

Circadian variation in expression of G₁ phase cyclins D₁ and E and cyclin-dependent kinase inhibitors p16 and p21 in human bowel mucosa

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Abstract

AIM: To evaluate whether the cellular proliferation rate in the large bowel epithelial cells is characterized by circadian rhythm.

METHODS: Between January 2003 and December 2004, twenty patients who were diagnosed as suffering from primary, resectable, non-metastatic adenocarcinoma of the lower rectum, infiltrating the sphincter mechanism, underwent abdominoperineal resection, total mesorectal excision and permanent left iliac colostomy. In formalin-fixed and paraffin-embedded biopsy specimens obtained from the colostomy mucosa every six hours (00:00, 06:00, 12:00, 18:00 and 24:00), we studied the expression of G₁ phase cyclins (D₁ and E) as well as the expression of the G₁ phase cyclin-dependent kinase (CDK) inhibitors p16 and p21 as indicators of cell cycle progression in colonic epithelial cells using immunohistochemical methods.

RESULTS: The expression of both cyclins showed a similar circadian fashion obtaining their lowest and highest values at 00:00 and 18:00, respectively ($P < 0.001$). A circadian rhythm in the expression of CDK inhibitor proteins p16 and p21 was also observed, with the lowest levels obtained at 12:00 and 18:00 ($P < 0.001$), respectively. When the complexes cyclins D₁-p21 and E-p21 were examined, the expression of the cyclins was adversely correlated to the p21 expression throughout the day. When the complexes the cyclins D₁-p16 and E-p16

were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high levels of p16 expression at 18:00.

CONCLUSION: Colonic epithelial cells seem to enter the G₁ phase of the cell cycle during afternoon (between 12:00 and 18:00) with the highest rates obtained at 18:00. From a clinical point of view, the present results suggest that G₁-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity.

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Key words: G₁ phase proteins; CDK inhibitors; Cell proliferation; Circadian rhythm

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INTRODUCTION

Eukaryotic cell division occurs in four phases of the cell cycle. The cells are prepared for DNA replication in G₁-phase. DNA is replicated during the S phase, a gap (G₂) period which allows preparation for mitosis before chromosome segregation and cytokinesis in M phase (mitosis). During development, differentiation, or growth factor withdrawal, cells can enter an inactive period (G₀-phase) before returning to G₁-phase^[1].

Cyclins represent important regulators of cell cycle process. There are at least 15 distinct cyclin genes in the human genome that fall into three categories, and each category regulates specific passage through the cell cycle^[2,3]. Passage through G₁ to S phase is regulated by cyclins C,

regulated by cyclins A and B₁₋₂^[4-7]. Their levels in cell cytoplasm increase or decrease depending on the stage of cell cycle^[2, 3, 8, 9]. Elevated nuclear expression of cyclins favour the progression from one phase to the next while their low expression decelerates this progression^[2-4, 7].

Cell cycle progression is mediated by the activation of a family of protein kinases, known as cyclin-dependent kinases (CDKs). CDKs constitute a large family of proteins, which act in a variety of key regulatory pathways, including control over the cell cycle and gene transcription^[2, 9]. They are also divided in G₁-phase CDK (CDK4), S-phase CDK (CDK2) and M-phase CDK (CDK1)^[8, 9]. Their levels in the cells remain fairly stable, but each must bind to the appropriate cyclin (whose levels fluctuate) in order to be activated^[3, 8]. Cyclin D₁ (an early G₁-phase cyclin) mainly binds to and activates CDK4 and CDK6^[8, 10] while cyclin E (a late G₁-phase cyclin) binds to and activates CDK2^[8, 10].

CDK inhibitors inhibit the passage through the various phases of the cell cycle^[11, 12]. The CDK inhibitors p16 and p21 protein families mediate regulation of cyclin/CDK activity^[11, 12]. p16 family contains four members and inhibits CDK4 and CDK6, forming binary complexes with CDK4 *in vitro*^[11, 12]. p21 family contains three members and interacts with both cyclin D and E complexes in G₁-phase and preferentially inhibits CDK2 activity^[10].

In mammals, physiological (e.g. cardiac rhythm) or biochemical (e.g. hormone levels) processes vary in a regular and predictable periodic manner, with respect to the time of the day, which is called endogenous circadian rhythm^[13, 14]. At the cellular level, each cell goes through the cell cycle in an orderly and controlled fashion, where the multiple steps associated with each phase should be successfully completed before progressing to the next phase^[15]. At the tissue level, experimental^[10, 16, 17] and clinical^[18-20] studies suggest that a greater proportion of cycling cells in a specific organ, enters S-phase and mitosis at specific times of the day.

The present study was designed to evaluate whether the cellular proliferation activity in the large bowel epithelial cells shows variation over the 24 hours of a day and if this variation is characterized by circadian rhythm. As indicators for cellular proliferation activity we examined the quantitative expression of cyclins D₁ and E, as well as the inhibitor proteins p16 and p21 in biopsy specimens taken from the normal bowel mucosa of a permanent colostomy at six-hour intervals.

MATERIALS AND METHODS

Patients

Between January 2003 and December 2004, eighty-six patients suffering from colon and rectal cancer were surgically treated in our department. Among them, there were twenty patients (twelve men and eight women, median age 67 years, interquartile range 55-78 years) who were diagnosed as suffering from adenocarcinoma of the lower rectum infiltrating the sphincter mechanism. In all patients, the preoperative and intraoperative staging work-up revealed primary, resectable, non-metastatic lower rectal cancer. In order to achieve an oncological procedure, all patients underwent abdominoperineal resection, total mes-

orectal excision and permanent left iliac colostomy. All patients were operated on electively and neither neoadjuvant nor adjuvant chemo-radiotherapy was administered during the period of the study.

Biopsies

Biopsy specimens of bowel mucosa were obtained from the site of colostomy of these twenty patients undergone an abdominoperineal resection. Biopsies were collected at the time when the bowel retained its normal function after the operation and the colostomy was fully functional. Patients received the usual in-hospital low fibre diet. In order to avoid disturbance of cell proliferation, no enemas, bowel preparations and paraffin oil were used^[18, 25]. All patients followed their usual sleep schedule (sleeping between 22:00 and 07:00) without receiving any sedatives^[18, 19, 22, 25]. Their sleep pattern was interrupted only at the time when the colostomy specimens were taken. Specimens of 2-4 mm² were collected every 6 hours from the colostomy mucosa, approximately 3 cm lower to the colostomy orifice, using biopsy miniforceps. The examinations were performed at 00:00, 06:00, 12:00, 18:00 and 24:00. The biopsies were fixed in 10% buffered formalin and embedded in paraffin wax using conventional techniques.

Immunohistochemistry

Immunostainings for cyclins and CDK inhibitors were performed using mouse monoclonal antibody for cyclin D₁, rabbit polyclonal antibody for cyclin E, mouse monoclonal antibody for p16 protein and mouse monoclonal antibody for p21 protein (Santa Cruz Biochemicals, Santa Cruz, California, USA) with a Vectastain Elite ABC-peroxidase kit (Vector Laboratories, Peterborough, United Kingdom) and a liquid DAB substrate-chromogen system (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. An additional step of antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was performed before antibody incubation for p21. The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). The percentage of positively stained cells in immunohistochemistry experiments was obtained by counting epithelial cells in each case by two independent observers.

Statistical analysis

All graphics were constructed using Microsoft Excel for Windows XT Professional. Statistical differences between the groups were determined by the Student's *t*-test. *P* < 0.05 was considered statistically significant. All statistical calculations were performed using the STATA statistical package (StataQuest Version 4.0, College Station, Texas, USA, 1995).

RESULTS

The median and interquartile range (IR) values of the percentage of the positively stained cells in immunohistochemistry for every single protein examined throughout the day of the present study, are presented in Table 1. The observed differences in the expression of the studied proteins between 00:00 and 24:00, could be explained by the fact that more bowel mucosa epithelial cells entered the

Table 1 Percentage of stained cells in immunohistochemistry for cyclin D1, cyclin E, protein p16 and protein p21 expression in bowel mucosa specimens

TIME	Cyclin D1 (Median + IR)	Cyclin E (Median + IR)	p16 (Median + IR)	p21 (Median + IR)
00:00	6 (4 - 8)	4 (2.75 - 5)	13 (11 - 15.25)	14 (10.75 - 16.25)
06:00	7 (5 - 8)	5.5 (5 - 7)	10 (8 - 11.25)	10 (8 - 11)
12:00	8 (6 - 9)	8.5 (7.75 - 10)	8 (6.75 - 8.25)	8 (6.75 - 8)
18:00	13 (11 - 15)	13.5 (11.75 - 16)	13.5 (9 - 15.25)	6 (5 - 7.25)
24:00	8 (6.75 - 10)	8 (6 - 9.25)	15 (12.75 - 16)	14 (11 - 15)

IR: Interquartile range.

proliferative activity as time passed.

The interindividual values for cyclin D₁ expression varied between 3% and 19%. Its expression gradually increased between 00:00 and 18:00 and gradually decreased between 18:00 and 24:00, obtaining the lowest and highest values at 00:00 and 18:00, respectively. The differences between the highest values of cyclin D₁ expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for cyclin E expression varied between 1% and 17%. Its expression gradually increased between 00:00 and 18:00 and then gradually decreased, obtaining its lowest and highest values at 00:00 and 18:00, respectively. Similarly to cyclin D₁, the differences between the values of cyclin E expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p16 expression varied between 4% and 19%. Its expression gradually decreased between 00:00 and 12:00 and gradually increased between 12:00 and 24:00, obtaining its lowest and highest values at 12:00 and 24:00, respectively. The differences between the low values of p16 expression at 12:00, as compared to the higher values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p21 expression varied between 6% and 14%. Its expression gradually decreased between 00:00 and 18:00 and gradually increased between 18:00 and 24:00, obtaining its lowest values at 18:00 and its highest values at 00:00 and 24:00. The differences between the lowest values of p21 expression at 18:00, as compared to the values at 00:00, 06:00 and 24:00, were highly statistically significant ($P < 0.001$).

The present study concluded that expression of both cyclins showed circadian rhythm in a similar fashion. The higher levels of both proteins were obtained between 12:00 and 24:00 (highest at 18:00), while the lower levels were observed between 00:00 and 12:00 (lowest at 00:00).

A circadian rhythm in the expression of inhibitor proteins p16 and p21 was also observed. The lower levels of p16 expression were obtained between 06:00 and 12:00 (lowest at 12:00), while the lower levels of p21 expression were obtained between 12:00 and 18:00 (lowest at 18:00).

When the complexes of cyclins D₁-p21 (Figure 1A) and E - p21 (Figure 1B) were examined, the present the

expression of both cyclins was adversely correlated to the p21 expression throughout the day. The highest levels of cyclins were correlated to the lowest levels of p21.

When the complexes of cyclins D₁-p16 (Figure 1C) and E-p16 (Figure 1D) were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high expression levels of p16 at 18:00.

DISCUSSION

How the circadian variation in proliferation at the tissue level relates to the control of the cell cycle, is a subject of continuous study^[22]. The predictable association between certain cell cycle proteins and defined events during the cell cycle may be used to study the timing of cell cycle phases in normal tissues^[22]. In malignant tissue, the expression level of cyclins and their inhibitors may have important prognostic and therapeutic implications, especially in the cell cycle phase-dependent toxicity of anticancer agents^[20, 21].

Bucchi *et al*^[18] are the first to investigate the presence or absence of rhythm in normal bowel mucosa proliferation. In their study, rectal biopsies were obtained every 2 or 3 h for 24 h using standard biopsy forceps during flexible sigmoidoscopy from 16 volunteers under fasting and fed conditions. Incorporation of [³H] thymidine was measured in each specimen. Both fed and fasting subjects showed circadian variation in DNA synthesis in rectal mucosa that peaked at 07:00. Although thymidine incorporation fell during fasting, the circadian rhythm remained intact.

Marra *et al*^[19] investigated the proliferation rhythm within the rectal crypt epithelium using the ³H-thymidine autoradiographic method and calculated the ratio (labeling index) of the S-phase cells to total cells in the crypt. By taking biopsies every 4 h using standard biopsy forceps via rectoscopy from 23 normally fed subjects, they found a circadian rhythm in the labeling index with a peak at 01:28 in the morning. The base of the crypt and the upper 40% (which contains mainly differentiated cells) did not show circadian variation in the labeling index.

Brandi *et al*^[25] studied the circadian variations of rectal cell proliferation in five patients by taking biopsies via proctoscopy from apparently normal mucosa 10 cm from the anal verge every 6h in a 24h period. Labeling index was evaluated as the percentage of labeled cells with respect to the whole cell population in the crypt. The results of the study suggest that rectal cell proliferation fluctuates during the day with the lower rates noted between 22:00 and 02:00.

By measuring the ratio of the S-phase cells to the whole cell population with the [³H] thymidine technique, all previously mentioned studies^[18, 19, 25] have demonstrated a rhythmicity in the rectal mucosa proliferation. Due to potential limitations of the [³H] thymidine technique^[27], most recent studies on the cellular proliferation rhythm in different tissues, are focused on the immunohistochemically quantitative expression of phase specific proteins. In the present study we examined the quantitative expression of the G₁-phase specific cyclins D₁ and E and the CDK

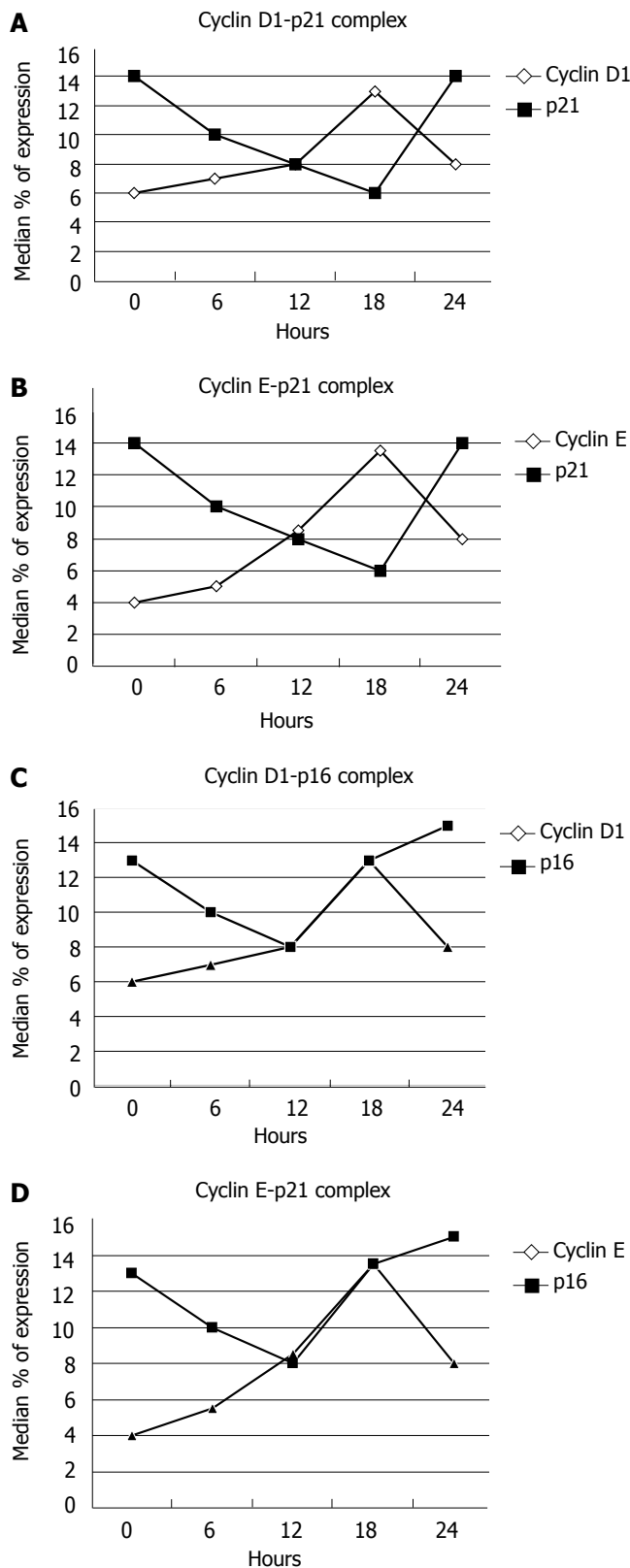


Figure 1 Graphical presentation of simultaneous quantitative expression of cyclin D1 and p21 (A), cyclin E and p21 (B), cyclin D1 and p16 (C), and cyclin E and p16 (D) in colostomy specimens sampled every 6 hours for 24 hours.

inhibitors p16 and p21 in normal large bowel mucosa.

Quantitative cyclin D₁ expression has been observed in normal bowel mucosa^[28], dysplastic and non-dysplastic adenomatous polyps of the small and large bowel^[18, 23, 29]. Its over-expression is related to adenocarcinoma of the colon

and rectum (independently of the differentiation or Dukes' stage)^[23], pancreatic^[30], esophageal^[30], endometrial^[30], head and neck cancers^[31].

Normal cells maintain strict control of cyclin E activity, while its deregulation plays a fundamental role in carcinogenesis. Cyclin E activities mainly consist of passage of cells through the restriction point "R" for cells entering the division from the resting state to G₁-phase^[32]. Cyclin E functions not only as a S-phase entry regulator, but also plays a direct role in the initiation of DNA replication, by inducing S-phase specific genes^[33, 34]. It is expressed higher than in many human tumors^[35, 36] and the accumulation of cyclin E is considered a marker for the transition from adenoma to adenocarcinoma^[37, 38].

p16 is a tumor suppressor gene and regulates cell proliferation by inhibiting CDK4 and CDK6 activities. Transient expression of p16 leads to inhibition of DNA synthesis by hypophosphorylating the retinoblastoma (Rb) gene protein^[39-42]. The p16/Rb tumor suppressor pathway is frequently defective in many human tumors either by inactivating p16 or Rb or by over-expressing cyclin D₁ or CDK4. In cases of colorectal cancer, only a low frequency of p16 mutation has been found^[39, 43]. Dai *et al*^[44] hold that p16 expression begins in the earliest detectable stages of human colonic neoplasia and exerts a continuous constraint of tumor growth. A recent experimental study^[45] showed that interaction of p16 expression and CDK4 may become a new prognostic marker in colorectal cancer. Tada *et al*^[46] studied the possible role of p16 in the development of colonic neoplasms and found that p16 is overexpressed in 98% of adenocarcinomas and that colorectal cancer with reduced p16 expression is more aggressive in lymphatic infiltration.

The p21 protein family can interact with both cyclin and CDK subunits. Members of the p21 family interact with cyclins D and E during the G₁-phase of the cell cycle, preferentially inhibiting CDK2 activity and promoting assembly of cyclin D/CDK 4 complex *in vivo* and *in vitro*^[47]. p21 constitutes the first molecule which is considered as a wild-type activated factor (WAF1) due to its upregulation by the tumor suppressor protein p53 and also as a cell-derived inhibitor of DNA synthesis^[48]. Expression of p21 has been detected *in vivo* by immunohistochemistry in cells of upper crypts and lower villus. The above mentioned areas are associated with enterocyte differentiation^[48] which is associated with a withdrawal from the cell cycle and the transcriptional activation of p21 either dependently or independently of the tumor suppressor p53^[48-50].

The results of the present study demonstrated that the expression of both cyclins showed a similar circadian fashion, with the higher levels obtained between 12:00 and 24:00 (highest at 18.00) and the lower levels between 00:00 and 12:00 (lowest at 00:00). These findings partly support the theory for coordinating and cascading activity between them during the G₁ phase of the cell cycle^[10]. As cyclin D₁ represents an early G₁ phase cyclin, while cyclin E constitutes a late G₁ phase cyclin, simultaneous increase and decrease of their expression during the same periods of time, require further investigation. A future study focusing on their expression at shorter intervals may disclose more accurately their fluctuation during the day. Circadian rhythm was also observed in the expression of

both inhibitor proteins, with the lowest values obtained at 12:00 and 18:00 for p16 and p21 expression, respectively. By comparing cyclins D1-p21 and E-p21 complexes, the lowest expression levels of p21 and the highest expression levels of both cyclins were observed at 18:00. When the complexes of cyclins D1-p16 and E-p16 were examined, the inhibitory action of p16 protein, successfully arrested the cell cycle during the night and early in the morning. Why high expression levels of p16 correlate to the highest expression levels of both cyclins at 18:00 remains unclear.

In conclusion, the expression of all examined parameters (which are involved in the progression from the G1 to S-phase of the cell cycle) is characterized by circadian rhythm. Colonic epithelial cells seem to enter the G1 phase of the cell cycle during afternoon between 12:00 and 18:00, because during that period the higher expression levels of cyclins D1 and E correlate to the lower expression levels of the expression of CDK inhibitor proteins p16 and p21. From a clinical point of view, the present results suggest that G1-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity^[2, 3, 51-53]. Further studies on the accurate circadian rhythm in anatomically intact human colonic epithelium and malignant tissues are required.

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