Loss or Altered Subcellular Localization of p27 in Barrett’s Associated Adenocarcinoma

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ABSTRACT

The cyclin-dependent kinase inhibitor p27 is a negative regulator of the cell division cycle. It is expressed at the highest levels during the quiescent (G0) and prereplicative (G1) phases, and its degradation is required for entry into the S phase. Because lack of p27 is associated with aggressive behavior in a variety of tumors of epithelial and lymphoid origin, we used immunohistochemistry and in situ hybridization to evaluate the expression of p27 in metastatic and dysplastic Barrett’s epithelium and to assess its prognostic significance in Barrett’s associated adenocarcinoma (BAA) of the esophagus.

In metastatic Barrett’s epithelium, p27 protein and mRNA were restricted to the superficial third of glands in all cases and extended to the lower third in 4 cases. In contrast, expression of p27 message and protein was both increased and full-thickness, in the 23 cases with high-grade dysplasia adjacent to BAA and in carcinoma in situ. Although all invasive carcinomas had elevated levels of p27 mRNA, 45 (83%) of 54 invasive carcinomas had low p27 protein levels (<50% positive tumor cells). Low p27 protein correlated with higher histological grade (P < 0.0001), depth of invasion (P = 0.0120), presence of lymph node metastasis (P = 0.05), and survival (P = 0.0197). In addition to the nuclear staining, cytoplasmic staining of p27 was noted in 11 of 23 (48%) of cases of dysplasia and in 14 of 54 (26%) adenocarcinomas and confirmed, in a subset of cases, by subcellular fractionation of protein lysates obtained from fresh tumor tissues. Cytoplasmic localization of p27 was also associated with decreased survival (P = 0.0239). Loss of p27 conferred poor prognosis independently of proliferative index, as assessed by Ki-67 (MIB-1) immunostaining, which was not significantly different in survivors versus nonsurvivors.

These results show that: (a) distribution of p27 message and protein parallel one another in metastatic and dysplastic Barrett’s epithelium, suggesting transcriptional regulation of the gene in the nonneoplastic setting; (b) p27 is inactivated in the majority of BAA as a result of either post-transcriptional modification or altered subcellular localization; and (c) loss of the cell cycle inhibitor p27 is associated with parameters of aggressive behavior and unfavorable outcome in BAA.

INTRODUCTION

Barrett’s esophagus is a condition in which normal squamous epithelium lining the distal portion of esophagus is replaced by metaplastic columnar epithelium, often acquired as a complication of chronic gastroesophageal reflux disease (1). Risk of occurrence of esophageal adenocarcinoma is 30 to 125 times greater in patients with Barrett’s esophagus as compared with the general population (2). It is recommended that patients with Barrett’s esophagus undergo endoscopic biopsy surveillance for early detection of dysplasia (1). Although histological criteria for diagnosis and grading of Barrett’s associated dysplasia are well established, there is considerable subjective variation in diagnosis of low-grade dysplasia and its distinction from nondysplastic Barrett’s epithelium or from reactive/regenerative changes (3). In addition, it is difficult to accurately predict the natural history of low-grade dysplasia. Consequently, surgical resection in patients with low-grade dysplasia is controversial (4). In contrast, high-grade dysplasia in Barrett’s esophagus is a recognized precursor event of BAA.

Cell cycle progression is regulated by a series of Cdk’s. Different Cdk’s in association with different cyclin-activating subunits are required at various stages of cell cycle (cyclin D-Cdk4-Cdk6 acting in the G1 phase; cyclin E-Cdk2 and cyclin A-Cdk2 in G1 and S; and cyclin A-cyclin B-Cdk1 at the G2-M transition; Refs. 5 and 6). Cyclin-Cdk activity is regulated by phosphorylation events and Ckis, which bind the Cdk-cyclin complexes and inhibit their activity (6). Ckis can be divided into two structurally related groups. One group forms the Ink4 family, for inhibitors of Cdk4, and consists of ankyrin repeats containing proteins (p15, p16, p18, and p19) that specifically inhibit cyclin D-Cdk4/6 complexes. Mutations and deletions, or inactivation by methylation of p16 and p15 genes, have been reported in various human malignancies and transformed cell lines (5–7). The other group forms the Cip/Kip family and includes p21, p27, and p57 proteins, which share partial structural homology and possess the ability to inhibit several cyclin-Cdk complexes in vitro but seem to target preferentially those containing Cdk2 (2, 6, 7). p21 (also known as Cip1 or Waf1) is regulated by several stimuli and transcription factors, including the p53 tumor suppressor gene (8). Although p53 gene mutations are common and found in a variety of malignancies (8), no molecular alterations have been reported for p21 gene (9).

Loss of p27 expression as assessed by immunohistochemistry is a powerful negative prognostic marker in different subsets of mammary, gastric, pulmonary, colorectal, and prostate carcinomas (10–17). However, as for p21, no structural alterations and only very rare genetic mutations, which do not affect its function, have been identified in the p27/Kip1 gene in human tumors (18–21). We demonstrated previously that p27 is at least in part regulated by proteolytic degradation by the ubiquitin-proteasome pathway (22) and that in colon and lung carcinomas, p27 is eliminated by enhanced degradation via this pathway (10, 14).

The aim of the present study was to characterize the expression of p27 in metaphasia and dysplasia associated with Barrett’s esophagus as well as in a series of BAAs. We show that p27 is up-regulated in dysplastic cells, whereas loss or cytoplasmic localization of p27 is associated with aggressive behavior in BAA.

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3 The abbreviations used are: BAA, Barrett’s associated adenocarcinoma; Cdk, cyclin-dependent kinase; Cki, Cdk inhibitor.

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MATERIALS AND METHODS

Patient Population and Histopathological Criteria. We performed a retrospective search through the surgical pathology files of the Beth Israel Deaconess Medical Center-West Campus, Boston, MA, from 1974 to 1995 for all esophagogastrectomy specimens from patients with Barrett's esophagus. Fifty-eight cases were randomly selected. Corresponding frozen tissue (normal and tumor) was available in five of these patients. H&E-stained tissue sections from the paraffin-embedded tissue block used for immunohistochemistry were reviewed for pathological features as follows: (a) presence of invasive adenocarcinoma and depth of invasion; (b) degree of differentiation; (c) presence of metaplastic Barrett's epithelium; and (d) presence of dysplasia adjacent to tumor. Twenty-one cases had metaplastic Barrett's epithelium. Dysplasia was classified according to the criteria published previously (3). There were 23 cases with high-grade dysplasia. In addition, 2 cases indeterminate for dysplasia, as well as 15 cases of low-grade dysplasia (7 of which were adjacent to areas of high-grade dysplasia) were available for evaluation. Fifty-four patients had invasive adenocarcinoma, whereas 4 had carcinoma in situ with no evidence of invasion. The tumors were staged according to the American Joint Committee on Cancer criteria (23). Follow-up data were obtained from the database of the Division of Cardio-Thoracic Surgery, which used patient's chart, telephone, and postal contact with patients, family members, or attending physicians and Tumor Registry records, when necessary.

The demographic data of patients are shown in Table 1. The study group had a striking predominance of men (93%). The patients' ages at diagnosis ranged from 37 to 81 years, with a mean of 61.5 years. Twenty-seven percent of the tumors were well differentiated, 51% were moderately differentiated, and the remaining 22% were poorly differentiated. The pathological stages were 7% stage 0 (i.e., 4 of 58 patients with in situ cancer), 10 (17%) stage I, 7 (12%) stage II, 7 (12%) stage III, 26 (45%) stage IV, and 4 (7%) stage IV. The follow-up interval ranged from 6 to 201 months (mean, 29 months), and the 5-year survival rate was 35.8%. None of the patients received postoperative adjuvant therapy.

Immunohistochemistry. Five-μm tissue sections were cut from the paraffin-embedded blocks, placed on charged glass slides, deparaffinized in xylene, and rehydrated through graded alcohol.

Immunohistochemistry was performed on an automated instrument (Ventana ES; Ventana Medical Systems, Tucson, AZ) as described previously (10, 12). Briefly, after antigen retrieval by microwave irradiation (10 mm sodium citrate buffer [pH 6.0] in a pressure cooker at 750-W for 30 min; Biogenex, San Ramon, CA), mouse monoclonal antibodies against p27 (Transduction Laboratories, Lexington, KY) and Ki-67 (Immunotech, Marseilles, France) were applied on the slides at the dilution of 1:200 and 1:25, respectively. Steps performed by the instrument included blocking with normal horse serum, application of a secondary antibody conjugated to the avidin-biotin peroxidase complex, and visualization with 3,3-diaminobenzidine as a substrate with hydrogen peroxide as a cooxidizing agent.
SUBCELLULAR LOCALIZATION OF P27

p27 Expression in Dysplastic Barrett's Epithelium and Carcinoma in Situ. Areas of Barrett's epithelium diagnosed as indeterminate for dysplasia expressed p27 in the nuclei of superficial cells (data not shown). Dysplastic nuclei exhibited strong immunostaining for p27 (Fig. 1f). In low-grade dysplasias, p27 was overexpressed in the cytologically atypical cells, regardless of their location (upper or lower portions of the crypt; data not shown). p27 immunoreactive nuclei were located in the lower third of the crypt in all cases of high-grade dysplasia (Fig. 1f). In 16 cases of high-grade dysplasia (70%), p27 immunoreactive nuclei also extended to the superficial cells (Fig. 1f). In five cases of severe dysplasia, p27 was localized both in the nucleus and in the cytoplasm. Six cases of severe dysplasia had exclusive cytoplasmic localization of p27. All cases of carcinoma in situ displayed full-thickness nuclear p27 staining (data not shown). p27 mRNA expression paralleled protein expression in all cases (Fig. 1g). Ki-67 expression also extended to the superficial cells in 93% of cases of high-grade dysplasia (Fig. 1h).

p27 Expression in BAA. Seventeen % of cases expressed p27 in >50% (3) of tumor cells (Fig. 2b), whereas low p27 protein expression (<50% positive cells) was observed in the majority (83%) of cases (Fig. 2d; 3b). All invasive adenocarcinomas expressed p27 mRNA in the majority of cells (Fig. 3a).

RESULTS

p27 Expression in Barrett's Epithelium. Metaplastic Barrett's epithelium adjacent to adenocarcinoma was available for evaluation in 21 cases. Cells expressing p27 protein (Fig. 1b) and mRNA (Fig. 1c) were located in the superficial, terminally differentiated portion of Barrett's epithelium in all cases. p27 was also expressed in the lower third of glands in 4 (19%) cases of benign (metaplastic) Barrett's epithelium. The staining was localized to the nuclei in all cases. Ki-67 staining was noted in the basal proliferative zone of Barrett's epithelium (Fig. 1d).

Subcellular Fractionation and Western Blot Analysis. Five esophageal tumor samples were finely diced with a surgical blade. Samples were washed twice with ice-cold PBS containing 1 mM MgCl₂ and spun down at 1000 rpm in a Eppendorf microcentrifuge for 5 min at 4°C to recover pellets after removing the supernatant. The pellets were resuspended in two volumes of ice-cold hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 300 mM sucrose, 1 mM EDTA, 1 mM DTT, 0.1 mM Na₃VO₄, and 0.1% NP40]. The following protease inhibitors were added: 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml L-1-chlor-3-(4-tosylamido)-4 phenyl-2-butanone, 5 µg/ml L-1-chlor-3(4-tosylamido)-7-aminono-2-heptanon-hydrochloride, and 1 µg/ml aprotinin. After swaying on ice for 20 min, plasma membranes were disrupted by repeated pipetting through a Gilson microtip. Cell breakage was assessed by microscopic observation. The samples were subsequently centrifuged at 3000 rpm in an Eppendorf microcentrifuge for 10 min at 4°C to recover a cytoplasmic fraction (supernatant). The pellets were resuspended in two-thirds of the starting volume in ice-cold hypertonic buffer [20 mM HEPES (pH 7.4), 250 mM NaCl, 1 mM EDTA, 0.5% deoxycholic acid, 1 mM DTT, 0.1 mM Na₃VO₄, 50 mM NaF, 1% Triton X-100, and 0.1% SDS] containing the above-listed protease inhibitors. After incubation on ice for 30 min, the samples were resuspended by repeated pipetting through a Gilson microtip and subsequently centrifuged at 3000 rpm in an Eppendorf microcentrifuge 10 min at 4°C to recover a nuclear fraction (supernatant). Identical amounts of protein were loaded on a gel for both fractions. Proteins were transferred from gels by semidy blotting, and immunoblotting was performed as described (10).

Statistical Methods. Results of immunohistochemistry for p27 and Ki-67 in the 54 invasive adenocarcinomas were compared with clinicopathological features using x² for qualitative data and Miettinen's modification of Fisher's exact test for categorical data. Probability was two-tailed, with P < 0.05 regarded as statistically significant. Adjusted survival was the time period of survival after resection to the date of last follow-up or death by disease. Survival rate was calculated for p27 and Ki-67 by the Kaplan-Meier method (24), and the difference in survival distribution was calculated using the log-rank test.
Of all clinicopathological parameters considered, low p27 correlated with higher histological grade, depth of invasion, and presence of lymph node metastases (Table 1). There was no correlation between p27 staining and distant metastases. The level of p27 expression was significantly associated with survival by actuarial analysis, with a median survival of more than 16.8 years in patients whose tumor displayed high p27 (≥50 p27-positive cells) and 2.1 years in tumors that had low p27 expression (<50 p27-positive cells; P = 0.0197; Fig. 4a).

Proliferative index ranged from 10 to 87%, as assessed by Ki-67-positive cells over total cells counted, with a median of 60%. When tumors were subdivided into two groups with low and high proliferative indices based on the median, the latter group significantly correlated only with lymph node metastases but not with depth of invasion, differentiation, or survival (Table 1; Fig. 4b). In addition, there was no correlation (positive or inverse) between p27 and Ki-67.

Abnormal Subcellular Localization of p27. In contrast to metaplastic epithelium, where p27 was localized exclusively in the nuclei, 11 cases of severe dysplasia also showed cytoplasmic staining of p27 (data not shown). In 6 of these cases of dysplasia with exclusively cytoplasmic localization of p27 (55%), the adjacent invasive neoplastic component also displayed the same subcellular localization for p27. Cytoplasmic staining for p27 with (Fig. Sc, two cases) or without (12 cases) concomitant nuclear p27 staining (Fig. Se) was present in 14 (26%) of the 54 cases of BAA. This group of patients had shorter survival in comparison with patients without cytoplasmic p27 (P = 0.0235), in spite of the fact that 2 of 14 (14%) were classified in the >50% group by nuclear staining (Fig. Sf).

Immunoblot analysis of fractionated lysates from five esophageal carcinomas with variable nuclear and cytoplasmic expression for p27 confirmed subcellular compartmentalization of p27 as observed by immunohistochemistry (Fig. 5a). Cases in which similar expression of p27 was observed in both the cytoplasm and nucleus of tumor cells are represented in the first four lanes (Fig. 5a). Cases with almost exclusive cytoplasmic immunoreactivity showed a predominant band in the cytoplasmic fraction by Western blot (Fig. 5a, Lanes 5–10). The faint band observed in the nuclear fraction after prolonged exposure (Fig. 5a).
occurred as a response to counteract proliferative signals. The loss of p27 in the majority of the cases as they become invasive, as a result of either increased degradation or altered subcellular localization, suggests a role for this Cki in preventing progression of Barrett’s esophagus to adenocarcinoma, and that the intensity and distribution of p27 expression may serve as a biomarker of increased risk of cancer development.

We and others found previously that lack of p27 was an independent negative prognostic marker in colon, stomach, lung, breast, and prostate carcinomas (10—17). In addition, recent studies have shown abundant expression of p27 in normal tissues and decreased expression in adenomas and carcinoma of endocrine and nonendocrine origin as well as in certain lymphomas (30, 31). In this study, we have shown that loss of p27 expression occurs in the majority of BAA and is associated with poor prognosis. Low p27 (<50% positive cells) expression also correlated with lymph node metastases, depth of invasion (T stage) and decreased survival, and inversely with the degree of differentiation. Although multivariate analysis could not be

5a. Lanes 6 and 8) in these cases probably represents p27 from quiescent stromal cells and/or lymphocytes.

DISCUSSION

The identification of the Ckis has been particularly instrumental in understanding the regulation of the transition from G1 to S phase in normal and neoplastic cells. Defective regulation of this important checkpoint may result in neoplasia (5). In fact, inactivation of several Ckis has been associated with transformation in a variety of tissues (5, 6). It has been shown that p27 gene knock-out mice have increased body weight and multiple organ hyperplasia, suggesting that p27 protein inhibits proliferation in vivo (26—28).

The incidence of BAA is rising more rapidly than that of any other carcinoma in the United States (29). Assessment of the presence and degree of dysplasia in esophageal biopsies after endoscopic surveillance is the present method of choice in assessing the risk of developing invasive cancer in patients with Barrett’s esophagus. It is important for epithelial cells to exit the cell cycle as they migrate upward toward the mucosal surface in their process of differentiation. Ki-67 staining extending to the superficial cells indicates that the cells in high-grade dysplasia have lost the ability to exit the cell cycle and to terminally differentiate. Interestingly, p27 expression was also increased in intensity and distribution throughout the dysplastic glands in Barrett’s esophagus. In addition, because mRNA and protein expression paralleled one another in both metaplastic and dysplastic epithelium, it is tempting to speculate that increased expression of p27 in this setting is a transcriptionally regulated phenomenon that might

![Fig. 4. Actuarial survival curve in 54 patients with invasive BAA according to p27 nuclear expression: low versus high (A). Actuarial survival curve in 52 patients with invasive BAA according to Ki-67 nuclear expression: low versus high (B).](image)

![Fig. 5. a, Western blot p27 analysis of cytoplasmic (C) and nuclear (N) fractions from five representative cases of esophageal BAAs with either co-expression of p27 in both nucleus and cytoplasm (Lanes 1—4) or exclusive cytoplasmic expression (Lanes 5—10). Lane 11, blank; Lane 12, purified, histidine-tagged p27. b and c, H&E (b) and immunohistochemistry (c) for p27 corresponding to case in Lanes 3 and 4. Note concomitant nuclear and cytoplasmic staining for p27 in tumor cells. d and e, H&E (d) and immunohistochemistry (e) for p27 corresponding to case in Lanes 7 and 8.](image)
performed due to the small sample size, loss of p27 was associated with all negative prognostic parameters used in routine clinical practice to predict behavior in BAA. The significant number of BAA in combination, correlate with survival in young breast cancer patients. Nat. Med., 3: 227—230, 1997.


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