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Proton pump inhibitors reduce cell cycle abnormalities in Barrett's esophagus

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Neoplastic progression in Barrett's esophagus is a multi-step process in which the metaplastic columnar epithelium sequentially evolves through a metaplasia-dysplasia-carcinoma sequence. The expression and DNA copy number of key cell cycle regulatory genes in paired normal and Barrett's esophagus samples was evaluated. Protein levels were evaluated in 60 formalin-fixed, paraffin-embedded human tissues by immunohistochemistry. DNA copy number from 20 fresh tissue pairs was analysed by Southern blot analysis. All normal mucosal samples expressed the $p27^{kip1}$ protein, but did not display appreciable nuclear staining for $p16^{kip4}$, $p21^{cip1}$ or cyclins D1 and E. Barrett's metaplastic specimens displayed increased expression levels of $p16^{kip4}$ (74%), $p21^{cip1}$ (89%) and cyclins D1 (43%) and E (37%). p27 protein was absent in three cases. There was a significant correlation between the expression of $p16^{kip4}$ and cyclin E, and $p21^{cip1}$ and $p27^{kip4}$ with cyclin D1. DNA analysis did not reveal any amplification or deletion of these genes. Acid suppression, however, was associated with significantly lower expression levels of key cell cycle proteins. Increased expression of key cell cycle regulatory genes appears to occur early in the neoplastic progression associated with Barrett's esophagus. Treatment with proton pump inhibitors appears to alter this increased expression. *Oncogene* (2001) **20,** 7987 – 7991.

Keywords: cell cycle; Barrett's esophagus; cyclins; cyclin-dependent kinase inhibitors; proton pump inhibitors

Introduction

Barrett's esophagus (BE) is a common condition in which part of the normal esophageal squamous epithelium is replaced by a metaplastic columnar epithelium as a result of chronic gastroesophageal reflux (Barrett, 1957; Spechler and Goyal, 1996). This specialized intestinal metaplasia is found in up to 10% of patients with severe gastroesophageal reflux disease. Furthermore, many individuals with mild or moderate reflux symptoms alone will have BE, which is associated with a 30 – 100-fold increased risk of development of adenocarcinoma of the esophagus (Spechler and Goyal, 1996). The pool of patients requiring surveillance endoscopy and biopsy for BE may constitute as much as 1 – 2% of the adult population in developed countries. Hence, the clinical problem of BE has important public health and medical-economic ramifications.

The exact pathophysiologic basis of progression from BE-associated metaplasia to frank carcinoma is still incompletely understood. Barrett's-associated cancers do not arise *de novo*, but rather evolve from a multi-step process in which the metaplastic columnar epithelium sequentially develops through a dysplasia-carcinoma sequence (Skinner *et al*., 1983; Hameeteman *et al*., 1989; Reid, 1991). As BE progresses to dysplasia and adenocarcinoma, increased and uncoordinated cell proliferation and decreased expression of differentiation markers have been observed (Fitzgerald and Triadafilopoulos, 1998). Molecular analyses of Barrett's epithelium may provide insight into the nature and significance of genetic events occurring during the multi-step process of esophageal adenocarcinogenesis. Thus, identification of these molecular genetic changes and cellular physiology would be of interest in the context of early detection, prevention and treatment strategies.

Uncontrolled cellular proliferation is one hallmark of cancer, and there is increasing evidence that tumor cells have acquired damage to genes that directly regulate their cell cycle (Pines, 1991; Hunter and Pines, 1994; Hall and Peters, 1996; Pardee, 1989; Sherr, 1994). Genetic alterations affecting the G_1 phase of the cell cycle are so frequent in human cancers that abnormalities in this pathway may actually be necessary for tumor development. Like the tumor suppressor protein

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p53, other proteins may participate in G_1 checkpoint functions regulating homeostatic tissue renewal throughout life. During the G_1 phase, cells respond to extracellular signals by advancing toward S phase and cell division, withdrawing from the cycle into a resting state, or undergoing apoptosis (Pardee, 1989; Sherr, 1994). Unlike transit through the S, G_2, and M phases, G_1 progression normally relies on stimulation by mitogens and can be blocked by anti-proliferative cytokines. Cancer cells tend to lose these normal control mechanisms and remain actively in the cell cycle. Oncogenic processes often target specific regulators of G_1 phase progression (Hunter and Pines, 1994; Hall and Peters, 1996).

To date, some of the earliest markers of tumor progression in Barrett's-associated neoplasia have been DNA content (ploidy) and mutant p53 protein (Gimenez *et al*., 1999). Other changes previously described in Barrett's lesions include amplification of the HER-2/neu, micro-satellite instability, Rb deletion, rare APC mutations, low expression of E-cadherin, high Src-specific activity, and increased expression of Bcl-2, cyclin D1, Fas ligand, TGF-alpha and PCNA (Sherr, 1994; Gimenez *et al*., 1999; Brien *et al*., 2000; Younes *et al*., 1999; Reid *et al*., 1993; Redd *et al*., 1994; Flejou *et al*., 1994; Bilir *et al*., 1994; Finkel *et al*., 1994; Kim *et al*., 1992; Arber *et al*., 1996a).

Adenocarcinomas arising in BE were once relatively uncommon. However, during the 1970s and 1980s, in white males, these cancers increased in incidence by 74%, a rate exceeding that of any other cancer in Europe and the United States (Blot *et al*., 1991, 1993; Pera *et al*., 1993; Chow *et al*., 1995). Adenocarcinomas now constitute more than 50% of all esophageal cancers in the developed world (Blot *et al*., 1991, 1993; Pera *et al*., 1993; Chow *et al*., 1995).

Epidemiological evidence suggests that patients with BE have longer exposure times to gastric acid than do patients with esophagitis or normal controls. There is sufficient data to support the importance of acid reflux in the appearance and length of BE (Chow *et al*., 1995). Anti-reflux therapy, i.e., with acid-suppressive drugs, generally relieves symptoms and heals concomitant esophagitis and Barrett's ulcers, and may prevent stricture formation. However, the role of acid reflux in the malignant transformation of the Barrett's epithelium is still not clear, and it remains to be seen whether acid-suppressive therapy can prevent transformation to esophageal adenocarcinoma (Gore *et al*., 1993; Clark and Demeetser, 1995).

An increasing number of clinical and experimental studies have suggested that normalization of intra-esophageal pH is the desired goal in the long-term treatment of patients with BE. Partial regression of BE, with the appearance and/or enlargement of squamous islands within the Barrett's epithelium, has been observed after treatment with proton pump inhibitors (Sampliner, 1994), anti-reflux surgery (Sagar *et al*., 1995), endoscopic laser ablation techniques (Lightdale, 1995; Overholt and Panjehpour, 1996), or thermal electrocoagulation (Sampliner *et al*., 1996). In an *ex vivo* BE culture model, short pulses of acid exposure increased proliferation (Fitzgerald *et al*., 1996). On the other hand patients with BE treated with even a high dose of PPI (80 mg qd of omeprazole) have demonstrated only a minor change in the length of Barrett's mucosa. It was suggested that up to 30% of these patients continue to have an abnormal acid exposure in the esophagus. These results suggest that acid-suppressive therapy needs to be powerful enough to completely eliminate any acid pulses in order to effectively inhibit inappropriate cell proliferation in BE epithelia *in vivo*.

The present study was undertaken in order to determine whether abnormalities in the expression of several key cell cycle control genes occur in BE-associated neoplastic progression. Protein levels and DNA copy number of the genes *p16, p21, p27, cyclin D1* and *cyclin E* were measured in a series of paired normal mucosal and BE samples. The current study showed that acid suppression decreases the expression of certain key cell cycle regulatory proteins, supporting the hypothesis that continuous long-term acid suppression decreases proliferation *in vivo* in BE patients.

Results

Samples were evaluated from 60 patients, 32 men and 28 women with a respective mean age of 63 ± 6 ($\pm$s.e.) and 61 ± 7 (range 31–84 years).

BE was diagnosed only if the specimen showed intestinal metaplasia, presenting as columnar mucosa with a villiform surface and intestinal-type crypt lined by mucus-secreting columnar cells and goblet cells. Low and high-grade dysplasia were seen in seven and two patients respectively.

All normal appearing mucosa expressed the p27 protein. None of them displayed appreciable nuclear staining for p16, p21 or cyclins D1 and E. Rarely, in about one cell per 20 crypts, was positive nuclear staining noted in a cell near the base of the crypt.

The abnormalities identified in BE are summarized in Table 1. A representative staining is shown in Figure 1. The most common alteration involved an increase in the $p21^{cip1}$ protein, which was overexpressed in 88% of cases. Increased p16 protein was observed in 73% of the samples and in three cases the $p27^{kip1}$ protein was not detected. Increased expression of cyclin D1 and cyclin E proteins were seen in 47 and 43% of the lesions respectively.

Increased expression of p21 was significantly higher in men (93%) than in women (76%) ($P<0.05$). There was a significant correlation between the expression of p16 and cyclin E, and cyclin D1 and p21 and p27. These findings were observed in both dysplastic and

Table 1 Cell cycle expression in Barrett's esophagus samples

	$p16^{kip4}$	$p21^{cip1}$	*p27KIP1*	*Cyclin D1*	*Cyclin E*
Normal mucosa (%)	0	0	100	0	0
BE mucosa (%)	73	88	95	47	43

non-dysplastic lesions, and were not associated with age, gender, cigarette or alcohol consumption.

There were no epidemiological differences between the American and Israeli populations, as well as in the expression of the different proteins.

Southern blot analysis (data not shown) did not reveal any amplification (>1.5) or deletion (<0.7) of *p16, p21, p27, cyclin* D1 and *cyclin* E genes.

Percentage of cell cycle abnormalities in BE samples in relation to drug consumption is summarized in Figures 2 and 3. Patients ($n=22$) consuming proton pump inhibitor (PPI) had significantly less cell cycle abnormalities than patients taking H_2 blockers ($n=18$) or antacids ($n=20$). All patients on PPI retained p16 activity, and only in 25% of the subjects was cyclin E overexpressed as compared to 79 and 41% for patients on H_2 blockers, and 45% and 65% in antacid consumers respectively. These differences were highly significant. Patients on PPI less often displayed increased expression of cyclin D1 and down-regulation of p21 and p27. Patients receiving antacids had the highest percentage of cell cycle abnormalities and patients taking H_2 blockers were between.

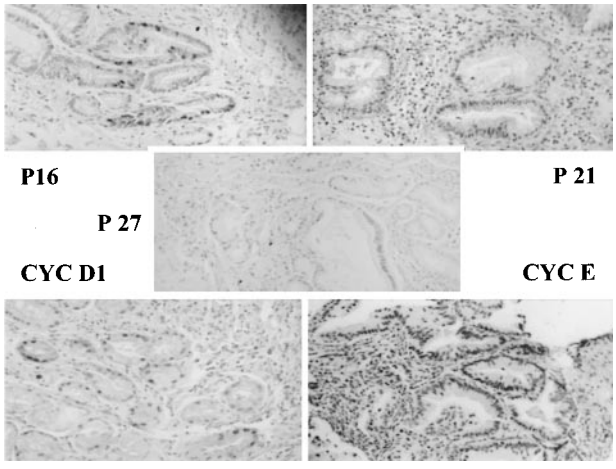

Figure 1 Example of p16 (**a**), p21 (**b**), p27 (**c**), cyclin D1 (**d**), cyclin E (**e**) positive nuclear staining. The immunoreactivity is located in the nuclei of a Barrett's esophagus lesion

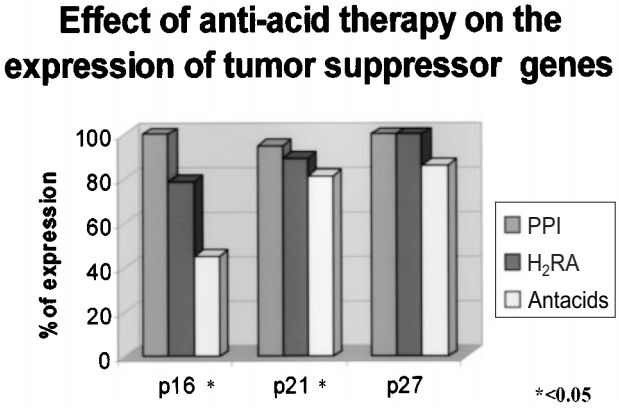

Figure 2 Percentage of CDK inhibitors in Barrett's esophagus samples in relation to drug consumption

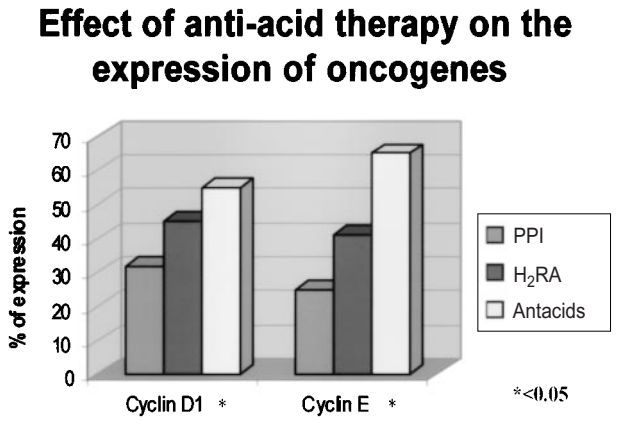

Figure 3 Percentage of cyclins D1 and E in Barrett's esophagus samples in relation to drug consumption

Discussion

Maintenance of normal epithelial differentiation and proliferation is an important goal in cancer chemoprevention. *Ex vivo* acid has a dynamic effect on cell proliferation/differentiation in BE. Effective intra-esophageal acid suppression favors differentiation and decreases proliferation (Quato-Lascar *et al*., 1999).

BE is associated with cell cycle abnormalities without a significant change in the respective DNA level. We suggest that increased nuclear expression of the oncogenes cyclin D1 (47%) and cyclin E (43%), and reduced levels of the tumor suppressor gene $p27^{kip1}$ (5%) might predispose the esophageal epithelium to malignant transformation. The increased expression of the cell cycle inhibitors $p16^{kip4}$ and $p21^{cip1}$ in BE may be a defense mechanism induced by the tumorigenic process. These results confirmed our previous observation regarding increased expression of cyclin D1 in 46% of 69 Americans with BE (Arbet *et al*., 1996a), and 71% of patients with esophageal adenocarcinomas (Arber *et al*., 1999). It appears that there is no molecular epidemiological difference between patients from different parts of the Western Hemisphere, namely Israel and the USA.

Reid *et al*. (1993) have suggested that an early event in neoplastic progression, in BE, is the mobilization of cells from the G_0 phase to G_1, loss of control of the G_1/S transition, and accumulation in G_2. It is suggested that cell cycle abnormalities, and in particular increased expression of cyclins D1 and E, are early events during the multi-step process of esophageal adenocarcinomas, since increased expression of this protein is found in an approximately similar percentage as in esophageal adenocarcinomas. Those changes might be responsible for initiating malignant cell transformation.

Acid exposure induces proliferation and inhibits apoptosis in BE and in esophageal adenocarcinoma

cells. Perhaps the most important clinical implication of this study can be seen in Figures 2 and 3. It is the first report demonstrating that suppression of acid exposure down-regulates the expression of cyclins D1 and E, and further increases the levels of $p16^{kip4}$ and $p21^{cip1}$. The molecular abnormalities were detected only on the protein level and not in DNA. This suggests that episodes of acid reflux might trigger proliferation and inhibit program cell death signaling pathways. Therefore, long-term therapy with PPI is desirable and can prevent or inhibit the malignant transformation in BE patients consuming PPI. The current studies suggest, for the first time, that episodes of acid reflux might trigger proliferation and inhibit program cell death signaling pathways. Thus, long term therapy with PPI is desirable and can prevent or inhibit the malignant transformation of BE. It is suggested that all the attempts, so far, to prevent this malignant transformation failed because of insufficient acid suppression.

Material and methods

Patients and samples

A total of 60 formalin-fixed, paraffin-embedded specimens of BE were obtained from the departments of pathology at Columbia University in NewYork and Tel Aviv Medical Center. In 20 of the samples, obtained in Tel Aviv, fresh biopsies from BE and normal adjacent mucosa were taken and snapped frozen in liquid nitrogen. Acid-lowering drugs were recorded on the day the biopsies were obtained. Patients were included in the study only if they had received, for at least 3 months, a standard regimen of acid inhibition (e.g. omeprazol 20 mg qd, lansoprazol 30 mg qd, ranitidine 300 mg qd or famotidin 40 mg qd). During 6 years of follow-up no adenocarcinoma developed in this group of patients.

Immunohistochemistry

All immunohistochemical analyses were performed with an avidin-biotin complex immunoperoxidase technique. Four μm tissue sections were mounted on poly-L-lysine coated slides. After deparaffinization in Americlear (Baxter, McGaw Park, IL, USA) and absolute ethanol, sections were hydrated through a series of graded alcohol, distilled water, and phosphate-buffered saline (PBS), at pH 7.4. Slides were then immersed in 10 mM citrate buffer (pH = 6) and microwaved at 750 W for a total of 10 min. After blocking with goat serum for 20 min, the primary antibodies were applied and incubated overnight at 4°C in a high-humidity chamber. These included mouse monoclonal anti-$p16^{ink4}$ (Pharmingen, San Diego, CA, USA), $p21^{waf1}$ (Santa Cruz Biotech, Santa Cruz, CA, USA), $p27^{Kip1}$ (Transduction Laboratories, Lexington, KY, USA), cyclin D1 (Immunotech, Inc., Freemont, CA, USA) and cyclin E (Santa Cruz Biotech, Santa Cruz, CA, USA). Subsequent steps utilized the Vectastain rabbit Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Color development was accomplished with a 0.375 mg/dl solution of a 3,3′-diamino-benzidine tetrahydro chloride (Sigma Chemical Co., St. Louis, MO, USA) containing 0.003% hydrogen peroxide. Slides were counter-stained with hematoxylin and dehydrated, and cover slips were applied using Acrytol mounting medium (Surgipath Medical Industries, Richmond, IL, USA).

Although all the concentrations of primary antibodies gave good nuclear staining, the optimal concentration that gave a minimal background was 5 mg/ml for cyclin D1 and 2 mg/ml for $p16^{ink4}$, $p21^{waf1}$, $p27^{Kip1}$ and cyclin E. Positive controls were breast adenocarcinoma for cyclin D1 and gastric adenocarcinoma for $p16^{ink4}$, $p21^{waf1}$, $p27^{Kip1}$ and cyclin E. As a negative control a duplicate section of each tissue sample was immunostained, but in the absence of the primary antibody. The specificity of the antibodies has been demonstrated (data not shown) by inhibition of immunohistochemical staining in positive controls by pre-incubating the antibody with 1 mg of immunizing peptides for 1 h at 4°C, representing approximately 100-fold excess of peptide over antibody.

Interpretation of immunohistochemical staining

Staining for cyclin D1 was interpreted by one author (H Habishoosh) and for p16, p21, p27 and cyclin E by another author (W Yasui). Both individuals are experienced surgical pathologists. Nuclear staining was considered positive if the chromogen was clearly detected in at least 10% of the nuclei within a microscopic field. Up to 5% of the nuclei at the base of the crypt were positive. This however represents less than 0.1% of the nuclei in an average microscopic field. Positive and negative controls were included within each batch of slides. To confirm reproducibility, 25% of the slides were randomly chosen and scored twice in the same batch, and all batches were coded and blindly scored at least twice. Duplicate slides gave similar results.

DNA preparation

In 20 of these cases two biopsies from BE mucosa and two biopsies, 5 cm proximal to the lesion, were taken from normal appearing mucosa and snapped frozen in liquid nitrogen. The lesions were stored at −80°C for further analysis. DNA was extracted from neoplastic and non-neoplastic biopsies as previously described (Arber *et al*., 1996b, Jiang *et al*., 1992).

Southern blot analysis

Ten μg of genomic DNA was digested to completion with the restriction enzymes *Hind*III or *Eco*RI. The digested DNA was electrophoresed through a 1% agarose gel. The DNA was then transferred to Hybond-N hybridization membranes as previously described (Arber *et al*., 1996b, Jiang *et al*., 1992). Fifty ng of *p16*^{ink4}, *p21*^{waf1}, *p27*^{Kip1}, *cyclin* D1 and *cyclin* E cDNA were used as probes. They were labeled with [α-^{32}P]-dCTP using the multi-prime DNA labeling system (Amersham, Arlington Heights, IL, USA) to a specific activity of 3×10^8 c.p.m/μl, and added to the hybridization reaction at 65°C for 16–18 h. Following hybridization, the filters were washed in 2 × SSC, 0.1% SDS, initially at room temperature for 20 min and subsequently for several additional washes at 65°C for 20 min each. The filters were then autoradiographed with Kodak XAR-5 X-ray film and Kodak 'lightning plus' intensifier screens for 1–7 days at −70°C. The autoradiograms were analysed by densitometric scanning. Equivalent loading in Southern blots was confirmed by the housekeeping gene actin, and by repeating all assays at least twice.

Statistical analysis

The proportions of samples expressing the different genes from different histological categories were computed and then compared across categories for selected factors (including gender, age, cigarette smoking, alcohol consumption, and presence of dysplasia). When comparing proportions positive for staining across histological categories, Fisher and Chi-squared tests were employed (Brier, 1980). McNemar's test (McNemar, 1994) was also conducted for testing asymmetry in the association between the different genetic markers.

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