Prospective Study of Cyclin D1 Overexpression in Barrett’s Esophagus: Association With Increased Risk of Adenocarcinoma

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Background: Esophageal adenocarcinoma commonly arises from a precancerous condition, Barrett’s esophagus, in which the normal squamous epithelium is replaced by a columnar cell-lined epithelium. Genetic alterations occurring in this process could serve as biomarkers for the risk of malignant progression, improve surveillance, and contribute to early diagnosis. We examined two potential biomarkers, cyclin D1 and p53, in a prospective cohort of Barrett’s esophagus patients. Methods: A total of 307 patients were enrolled in an endoscopic surveillance cohort, and esophageal biopsy specimens were collected at each endoscopy. Incident cases of adenocarcinoma were matched to control patients within the cohort by duration of follow-up, age, sex, and length of columnar cell-lined epithelium at recruitment. Biopsy specimens were analyzed for cyclin D1 and p53 protein levels by immunohistochemistry. Statistical tests were two-sided. Results: A total of 12 cases of adenocarcinoma occurred within the follow-up period, and tumor biopsy specimens from 11 cases stained positive for cyclin D1. Biopsy specimens from eight of these patients taken at recruitment also stained positive for cyclin D1. A case–control analysis of biopsy specimens obtained at recruitment revealed a statistically significantly increased risk of progression to adenocarcinoma in Barrett’s esophagus patients whose biopsy specimens were cyclin D1 positive (odds ratio [OR] = 6.85; 95% confidence interval [CI] = 1.57–29.91; \( P = .0106 \)) but not in patients whose biopsy specimens were p53 positive (OR = 2.99; 95% CI = 0.57–15.76; \( P = .197 \)). Conclusions: Cyclin D1-positive staining could be a useful biomarker in identifying Barrett’s esophagus patients at high risk of esophageal adenocarcinoma. Given the complexity of genetic alterations in the natural history of this cancer, additional biomarkers will be required to increase the sensitivity and specificity of molecular diagnosis. [J Natl Cancer Inst 2000;92:1316–21]
and a reduction in the cost-effectiveness of endoscopic surveillance. Biologic markers that would permit surveillance to be focused on a subgroup of BE patients who are at high risk of esophageal adenocarcinoma would, therefore, be of considerable value in the clinical management of this condition.

A number of genetic alterations have been associated with the early stages of the natural history of adenocarcinoma of the esophagus (10–12), and there has been considerable interest in identifying molecular markers, which could be of greater diagnostic value than dysplasia. In particular, overexpression of cyclin D1, loss of heterozygosity on chromosome 9p21, hypermethylation and mutation of CDKN2, aneuploidy, and p53 mutations have all been reported in BE tissue (13–16). In this study, we examined the overexpression of cyclin D1 and the presence of p53 protein by immunohistochemistry in a nested case–control study of esophageal adenocarcinoma. This analysis was conducted between 1984 and 1995 within the context of an annual endoscopic surveillance cohort of BE patients.

### METHODS

#### Surveillance Cohort and Study Design

A total of 357 individuals were recruited from January 31, 1984, through January 31, 1995, into the annual surveillance program at the Leeds General Infirmary, U.K., on the basis of the endoscopic and histologic diagnosis of the presence of BE. At the start of the surveillance program, BE was defined as the presence of specialized columnar cell-lined epithelium above the gastroesophageal junction (307 patients) or as a greater than 3-cm columnar segment without specialized epithelium (50 patients). However, according to current criteria for BE, only the first category of patients would be considered as having BE; therefore, only this group was considered further in the study. Indeed, no adenocarcinomas occurred in the 50 patients without specialized epithelium. Patients were excluded from the cohort if they were more than 80 years of age at first visit, had other major diseases or malignancies, or died before the first follow-up visit. Patients who were diagnosed with esophageal adenocarcinoma within 6 months of the endoscopic diagnosis of BE were also excluded from the cohort. A total of 179 males and 128 females were recruited, with mean ages of 58 and 65 years, respectively. The duration of follow-up was defined as the time between the date of the first endoscopy at which the initial diagnosis of BE was established at Leeds General Infirmary to the date of the last surveillance endoscopy (up to December 31, 1996). Each patient gave written informed consent prior to each endoscopy in accordance with the requirements of the United Leeds Teaching Hospital Trust.

At each endoscopy, multiple biopsy specimens were collected. Although a systematic 2-cm quadrant biopsy protocol has now been adopted, during the period of this study, the number and site of biopsies were not standardized. From two to 10 biopsy specimens were taken at any one endoscopy. All biopsy specimens from a given visit were analyzed together by immunohistochemistry (see below). For patients who were diagnosed with esophageal adenocarcinoma during the follow-up, specimens of the tumor were also analyzed. All tumors were localized within the tubular esophagus. Following a histologic diagnosis of invasive adenocarcinoma, the tumors were staged with thoracoabdominal computed tomography scanning by use of the Union Internationale Contre le Cancer Tumor–Node–Metastases (UICC TNM) system (17). For each cancer case, up to six cancer-free control BE patients (total, 49) were selected from the surveillance cohort matched on sex, age at first biopsy (single year), length of columnar cell-lined epithelium at first biopsy, and length of follow-up. Only one case patient and no control patients had evidence of high-grade dysplasia at recruitment. Biopsy specimens (and resected tumors) from the esophageal adenocarcinoma patients, taken from the time of recruitment to the time of clinical diagnosis of cancer, were analyzed for cyclin D1 and p53 protein expression by immunohistochemistry. The first and last biopsy specimens obtained from each control subject were also stained for these antigens. For p53 staining, there was insufficiency of tissue remaining to perform the analysis on a few of the histologic blocks used for immunohistochemical analysis after completion of cyclin D1 staining.

#### Immunohistochemistry

Sections (5 μm) were cut from formalin-fixed, paraffin-embedded biopsy specimens and resected esophageal tissue samples. Sections were deparaffinized in xylene and then rehydrated and equilibrated in Tris–buffered saline (TBS: 50 mM Tris and 145 mM NaCl [pH 7.6]). Sections were treated with 0.5% hydrogen peroxide in methanol for 30 minutes to remove endogenous peroxidase activity. Antigen retrieval was performed by pressure-cooking the sections for 90 seconds in antigen-unmasking solution (Vector Laboratories Ltd., Peterborough, U.K.). To block nonspecific protein binding, we incubated sections for 10 minutes at room temperature in 2% normal goat serum in the case of cyclin D1 or in 10% casein solution (Vector Laboratories Ltd.) in the case of p53. Nonspecific binding reactions were further blocked by incubating the sections with an avidin–biotin blocking kit (Vector Laboratories Ltd.) according to the manufacturer’s instructions prior to the application of the primary monoclonal antibody. Duplicate sections were incubated (1 hour at room temperature) either with NCL-cyclin D1 antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.), which was diluted 1:30 in 0.01% Tween 20–TBS, or with DO-7-p53 antibody (Novocastra Laboratories Ltd.), which was diluted 1:100 in 2% casein solution–TBS. Sections were washed in TBS and incubated with biotinylated rabbit anti-mouse immunoglobulin (Vector BA-2000; Vector Laboratories Ltd.; diluted 1:100 or 1:200 in TBS) for 30 minutes at room temperature. Detection of bound secondary antibody was performed by use of a Vectorstain Elite avidin–biotin complex kit (Vector Laboratories Ltd.) in conjunction with horseradish 3,3′-diaminobenzidine according to the manufacturer’s instructions.

Throughout the study, sections from a breast carcinoma specimen known to stain positive for cyclin D1 and a colon tumor specimen known to stain positive for p53 protein were analyzed in parallel to serve as positive controls. Omission of the primary antibody from these samples acted as a negative control. All sections were counterstained with hematoxylin and examined by light microscopy.

#### Histologic and Immunohistochemical Assessment

Slides were assessed in a blinded fashion by a consultant pathologist (N. Mapstone). Dysplasia was graded according to the criteria reviewed by Haggitt (18), and biopsy specimens were graded by the most severe change observed. Immunohistochemical staining of slides was rated by the pathologist in the following way: Intraocular cyclin D1 staining was graded as 0 (no visible staining), 1+ (any identifiable staining), 2+ (widespread strong staining), or 3+ (widespread intense staining). In this study, we defined any positive staining (1+, 2+, or 3+) as overexpression of cyclin D1. p53 staining was graded as described by Symmans et al. (19) because these authors used the same antibody as in the current study: 0 (no visible staining), 1+ (low-intensity staining in at least part of a section), or 2+ (high-intensity staining in at least part of the section). However, because most p53 staining was of low intensity and focal, the data were dichotomized to + (positive) and 0 (no visible staining) for statistical analysis. Staining was evaluated in columnar epithelial or tumor cells; for both proteins, the result was considered to be positive only if nuclear staining was observed.

#### Statistical Analysis

Because of the relatively small size of the overall cohort, the selected control patients did not always correspond closely with their matched case patients on some or all of the matching criteria. As a result, an unmatched analysis was performed with adjustment for relevant covariates. Odds ratios (ORs) were, therefore, calculated by use of unconditional logistic regression (EGRET; CYTEL Software Corporation, Cambridge, MA). Statistical tests were two-sided. ORs compared case and control patients for the presence or absence of cyclin D1 or p53 staining in biopsy specimens taken at recruitment to the study cohort after adjustment for sex, age at first biopsy (single year), length of follow-up (months), length of columnar cell-lined epithelium at first biopsy (cm), and number of biopsy specimens taken at study entry. One of the subjects was recruited 3 years prior to the diagnosis of adenocarcinoma, but a biopsy specimen was available for analysis only in the year prior to diagnosis.

#### RESULTS

Twelve patients within the surveillance cohort (11 males and one female) developed adenocarcinoma of the esophagus over the follow-up period. Ten of these patients underwent esophagectomy, and the tumors were staged by use of the UICC TNM...
system (17); there were five stage I tumors, three stage II, and two stage III. Two of the patients did not have surgery because of other medical reasons in one case and advanced disease in the other. The mean duration of follow-up, sex distribution, mean age, and mean length of columnar cell-lined epithelium at recruitment and the number of individual biopsy specimens obtained at endoscopy were all similar in case and control patients (Table 1).

Almost all of the adenocarcinomas of the esophagus overexpressed cyclin D1. Of the 12 tumors, 11 (92%) stained positive for cyclin D1 (1+, 2+, or 3+); an example of strong positive staining is given in Fig. 1, A. In contrast, of the final biopsy specimens taken from the control patients, only 14 (29%) of 49 showed positive staining for cyclin D1. For 12 of these 14 control patients, both the recruitment and the final biopsy specimens were positive for cyclin D1 (data not shown).

Cyclin D1 overexpression in columnar cell-lined epithelium occurred far more frequently in BE patients who later developed adenocarcinoma than in those who did not. The case–control comparison of cyclin D1 staining in the BE biopsy specimens taken at recruitment is shown in Table 2, with an example of positive staining in columnar epithelium shown in Fig. 1, B. Of the BE patients who later developed adenocarcinoma, 67% (eight of 12) of them had biopsy specimens that stained positive for cyclin D1 at recruitment compared with only 29% (14 of 49) of the BE patients who did not develop adenocarcinoma (OR = 6.85; 95% confidence interval [CI] = 1.57–29.91; P = .0106; adjusted for age, sex, length of follow-up, length of columnar cell-lined epithelium, and number of biopsy specimens) (Table 2). If more stringent criteria for positive staining were applied and only biopsy staining of grades 2+ or 3+ was taken as positive for cyclin D1 overexpression, then six case patients (50%) and seven control patients (14%) were scored as positive, resulting in a similar OR as above (adjusted OR = 6.97; 95% CI = 1.58–30.74; P = .0103).

Analysis of biopsy specimens collected throughout the follow-up period from the 12 BE patients who went on to develop adenocarcinoma showed that, in 11 patients, at least one of these biopsy specimens stained positive for cyclin D1 prior to diagnosis of cancer (data not shown). In the two patients with the longest follow-up (10 and 14 years, respectively), cyclin D1 overexpression was observed in the first biopsy in the first patient (10 years prior to diagnosis) and 2 months after the first biopsy in the second patient (13 years and 10 months prior to diagnosis). The profile of cyclin D1 for the first patient, shown in Fig. 2, also demonstrates that staining for cyclin D1 was consistent over the follow-up period.

The p53 immunohistochemistry revealed five (45%) of the 11 tumors analyzed to be positive for p53 expression. A smaller percentage of the biopsy specimens obtained at recruitment stained positive for p53 than for cyclin D1: Only four (36%) of 11 of the patients with BE who later developed adenocarcinoma had p53-positive biopsy specimens compared with seven (17%) of 41 control patients. (Not all biopsy and tumor specimens were analyzed for p53 protein because of the limited amount of speci-

### Table 1. Comparison of case and control patients for parameters used in matching*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case patients (n = 12)</th>
<th>Control patients (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>11/1</td>
<td>46/3</td>
</tr>
<tr>
<td>Age, y, mean ± SD</td>
<td>60.3 ± 11.4</td>
<td>61.5 ± 11.5</td>
</tr>
<tr>
<td>Length of columnar cell-lined epithelium at recruitment, cm, mean ± SD</td>
<td>7.8 ± 3.5</td>
<td>7.4 ± 3.7</td>
</tr>
<tr>
<td>No. of biopsy specimens at recruitment, mean ± SD</td>
<td>3.4 ± 1.7</td>
<td>3.6 ± 1.7</td>
</tr>
<tr>
<td>Mean duration of follow-up ± SD, mo</td>
<td>52.0 ± 48.1</td>
<td>56.5 ± 35.1</td>
</tr>
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*The data for the remaining 246 patients in the cohort were as follows: sex—122 males and 124 females; mean age ± SD = 61 ± 12.4 years; mean length of columnar cell-lined epithelium ± SD = 9.0 ± 3.5 cm; and mean duration of follow-up ± SD = 39.2 ± 57.3 months. The number of biopsy specimens at recruitment were not used for matching but are shown for comparison purposes. SD = standard deviation.

### Table 2. Case–control comparison of cyclin D1 and p53 staining of the biopsy specimens obtained at recruitment into the surveillance cohort

| Immunohistochemical staining* | Case patients, No.† | Control patients, No.‡ | OR (95% CI)§ | P|| |
|------------------------------|----------------------|------------------------|--------------|-----|
| Cyclin D1 positive           | 8                    | 14                     | 6.85 (1.57–29.91) | .0106 |
| Cyclin D1 negative           | 4                    | 35                     | 1.0          |     |
| p53 positive                 | 4                    | 35                     | 2.99 (0.57–15.76) | .197 |
| p53 negative                 | 7                    | 34                     | 1.0          |     |

*Positive staining was classified as a score of 1 or greater (see the "Methods" section).
†Only 11 of the 12 case patients were available for p53 staining because of the small size of the biopsy specimen from one patient.
‡Only 41 of the 49 control patients were available for p53 staining because of the limited size of some biopsy specimens.
§OR = odds ratio; CI = confidence interval.
||P = .0103.

**Fig. 1.** Nuclear cyclin D1 staining detected by immunohistochemistry. **Panel A:** An esophageal tumor section from a patient with adenocarcinoma exhibiting intense (3+) staining. **Panel B:** A section of specialized columnar cell-lined epithelium from a patient with Barrett’s esophagus exhibiting strong (2+) staining. Staining was performed as described in the "Methods" section. Original magnification x325.
than from control patients, the elevated OR was not statistically overexpression was observed more than 10 years before diag-
lent histopathology at recruitment but whose biopsy specimens
more likely to develop the malignancy than patients with equiva-
 mens stained positive for cyclin D1 were six to seven times
esophagus among BE patients. BE patients whose biopsy speci-
cum of risk for the development of adenocarcinoma of the
overexpression could prove to be useful as a predictive biomar-

DISCUSSION

This prospective cohort study demonstrates that cyclin D1 overexpression could prove to be useful as a predictive biomarker of risk for the development of adenocarcinoma of the esophagus among BE patients. BE patients whose biopsy specimens stained positive for cyclin D1 were six to seven times more likely to develop the malignancy than patients with equivalent histopathology at recruitment but whose biopsy specimens were negative for the biomarker. In some individuals, cyclin D1 overexpression was observed more than 10 years before diag-

nosis of the tumor. Although positive p53 staining was more prevalent in recruitment biopsy specimens from case patients than from control patients, the elevated OR was not statistically significant.

Diagnosis of BE is the subject of much debate, and criteria for diagnosis have been reviewed thoroughly (20). In this study, which began in 1984, the initial criterion for diagnosis of BE was columnar cell-lined epithelium (with or without specialized intestinal epithelium) situated above the gastroesophageal junction, the position of the junction being determined endoscopically. However, because specialized intestinal-type epithelium, including the presence of mucin-secreting goblet cells, is now generally considered to be necessary for the diagnosis of BE and is associated with the highest risk of adenocarcinoma (3,20), only these subjects were included in the study.

Overexpression of cyclin D1 has been suggested to be an early event in esophageal carcinogenesis, based on its occurrence in BE (13). In other tumor types, cyclin D1 overexpression can arise via a number of mechanisms, including modification of messenger RNA stability and disruption of promoter structure (21). However, the most common mechanism is amplification of the 11q13 chromosomal region, whereby the copy number of the cyclin D1 gene (CCND1) can be increased from threefold to 10-fold. The mechanism underlying cyclin D1 overexpression in BE and adenocarcinoma has yet to be elucidated, although pre-
liminary evidence suggests that gene amplification may not be responsible (13,22).

To date, there are relatively few studies of cyclin D1 expression in BE patients in the absence of concurrent adenocarcinoma. In a study of cyclin D1 expression in BE patients with intestinal metaplasia (13), tissues from approximately 30% of the patients stained positive for cyclin D1, similar to the prevalence in our control group (29%). However, in the majority of studies of molecular alterations in the columnar cell-lined epithelium of BE patients, tissue specimens were obtained from patients undergoing resection for esophageal adenocarcinoma. This approach raises the problem of selection bias in that the patients studied are, by definition, those who have developed cancer. The current study avoided selection bias because it was prospective in nature. Only one of the 12 esophageal cancer patients was diagnosed as having high-grade dysplasia at recruit-
ment (this patient was also cyclin D1 negative at recruitment), and excluding this subject from the analysis had no marked effect on the OR for developing esophageal cancer associated with the expression of either cyclin D1 (OR = 8.11; 95% CI = 1.72–38.36; P = .008) or p53 (OR = 2.41; 95% CI = 0.42–13.70; P = .323).

Multiple genetic alterations occur in the natural history of esophageal adenocarcinoma (10–12). Given the complexity of these alterations, a single event, such as cyclin D1 overexpression, would not be expected to have 100% sensitivity or specificity in identifying high-risk BE patients. In this study, the sensitivity and specificity of cyclin D1 overexpression as a bio-
marker for the development of esophageal adenocarcinoma were high (67% and 71%, respectively), but there were some false negatives and false positives. One potential difficulty in a sur-
veillance study of BE, which could reduce the apparent sensi-
tivity of any biomarker, is that of obtaining representative sam-
ping by biopsy. In our cohort, there was no systematic application of a biopsy protocol, which could have led to sam-
ping errors. However, for the majority of BE patients in our study, multiple biopsy specimens were available from each visit; in addition, the biopsy specimens taken over time for a given patient gave consistent results for cyclin D1 staining (e.g., Fig.

2). Furthermore, adjusting for the number of biopsy specimens in the case–control analysis had no effect on the OR. It should also be noted that the entire cohort of BE patients is at one to two orders of magnitude increased risk of esophageal adenocarci-
noma compared with the general population (3); therefore, we cannot exclude the possibility that some patients chosen as controls from the cohort may develop adenocarcinoma at a later date. In fact, one subject selected as a control, who stained positive for cyclin D1 at recruitment and at final biopsy, has since been diagnosed with adenocarcinoma of the esophagus. In this sense, the data that we present may be a conservative esti-
mate of the predictive value of the cyclin D1 biomarker.

The sensitivity of cyclin D1 as a biomarker for future develop-
ment of esophageal adenocarcinoma may be improved if it is used in combination with other biomarkers that indicate early events in the progression from BE to adenocarcinoma. A number of candidate biomarkers relevant to cyclin D1, cell cycle control, and apoptosis have been shown to be altered in the columnar cell-lined epithelium of patients with BE (11–14,16,22). For example, the role of cyclin D1 in cell cycle control is mediated

Fig. 2. Temporal analysis of cyclin D1 staining in a Barrett’s esophagus (BE)
patient who developed adenocarcinoma of the esophagus 10 years after recruit-
ment into the surveillance cohort. Staining intensity was graded as described in
the “Methods” section. The shading of the bars indicates histologic diagnosis
throughout the surveillance period. Open bars = BE; gray bars = high-grade
dysplasia; and solid bar = adenocarcinoma of the esophagus. HGD = high-
grade dysplasia; ADC = adenocarcinoma of the esophagus.

men remaining on the histologic blocks after cyclin D1 analy-
sis.) The resulting adjusted OR was 2.99 (95% CI = 0.57–
15.76; P = .197). The OR was slightly reduced when cyclin D1
status was also included in the multivariate model (OR = 2.47;
95% CI = 0.40–15.17; P = .329).

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via cyclin D1–cyclin-dependent kinase (cdk) complexes, which phosphorylate the retinoblastoma protein, resulting in enhanced transcription of growth-promoting genes. Cyclin D1–cdk complex formation is subject to negative regulation by a number of cdk-inhibitory proteins, including p16, p15, and p21 (23). Thus, it is likely that the development of a panel of biomarkers within the context of understanding cell cycle control will hold the key to refining the identification of BE patients whose disease is at high risk of progression to malignancy.

Because previous studies (24,25) have suggested that alterations in the p53 tumor suppressor gene may represent an early event in the progression of BE to adenocarcinoma, we also analyzed p53 protein expression by immunohistochemistry in BE patients. The presence of p53-positive staining could reflect p53 protein stabilization by sequence mutations or other mechanisms. Although p53 staining was more prevalent in the recruitment biopsy specimens from patients who later developed adenocarcinoma than from patients who did not, the OR was not statistically significant (Table 2), even when the p53 data were adjusted for cyclin D1 status (see the “Results” section). Of the four adenocarcinoma patients whose recruitment biopsy specimens were p53 positive, three of these had specimens that were also cyclin D1 positive, the exception being the patient who was diagnosed with high-grade dysplasia. The presence of p53 mutation and high levels of p53 protein are known to be common in dysplasia of the esophagus (25).

Surveillance of BE patients would be improved if high-risk categories of patients could be identified by biomarkers, such as cyclin D1 overexpression in the columnar cell-lined epithelium. However, these patients are not the only category of BE patients at high risk for the development of esophageal adenocarcinoma. Another high-risk category includes males. The incidence of esophageal adenocarcinoma in patients with BE is of the order of one per 100 patient-years overall, but the rate is higher in males than in females (3). Although BE appears to be two to four times as prevalent in males than in females (26), the sex ratio for adenocarcinoma is approximately 10:1 (1). In our study cohort, the overall incidence of esophageal adenocarcinoma in BE patients with specialized epithelium was one per 95 patient-years, but one per 61 patient-years in males and one per 468 patient-years in females. The reason for this sex difference is not yet known, but it may provide clues to the etiology and natural history of the disease and deserves further study.

Our findings show that the addition of biomarkers such as cyclin D1 overexpression to endoscopic surveillance of BE patients may further help identify those individuals at highest risk of progression to adenocarcinoma and thus permit surveillance to be focused on these individuals. The prevalence of cyclin D1 staining in control patients at recruitment (29%) means that, if the biomarker had been applied to all 295 noncancer patients in this cohort in a cross-sectional analysis, 85 would be expected to be positive and thus be included in a targeted category of individuals for increased surveillance. Given that an additional eight cases of esophageal adenocarcinoma were positive for cyclin D1 staining at recruitment, the positive predictive value of the biomarker in identifying the cancer cases within this category of highest-risk individuals would be 8.6% (eight of 93). It should be noted that this study is, to our knowledge, the first to show a positive association between cyclin D1 staining and risk of development of adenocarcinoma in BE patients. Further independent investigations will, therefore, be valuable, both in confirming this positive association and in providing a more precise estimate of the risk associated with a positive cyclin D1 result. The possibility of using molecular markers to identify high-risk individuals to target with more frequent surveillance would represent a valuable advance in the clinical management of BE and provide an opportunity to improve prognosis of esophageal adenocarcinoma, a disease that is continuing to increase in public health relevance.

REFERENCES


NOTES

K. Bani-Hani would like to thank Mrs. Sally Gray and Mr. Peter Jackson for his training in immunohistochemistry.

Manuscript received November 3, 1999; revised May 26, 2000; accepted June 16, 2000.