
Expression of COX-2, but not that of COX-1, has been shown to be elevated
in Barrett’s metaplasia, dysplasia and adenocarcinoma while the nonneoplastic
squamous epithelium is virtually negative.22,23 Expression of COX-2 is primarily
localized in the cytoplasm of the neoplastic cells in invasive adenocarcinoma.
In our cancer specimens, metaplastic lesions were either negative or weakly
positive (primarily in the epithelial compartment). Thus, it seems that
expression of COX-2 is progressively elevated during progression from
metaplasia to invasive carcinoma, as has been recently published for both the
oesophagus and the stomach.24,25,26 In our series, tumour stroma was positive
for COX-2 only at sites of mucosal injury or around necrosis, which often
occurs in these tumours. Thus, the frequency of COX-2 positive tumours might be overestimated when whole tissue preparations are analysed consisting of both epithelial and stromal components (e.g. Western blots for proteins and Northern blots or RT-PCR for mRNA, in which histology is no longer available), since a proportion of the signal can originate from nonneoplastic cells. This is especially important when using superficial biopsy specimens from tumours that express low or non-detectable levels of COX-2, since we found COX-2 positive mucosal injuries in 43% of the samples in the ‘COX-2 low’ category. Epidemiological data indicate that there is a reduced risk for developing oesophageal cancer in patients who regularly take NSAIDs.\textsuperscript{27-29} The precise mechanism of a chemopreventive effect of NSAIDs is not known, but COX-2 enzyme does not seem to be necessary for malignant transformation in some systems.\textsuperscript{30} However, COX-2 is necessary in the development of intestinal neoplasias in certain knockout mouse models\textsuperscript{31,32} and sufficient for mammary gland tumourigenesis in a transgenic mouse model.\textsuperscript{33} COX-2 expression may participate in intestinal carcinogenesis by inhibiting apoptosis, by inducing angiogenesis and by promoting metastases\textsuperscript{34,35} and a selective COX-2 inhibitor suppressed growth and induced apoptosis in human oesophageal adenocarcinoma cells.\textsuperscript{36} This suggests that COX-2 might increase the metastatic potential of malignant cells, and is consistent with our finding that the expression of COX-2 is related to the development of distant metastases. It is not known why COX-2 is upregulated in adenocarcinomas of the oesophagus, but since bile acids induce COX-2 expression in an oesophageal adenocarcinoma cell line\textsuperscript{37} and in organ cultures of Barrett’s epithelium\textsuperscript{23}, it is possible that reflux disease induces the early expression of COX-2 during the development of the Barrett’s metaplasia. It is however, unlikely that reflux would be a precondition for the expression of COX-2 in invasive cancer cells. A more likely explanation is that neoplastic cells are either intrinsically more active in expressing COX-2 than the non-neoplastic cells, for example due to the activation of oncogenes or the inactivation of tumour suppressor genes, or that they are stimulated by stromal cell derived factors.

In conclusion, this is the first report on reduced survival with elevated expression of COX-2 in adenocarcinoma of the oesophagus. The present findings support efforts to initiate clinical studies on the effect of COX-2 inhibitors in (adjuvant) treatment of adenocarcinoma developed in Barrett’s oesophagus.\textsuperscript{39}
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