Regulation of Genes of the Circadian Clock in Human Colon Cancer: Reduced Period-1 and Dihydropyrimidine Dehydrogenase Transcription Correlates in High-Grade Tumors

Walter Krugluger,1 Anita Brandstaetter,1 Enikő Kállay,5 Johann Schueller,2 Elisabeth Krexner,2 Stefan Kriwanek,3 Elisabeth Bonner,4 and Heide S. Cross4

Abstract
Expression of dihydropyrimidine dehydrogenase (DPD) displays a regular daily oscillation in nonmalignant cells. In colorectal cancer cells, the expression of this 5-fluorouracil–metabolizing enzyme is decreased, but the reason remains unclear. In this study, we analyzed by real-time reverse transcription-PCR (RT-PCR) the expression of DPD and of members of the cellular oscillation machinery, period 1 (Per1), period 2 (Per2), and CLOCK, in primary colorectal tumors and normal colon mucosa derived from the same patients. Analysis of tumors according to differentiation grade revealed a 0.46-fold (P = 0.005) decrease for DPD mRNA and a 0.49-fold (P = 0.004) decrease for Per1 mRNA in undifferentiated (G3) tumors compared with paired normal mucosa. In this tumor cohort, the correlation between DPD and Per1 levels was r = 0.64, P < 0.01. In moderately differentiated (G2) colon carcinomas, reduction of DPD and Per1 mRNA levels did not reach significance, but a significant correlation between the respective mRNA levels was detectable (r = 0.54; P < 0.05). The decrease and correlation of DPD and Per1 mRNA levels were even more pronounced in female (G3) patients (DPD: female, 0.35-fold, P < 0.001 versus male, 0.58-fold, P < 0.05; and Per1: female, 0.47-fold, P < 0.01 versus male, 0.52-fold, P < 0.01). The highly significant correlation of DPD mRNA with Per1 mRNA expression suggests control of DPD transcription by the endogenous cellular clock, which is more pronounced in women. Our results also revealed a disturbed transcription of Per1 during tumor progression, which might be the cause for disrupted daily oscillation of DPD in undifferentiated colon carcinoma cells. [Cancer Res 2007;67(16):7917–22]

Introduction
Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of fluoropyrimidines and, therefore, greatly influences the metabolism of the anticancer drug 5-fluorouracil (5-FU) commonly used in the chemotherapy of colorectal cancer (1, 2). The importance of this enzyme in 5-FU therapy for treatment of advanced colorectal cancer (13–16).

The circadian rhythm is generated by a transcription-translation–based oscillatory loop and involves a set of clock genes encoding the key molecules period 1 (Per1) and period 2 (Per2), CLOCK, BMAL1, CRY1 and 2, and TIM (reviewed in refs. 17, 18). The transcription of the main oscillators Per1 and Per2 is positively regulated by the CLOCK/BMAL1 complex. A complex of CRY1/2, PER1/2, and TIM negatively regulates the transcription of Per1 and Per2 by binding the positive factors CLOCK/BMAL-1 (17, 18).

Originally, the circadian oscillations were thought to occur only within the suprachiasmatic nucleus (SCN), where it regulates circadian variation in gene transcription and behavior. Recent studies have found molecular clocks in cells of many peripheral organs, including the gastrointestinal tract (19–22). It has been shown that a circadian oscillation of Per1 and Per2 is found in the oral mucosa as well as in colon crypt cells, and that this oscillation is independent from the SCN (22, 23).

The mechanism for altered DPD transcription in colon cancer is yet unknown. It has been shown that DPD and other genes that are expressed in the gastrointestinal tract are under the regulation of a peripheral clock that may be entrainable by food (21, 22). A disruption of this regulation in malignant tissue might therefore be a cause for the altered gene expression of DPD in colorectal tumor tissue. In this study, we have investigated mRNA levels of members of the clock genes and of DPD in colon tumors and in the adjacent mucosa derived from the same patients.

Materials and Methods
Patients. The study was approved by the ethical board of Vienna, and all patients gave written informed consent. DPD, Per1, Per2, and CLOCK mRNA levels were evaluated in tumor tissue and adjacent normal tissue of 30 patients (15 men and 15 women) undergoing surgery for primary colorectal cancer. Patient characteristics are shown in Table 1. No tumors with grade G1 were included in this study because of the low number of patients with colorectal tumors grade G1 in our cohort of patients. All specimens were observed in the liver (5). In advanced colon cancer, both DPD activity and expression are decreased in malignant cells when compared with the surrounding normal mucosal tissue (6, 7). On the other hand, DPD activity and expression is increased in corresponding metastases in the liver and lymph node (8, 9). DPD activity in colon cancer cells shows interindividual differences, and recent studies have suggested that altered DPD activity and mRNA expression in tumor cells determine the efficiency and outcome of 5-FU therapy (10–12).

The existence of a circadian rhythm in the expression and activity of DPD in normal tissues has led to the introduction of so-called chronomodulated chemotherapy regimens with variable rate infusions of 5-FU for treatment of advanced colorectal cancer (13–16).
obtained between 9:00 a.m. and 12:00 p.m. of the same day (3 h), and viable tumor tissue and adjacent normal tissue were dissected immediately and snap-frozen in liquid nitrogen.

RNA extraction and first-strand cDNA synthesis. Preparation of total RNA from tissue samples was done using the TRIzol reagent (Invitrogen). The amount of total RNA was determined by UV spectrophotometry, and RNA integrity was assessed by agarose gel electrophoresis. First-strand cDNA was prepared by reverse transcription with oligo-dT primers from 2.5 μg of total RNA using a commercial cDNA synthesis kit (Advantage RT-forPCR Kit; BD Biosciences).

Real-time reverse transcription-PCR (RT-PCR). About 5 μl of the cDNA was amplified for 40 cycles with specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DPD, Per1, Per2, and CLOCK (Table 2). PCR reactions containing SYBR-green were amplified on a Corbett Real Time PCR machine (Rotor gene 2000, Corbett Research). Data were acquired as threshold cycle (Ct) value. As internal standard to normalize mRNA levels for differences in sample concentration and loading, amplification of GAPDH was used. Because the amplification efficiencies of target genes and internal control were equal, the relative change of target gene expression in tumor cells compared with normal colon mucosa (ΔCt (calibrator)) was calculated using the equation 2−ΔΔCt, where ΔCt = C(tumor) − C(normal), and ΔΔCt = C(tumor) − C(normal) (24). The ΔCt values were determined by subtracting the average GAPDH Ct value from the average target gene Ct value. The SD of the difference was calculated from the SDs of target gene and GAPDH values (24).

After each real-time RT-PCR, a melting profile as well as agarose gel electrophoresis of each sample was done to rule out nonspecific PCR products and primer dimers.

Statistical analysis. Comparison of mRNA levels between normal mucosa and tumor tissue of patients was done by paired Student’s t test. Results from correlation analyses are expressed by Pearson’s correlation coefficient. A P value <0.05 was considered significant.

Results

Evaluation of Per1, Per2, CLOCK, and DPD mRNA levels in colorectal tumors. Per1, Per2, CLOCK, and DPD mRNA levels were quantified in 10 pairs of tumor tissue/normal mucosa from moderately differentiated (G2) colorectal tumors and 20 pairs of tumor tissue/normal mucosa from undifferentiated (G3) colorectal tumors. Median values, 25th and 75th percentile, and extremes of GAPDH-Ct value/target gene-Ct value are shown in Fig. 1.

DPD mRNA levels were significantly decreased in undifferentiated (G3) tumors when compared with their paired normal

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<th>Table 1. Patient characteristics</th>
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<tr>
<td>Patients’ tumor grade G2</td>
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<tr>
<td>Tumor stage; lymph nodes</td>
</tr>
<tr>
<td>Female</td>
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<tr>
<td>MA: 72</td>
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<td>MA: 73</td>
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NOTE: MA: Medium age (y). Four patients of the whole cohort had known metastasis at time of surgery.

<table>
<thead>
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<th>Table 2. Primer pairs used for real-time RT-PCR</th>
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<tr>
<td>Forward primer (5’-3’)</td>
</tr>
<tr>
<td>GAPDH</td>
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<tr>
<td>DPD</td>
</tr>
<tr>
<td>Per1</td>
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<td>Per2</td>
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mucosa (0.46-fold, \( P = 0.005 \), Fig. 2A). In moderately differentiated (G2) colorectal tumors, the decrease in DPD mRNA levels compared with that in paired normal mucosa was not significant (0.84-fold, \( P = \text{n.s.} \); Fig. 2A). In addition, the decrease in DPD mRNA levels in G3 colorectal tumors was more pronounced in tumors of female patients as compared with that in male patients (0.35-fold, \( P < 0.001 \) versus 0.58-fold, \( P < 0.05 \), respectively; Fig. 2B and C).

Similarly, a significant decrease of Per1 mRNA levels was observed in undifferentiated (G3) colorectal tumors as compared with the paired normal mucosa (0.49-fold, \( P = 0.004 \), Fig. 2A). In G2 colorectal tumors, the decrease did not reach significance (0.98-fold, \( P = \text{n.s.} \); Fig. 2A). Again, the decrease in G3 tumors was more pronounced in female patients compared with that in male patients (0.47-fold, \( P < 0.01 \) versus 0.52-fold, \( P < 0.01 \), respectively; Fig. 2B and C).

No significant changes of Per2 and CLOCK mRNA levels between normal mucosa and colorectal tumors were found (Fig. 2).

**Correlation of DPD mRNA levels with Per1, Per2, or CLOCK.**

The decreased DPD mRNA levels observed in colon adenocarcinomas, as compared with normal mucosa, correlated strongly with the decrease of Per1 mRNA levels in the analyzed tumor samples (\( r = 0.66, P < 0.01 \); Fig. 3A). In moderately differentiated (G2) tumors, the correlation was weak but significant (\( r = 0.54, P < 0.05 \)), whereas in undifferentiated (G3) tumors, the correlation was very pronounced (\( r = 0.64, P < 0.01 \), respectively). This was mainly due to contribution from the female cohort. The correlation of Per1 and DPD changes in the tumors was more pronounced in female patients compared with male patients (all tumors: \( r = 0.84, P < 0.001 \) versus m: \( r = 0.49, P < 0.05 \); G2 tumors: \( r = 0.97, P < 0.001 \) versus m: \( r = 0.20, P = \text{n.s.} \); and G3 tumors: \( r = 0.72, P < 0.01 \) versus m: \( r = 0.56, P < 0.05 \), see Fig. 3).

**Discussion**

The cellular clock regulates a variety of physiologic processes including gene transcription, the activity of metabolic pathways and cell growth and differentiation (17, 25). In mammals, the master circadian pacemaker resides in the SCN and generates cycles of ~24 h in the absence of external time cues (17, 18). This rhythm is dependent on a protein system with the key oscillatory molecules Per, CLOCK, BMAL, Cry, and Tim (17, 18). In addition, the CLOCK/BMAL1 system can interfere with promoter regulation by other, noncircadian transcription factors, including N-MYC and ETS, which in turn leads to attenuation or abrogation of transcription of CLOCK/BMAL1-controlled stress-induced genes (26). Circadian oscillations derived from the same protein system as in the SCN have been found in most cell types and organs of the body (20, 25). Under normal conditions, the peripheral clock systems are synchronized by the SCN pacemaker through neuronal and humoral signals (25, 27).
An important peripheral clock is the feeding-entrained oscillator (FEO), which underlies the food-anticipatory activity characterized by an increase of locomotion and core body temperature that precedes a daily meal (19, 20). This biological circadian rhythm is independent from the SCN because complete lesions of the SCN do not abolish the FEO (28, 29), and recent studies have suggested the liver or gastrointestinal tract to be the site of the FEO (21). However, recent studies also suggest the dorso medial hypothalamic nucleus as a potential central FEO in the feeding-mediated regulation of circadian behavior (30). This suggestion is supported by the detection of clock proteins in cells of the liver and of the gastrointestinal tract with a circadian expression profile similar to the SCN (21, 22).

Among the many genes controlled by the circadian clock (25, 27) are genes involved in the proliferation of the gastrointestinal epithelium as well as in cellular metabolism (21, 22). As an example, the activity of thymidylate synthase (TS), which influences the resistance of colon tumor cells to chemotherapy with 5-FU, has been shown to be driven by the endogenous circadian clock (22). Furthermore, the peak expression of Per1 coincides with the peak of the G1 phase marker p53, implicating a role of the circadian clock in physiologic cell cycle progression (22). It has been recently recognized that the circadian clock is a control point in tumor progression. An alteration in daily rhythms of motor activity and adrenocortical secretion is associated with poor survival of patients with metastatic colorectal (31, 32) or breast cancer (33). If the SCN was destroyed in mice, transplanted tumors grew twice as fast as in sham-operated mice (34). When rhythms of clock genes were suppressed in jet lagged mice, down-regulation of p53 and overexpression of c-myc occurred, and both effects also favor cancer growth (35). Down-regulation of circadian gene expression may actually occur by epigenetic mechanisms: in endometrial cancers, Cpg methylation in promoter sequences of PER1, PER2, and CRY1 was detected (36).

In this study, we have evaluated the expression of DPD and of members of the cellular clock, Per1, Per2, and CLOCK in colorectal tumors and adjacent mucosa. Samples were obtained in the morning hours when Per1 expression is usually high and fluctuations can be observed more readily. We have found a significant reduction of DPD and Per1 mRNA in undifferentiated (G3) colon cancer cells as compared with normal mucosa from the same patient. In addition, the reduced expression of DPD mRNA was strongly correlated to the expression of Per1 mRNA. Per2 and CLOCK mRNA levels were not significantly altered in tumor tissue. Recent studies revealed a circadian rhythm in expression and enzyme activity of DPD in mouse liver (13). Porsin et al. have shown in the liver of synchronized mice that maximum DPD activity occurred 16 h after the maximum observed DPD mRNA expression. In the healthy mouse liver, DPD activity seems to be sustained by a circadian rhythm at the transcriptional level (13). In humans, a circadian rhythm is not as easy to determine as in synchronized mice: Harris et al. (15) found a peak in DPD activity in circulating mononuclear cells of both healthy and cancer patients between 1000 p.m. and 100 a.m. In another study, it was shown that a circadian rhythm apparently exists in healthy subjects, whereas in patients with advanced gastrointestinal carcinomas, this rhythm was disturbed (14). However, although there were large intersubject differences, in bladder cancer patients, a circadian rhythm apparently existed in healthy subjects, whereas in patients with advanced gastrointestinal carcinomas, this rhythm was disturbed (14). However, although there were large intersubject differences, in bladder cancer patients, a circadian rhythm apparently exists in healthy subjects, whereas in patients with advanced gastrointestinal carcinomas, this rhythm was disturbed (14). 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Figure 3. Correlation of fold change of DPD mRNA expression in tumor tissue versus normal mucosa (NM) and fold change Per1 expression in tumor tissue versus NM. A, whole tumor cohort (tumor grades G2 + G3); B, patients' tumor grade G2. C, patients' tumor grade G3. a, all patients; f, ▲, female patients; m, △, male patients.
sex hormone–dependent organ like the mammary gland and the uterus, it should, however, be recognized that the colon is also positive for estrogen receptor-β (ER-β) and ER-α in both men and women (see, e.g., ref. 40). It has been long recognized that estrogen alters circadian rhythms in behavior and reproductive function, although molecular mechanisms are not well established yet. However, it has been shown that chronic treatment of ovariectomized rats with 17β-estradiol changes expression of Per1 as well as of Per2 mRNA in both brain and peripheral tissues such as the liver in a differential manner. In the liver, 17β-estradiol delayed the phase and increased the amplitude of Per1 mRNA expression (41). This could conceivably lead to impairment of normal entrainment because constitutive overexpression of Per1 in transgenic rats was shown to result in dampening of its rhythmic expression (42). On the other hand, it is quite clear that in hormone-dependent cancers such as that of the endometrium (43) and of the mammary gland (44), expression of Per genes is deregulated, probably by promoter methylation.

A recent multicenter, randomized, phase III study has shown that chronomodulated delivery, as compared with conventional delivery, of combined fluorouracil, leucovorin, and oxaliplatin to metastatic colorectal cancer patients was beneficial only for men but not for women: whereas the risk of death was decreased by 25% in men, in women, it was increased by 38% (45). Our results showing a stronger down-regulation of both Per1 and DPD expression in women with G3 tumors might be one of the explanations why chronomodulated delivery has no advantage in women. The conventional delivery, however, was slightly better for women. Mechanisms might involve gender dependencies in drug chronopharmacology, e.g., lower DPD activity in women. Indeed, our results suggest that women have a tendency to express lower levels of Per1 and DPD than men (see Fig. 2). In addition, estrogen might dampen rhythmic expression of Per1 as already described.

It has been shown that the expression of DPD and TS greatly influences prognosis and therapy outcome in colorectal cancer (46, 47). Well-advanced colon cancer patients with lower DPD expression in the tumor tended to have a longer overall and disease-free survival than patients with high DPD expression. These patients had received systemic chemotherapy with 5-FU by conventional delivery (9, 48). In our study, this seems to apply particularly to female G3 patients. Because expression of DPD and TS is under the control of the oscillating circadian clock, alterations of the cellular clock machinery might have considerable impact on therapy resistance and tumor progression. During 5-FU chronotherapy regimens, 5-FU administration near daily awakening seems to result in least damage to bone marrow and gut, greatest antitumor effect, and best survival. This time of day is associated with the highest levels of tumor nuclear BMAL-1 (49).

In accordance with such findings, our demonstration of a parallel decrease of Per1 and DPD mRNA levels in colon tumor tissue suggests an increased susceptibility of these cells to 5-FU toxicity, which is caused by a disturbed function of the cellular clock in colon tumor cells. This susceptibility might be further enhanced in women due to even lower levels of DPD and maintenance of the cellular rhythm.

Circadian clock gene expression also gates tumor cell proliferation. Per1 provides an important link between the circadian system and the cell cycle system. Overexpression of Per1 sensitizes human cancer cells to DNA damage–induced apoptosis, whereas inhibition of Per1 blunts apoptosis. Also, Per1 apparently interacts with checkpoint proteins. Transfection of human cancer cell lines with Per1 expression vectors leads to cessation of growth (50). Consequently, low levels of Per1 and DPD, and therefore, high proliferation of tumor cells, would constitute an optimal situation for 5-FU chemotherapy of high-grade human colorectal cancer.

There is a stringent need to better understand the interactions between the circadian timing system, the cell division cycle, and pharmacology, especially during less advanced malignancy. Although it is evident that there is a strong sex dependency of optimal scheduling of 5-FU related to the patients’ molecular clock, it should also be recognized that peaks and troughs of DPD activity vary even within single patients. Because fluctuations in DPD activity influence tolerability of fixed rate infusions of 5-FU, such treatment may not be suitable for all patients nor for any individual that needs treatment for several months. Thus, further studies are necessary to confirm the survival benefit of chronotherapy in men, as compared with conventional delivery, and to develop further optimal scheduling guidelines for women.

Acknowledgments

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