Expression of cyclin E in stage III colorectal carcinoma

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Abstract. Carcinogenesis is characterized by an abnormal regulation of the cell cycle. Regulators of the cell cycle such as cyclin E play an important role in neoplasia and may be correlated with prognosis. The clinical significance of the expression of cyclin E in stage III colorectal carcinoma has not yet been investigated. The expression of cyclin E was evaluated in 49 patients. Using a multivariate analysis, the expression of cyclin E in the tumor at diagnosis was compared with various clinicopathological variables, including age, gender, tumor site, tumor size, tumor differentiation and lymph node involvement. There were more node-positive cases in the cyclin E-negative group than in the cyclin E-positive group (P=0.003). However, there was no correlation between the degree of cyclin E expression and the clinical data. In conclusion, our data suggest that overexpression of cyclin E does not predict the clinical outcome in colorectal cancer stage III. Negative cyclin E staining may be associated with lymph node involvement.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and females. In 2011, 141,210 new cases were diagnosed and 49,380 mortalities from CRC occurred (1). Colorectal cancerogenesis is a result of complicated molecular mechanisms and disturbances of the cell cycle and requires a number of genetic alterations (2-4). One of the main characteristics of malignant tumors is abnormal regulation of cell growth. Cell cycle abnormality is the leading factor in tumor progression. Cyclins are a family of proteins that control the progression of cells through the cell cycle by activating cyclin-dependent kinase (CDK) enzymes (5,6). Cyclin E, through binding and activation of CDK2, facilitates cell transition from G1 to S phase. Conversely, impaired activity of the cyclin E/CDK2 complex arrests the cell before new DNA is synthesized. The precisely timed degradation of cyclins is one of the mechanisms that regulate CDK activity, which is necessary for coordination of the cell cycle and may prevent the development of cancer cells (7,8). Cyclin E overexpression increases cell proliferation via enforced progression to S phase and may lead to carcinogenesis (9). The cyclin E gene is overexpressed, and protein concentrations and related kinase activity are often altered, in numerous human tumors. Impaired expression of cyclin E has been identified in breast, ovarian, gastric, colorectal and non-small lung cancers, leukemias, lymphomas and melanomas (7,10-17).

An additional significant finding is the overexpression of cyclin E mRNA in three CRC cell lines (LoVo, CaCo2 and HT29) with an aberrant number of chromosomes (18). Simone *et al* proposed that overexpressed cyclin E mRNA may maintain the abnormal chromosome number and support cells with high indices of proliferation and altered genotypes (18). The activation of cyclin E in neoplasia may involve mutations in regulatory pathways, supporting abnormal proliferation and the development of cancer. During the last decade, efforts have been made to discover new predictive and prognostic markers in gastrointestinal and CRCs (19).

The role of cyclin E as a prognostic marker in colorectal carcinoma is controversial. The purpose of our study was to evaluate cyclin E expression in patients with stage III colorectal carcinoma.

Materials and methods

Clinicopathological data. The study was approved by the IRB of the Rabin Medical Center. The study included 49 patients with metastatic CRC (29 males and 20 females; mean age, 66.2 years; range, 50-81 years). All patients underwent surgical resection of the tumor at the Rabin Medical Center between 1996 and 2005. Patients with stage III cancers according to the AJCC (American Joint Committee on Cancer) (20,21) classification were selected. All patients had metastatic disease, either at diagnosis or during the course of their disease. The median follow-up was 71 months. All patients received the same standard first-line treatment for metastatic CRC and the response to this treatment was determined. The first-line treatment in all cases was a standard combination of 5-FU, leucovorin (LV) and irinotecan (FOLFIRI regimen). Tumor samples from these patients were examined. Each sample

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contained an area of adenocarcinoma with some adjacent normal colonic mucosa.

Immunohistochemistry. Cyclin E protein expression was evaluated by immunohistochemistry using a monoclonal antibody clone (Abcam, Cambridge, UK) that recognizes an intracellular domain of the cyclin E protein. Tissue sections (4 μm thick) were cut, deparaffinized in xylene, rehydrated with graded ethanol and immersed in hydrogen peroxide for 20 min. The slides were heated in a microwave oven at 300 W and left in the Coplin jar at room temperature for 30 min. To block non-specific binding of the primary antibody, a normal goat serum was used for 15 min. After removing the blocking solution, the primary antibody for cyclin E (Abcam) was applied at a dilution of 1:200 for 15 min in a modified chamber at room temperature. Negative control studies were performed without applying the primary antibody. The sections were washed with PBS, incubated with polymer detection kit (HRP broad spectrum; Zymed, Carlsbad, CA, USA) for 15 min at room temperature, then rinsed with washing buffer and incubated with diaminobenzidine tetrahydrochloride (DAB) at room temperature for 5 min and rinsed with running water for 3 min. Counter staining was carried out with Mayer's hematoxylin for 5 min, dehydrated in a series of ethanol and mounted with glass cover slips using Permount. In each case, the entire section was systemically examined for cyclin E immunoreactivity.

The cyclin E labeling index was defined as the percentage of tumor cells exhibiting nuclear immunoreactivity and was calculated by counting cyclin E nuclear stained tumor cells in 1,000 tumor cells. A single representative section from each sample was surveyed microscopically at x100 for at least two areas of the highest cyclin E intensity of positive cells. Cell counts were carried out at x400 in at least five fields in these areas. Tumor samples were judged to be negative (<2%) or positive (>2%) according to the percentage of the cells showing the nuclear stain pattern (22). A positive control slide was stained with each series.

Statistical analysis. Univariate and multivariate analysis were used to determine the correlation between clinicopathological variables and the cyclin E staining positivity. The Chi-square test was used to evaluate the correlation between the node status and cyclin E positivity. P<0.05 was considered statistically significant.

Results

The specimens were obtained from 29 male and 20 female patients. A total of 37 tumors (75.5%) were in the colon while 12 (24.5%) were located in the rectum. All tumors were stage III. N0 was observed in 14, N1 in 19 and N2 in 16 patients. The mean follow-up time was 71 months (range, 22-91).

We identified positive cyclin E staining in the tumor tissue. In 28 patients the tumors had no cyclin E expression, while the remaining 21 cases had various degrees of cyclin E positivity, from 3 to 50%. The positive staining was observed in the nuclei of the malignant epithelial cells. There was also a positive nuclear stain in the nuclei of small lymphocytes in the stroma of the tumors, which were ignored in the count.

Table I. Correlation between cyclin E and node status.

Cyclin E	Node-negative (%)	Node-positive (%)	Total number	P-value
Negative	3 (10.7)	25 (89.3)	28	0.003
Positive	11 (52.4)	10 (47.6)	21	

Results were obtained using a Chi-square test.

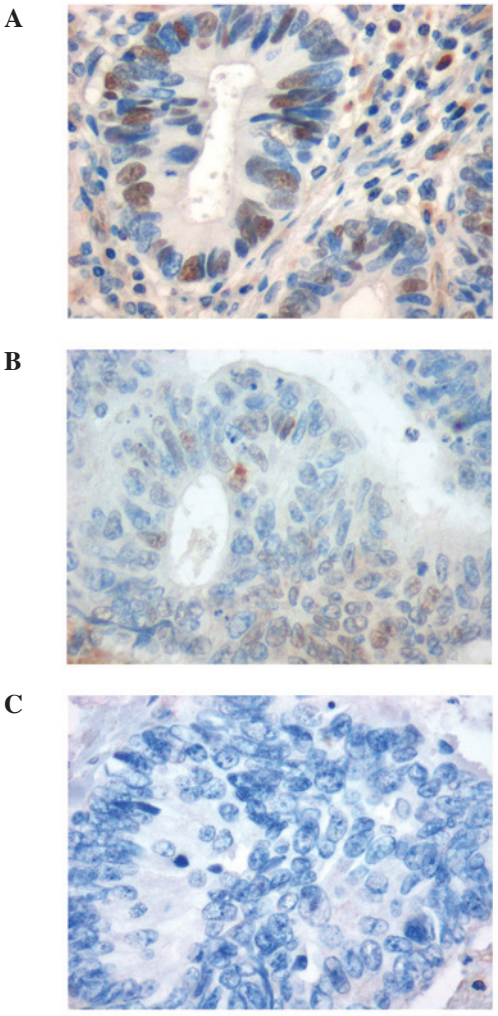

Figure 1. Cyclin E-positive stain in (A) 30% and (B) 3% of tumor nuclei. (C) Cyclin E-negative stain in the tumor nuclei. Magnification, x40.

The degree of cyclin E expression varied in the tumor tissue of the different patients as shown in (Fig. 1).

There was no correlation between the degree of cyclin E expression and the clinical data. A statistically significant correlation was identified when the N-stage was compared with cyclin E expression (P=0.003; Table I).

Discussion

The degree of invasion and nodal status are the main prognostic indicators of the outcome in CRC. In the TN staging

category, the prognosis of stages I and IV is predictable while the outcomes of stages II and III have a broad spectrum. The 5-year survival rate in stage III CRC is approximately 75% (21). There is a need for the identification of new prognostic biomarkers which may be implemented in clinical practice for the identification of patients with stage III CRC who have higher survival potential (19). The detection of such markers may aid in risk stratification and cyclin E may be included in the list of possible candidates.

The role of cyclin E in colon tumorgenesis is supported by certain studies which revealed overexpression of cyclin E in early stages of tumor development (17,23). Positive cyclin E staining was correlated with Ki-67 antigen (a marker of proliferative activity of malignant cells) and p53 protein level in these cells (3,24). This finding supports the contribution of cyclin E to abnormal regulation of the cell cycle, affecting colorectal carcinogenesis (7). Yasui *et al* found expression of cyclin E in 25% of human colorectal adenomas and 56% of adenocarcinomas (15). Increased levels of cyclin E in certain cases indicate gene amplification (25,26). Corin *et al* revealed a significant correlation between high total cyclin E expression and cancer-specific survival in patients with colon cancer (17). Zhou *et al* found that cyclin E overexpression is a strong negative predictor for survival in patients with rectal cancer (27). The results of the study by Kitahara *et al* support the possibility that cyclin E gene amplification may play a role in the pathogenesis of CRC (26). Iwatsuki *et al* studied FBXW7, a cell cycle regulating gene involved in cyclin E degradation, and found that in the case of low FBXW7 protein expression there was strong cyclin E protein expression in CRC (28).

The current knowledge regarding the role of cyclin E as a prognostic marker in CRC is controversial. In a group of patients younger than 45 years old with early-onset CRC, a lack of cyclin E expression was correlated with higher mortality (29).

Cyclin E was absent in 86% of microsatellite-stable CRCs in a younger group (29). Sutter *et al* revealed that cyclin E was overexpressed in colorectal tumors with high microsatellite instability, and suggested that increased expression of cyclin E may contribute to the mutator phenotype of CRC (30). Cells from colorectal cell lines with an aberrant number of chromosomes had a higher cyclin E mRNA expression. This finding supports the role of cyclin E as a factor involved in the chromosome instability phenotype *in vitro* (18). However, certain studies revealed that cyclin E overexpression was correlated with certain clinicopathological features, but could not be used as a prognostic marker of early recurrence and survival (23). Lim *et al* revealed that cyclin E expression did not predict poor prognosis in patients with stage II colorectal carcinomas (31). Li *et al* demonstrated that the three-year survival rate of a cyclin E-negative group was significantly lower than that of the cyclin E-positive group (32). The findings of Wang *et al* support the theory of overexpression of cyclin E in CRC without a significant correlation with clinical and pathological characteristics (33). Bondi *et al* did not identify a significant correlation between cyclin E expression and gene amplification in primary colon adenocarcinomas and clinical outcome (34). Cyclin E was found to be higher in rectal cancer compared with colon cancer in a study from Norway (35). In light of the above, the possible role of the expression of cyclin E as a prognostic marker in CRC is unclear. Cyclin E overexpression was observed in certain studies, while negative cyclin E staining was revealed in others. Thus, there are insufficient data to apply cyclin E for clinical and prognostic purposes in CRC. In the present study, in patients with T3 CRC, there were more lymph node-positive cases in the cyclin E-negative group than in the cyclin E-positive group (P=0.003) when we compared the node status with the cyclin E expression. Our data support the findings of Li *et al* (32). In their study of 304 patients with colorectal tumors, low levels of cyclin E were significantly correlated with neoplasm size, deep invasion, multiple metastases and poor prognosis (32). According to their interpretation of the data, tumor progression is associated with loss of cyclin E expression. We did not identify a significant correlation between the degree of cyclin E expression and the clinical data in our patients with T3 CRC.

Thus, the role of cyclin E as a prognostic marker in gastrointestinal cancer requires further study, with focus on the lymph node status and cyclin E expression.

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