Increased Expression of the Cyclin D1 Gene in Barrett’s Esophagus

Abstract
Previous studies have found a 3–10-fold amplification and overexpression of the cyclin D1 gene in about 32% of human esophageal squamous cell carcinoma. The purpose of this study was to evaluate the prevalence of increased expression of the cyclin D1 protein in Barrett’s esophagus. Using 69 formalin-fixed and paraffin-embedded human esophageal specimens, which had been removed endoscopically or obtained at surgery during 1993 and 1994, all immunohistochemical analyses were performed using an avidin-biotin complex immunoperoxidase technique. Increased nuclear expression of the cyclin D1 protein was noted in 32 of 69 samples (46%; 44% of the samples from males and 50% of the samples from females). Positive nuclear staining for the cyclin D1 protein in Barrett’s disease with intestinal metaplasia was found in 38% of the male cases and 25% of the female cases, whereas in gastric metaplasia it was positive in 33% of men and 48% of women. Nuclear accumulation of the cyclin D1 protein was also found in both dysplastic and nondysplastic lesions, and it was not associated with sex, age, or cigarette or alcohol consumption. Samples from patients taking proton pump inhibitors tended to be less frequently positive (32%) for cyclin D1 nuclear staining when compared to patients taking H2 antagonists (45%) or antacids (55%). These studies suggest that increased expression of cyclin D1 is an early event in the tumorigenic process of esophageal adenocarcinomas and that the increased expression of this gene might predispose the epithelium to malignant transformation.

Introduction
Adenocarcinomas arising in Barrett’s esophagus were once rare, but in the 1970s and 1980s their incidence increased more rapidly than that of any other cancer in the United States. Adenocarcinomas now constitute up to 34% of all esophageal cancers. They are especially common in white males, in whom adenocarcinoma of the esophagus is now as frequent as squamous cell carcinoma (1–3).

The exact pathological progression from Barrett’s metaplasia to carcinoma is still unknown, although progression from metaplasia to low-grade dysplasia through high-grade dysplasia and finally to adenocarcinoma has been suggested (4–6). Thus, identification of changes in the molecular genetics and cellular physiology that take place in the progression from Barrett’s esophagus to esophageal adenocarcinoma would be of interest for developing prevention and treatment strategies.

Progression of cells through the cell cycle is governed by the sequential formation, activation, and degradation of a series of cyclins and CDK3 complexes. These complexes play a critical role in cell proliferation and differentiation. There are at least 11 distinct cyclin genes in the human genome that can bind to and activate a series of at least seven CDKs (for review, see Ref. 7).

Rearrangement, amplification, and/or increased expression of the cyclin D1 gene have been reported in human parathyroid adenomas, B-cell lymphomas, breast, colon, lung, and bladder cancers, hepatomas, and squamous cell carcinomas of the esophagus and head and neck (7–11). Thus, cyclin D1 may act as a cellular oncosgene. A 3–10-fold amplification and overexpression of the cyclin D1 gene was found in 32% of human esophageal squamous cell carcinomas and in two out of four esophageal carcinoma cell lines (8). Using immunohistochemistry, we have noted overexpression of this protein in 64% of a series of 22 samples of esophageal adenocarcinomas.4 Therefore, we undertook the present study to evaluate the levels of expression of the cyclin D1 protein in a series of biopsies of Barrett’s esophagus lesions.

Materials and Methods
A total of 84 tissue sections from 69 patients with Barrett’s esophagus were studied. Formalin-fixed, paraffin-embedded specimens were obtained from the Department of Pathology at Columbia-Presbyterian Medical Center in New York. The tissues had been removed endoscopically or obtained at surgery during 1993 and 1994 and had been processed by routine clinical histopathological methods. Barrett’s esophagus was diagnosed if biopsies showed either intestinal or gastric metaplasia. Intestinal metaplasia was defined as columnar mucosa with a villiform surface and intestinal-type crypts lined by mucus-secreting columnar cells and goblet cells. Gastric meta-
plasia was defined as columnar lined mucosa with cardiac-type mucus glands, or gastric glands with chief and parietal cells. All immunohistochemical analyses were performed with an avidin-biotin complex immunoperoxidase technique. Tissue sections 5 µm thick were mounted on poly-L-lysine coated slides. After deparaffinization in AmeriClear (Baxter, McGaw Park, IL) and absolute ethanol, sections were hydrated through a series of graded alcohols, distilled water, and PBS at pH 7.4. Slides were then immersed in 10 mm citrate buffer (pH 6) and microwaved at 750 W (to enhance antigen exposure) for a total of 10 min. After blocking with goat serum for 20 min, the primary antibody, polyclonal IgG rabbit antihuman cyclin D1 (Upstate Biotechnology, Lake Placid, NY) was applied and incubated overnight at 4°C in a high-humidity chamber. As a negative control, a duplicate section of each tissue sample was incubated overnight at 4°C in a high-humidity chamber. As a negative control, a duplicate section of each tissue sample was immunostained in the absence of the primary antibody. A breast carcinoma with known cyclin D1 overexpression served as a positive control. Subsequent steps used the Vectastain Elite ABC kit (Vector laboratories, Burlingame, CA) according to the manufacturer’s instructions. Color development was accomplished with a 0.375 mg/dl solution of 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO), containing 0.0035% hydrogen peroxide. Slides were counterstained with hematoxylin and dehydrated, and coverslips were applied using Acrytol mounting medium (Surgipath Medical Industries, Richmond, IL). The specificity of the antibody has been demonstrated (data not shown) by inhibition of immunohistochemical staining in positive controls by preincubating the antibody with 1 µg of cyclin D1 immunizing peptide (M, 1413, amino acids 285–295) for 1 h at 4°C (Upstate Biotechnology, Lake Placid, NY, Catalog No. 12-167), representing an approximately 100-fold excess of peptide over antibody. We should emphasize that the polyclonal antibodies used in this study recognize both the cyclin D1 and cyclin D2 proteins. However, Jiang et al. (8, 12) showed that increased expression in esophageal tumors is mainly cyclin D1. It is therefore likely that the immunostaining results obtained in the present study mainly reflect the abundance of the cyclin D1 protein.

All slides were independently interpreted by two of the authors (H. R. and N. A.). Positive and negative control slides were included within each batch of immunostained slides. Twenty-five % of the slides were randomly chosen and scored twice in the same batch. All batches were coded and scored blind twice. All duplicate slides resulted in similar interpretations. Nuclear staining was considered positive if the chromogen was detected in at least 5% of the nuclei within a microscopic field. Staining intensity included four scales: no staining (scale 0), weakly positive compared to adjacent nonneoplastic epithelium (scale 1), moderately positive (scale 2), and strongly positive (scale 3). Scales 0 and 1 were regarded as negative and scales 2 and 3 as positive. Nonneoplastic epithelial cells and stromal and inflammatory cells were also evaluated for cyclin D1 expression.

Results and Discussion

Samples from 69 patients were evaluated. In 15 patients, 2 biopsies from different levels of the lesions were evaluated. In all of these cases the same results were obtained in the two samples. The patients were 39 men and 30 women with a mean age ± SE of 65.8 ± 7.9 and 63.7 ± 8.5 years, respectively (range, 27–89 years). As can be seen in Table 1, increased nuclear expression of the cyclin D1 protein (Fig. 1) was noted in 32 patients (46%), 44% of the males and 50% of the females. Positive nuclear staining for cyclin D1 protein in lesions displaying intestinal metaplasia was found in at least 5% of the nuclei within a microscopic field. Staining intensity included four scales: no staining (scale 0), weakly positive compared to adjacent nonneoplastic epithelium (scale 1), moderately positive (scale 2), and strongly positive (scale 3). Scales 0 and 1 were regarded as negative and scales 2 and 3 as positive. Nonneoplastic epithelial cells and stromal and inflammatory cells were also evaluated for cyclin D1 expression.

Table 1

<table>
<thead>
<tr>
<th>Race</th>
<th>No. of cases</th>
<th>Cyclin D1 nuclear staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>39</td>
<td>17 (44)</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>38</td>
<td>15 (38)</td>
</tr>
<tr>
<td>Gastric metaplasia</td>
<td>36</td>
<td>12 (33)</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>15 (50)</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>24</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Gastric metaplasia</td>
<td>29</td>
<td>14 (48)</td>
</tr>
<tr>
<td>Drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>22</td>
<td>7 (32)</td>
</tr>
<tr>
<td>H2 antagonist</td>
<td>20</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Antacids</td>
<td>22</td>
<td>12 (55)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>23</td>
<td>13 (57)</td>
</tr>
<tr>
<td>Never</td>
<td>23</td>
<td>12 (52)</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>19</td>
<td>9 (47)</td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>13 (59)</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>32 (46)</td>
</tr>
</tbody>
</table>

Fig. 1. Example of cyclin D1-positive nuclear staining. The immunoreactivity is located in the nuclei of a Barrett’s esophagus lesion displaying gastric metaplasia. ×250.
of positive cells was restricted to the basal layer. Only 32% of the samples from patients treated with proton pump inhibitors showed increased expression of cyclin D1, as compared to 45 and 55% of samples from patients treated with H2 antagonists or other drugs, respectively. However, the series was too small to demonstrate that these differences were statistically significant. Nuclear staining was not significantly associated with age, gender, race, alcohol, smoking, type of metaplasia, or degree of dysplasia. Thus, about 46% of Barrett’s esophagus samples showed increased nuclear expression of the cyclin D1 protein when compared to normal esophageal mucosa. This was found in both dysplastic and nondysplastic lesions, and in lesions with intestinal or gastric metaplasia. An approximately similar percent of esophageal squamous cell carcinomas and adenocarcinomas also display increased expression of cyclin D1 (8). Previous studies of squamous carcinomas of the esophagus, obtained from patients in China, Italy, France, South Africa, or the United States, indicated that about 30–50% of these cases display a 3 to 10-fold amplification of the cyclin D1 gene, as well as increased expression of this gene at the mRNA and protein levels (8, 12–14). Similar findings have been seen with cell lines established from squamous carcinomas of the esophagus (12, 14). On the other hand, a study by Wang et al. (15) detected only a slight increase in cyclin D1 protein in squamous carcinomas of the esophagus and adenocarcinomas of the gastric cardia obtained from China, but their immunostaining method may not have been sufficiently sensitive.

The present study suggests that increased expression of cyclin D1 may be an early event in the development of adenocarcinomas of the esophagus. Other changes described previously in Barrett’s lesions include microsatellite instability; loss of p53; APC mutations; and increased expression of C-NEU, microsatellite instability in esophageal cancer and Barrett’s esophagus. Gastroenterology. 106: 1249–1256, 1989.

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