Immunohistochemical Evaluation of a Panel of Tumor Cell Markers During Malignant Progression in Barrett Esophagus

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Abstract

Histopathologic grading of dysplasia in Barrett esophagus (BE) shows substantial interobserver and intraobserver variation. We used immunohistochemical analysis with a set of tumor cell markers, ie, epidermal growth factor receptor (EGFR), ERBB2 (HER2/neu), MYC, CDKN2A (p16), SMAD4, MET, CCND1 (cyclin D1), CTNNB1 (β-catenin), and TP53 (p53), in histologic sections of endoscopic biopsies of 86 patients with BE in various stages of neoplastic progression. The markers, except SMAD4, were scored as 0 (<1% of cells stained), 1 (1%-25%), 2 (26%-50%), or 3 (>50%). All markers, except EGFR, showed a significant trend for immunohistochemical protein overexpression during malignant progression in BE (P < .01). When the successive stages along the metaplasia–low-grade dysplasia (LGD)–high-grade dysplasia (HGD)–adenocarcinoma axis were compared, protein overexpression of β-catenin separated LGD from metaplasia, whereas protein overexpression of cyclin D1 and p53 discriminated HGD from LGD (all P < .001). β-Catenin can be helpful for a diagnosis of LGD in BE, although it stains positively in a subset only, whereas p53 remains an appropriate marker to define HGD. In case of doubt, cyclin D1 can be added to separate LGD from HGD in BE.

Barrett esophagus (BE) is the major precursor of esophageal adenocarcinoma and is endoscopically characterized by a salmon pink, velvety appearance of the distal esophageal lining. Histopathologically, BE is defined by the presence of a specialized columnar epithelium with the presence of goblet cells, which is referred to as intestinal metaplasia.1,2 Histopathologically, the development of an adenocarcinoma seems to be preceded by epithelial dysplasia. Dysplasia is defined as neoplastic proliferation within epithelial glands without affecting the basement membrane. Dysplastic changes might be taken as early indicators of incipient malignancy. This is important, because on the one hand, patients with BE have a 30- to 40-fold increased risk for esophageal adenocarcinoma, but on the other hand, cancer eventually develops in only a low percentage of patients with BE.3,4

The incidence of esophageal adenocarcinoma is rising rapidly in the Western world.5 The development of adenocarcinoma follows a multistep sequence from intestinal metaplasia to low-grade dysplasia (LGD), high-grade dysplasia (HGD), and, finally, adenocarcinoma.1,6,7 However, histopathologic grading of dysplasia is subject to intraobserver and interobserver variation.8,9 LGD is rather indolent and not a reliable hallmark for malignancy.10,11 Moreover, dysplastic BE is often multifocal within the Barrett segment.12 There is no doubt that HGD is an indication for surgery or endoscopic mucosectomy because it confers a high risk of developing into adenocarcinoma, but some controversy concerns the extent of HGD and the risk of adenocarcinoma.13 Buttar et al14 investigated whether a limited extent of HGD had the same potential for cancer development as diffuse HGD. They found that patients with focal HGD are...
less likely to develop adenocarcinoma than patients with diffuse HGD.\textsuperscript{14} Weston et al\textsuperscript{15} monitored the fate of patients with focal HGD. In approximately 50\% of the patients, the disease progressed to multifocal HGD or cancer.

Cell biologic markers have been studied in the progression of BE to adenocarcinoma. Malignant progression has been assessed by determining cell cycle parameters and by studying growth factors. For example, the growth fraction was studied by staining for the proliferative cell nuclear antigen or Ki-67.\textsuperscript{16,17} These investigators found a high proportion of cycling cells in intestinal-type epithelium with expansion of the proliferative compartment. Reid et al\textsuperscript{18} documented by flow cytometry that cell cycle abnormalities occur in the development of adenocarcinoma in BE. Moreover, polyploidy and aneuploidy have been reported as an early event in BE.\textsuperscript{19,20} Alteration of the p53 tumor suppressor gene is likely the most frequent genetic lesion in BE and adenocarcinoma. In metastatic BE, p53 protein overexpression is generally not observed, whereas p53 accumulation increases during progression from LGD to HGD.\textsuperscript{21,22} p16 is a tumor suppressor gene, which is also known as cyclin-dependent kinase inhibitor 2A (CDKN2A). Loss of heterozygosity is the predominant mechanism for inactivation of 1 of the p16 alleles, occurring in approximately 75\% of Barrett carcinomas.\textsuperscript{23} The value of cell cycle regulator cyclin D1 as a biomarker for malignant progression in BE is unclear. Conflicting results were reported concerning the usefulness of this protein.\textsuperscript{24,25} This also accounts for Wnt pathway–associated gene β-catenin. A decrease of membranous and an increase of nuclear β-catenin staining has been observed during progression to adenocarcinoma in BE.\textsuperscript{26,27} However, in a case-control study by Murray et al,\textsuperscript{28} increased nuclear β-catenin expression in carcinomas was not significantly different from that in control samples. This was also found for p53 and cyclin D1.\textsuperscript{28} Involvement of the oncogenes MYC, EGFR, HER2/neu (ERBB2), and MET has been investigated in BE-related cancer but not in preneoplasias (reviewed by Koppert et al\textsuperscript{29}). Likewise, for SMAD4, also termed DPC4, only DNA analyses have been described.\textsuperscript{30} This lack of practical information prompted us to investigate the usefulness of a large panel of tumor-associated protein markers for the histopathologic assessment of malignant progression in BE.

Materials and Methods

Patient Samples

We studied samples from 86 patients (64 men and 22 women) with BE, ie, intestinal metaplasia without dysplasia (BAR; n = 22), LGD (n = 19), HGD (n = 20), and adenocarcinoma (n = 25). The mean age of the patients was 64 years (range, 38-82 years). The following inclusion criterion was applied: endoscopic BE with specialized intestinal metaplasia on histologic examination. All biopsy specimens were formalin-fixed and paraffin-embedded and stained with H&E. Using standard criteria, all histologic diagnoses were made by 2 experienced gastrointestinal pathologists (H.v.D. and H.vd.V.). This study was approved by the review board of the Erasmus Medical Center, Rotterdam, the Netherlands.

Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded, routine 5-μm tissue sections were mounted on aminoacetylsilane-coated slides (Starfrost, Berlin, Germany), and immunostaining was performed using the EnVision system (DAKO, Glostrup, Denmark) as described.\textsuperscript{31,32} In brief, the sections were deparaffinized in xylene and dehydrated in alcohol. Endogenous peroxidase activity was blocked by incubation with 3\% hydrogen peroxide in methanol for 20 minutes. For all antibodies, microwave (700 W) pretreatment in tris(hydroxymethyl)aminomethane-EDTA, pH 9.0, was performed for 15 minutes. All primary antibodies, ie, epidermal growth factor receptor (EGFR; clone 31G7, diluted 1:50; Zymed, San Francisco, CA), HER2/neu (clone SP3, diluted 1:50; Lab Vision NeoMarkers, Fremont, CA), MYC (clone 9E10, diluted 1:600; Santa Cruz Biotechnology, Santa Cruz, CA), p16 gene product (clone 6H12, diluted 1:100; Novocastra Laboratories, Newcastle upon Tyne, England), SMAD4 (clone B8, diluted 1:100; Santa Cruz Biotechnology), MET (clone C-12, diluted 1:100; Santa Cruz Biotechnology), cyclin D1 (clone SP4, diluted 1:50; Lab Vision NeoMarkers, Fremont, CA), β-catenin (clone 14, diluted 1:100; BD Transduction Laboratories, San Jose, CA), and p53 (clone DO-7, diluted 1:200; DAKO) were incubated overnight at 4°C, followed by the secondary step of the EnVision system. A pan-keratin antibody was used as a positive control (clone AE1-AE3; diluted 1:200; BioGenex, San Ramon, CA), and negative control experiments were done by omitting the first antibody. Diaminobenzidine tetrachloride from the EnVision kit was used as the chromogen for visualization. Tissues were counterstained with hematoxylin. Finally, the slides were dehydrated, cleared in xylene, and mounted in Malinol (Chroma-Gesellschaft, Köngen, Germany).

The immunoreactivity pattern was evaluated independently by 2 observers. Nuclear immunostaining was scored for MYC, p16, SMAD4, cyclin D1, β-catenin, and p53. For EGFR, HER2/neu, and MET only membranous expression was counted. For each marker, at least 200 cells were scored in representative areas, ie, longitudinally sectioned Crypts in metaplasia and dysplasia. All markers, except SMAD4, were scored as 0 (<1\% of cells stained), 1 (1%-25\% of cells), 2 (26%-50\% of cells), or 3 (>50\% of cells). For SMAD4, 0, 1, 2, and 3 referred to more than 99\% of positive cells, 75\%
to 99% of cells, 50% to 74% of cells, and fewer than 50% of positively stained cells, respectively. No discrepancies emerged using this procedure.

Statistical Evaluation

The Mann-Whitney Test was applied for comparison of immunostaining results (scores 0-3) between groups. The Spearman correlation coefficient was used to evaluate significant trends along the metaplasia-LGD-HGD-adenocarcinoma axis. A P value equal to .05 (2-tailed) was taken as the limit of significance. A P value between .05 and .10 was considered a statistical trend (borderline significance).

Results

We used immunohistochemical analysis with a set of tumor cell markers, ie, EGFR, HER2/neu, MYC, p16, SMAD4, MET, cyclin D1, β-catenin, and p53, to evaluate dysplasia in histologic sections of endoscopic biopsy specimens of 86 patients with BE. For this purpose, we selected 22 cases of intestinal metaplasia without dysplasia, 19 of LGD, 20 of HGD, and 25 of esophageal adenocarcinoma.

Nuclear immunostaining was scored for MYC, p16, SMAD4, cyclin D1, β-catenin, and p53. For EGFR, HER2/neu, and MET only membranous expression was counted. All markers, except EGFR, showed a significant trend for immunohistochemical protein overexpression during malignant progression in BE. The P values for SMAD4, MET, cyclin D1, β-catenin, and p53 were less than .001, whereas HER2/neu, MYC, and p16 revealed P values less than .01, indicating accumulating patterns of aberrant protein expression in the subsequent stages of malignant progression. When the successive stages along the metaplasia-LGD-HGD-adenocarcinoma axis were compared, protein overexpression of β-catenin was found to separate LGD from HGD, whereas protein overexpression of cyclin D1 and p53 discriminated HGD from LGD (all P < .001; Table 1, Image 1).

Table 1 demonstrates the mean immunoscore patterns of the tested antibodies. It illustrates the increasing number of immunopositive epithelial cells during Barrett carcinogenesis and progression. However, in daily practice, it might be more appropriate to use a window of “clearly” positive staining (immunoscores 2 + 3 combined, ie, >25% of the target cells) with this threshold, β-catenin is positive in about 25% of LGD cases. It should be noted that for β-catenin, only nuclear immunostaining should be evaluated. Cyclin D1 is “clearly” positive (>25% of the target cells) in 45% of HGD cases, whereas p53 delineates 70% of the cell nuclei. Therefore, p53 is first choice to help establish a diagnosis of HGD. Furthermore, p53 was an easier target to assess than cyclin D1, which weakly stains the proliferative compartment. In case of difficulty, cyclin D1 can be added to p53 staining to separate LGD from HGD in BE.

Discussion

We evaluated a panel of 9 cancer-related proteins for delineation of morphologic changes during neoplastic progression in BE. This study was not designed to provide independent biomarkers for malignant progression in BE but to assess the usefulness of the cancer-related proteins for decision making about pathologic findings. The oncogenic proteins EGFR, HER2/neu, MYC, and MET, as well as tumor suppressor-related proteins p16 and SMAD4, were predominantly aberrant within the HGD and adenocarcinoma groups but could not be used to improve a specific diagnosis. In contrast, immunostaining with β-catenin, cyclin D1, and p53 was useful for establishing diagnoses of LGD and HGD in BE. These 3 antibodies are strongly immunoreactive and relatively easy to score. However, it needs to be mentioned that for β-catenin, only nuclear protein expression should be evaluated. Furthermore, only a subset of LGD is positive, limiting broad application of this protein for a diagnosis of LGD in BE. Cyclin D1 shows a faint staining of proliferating cells, which might interfere with a correct interpretation. Nevertheless, as shown herein, these 3 antibodies can be used as an adjunct to come to a diagnosis of LGD or HGD in BE. In the LGD group, 7 (of 19) biopsy specimens were initially graded “indefinite for dysplasia.” The immunoprofiles for
β-catenin, cyclin D1, and p53 did not differ from the overall immunoscore of this group, suggesting that indefinite for dysplasia indeed correlates with LGD and that these 3 markers can be used for classification of difficult cases.

The p53 gene is involved in controlling cell proliferation. Mutations of p53 are associated with an increased half-life of the p53 protein, resulting in its accumulation in the cell nucleus to levels that can be detected immunohistochemically. Several studies have shown a stepwise overexpression of p53 with increasing grades of dysplasia in BE. Younes et al suggested that p53 accumulation might even occur before the phenotypic changes characteristic of dysplasia and malignancy become obvious because they also discerned normal-appearing nondysplastic glands adjacent to dysplastic glands or carcinoma positive for p53. This phenomenon was not seen in our series. p53 as a marker for malignant progression in BE was reported in other studies, but the sensitivity of this marker alone appeared too low to predict cancer risk. Thus, although not capable of predicting malignant progression, p53 immunohistochemical analysis can be adequately used for diagnostic purposes.

We detected increasing patterns of protein expression of cyclin D1 and β-catenin along the metaplasia-dysplasia-carcinoma axis in BE. In a prospective study by Bani-Hani et al, immunohistochemically detected cyclin D1 was found significantly overexpressed in almost all Barrett carcinomas. In
addition, a high proportion of biopsy specimens obtained at earlier times showed cyclin D1 overexpression, compared with about one third of biopsy specimens from control subjects without malignant progression in BE. It was then suggested that cyclin D1 staining could be a useful biomarker in identifying patients with BE with an increased risk of neoplastic progression.\textsuperscript{24} These results were, however, contradictory to results of other studies in which cyclin D1 was not significantly associated with risk of malignant progression.\textsuperscript{25,28} The adenomatous polyposis coli (\textit{APC}) tumor suppressor gene regulates intracellular concentration of \(\beta\)-catenin by causing its degradation. When the \textit{APC} tumor suppressor gene is mutated, membranous \(\beta\)-catenin decreases and the molecule accumulates in the nucleus, promoting cellular proliferation. In BE, a decrease of membranous \(\beta\)-catenin on the one hand and an increase of nuclear \(\beta\)-catenin on the other has been observed during progression from BE to cancer in most but not all studies.\textsuperscript{26-28,37}

We created a map of protein changes from well-known oncogenes and tumor suppressor genes during neoplastic progression in BE. We found adequate statistical associations between grades of dysplasia and immunostaining profiles for \(\beta\)-catenin, cyclin D1, and p53. These cancer-related proteins might thus serve as an aid to standard histologic examination for the grading of dysplasia in Barrett epithelium.

\textbf{E-H.} Low-grade dysplasia. Nuclear protein overexpression is detected for \(\beta\)-catenin (\textbf{F}) but not for p53 (\textbf{G}) or cyclin D1 (\textbf{H}). Cyclin D1 shows weak expression in the proliferative compartment of the squamous epithelium.
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References


Image II (cont) I-L. High-grade dysplasia. Nuclear protein overexpression is seen for β-catenin (J), p53 (K), and cyclin D1 (L).
Adenocarcinoma. Membranous expression is present for HER2/neu (N), whereas loss of (nuclear) expression of SMAD4 is shown in cancer cells but not in stromal cells (P) (A, E, I, M, and O, H&E, ×100; B-D, F-H, J-L, N, and P, immunostains, ×200).
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