Annals of Surgical Oncology, **6**(3):298–304
Published by Lippincott Williams & Wilkins

Significance of Fas and Retinoblastoma Protein Expression During the Progression of Barrett's Metaplasia to Adenocarcinoma

Domenico Coppola, MD, Robert H. Schreiber, MD, Linda Mora, MD, William Dalton, MD, PhD, and Richard C. Karl, MD

Background: Barrett's esophagus (BE) is a premalignant lesion characterized by replacement of normal squamous epithelium with columnar epithelium. This lesion can progress to dysplasia and adenocarcinoma. Recently, the Fas receptor and retinoblastoma (Rb) protein have been described as important mediators of apoptosis and tumor suppression, respectively. This study was undertaken to examine their expression during the progression of metaplasia to adenocarcinoma in BE.

Methods: In a review of 56 adenocarcinomas arising in BE, the specimen blocks were examined using the immunohistochemical avidin-biotin-peroxidase complex technique. For each specimen, areas of normal epithelium were compared with areas of metaplasia, dysplasia, or carcinoma (when present). Monoclonal mouse anti-human antibodies were used to identify Rb protein (Rb-Ab5, 1/50 dilution; Oncogene Science) and the 40–50-kDa cell membrane Fas protein (APO-1/Fas, 1/5 dilution; DAKO Corp.).

Results: Loss of Rb staining was observed as the metaplasia progressed to dysplasia and carcinoma, indicating accumulation of unstainable aberrant protein. Conversely, Fas protein staining was undetectable or weak in normal or metaplastic epithelium, increasing in the areas of high-grade dysplasia and carcinoma. These differences were statistically significant ($P < .001$).

Conclusions: The accumulation of abnormal Rb protein during the progression of Barrett's metaplasia to carcinoma leads to unsuppressed tumor growth. Fas overexpression may represent a cellular attempt to balance the uncontrolled tumor proliferation by promoting apoptosis.

Key Words: Barrett's esophagus—Adenocarcinoma—Fas—Retinoblastoma.

Barrett's esophagus (BE) is defined as the replacement of the normal stratified squamous epithelium of the lower esophagus with columnar epithelium. This is a premalignant condition that can progress to dysplasia and adenocarcinoma. The incidence of BE has been increasing in recent years; consequently, adenocarcinoma arising in BE has been the most rapidly increasing cancer in the last two decades.[1–4] It is estimated that approximately 10–20% of patients with symptomatic gastroesophageal reflux who undergo endoscopy have BE,[1] and they have a 30–125-fold increased risk of developing adenocarcinoma.[5] However, the natural history of BE is unknown, and the treatment of patients with BE is complicated by the inability to determine which patients will experience progression to adenocarcinoma.[6] The identification of patients at high risk of developing adenocarcinoma would be an important advance in the management of this disease, allowing closer follow-up monitoring and earlier intervention.[1–18] At this time, patients who present with BE-associated adenocarcinoma (BAAC) are usually at an advanced stage of disease, with very poor prognoses.[1] Overall 5-year survival rates for esophageal cancer are <20%.[19]

The expression of several oncogenes, cell surface receptors, and tumor suppressor genes has been correlated with tumor progression in BAAC; however, the results

Received March 27, 1998; accepted September 10, 1998.

From the Departments of Pathology (DC, LM), Surgery (RHS, RCK), and Internal Medicine (WD), H. Lee Moffitt Cancer and Research Institute and University of South Florida, Tampa, Florida.

Address correspondence and reprint requests to: Richard C. Karl, MD, Department of Surgery, H. Lee Moffitt Cancer and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612; Fax: 813-979-7229.

have been inconsistent.[7–18,20] It is well established that malignant transformation follows alterations in cell proliferation, cell cycle regulation, and programmed cell death. Despite recent advances in the understanding of apoptosis, studies using apoptotic markers in BAAC have been rare.[21,22] The Fas receptor, a 36-kDa protein and member of the tumor necrosis factor/nerve growth factor receptor family, has recently captured the attention of several investigators as an apoptosis-signaling surface receptor that is able to trigger programmed cell death.[23,24]

The retinoblastoma (Rb) protein is capable of modulating the expression of genes involved in the regulation of the cell cycle. It exerts this function by binding and inactivating the transcription factors (i.e., E2F) of such genes. Recently, Rb protein has been reported to be altered in BAAC.[14] However, this result has not been confirmed. In this investigation, we studied the expression of Fas receptor and Rb protein in a group of 56 patients who underwent esophagogastrectomy for treatment of BAAC.

MATERIALS AND METHODS

Immunohistochemical Assay Procedures

Tumor specimens from 56 patients who underwent gastroesophageal surgical resection for treatment of BAAC at Moffitt Cancer Center (Tampa, FL), between 1988 and 1997, were obtained. The hematoxylin/eosin-stained slides from the resected specimens were reviewed. Two representative sections for each case were selected for the study. Clinical information, including patient age, gender, tumor stage, and therapy, was obtained from the medical records. The specimens were fixed in formalin for not more than 24 hours and embedded in paraffin. Three-micron serial sections were cut and mounted on positively charged slides. Slides were deparaffinized with xylene and rehydrated with a series of ethanol washes. Sections from all specimens were immunohistochemically stained using mouse anti-human monoclonal antibodies to identify undetermined epitopes of Rb protein (Rb-Ab5, 1/50 dilution; Oncogene Science) and to identify the 40–50-kDa cell membrane Fas receptor (APO-1/Fas, 1/5 dilution; DAKO Corp.). Nonenzymatic antigen retrieval was performed as previously described.[25] Staining was performed manually, using the avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector, Burlingame, CA). This three-step indirect immunoperoxidase staining procedure was performed at room temperature. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 3% aqueous hydrogen peroxide for 10 minutes. After being washed with phosphate-buffered saline (PBS) for 5 minutes, slides were blocked with normal serum for 20 minutes, followed by incubation with each of the aforementioned antibodies for 60 minutes. After being rinsed with PBS for 5 minutes, sections were incubated with a biotinylated secondary antibody for 20 minutes. After being washed with PBS for 5 minutes, slides were incubated with avidin-biotin complex for 30 minutes and washed again. Chromogen was developed with 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma), diluted in 12 ml of Tris buffer at pH 7.6, for 2 minutes. All slides were lightly counterstained with Mayer's hematoxylin for 30 seconds before dehydration and mounting. Positive controls and nonimmune protein-negative controls were used for each section with each antibody.

Analysis of Immunohistochemical Data

The stained slides were examined microscopically by two independent observers (DC and RHS), using the following parameters and semiquantitative criteria: 0, negative; 1+, <25% positive staining area; 2+, 25 to <50% positive staining area; 3+, 50 to <75% positive staining area; 4+, 75 to 100% positive staining area. Positive staining was also graded in intensity (0 to 3+). A combined preliminary score of 0 to 7 was assigned. This was then divided by 2 to assign a final score of 0 to 3.5. Tumors given a score of 0 to <1 were classified as negative, those given a score of 1 to <2 were classified as weakly positive, those given a score of 2 to <3 were regarded as moderately positive, and those given a score of 3 to >3 were considered strongly positive. The immunohistochemical reaction was also independently evaluated using a Cell Analysis Systems (CAS) 200 image-analysis system (Cell Analysis Systems, Elmhurst, IL). A mean of 500 cells within at least 10 adjacent fields, at a magnification of ×400, were analyzed for each section.

Statistical Analysis

The results obtained with the CAS measurements were analyzed using the Friedman's test to determine differences between groups. The Wilcoxon signed-rank test was used to determine differences between individual score cohorts, e.g., BAAC vs. high-grade dysplasia (HGD) vs. low-grade dysplasia (LGD). Values were considered significant at .005, using the Bonferroni procedure for multiple comparisons. Correlation between Fas and Rb staining in BAAC was assessed using Spearman's correlation coefficient.

RESULTS

Clinicopathological Features of the Tumors Studied

The age of the patients ranged between 36 and 82 years (average, 65 years; SD, 9.3 years) (Table 1). Fifty-two patients were male and four were female. All patients underwent Ivor-Lewis esophagogastrectomy, including a standard lymph node dissection. The size of the resected tumors ranged between 0.5 and 11.7 cm (mean, 4 cm; SD, 3 cm). All tumors were invasive adenocarcinoma arising in a background of goblet-cell intestinal metaplasia and were located in the lower one-third of the esophagus. Two representative sections for each case were studied. In 32 cases intestinal metaplasia and in 34 cases HGD and LGD were present adjacent to the invasive adenocarcinoma. In 38 cases, histologically normal squamous mucosa was identified in the same section containing the invasive adenocarcinoma and/or the dysplastic or metaplastic epithelium. Three (5.4%) tumors were well differentiated, 28 (50%) moderately differentiated, and 25 (44.6%) poorly differentiated. Four (7.1%) patients had stage I, 18 (32%) stage II, 24 (43%) stage III, and 10 (18%) stage IV disease. Five patients received postoperative chemotherapy. For eight patients, radiation and chemotherapy were administered after surgical resection; only two patients received preoperative chemoradiation.

Immunohistochemical Analysis

Immunohistochemically, diffuse and strong to moderate nuclear Rb positivity was detected in 11 (20%) BAAC cases, and focal/weak staining was identified in 23 (41%) BAAC cases (Table 2). Rb staining was negative in 22 (39%) cancers. In contrast to the constant (90%), diffuse, and strong Rb nuclear staining decorating the basal proliferative layer of the normal squamous mucosa, fading of the Rb staining was observed during the progression from LGD (CAS mean score, 29.5%; SD, 9.1%) to HGD (CAS mean score, 17.8%; SD, 7.8%) to BAAC (CAS mean score, 11.5%; SD, 9.8%) ($P < .0001$) (Fig. 1). Conversely, intense but patchy Fas staining decorated the cellular membrane of the tumor cells in 32 (57%) BAAC cases. Staining was weak in 15 (27%) cases and negative in nine (16%) cases. Fas stained the normal squamous mucosa (CAS mean score, 0.3%; SD, 0.4%) and the LGD (CAS mean score, 1.6%, SD, 3.4%) in only a minority of cases. However, increasing cytoplasmic Fas expression was detected during the progression to HGD (CAS mean score, 8.6%; SD, 5.9%) and to BAAC (CAS mean score, 16%; SD, 7.6%) ($P < .0001$) (Fig. 2, A and B). Interestingly, these Fas-negative tumors always strongly and diffusely expressed Rb. Although inversely correlated (Spearman's correlation coefficients of $-.095$ and $-.06$ for CAS and semiquantitative methods, respectively), these correlations were not statistically significant ($P = .48$ and $P = .66$, respectively). Negative controls did not show any staining, and background staining was insignificant. At follow-up examinations, 34 patients were dead (31 with evidence of disease). Twenty-one patients were alive (15 with no evidence of disease). One patient was lost to follow-up monitoring. The median survival time was 1.24 years, and the mean survival time was 1.70 years. Using a Cox (proportional-hazard) regression analysis, neither Fas ($P = .59$) nor Rb ($P = .73$) correlated with postsurgical survival rates. Figure 3 summarizes, in graphical form, the results of Rb staining, and Figure 4 presents the results of Fas staining.

TABLE 1. *Clinicopathological features*

	No. of tumors
Stage	
I	7 (12.5%)
II	18 (32%)
III	24 (42.9%)
IV	7 (12.5%)
Total	56 (100%)
Grade	
Well differentiated	3 (5.4%)
Moderately differentiated	28 (50%)
Poorly differentiated	25 (44.6%)
Total	56 (100%)

Age at diagnosis, 36–82 years; tumor size, 0.5–11.7 cm.

TABLE 2. *CAS and semiquantitative analysis data*

	Positivity (%)					
	CAS			Semiquantitative		
	n	Mean	SD	n	Mean	SD
Fas staining						
Adenocarcinoma	56	16	7.6	56	2.2	1.3
HGD	34	8.3	5.9	34	2.4	1.34
LGD	34	1.6	3.4	34	0.72	0.96
Metaplasia	32	0.4	0.4	32	0.64	1.01
Normal tissue	38	0.28	0.38	38	0.5	0.97
Rb staining						
Adenocarcinoma	56	11.5	9.8	56	1.8	0.87
HGD	34	17.8	7.8	34	2.3	0.71
LGD	34	29.5	9.1	34	2.8	0.66
Metaplasia	32	13.1	9.5	32	1.7	0.98
Normal tissue	38	32.2	8.9	38	3	0.48

DISCUSSION

The search for molecular markers to identify patients with BE that is destined to progress to cancer is ongoing. Growth factors and their receptors, oncogenes, tumor suppressor genes, and cell cycle regulators have all been tested, with inconsistent results.[7–18,20] To date, the most

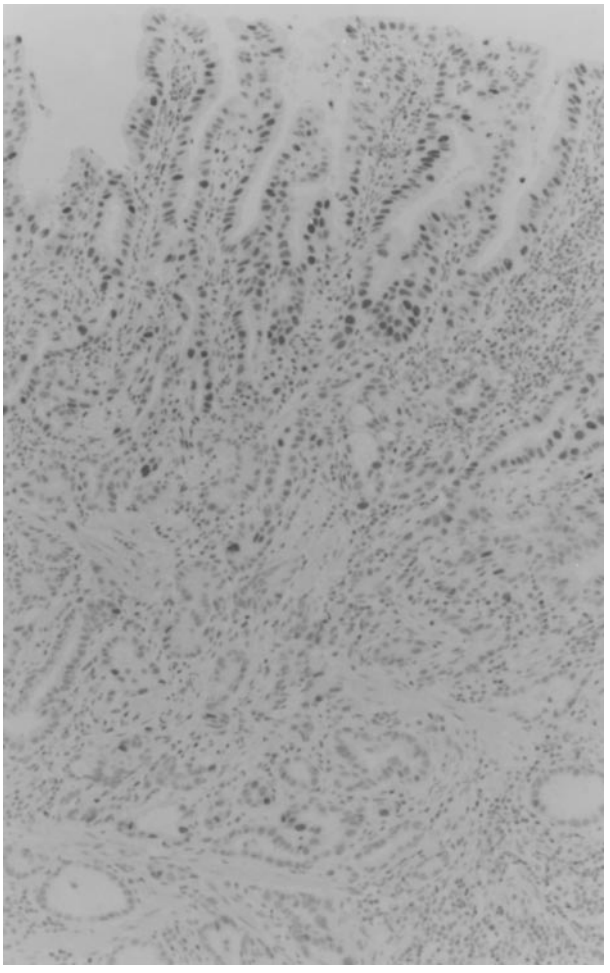

FIG. 1. Fading of Rb nuclear staining during the transition from LGD (mucosal surface) to HGD and invasive adenocarcinoma (immunostaining; original magnification, ×100).

reliable marker of tumor progression in BE has been DNA ploidy. Reid et al.[26,27] and others[28] have reported that premalignant lesions of BE (dysplasia) are commonly associated with aneuploidy. However, the endoscopic distinction between metaplastic and dysplastic mucosa is difficult, rendering appropriate correlation and reproducible sampling for flow cytometric analysis difficult. Immunohistochemical and/or in situ hybridization analyses seem more reliable techniques, allowing objec-

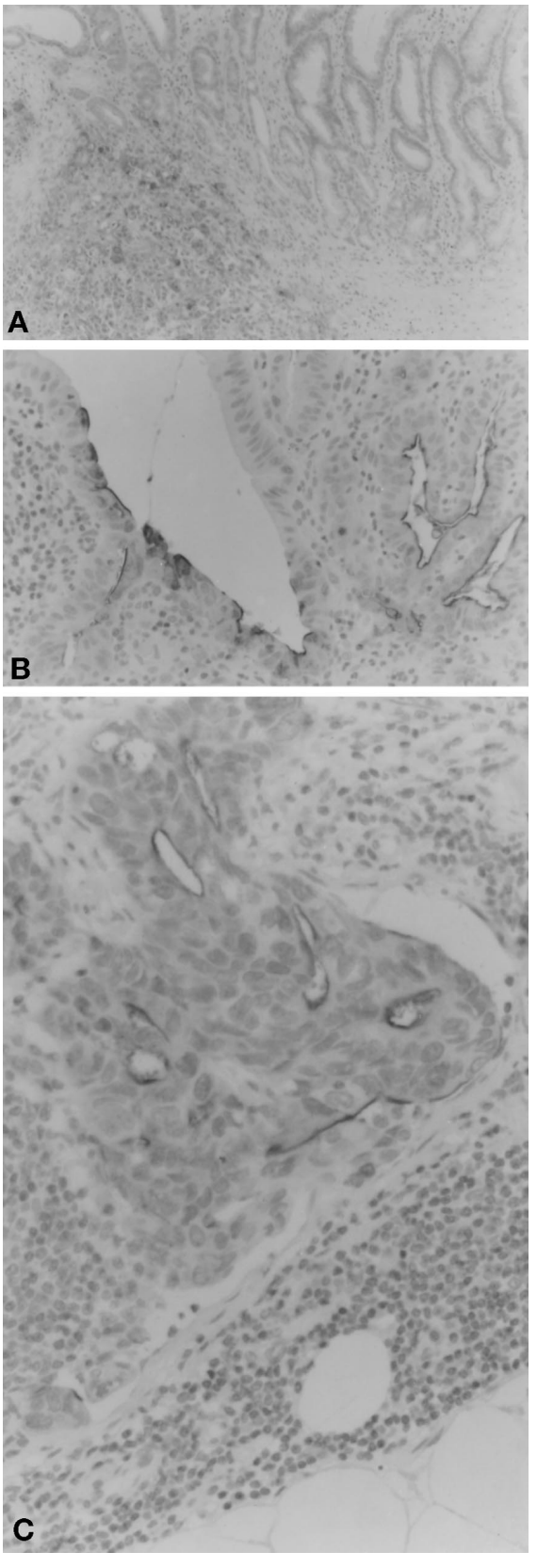

FIG. 2. (**A**) Metaplastic esophageal mucosa showing weak Fas cytoplasmic staining, compared with the strong and diffuse Fas-positive BAAC (immunostaining; original magnification, ×100). (**B**) Metaplastic gland in transition to HGD. Note the contrast between Fas-negative LGD and strongly Fas-positive HGD (immunostaining; original magnification, ×300). (**C**) BAAC, metastasized to a lymph node, exhibiting strong membranous Fas immunoreactivity (immunostaining; original magnification, ×300).

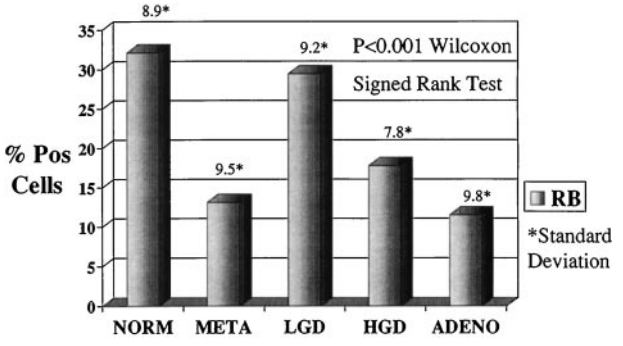

FIG. 3. Rb staining. *NORM*, normal tissue; *META*, metaplasia; *ADENO*, adenocarcinoma; *Pos*, positive.

tive correlation between test positivity and histological characteristics of the lesion tested.

In this study we used immunohistochemical analyses to detect changes in Rb and Fas protein expression during the progression of Barrett's metaplasia to dysplasia and to adenocarcinoma. Our data indicate that Rb protein becomes abnormal, and therefore immunohistochemically undetectable, as the metaplasia progresses to dysplasia and carcinoma. Conversely, we report the novel finding of increased Fas protein expression in BAAC.

It is well established that malignant transformation follows alterations in cell proliferation, cell cycle regulation, and programmed cell death. Therefore, in this study we focused on two molecular markers with pivotal roles in either cell cycle regulation (Rb) or programmed cell death (Fas).

The *Rb* gene was the first tumor suppressor gene to be identified.[29] It is located on chromosome 13q14 and encodes a nuclear protein with a pivotal role in cell cycle regulation.[30] To be functional, Rb protein must be phosphorylated. Cyclin D1/cdk4 or cyclin D1/cdk6 complexes regularly phosphorylate Rb protein during the mid-G_1 phase of the cell cycle.[31] The functional consequence of Rb hyperphosphorylation is the release of transcriptional factors, such as E2F, that modulate the expression of genes involved in cell cycle progression. Conversely, in the nonphosphorylated or hypophosphorylated form, Rb represses cell proliferation by complexing with the transcription factors (E2F) and enabling them to exert their function.[32] Loss of heterozygosity at the *Rb* locus and mutated Rb protein expression have been described in carcinoma of the esophagus.[14] Similarly, we report the presence of altered Rb protein expression (negative or weak focal staining) in 80% of BAAC cases. Interestingly, the histological sections taken from these tumors showed a gradual loss of Rb immunoreactivity during the progression from metaplasia to dysplasia to carcinoma. This finding indicates that, in a large proportion of BAAC cases, alterations in Rb function underlie the unregulated cell proliferation and reflect the selective advantage of the mutated Rb phenotype during tumor progression. In the metaplastic epithelium, Rb staining was usually confined to the basal crypts, which, because of embedding artifacts, were not always evident in the sections studied. This may account for the lower levels of Rb staining detected in the metaplastic epithelium, compared with LGD. In a subset of BAAC cases, however, the Rb protein was functional (strong immunostaining), indicating that in this group of patients an alternative alteration must exist to account for the malignant transformation (i.e., p53).

Fas (APO-1, CD95) is a 48-kDa cellular membrane protein belonging to the tumor necrosis factor/nerve growth factor receptor family. When activated, this receptor is able to trigger apoptosis. The binding of the Fas ligand to the Fas receptor induces trimerization of the receptor and activation of the death-inducing signaling cascade, which ultimately stimulates the death effector molecule interleukin-1β-converting enzyme, inducing cell death.[23,24] Fas was initially detected on activated T lymphocytes, functioning as a modulator of cell-mediated immunity by inducing apoptosis in autoreactive lymphocytes.[33] Later, this receptor was also identified in a large variety of normal and neoplastic tissues.[34] Only recently has the expression of Fas protein been investigated in BAAC.[21] Hughes et al.[22] reported decreased levels of Fas protein in esophageal adenocarcinomas, compared with Barrett's metaplasia and dysplasia. However, the authors noted that some of the tumors, despite the clear presence of Fas protein within the cells (detected by Western blotting), exhibited a decreased immunohistochemical staining pattern.[22] As suggested by the authors, this finding may be ascribed to loss of cellular antigens during manipulation and storage of the tissue or to inadequate anti-Fas antibodies. The possibility that the

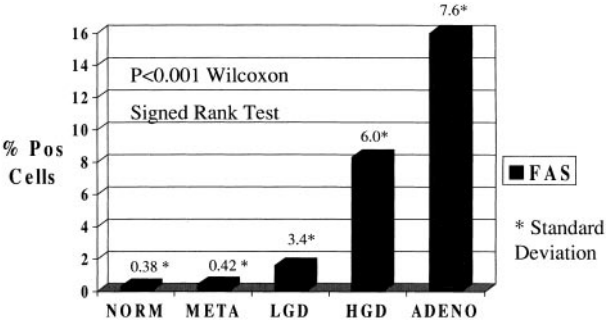

FIG. 4. Fas staining. *NORM*, normal tissue; *META*, metaplasia; *ADENO*, adenocarcinoma; *Pos*, positive.

binding epitopes are not exposed in the cytoplasmic (immature) protein is valid; however, the antibody we used yielded both cytoplasmic and membranous staining. In our study we noted that Fas staining in most of the tumors was patchy but strong and was usually more intense on the cellular membrane. The normal squamous epithelium, when present, was Fas negative. Weak staining, usually localized to the surface, was observed in only a few cases. This most likely represents nonspecific staining of the superficial, dead, squamous cells. In the carcinomas, the patchiness of the staining accounts for the false lower scores obtained using CAS analysis, compared with the semiquantitative method. The latter yields a more objective estimation of the percentage of positive tumor cells in each case. However, independently of the method, we noted increased Fas protein expression in BAAC, compared with metaplasia and dysplasia. This finding is puzzling and may represent a compensatory mechanism by which the tumor cells counteract their unregulated proliferation. It is interesting that all of the Rb-negative tumors were strongly and diffusely positive for Fas. This observation is in agreement with the findings of Katada et al.,[21] who found Bcl-2 overexpression in 72% of Barrett's metaplasia cases and 100% of LGD cases but in only 20–40% of carcinomas. The inverse relationship between Fas and Bcl-2 protein expression in BAAC is not surprising, because Bcl-2 protein inhibits apoptosis, possibly via modifications of cytochrome *c* translocation and blocking of caspase activation.[35] Katada et al.,[21] in their study, noted that Bcl-2 was preferentially localized to the basal proliferative zone of the esophageal squamous epithelium, suggesting a function of Bcl-2 in protecting the stem cells of the esophageal epithelium. Those authors also noted increased apoptotic bodies at the surface of the esophageal squamous epithelium in reflux esophagitis, and they hypothesized that increased apoptosis in this context may represent a protective mechanism, counteracting increased epithelial cell proliferation.[20] We think that a similar mechanism may be active in BAAC. Despite the lack of correlation, in the cases studied, of either Rb or Fas receptor expression with survival rates, conclusions regarding the significance of these proteins as predictors of tumor behavior cannot be drawn. In fact, the patients in our study had all reached the end stage of Barrett's progression. This issue deserves a more thorough investigation in a prospective study.

In conclusion, we studied the expression of Fas and Rb protein expression in a group of esophagogastrectomy specimens from patients with BE. We report the novel accumulation of Fas and abnormal Rb proteins during the progression of Barrett's dysplasia to carcinoma. Fas overexpression in BAAC may represent an attempt by tumor cells to balance the uncontrolled cell proliferation promoted by nonfunctional Rb.

REFERENCES

1. Blot WJ, Devesa SS, Kneller RW, Fraumeni JF Jr. Rising incidence of adenocarcinoma of the esophagus and gastric cardia. *JAMA* 1991;265:1287–9.
2. Pera M, Cameron AJ, Trastek VF, et al. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. *Gastroenterology* 1993;104:510–3.
3. Spechler SJ, Goyal RK. Barrett's esophagus. *N Engl J Med* 1986; 315:362–71.
4. Van der Veen AH, Dees J, Blankensteijn JD, et al. Adenocarcinoma in Barrett's oesophagus: an overated risk. *Gut* 1989;30: 14–8.
5. Dent J, Bremner CG, Collen MJ, Haggitt RC, Spechler SJ. Barrett's esophagus: Working Party report to the World Congress of Gastroenterology, Sydney, Australia, 1990. *J Gastroenterol Hepatol* 1991;6:1–22.
6. Mangla JC. Barrett's epithelium: regression or no regression? *N Engl J Med* 1980;303:529–530.
7. Rustgi AK. Biomarkers for malignancy in the columnar-lined esophagus. *Gastroenterol Clin North Am* 1997;26:599–606.
8. Wang LD, Shi ST, Zhou Q, et al. Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastria-cardia carcinogenesis. *Int J Cancer* 1994; 59:514–9.
9. Hickey K, Grehan D, Reid IM, O'Briain S, Walsh TN, Hennessy TPJ. Expression of epidermal growth factor receptor and proliferating cell nuclear antigen predicts response of esophageal squamous cell carcinoma to chemoradiotherapy. *Cancer* 1994;74:1693–8.
10. Casson AG, Kerkvliet N, O'Malley F. Prognostic value of p53 protein in esophageal adenocarcinoma. *J Surg Oncol* 1995;60:5–11.
11. Ireland AP, Clark GWB, DeMeester TR. The significance of p53 in clinical practice. *Ann Surg* 1997;225:17–30.
12. Soslow RA,Ying L, Altorki NK. Expression of acidic fibroblast growth factor in Barrett's esophagus and associated esophageal adenocarcinoma. *J Thorac Cardiovasc Surg* 1997;114:838–43.
13. Swami S, Kumble S, Triadafilopoulos G. E-Cadherin expression in gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma: an immunohistochemical and immunoblot study. *Am J Gastroenterol* 1995;90:1808–13.
14. Boynton RF, Huang Y, Blount PL, et al. Frequent loss of heterozygosity at the retinoblastoma locus in human esophageal cancers. *Cancer Res* 1991;51:5766–9.
15. Ray GS, Lee JR, Nwokeji K, Mills LR, Goldenring JR. Increased immunoreactivity for Rab11, a small GTP-binding protein, in low-grade dysplastic Barrett's epithelia. *Lab Invest* 1997;77:503–11.
16. Barrett MT, Sanchez CA, Galipeau PC, Neshat K, Emond M, Reid BJ. Allelic loss of 9p21 and mutation of the *CDKN2/p16* gene develop as early lesions during neoplastic progression in Barrett's esophagus. *Oncogene* 1996;13:1867–73.
17. Al-Kasspooles M, Moore JH, Orringer MB, Beer DG. Amplification and overexpression of the *EGFR* and *erbB-2* genes in human esophageal adenocarcinomas. *Int J Cancer* 1993;54:213–9.
18. Kumble S, Omary MB, Cartwright CA, Triadafilopoulos G. Src activation in malignant and premalignant epithelia of Barrett's esophagus. *Gastroenterology* 1997;112:348–56.
19. Daly JM, Karnell LH, Menck HR. National cancer data base report on esophageal carcinoma. *Cancer* 1996;78:1820–8.
20. Shimada Y, Imamura M, Shibagaki I, et al. Genetic alterations in patients with esophageal cancer with short- and long-term survival rates after curative esophagectomy. *Ann Surg* 1997;226:162–8.
21. Katada N, Hinder RA, Smyrk TC, et al. Apoptosis is inhibited

early in the dysplasia-carcinoma sequence of Barrett esophagus. *Arch Surg* 1997;132:728–33.
22. Hughes SJ, Nambu Y, Soldes OS, et al. Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma. *Cancer Res* 1997;57:5571–8.
23. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267:1456–62.
24. Nagata S, Golstein P. The Fas death factor. *Science* 1995;267: 1449–56.
25. Shi SR, Key ME, Kalza KL. Antigen retrieval in formalin-fixed paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;39:741–8.
26. Reid BJ, Blount PL, Rubin CE, Levine DS, Haggitt RC, Rabinovitch PS. Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective endoscopic surveillance of a cohort. *Gastroenterology* 1992;102:1212–19.
27. Reid BJ, Sanchez CA, Blount PL, Levine DS. Barrett's esophagus: cell cycle abnormalities in advancing stages of neoplastic progression. *Gastroenterology* 1993;105:119–29.
28. Nakamura T, Nekarda H, Hoelscher AH, et al. Prognostic value of DNA ploidy and c-ErbB-2 oncoprotein overexpression in adenocarcinoma of Barrett's esophagus. *Cancer* 1994;73:1785–94.
29. Knudson AG. Mutation and human cancer. *Adv Cancer Res* 1973; 17:317–52.
30. Lee WH, Shew JY, Hong F, et al. The retinoblastoma susceptibility gene product is a nuclear phosphoprotein associated with DNA binding activity. *Nature* 1987;329:642–5.
31. Niculescu AB III, Chen X, Smeets M, Hengst L, Prives C, Reed SI. Effects of p21 (Cip1/Waf1) at both the G_1/S and the G_2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol Cell Biol* 1998;18:629–43.
32. Lundberg AS, Weinberg RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-Cdk complexes. *Mol Cell Biol* 1998;18:753–61.
33. Debatin KM, Goldman CK, Bamford R, Waldmann TA, Krammer PH. Monoclonal antibody mediated apoptosis in adult T cell leukemia. *Lancet* 1990;335:497–500.
34. Leithäuser F, Dhein J, Mechtersheimer G, et al. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. *Lab Invest* 1993;69:415–29.
35. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;275:1132–6.