The Prognostic Value of G1 Cyclins, p21 and Rb Protein in Patients With Colon Cancer

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Abstract. *Background/Aim:* Cyclins D1 and E play different roles in the cell cycle. Cyclin E promotes chromosome instability, whereas cyclin D1 regulates apoptosis of cells. This study evaluated the prognostic significance of G1 cyclins, p21 and pRb in tumor proliferation. *Patients and Methods:* A total of 102 patients with colon cancer were operated on and staged according to TNM. Follow-up was 2 to 68 months (mean 38.3±16.7 months). Expression of cyclin E and D1 were evaluated using immunohistochemistry. *Results:* Levels of cyclin E expression were correlated with cyclin D1 expression ($p=0.038$), p21 expression ($p=0.047$), and pRb expression ($p=0.004$). The 5-year survival rate along with prognosis of patients with advanced stage (III, IV) colon cancer and cyclin D1 positive tumors, were significantly worse ($p=0.009$). Statistically significant association was observed between tumor proliferative capacity Ki-67, cyclin D1 ($p=0.009$), pRb ($p=0.031$) and p21 ($p=0.050$). *Conclusion:* Cyclin D1 is highly expressed in advanced stage colon cancer patients, implying a potential prognostic value.

Colorectal cancer (CRC) is one of the main challenges for public health representing the third most common cancer worldwide, following prostate and lung cancer in males and breast and lung cancer in females (1, 2). According to the World Health Organization, in 2018 there were 1.8 million colorectal cancer cases of which 861,000 resulted in death (3). In the United States, 1,762,450 new cancer cases and 606,880 cancer deaths are projected to occur in 2019 (4). The annual estimation of new cases diagnosed with large

bowel cancer is approximately 145,600 (4). Among these, 101,420 are colon and the remainder are rectal cancers, while CRC-related deaths are approximately 50,630.

Currently, advances in chemotherapy either as adjuvant in colon cancer, or as neoadjuvant with radiotherapy in rectal cancer, require the precise determination of the subgroup of patients which may have a survival benefit (5). In this respect, the identification of specific, early expressed biomarkers may contribute to a better prognosis of patients with intermediate grade tumors.

Cyclins and the cyclin-dependent kinases, are key regulators of the cell cycle (6). Errors in the normal process of the cell cycle result in deregulated DNA replication and mitosis (7) leading to increased resistance to apoptosis and the induction of cancer (8, 9). A major cell cycle regulatory point in most animal cells called the restriction point, occurs late in G₁ and controls progression from G₁ to S (10). The two main aspects of cell cycle regulation are the DNA structure checkpoints, which arrest the cell cycle in response to DNA damage or incomplete replication, and the existence of the restriction point (11). Once cells have passed the restriction point, they are normally committed to proceed and divide, hence, the so called G1 cyclins have a prevailing role in promoting cell division (12). The G1-S transition of the cell cycle, is mainly regulated by cyclins D1 and E, each of them playing a different and discrete role (13). Cyclin E has been involved in various cancer cell lines (14), in breast carcinomas (15) and in about 10% of colorectal cancer cases (16). Keyomarsi *et al.*, (1994) reported a correlation between cyclin E expression and advanced stage breast carcinomas, implying its potential role as a prognostic marker (17).

The retinoblastoma (Rb) protein is a tumor suppressor, and a key negative regulator of the cell cycle (18) by suppressing G₁ phase progression (19). Rb protein is active in the early G₁ phase in its hypophosphorylated form, whereas in mid/late G₁ phase the protein becomes

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inactivated by phosphorylation. Cyclins D1 and E, along with their catalytic partners, the cyclin-dependent kinases (cdk), are responsible for this phosphorylation of pRb and also regulate progression of the cell cycle from G₁ to S-phase (20). Moreover, p21 acts by binding cdks in the G₁ phase of the cell cycle and leading to the inhibition of phosphorylation of other proteins like Rb, necessary for cell cycle progression (21). The protein product of Rb leads to the inhibition of the cyclin D1 and cyclin E promoter activity by binding to E2F transcription factors. In mid-to-late G₁ phase, where Rb is initially phosphorylated, probably by cdk-cyclin D complexes, E2F transcription factors are produced, which promote transcription of the cyclin D1 and cyclin E genes (22-24). Inactivation of the *Rb* gene is involved in various types of cancers (25, 26), however in colorectal carcinomas loss or inactivation of Rb seems to be rare (27). In contrast to the fact that loss of pRb activity is observed in various tumors, other studies have depicted increased levels of Rb transcripts in colorectal cancer, implying a potential mitotic role of pRb (28, 29). Moreover, increased cyclin D1 and cyclin E expression endures Rb phosphorylation. As a consequence, additional E2Fs are released that pursue cyclin D1 and cyclin E transcription. Overexpression of cyclin D1 or cyclin E enhances phosphorylation of Rb and promotes cellular proliferation suppressing pRb activity. This concept of cyclin D1 and cyclin E as positive regulators of cell proliferation is supported by various studies where microinjection of anti-cyclin D1 and E antibodies prevent cells from entering the S-phase of the cell cycle (30-32).

Overexpression of cyclin D1 or cyclin E is positively involved in carcinogenesis as increased levels of cyclin D1 and E have been shown to induce cell growth by shortening the duration of the G₁ phase of the cell cycle (13, 32). Mermelshtein *et al.* reported a correlation between cyclins D1 and/or D2, but not cyclin D3, and TNM stage of colon carcinomas. In addition, overexpression of cyclin D2 may be strongly related to a higher TNM stage of tumors (33). In another prospective patient cohort study by Wangefjord *et al.*, it was depicted that cyclin D1 expression in colorectal cancer has a different effect in males and females. It was shown that cyclin D1 was a favorable prognostic factor in men compared to women (34). A meta-analysis in 2014, of 22 studies and a total of 4150 patients, indicated that cyclin D1 is an unfavorable prognostic factor for colorectal cancer. Cyclin D1 overexpression might be associated with a poor clinical outcome and clinical and pathological data such as age, tumor, lymph nodes and distant metastasis in colorectal cancer patients (35). Al-Maghrabi J *et al.* have also published that expression of cyclin D1 in colorectal carcinomas is not related to overall survival. No significant correlation with clinicopathological features was presented, except from lympho-vascular invasion (36).

We can assume that overexpression of cyclins, may not only contribute to tumorigenesis but may also have a potentially predictive role in the clinical aggressiveness of colon carcinomas. In the present study, we investigated immunohistochemically the expression of both G₁ cyclins (D1 and E) in relation to Ki-67 expression, in human colon cancer specimens. The above data support that cyclins may be used as sensitive and accurate markers in the diagnosis of colorectal cancer (37). The present study aimed to identify the involvement of G₁ cyclins in the cell cycle regulation of human colon cancer cells and whether there is any prognostic significance in subgroups of patients, according to staging.

Patients and Methods

Study population. The study group consisted of 102 patients with newly diagnosed and histologically confirmed colon cancer, 60 men (58.8%) and 42 (41.2%) women. All patients enrolled, received surgical resection treatment with curative intent. The age composition of our study population was 42-94 years with a mean age of 70.6 years. Patients with rectal cancer and those who refused follow-up were excluded from the study.

Histology and immunohistochemistry. *Histology:* Tissue samples were fixed in 10% formalin and embedded in paraffin A. Five µm thick paraffin sections were produced using a microtome. Dewaxed sections were stained with hematoxylin and eosin as previously described (38).

Immunocytochemistry: Sections were incubated in 10 mM citrate buffer (pH 6.0) for 15 min, and then exposed to freshly prepared 0.3% hydrogen peroxide in methanol, for 30 min, at room temperature (RT). Blocking was performed using blocking reagents (Snipper, Biocare Biochemicals, Walnut Creek, CA, USA), for 10 min. Sections were incubated for 1 h, at RT, with the primary antibodies diluted in 1:100 phosphate buffered saline (PBS). Slides were incubated with secondary biotinylated antibodies for 20 min at RT. Peroxidase-streptavidin labelling (AB Complex, Dakopatts) was performed for 20 min, at RT. Then, the colour was developed in 0.5% (v/v) 3,3'-diaminobenzidine hydrochloride (DAB, Sigma, St Louis, MO, USA) in PBS containing 0.03% (v/v) hydrogen peroxide for 2 min. Antigens were retrieved only for Ki-67, by the use of microwaves. All immunohistochemical studies were blinded to the clinical data. The primary antibodies used were: mouse (IgG1k) monoclonal antibodies anti-cyclins D1 and E (Santa Cruz Biochemicals, CA, USA), anti-human pRb (Santa Cruz Biochemicals), and anti-Ki-67 (Dakopatts, Glostrup, Denmark). Secondary antibodies included the Rodent immunoglobulins (Dakopatts) for detection of cyclin D1, cyclin E and Ki-67 and the swine anti-rabbit immunoglobulins for pRb, and were diluted 1:200 in PBS.

Statistical analysis. We evaluated the correlation between cyclin E, cyclin D1, p21, pRb and Ki-67 expression and various clinical and pathological variables (age, location, stage and grade, lymph node metastasis, liver metastasis and vascular invasion) by using the chi-square test (X²). Kaplan–Meier survival curves were compared using the log rank test. The effect of each potential prognostic factor

Figure 1. Section of a colon cancer specimen stained positive for Cyclin E. Magnification: $\times 20$

Figure 2. Section of a colon cancer specimen stained positive for pRb. Magnification: $\times 20$

Table I. The expression of cyclins D1, E, p21 and their correlation to clinical and pathologic characteristics of colon cancer (X^2 test).

	Cyclin E		p-Value	Cyclin D1		p-Value	p21		p-Value
	(+)	(-)		(+)	(-)		(+)	(-)	
Age									
<70 years	21	26	0.321	34	13	0.136	26	21	0.122
≥70 years	30	25		32	23		22	33	
Gender									
Male	25	35	0.044	36	24	0.235	28	32	0.924
Female	26	16		30	12		20	22	
Staging									
I+II	30	25	0.321	36	19	0.864	29	26	0.215
III+IV	21	26		30	17		19	28	
Grade									
Good/moderate	39	43	0.318	50	32	0.110	40	42	0.481
Poor	12	8		16	4		8	12	
Location									
Proximal	17	18	0.835	24	11	0.555	19	16	0.291
Distal	34	33		42	25		29	38	
Lymph nodes									
Positive	20	20	1.000	24	16	0.424	15	25	0.120
Negative	31	31		42	20		33	29	
Liver metastasis									
Positive	9	7	0.586	13	3	0.132	5	11	0.168
Negative	42	44		53	33		43	43	
Vascular infiltration									
Positive	16	11	0.262	19	8	0.473	11	16	0.443
Negative	35	40		47	28		37	38	

on patients' overall survival was evaluated by using Cox regression analysis. Results were considered statistically significant when p -value was less than 0.05 ($p < 0.05$).

Results

Patients tumor, node, metastasis (TNM) staging classification. The total of 102 patients included in this study had been diagnosed with colon cancer. None of the patients had received any preoperative chemotherapy. Anatomic subsites of tumors were located in the right, cecum and the ascending colon in 28 cases (27.5%), in the transverse colon in 7 cases (6.9%), in the descending colon in 9 cases (8.8%) and in the sigmoid in 58 cases (56.8%). Patients were operated with an open or laparoscopic technique and the collected tumor samples were histopathologically staged according to the 6th edition of the TNM classification system as: T1-2N0, 16 cases (15.7%); T3-4N0, 39 cases (38.2%); T1-4 N1-2, 24 cases (23.5%); and T1-T4 N0-2M1, 23 cases (22.5%). According to the differentiation stage of tumors (grade), samples were classified as, well differentiated (low grade), moderately differentiated (intermediate grade) and poorly differentiated (high grade), corresponding to 8 (7.8%), 74 (72.5%) and 20 (19.6%) patients, respectively. All patients were followed-up for 2 to 68 months, (mean

38.3±16.7 months) and the median follow-up time for all patients was 38 months.

E and D1 cyclin expression in colon cancer patients. Sections were evaluated as either "positive" for cyclin D1, p21 or pRb when the majority of the cells in the selected sections were positively stained compared to the control, normal colon tissue, negative for cyclin D1. Five percent (5%) was used as the cutoff value as previously published (39-42). For cyclin E and Ki-67, we defined labeling index (LI) (%) as the percentage of tumor cells which displayed nuclear immunoreactivity. Overall, 64.7% and 50% of tissue samples from colon cancer patients were found positive for cyclin D1 and E (Figure 1), respectively. In 22.7% of cases, cyclin D1 expression was nuclear, 4.5% cytoplasmic and 72.7% mixed (cytoplasmic and nuclear localization of cyclin D1). All samples positive for cyclin E expression revealed a high level of expression ($LI \geq 30\%$), while D1 immunostaining was evaluated as low in 32 out of the 66 cases (48.5%), moderate in 24 out of 66 (36.4%), and high in 10 out of 66 (15.1%). However, when the pattern and the intensity of cyclin D1 were compared to tumor or patient characteristics (Table I), no correlation was observed. Moreover, no significant association of the cyclin D1 pattern was observed when compared with the Rb expression.

We further analyzed patient's colon cancers for p21 expression. A total of 48 patients (47%) presented a positive staining, however, no significant association between p21 expression and TNM classification, survival rate or Rb protein expression was detected (Table I). Nevertheless, cyclin E expression was correlated with cyclin D1 ($p=0.038$), p21 ($p=0.047$), and Rb expression ($p=0.004$) (Table II). Among the 64 cases of tumors with cyclin D1 positive staining, 38 of them expressed cyclin E at high levels. Similarly, among the 48 cases with p21 positive tumors, 29 highly expressed cyclin E.

The intensity of pRb staining (Figure 2) was assessed as low in 27 out of 64 (42.2%), as moderate in 18 out of 64 (28.1%) and as high in 19 out of 64 cases (29.7%). Among the 38 cases of tumors with pRb negative staining, 17 of them were cyclin D1 negative, however this was not statistically significant. However, no relation between pRb expression and age, tumor location, histological stage, grade, lymph node, liver metastasis and venous invasion was detected.

Follow-up of the patients did not show any effect of cyclin D1, cyclin E and pRb on the 5-year survival rate. Patients with advanced stage disease (stage III and IV) and positive for cyclin D1 were found to have significantly poorer survival rates compared those with a negative cyclin D1 expression ($p=0.009$). In addition, the survival of patients who were less than 70 years old, was significantly correlated with a positive cyclin D1 expression ($p=0.008$).

To determine the proliferative capacity of colon cancer cases with abnormal expression of Rb, p21 and cyclins E and D1, we performed immunostaining for Ki-67 (Table III). We observed a statistically significant association between tumor proliferative capacity expressed as Ki-67 labeling index (LI) and positive cyclin D1 staining ($p=0.009$), positive pRb staining ($p=0.031$), and positive p21 staining ($p=0.050$). Tumors with low levels of cyclin E expression presented higher Ki-67 LI compared to tumors with high levels of cyclin E ($p=0.322$).

Among the 66 tumors positive for cyclin D1 expression, 38 were assessed to have less than 30% Ki-67 positive cells, and 38 had $\geq 30\%$. Among the 64 patients presenting Rb positive tumors, 36 were assessed to have more than 30% Ki-67 positive cells, and 28 had less than 30% ($p=0.031$), (Table III).

Discussion

In the present study, we have demonstrated the different expression patterns of cyclin D1 and cyclin E in the cell proliferation and clinical outcome of colon cancer. Cyclin D1 expression was positively correlated with cell growth as determined by Ki-67 expression. However, it was not an independent unfavorable prognostic factor. On the other hand,

Table II. Expression of cyclin E in colon cancer patients' samples and correlation with cyclin D1, p21 and pRb expression.

	Cyclin E		<i>p</i> -Value
	High ^a	Low ^b	
Cyclin D1			
Positive	38	28	0.038
Negative	13	23	
p21			
Positive	29	19	0.047
Negative	22	32	
pRb			
Positive	39	25	0.004
Negative	12	26	

^aHigh expression of Cyclin E. Cyclin E LI $\geq$ 30% (Figure 1); ^bLow expression of Cyclin E. Cyclin E LI<30%.

Table III. Cyclin E, cyclin D1, pRb and p21 and their correlation with Ki-67 in colon cancer.

	No	Ki-67		<i>p</i> -Value
		<30%	$\geq 30\%$	
Cyclin E				
High ^a	51	24	27	0.322
Low ^b	51	29	22	
Cyclin D1				
Positive	66	28	38	0.009
Negative	36	25	11	
pRb				
Positive	64	28	36	0.031
Negative	38	25	13	
p21				
Positive	48	20	28	0.050
Negative	54	33	21	

^aHigh expression of Cyclin E. Cyclin E LI $\geq$ 30% (Figure 1); ^bLow expression of Cyclin E. Cyclin E LI<30%.

cyclin E expression was not associated neither with the Ki-67 LI nor with clinical outcome. Our study showed that cyclin D1 expression is involved in cell growth and is highly expressed in young colon cancer patients with advanced stage disease, suggesting that it may have a great value in identifying high-risk colon cancer patients. Similarly, in a cohort study of 602 colon cancer cases, cyclin D1 overexpression was associated with a low cancer-specific mortality in Kaplan–Meier analysis ($p=0.006$) or in other words with longer survival (43).

Cyclin E, p21 and Rb protein accumulation were not of any significant prognostic value. Cyclin E expression and the cellular marker of proliferation Ki67 have been shown to be

positively correlated. On the contrary, p27^{kip1} protein expression and Ki67 were negatively correlated.

These results were closely related to the TNM stage and degree of differentiation. Peculiarly, these results were also associated with prognosis, progression-free survival and median survival.

Although these observations emphasize the importance of cyclins in tumorigenesis and prognosis, the question remains and further studies are needed to further evaluate the role of cyclins on the onset of colon cancer or other malignancies. In conclusion, cyclins can be used as sensitive and accurate markers for the diagnosis of colorectal cancer (37).

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

PP, GK, DC: conception and design of the study; PP, DC: acquisition of data, drafting the article; ST: histological study, analysis of histological scores; GK, DC: drafting the article, revising it critically for important intellectual content; GK: final approval of the version to be submitted.

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