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Deregulated expression of the *per2* gene in human colorectal carcinoma

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Abstract. The circadian system is involved in the control of cell proliferation and apoptosis. The aim of this study was to analyze expression of the human *per2* gene in patients who underwent surgery for colorectal carcinoma. The study included 25 patients of both genders. Patients were exposed to light from 6:00 a.m. until 9:00 p.m. according to standard hospital practice. Tissue samples were taken from the tumor as well as from the proximal and distal areas of the resected colon at the time of surgery. Surgery was performed during the morning hours. Expression of *per2* mRNA was measured by real-time PCR. There was a significant negative correlation between *per2* gene expression and tumor staging. Expression of *per2* mRNA did not correlate with whether the tumor was localized in the colon or rectum. In comparison with ectomized tissue without malignancy from patients with colorectal carcinoma, our data demonstrate *per2* mRNA deregulation in tumor tissue, and suggest a way in which the circadian system can influence tumorigenesis.

Introduction

Alteration of the natural 24-h light/dark cycle and subsequent disruption of the circadian rhythms is involved in increased susceptibility to different kinds of cancer in humans. Epidemiological studies have demonstrated an increased risk of colorectal (1) and breast (2-4) cancer in women exposed to shift work over prolonged periods of time. It is expected that disturbed biological rhythms, resulting from prolonged shift work and/or light exposure at night, represent important risk factors for cancer development.

Biological rhythms with a period close to 24 h (circadian rhythms) are efficiently orchestrated by the circadian timing system, consisting of the central and peripheral oscillators. The central oscillator is localized in the suprachiasmatic nucleus (SCN) of the hypothalamus, while peripheral oscillators are present in most of the cells of the body (5). The central oscillator governs the peripheral oscillators; however, in some circumstances the peripheral oscillators can become disconnected from the SCN and run independently (6-8). The generation of biological rhythms is based on the molecular oscillations of clock genes, which create interacting positive and negative transcriptional feedback loops. Several clock genes have been identified in mammals. Of these, the *per* genes (*per1*, *per2*, *per3*) play a key role as negative regulators (9). PER proteins create heterodimers with the protein products of *cry* genes and, in this form, are translocated to the nucleus. In the nucleus, PER:CRY interacts with another heterodimer created by transcriptional factors, BMAL:CLOCK. BMAL:CLOCK (or its functional homologue) stimulates *per* and *cry* gene expression via the E-box in their regulatory regions, while the PER:CRY complex inhibits this process. When the transcription and subsequent translation of *per* and *cry* genes is efficiently down-regulated, the inhibition of BMAL:CLOCK is lifted due to the absence of the PER:CRY heterodimer. The transcription of the *per* and *cry* genes is then restarted. This chain of events takes place over a period close to 24 h, and determines circadian oscillator function (5).

Interconnectivity between the clock function and the cell cycle is presumably mediated by clock controlled genes and transcriptional factors, since disruption of the clock function has a significant impact on cell division (10-12). Hyperplasia can either result from a greater proliferative rate or from a lower rate of apoptosis. PER proteins seem to be involved in both processes. Mice deficient in the *per2* gene show higher sensitivity to ionizing radiation and a higher incidence of spontaneous cancer in comparison with wild-type mice (10). The E-box mediated mechanism participates in the activation of the Wee1 kinase, which negatively controls cyclin-dependent kinase 1 activity and the G2-M transition of cell division (11). Another known functional connection between the circadian clock and cell proliferation involves the proto-oncogene *c-myc*, which plays a role in the regulation of several cell cycle transitions. Expression of the *c-myc* gene was found up-regulated in the liver of *per2* gene deficient mice and, via the E-box mediated mechanism, basic clock components were able to influence *c-myc* expression (10).

Deregulated *per* gene expression has been observed in mice following tumor transplantation (13). Exposure of the animals to an irregular light/dark regime resulted in further

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disturbance of clock gene expression (14,15). Up-regulation of PER protein leads to the suppression of tumor growth (16-20), thus implicating PER in cell cycle regulation.

Expression of clock genes has previously been demonstrated in human healthy and cancerous tissue of the gastrointestinal tract (21,22). We hypothesize that decreased expression of the *per2* gene may contribute to colorectal cancer development. The aim of this study was therefore to determine the expression of the *per2* gene in colonic tumors and in the distal and proximal parts of resected tissue that exhibited no signs of malignancy. We correlated *per2* gene expression with tumor localization and stage and with mutations in the human proto-oncogene *K-ras,* which is frequently mutated in colorectal carcinoma and contributes to the early developmental stages of this cancer.

Materials and methods

The study comprised 25 patients of both genders (age 71.88± 1.47, range 59-85 years) with colonic cancer. Patients were exposed to light from 6:00 a.m. to 9:00 p.m. according to standard hospital practice (First Surgery Department, Medical Faculty and Hospital, Comenius University, Bratislava). The protocol was explained to each patient and informed consent was obtained.

Histopathological examinations were performed by a hospital pathologist. Tissue samples were taken during surgery, collected from the tumor as well as from the proximal (≥10 cm from the tumor) and distal (≥2 cm from the tumor) areas of the resected gut. Surgery was carried out between 10.00 a.m. and 1.00 p.m. The removed samples were collected in liquid nitrogen and stored at -80˚C until use for RNA extraction.

Gene expression. Total-RNA was isolated from the tissue using Tri-reagent (MRC, USA) according to the manufacturer's instructions. Synthesis of cDNA was performed with ImProm-II Reverse Transcription System (Promega, USA) according to the manufacturer's instructions.

Quantification of cDNA was performed by real-time PCR with the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) and the ABI PRISM®7900HT Sequence Detection System (Applied Biosystems, USA). The primer pairs used for the amplification of each product were: *per2* (GenBank accession no. NM022817.1) sense 5'-AATGCCGATATGTTTGC GGT-3', antisense 5'-GCATCGCTGAAGGCATCTCT-3'; *mrpl19* (GenBank accession no. NM014763.3) sense 5'-GGG ATTTGCATTCAGAGATCAG-3', antisense 5'-GGAAGG GCATCTCGTAAG-3' (23). Real-time PCR conditions were: activation of hot start polymerase at 95˚C for 15 min followed by 50 cycles at 94˚C for 15 sec, 49˚C for 30 sec and 72˚C for 30 sec. Amounts of reverse transcription product were 1.6 and 0.8 μl for *per2* and *mrpl19*, respectively. The specificity and identity of the PCR products were validated by melting curve analysis and gel electrophoresis. The fluorescence dye ROX served as an internal reference for normalization of the SYBR Green I fluorescent signal. Samples to be compared were assayed using the same mastermix on one plate.

Detection of K-ras mutation in codons 12 and 13. Frozen tissue (65-75 mg) was dissected using a sterile scalpel into 1-2 mm sections. Samples were incubated overnight at room temperature in 0.5 ml DNAzol (MRC) with 50 μg proteinase K (Fermentas, Canada). The next day, DNA was isolated with DNAzol according to the manufacturer's instructions. Samples were dissolved in 0.8 mM NaOH. DNA was amplified by mutagenic PCR assay (24). A mismatched upstream primer for codon 12 and downstream primer for codon 13 were used to introduce the *Mva*I and *Bsu*RI restriction sites, respectively. The primer pairs used for amplification were: K-ras codon 12 sense 5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3', antisense 5'-CTGTATCAAAGAATGGTCCTGCACCA GTA-3'; K-ras codon 13 sense 5'-GTACTGGTGGAGTAT TTGATAGTGTATTAA-3', antisense 5'-GTATCGTCAAG GCACTCTTGCCTAGG-3' (the underlined bases represent mismatches).

The PCR reaction (50 μl) contained: 200-350 ng DNA, 1X reaction buffer with KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 μM specific primers and 1 U Taq DNA polymerase (Fermentas). PCR conditions for K-ras codon 12 were: initial denaturation at 95˚C for 5 min followed by 35 cycles at 94˚C for 40 sec, 60˚C for 30 sec and 72˚C for 30 sec. For K-ras codon 13, the annelation step was performed at 50˚C and the final extension step lasted 3 min. The size of the PCR products was validated by electrophoresis on 2% (w/v) agarose gel.

Amplified samples (10 μl) were digested with 5 U *Mva*I (Fermentas) for K-ras codon 12 and 5 U *Bsu*RI (Fermentas) for K-ras codon 13 at 37˚C overnight. Restriction fragments were separated by electrophoresis performed in 3.5% (w/v) MetaPhor agarose (Cambrex, USA) in Trisborate-EDTA buffer. The product was visualized by UV transillumination following ethidium bromide staining.

Statistical evaluation. Differences in *per2* gene expression were compared using the unpaired Student's t-test or ANOVA, depending on the number of groups to be compared. The Spearman rank correlation coefficient test was used to reveal the relationship between *per2* gene expression and tumor staging.

Results

Patients were divided into three groups according to cancer staging (Table I). The first group comprised patients with primary tumors confined to the gut with no lymph node or distant metastases ($T_{1-3}N_0M_0$; n=16); the second group comprised patients with lymph node but without distant metastases ($T_{2-3}N_1M_0$; n=5), and the third group comprised patients with distant metastases ($T_{3-4}N_{1-2}M_1$; n=4).

Relative values of *per2* gene expression in tumor tissue (Fig. 1) show a significant negative correlation between *per2* gene expression and tumor staging ($P<0.05$; $R=0.44$). Tumor localization in patients is described in Table I. No significant difference between *per2* gene expression in the colon and rectum was found (Fig. 2).

To analyze *per2* mRNA expression in cancer and adjacent tissues in relation to tumor staging, patients were divided into three groups (Fig. 3, Table I). The relative expression of the *per2* gene was evaluated as the ratio of expression in cancer tissue to expression in the adjacent part of the colon. In patients with no distant metastases, the ratio of cancer/adjacent tissue

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umor distribution.

	No.	Women	Men	Ascendent colon	Descendent colon	Rectum
$T_{1-3}N_0M_0$	16	6	10	9	0	7
$T_{2-3}N_1M_0$	5	2	3	3	1	1
$T_{3-4}N_{1-2}M_1$	4	3	1	2	2	0
Total	25	11	14	14	3	8

$T_{1-3}N_0M_0$, patients with primary tumors confined to gut, with no lymph node or distant metastases; $T_{2-3}N_1M_0$, patients with lymph node metastases but with no distant metastases; $T_{3-4}N_{1-2}M_1$, patients with distant metastases.

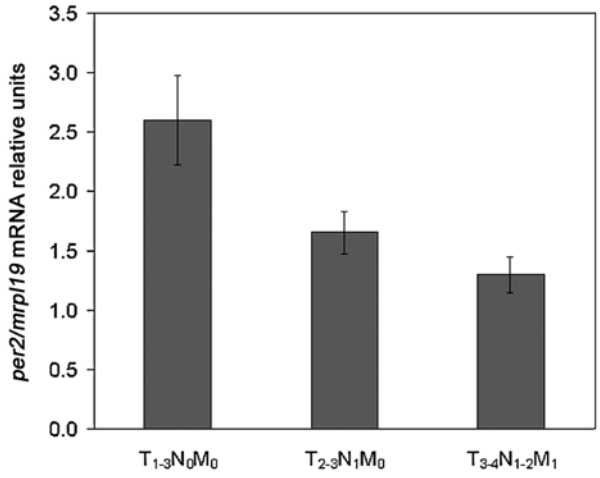

Figure 1. Correlation between *per2* mRNA expression in cancer tissue and tumor staging. $T_{1-3}N_0M_0$, patients with primary tumors confined to gut, with no lymph node or distant metastases; $T_{2-3}N_1M_0$, patients with lymph node metastases but with no distant metastases; $T_{3-4}N_{1-2}M_1$, patients with distant metastases. Values are the means ± SEM.

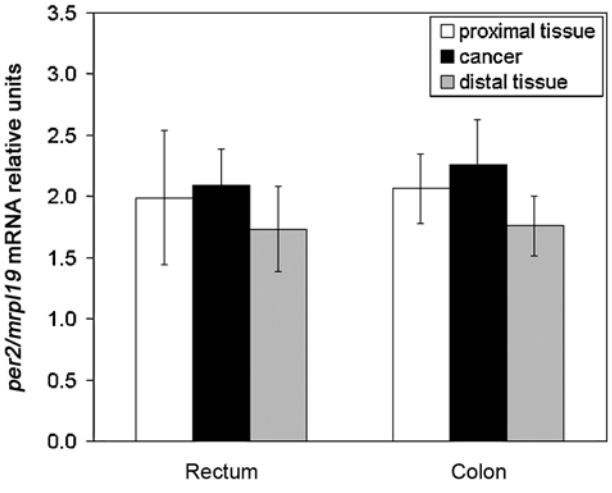

Figure 2. Expression of *per2* in the tumor, proximal and distal areas of the resected rectum and colon. Values are the means ± SEM.

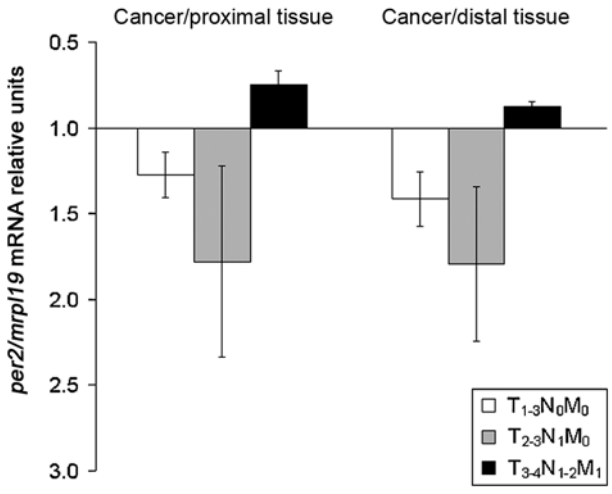

Figure 3. Ratio of *per2* mRNA expression in cancer and adjacent proximal or distal tissue. Values are the means ± SEM. See Fig. 1 for tumor staging.

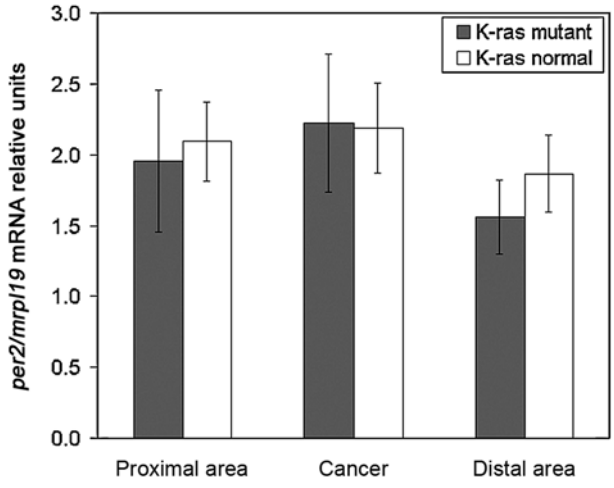

Figure 4. Expression of *per2* in the tumor, proximal and distal areas of the resected tissue in relation to the presence of K-ras mutation in codon 12 (n=10) and K-ras non-mutated codon 12 (n=15). Values are the means ± SEM.

was >1, implying lower *per2* gene expression in adjacent tissue than in tumor tissue. In patients with distant metastases, the above-mentioned ratio was <1, indicating lower *per2* gene expression in tumor tissue.

K-ras mutations in codons 12 and 13 were detected in the cancer tissues (Fig. 4). The size of the PCR product was, as expected, 162 and 159 bp for codons 12 and 13, respectively. *Mva*I digestion of the wild-type *K-ras* codon 12 allele generated two bands of 133 and 29 bp, while mutant type remained intact (162 bp). Wild-type *K-ras* codon 13 PCR

Table II. *K-ras* mutation frequency in cancer tissue depending on tumor stage.

		K-ras codon 12 (%)		K-ras codon 13 (%)	
	No.	Mutant	Normal	Mutant	Normal
$T_{1-3}N_0M_0$	16	6 (37.5)	10 (62.5)	0 (0.0)	16 (100)
$T_{2-3}N_1M_0$	5	3 (60.0)	2 (40.0)	1 (20.0)	4 (80.0)
$T_{3-4}N_{1-2}M_1$	4	1 (25.0)	3 (75.0)	0 (0.0)	4 (100)
Total	25	10 (40.0)	15 (60.0)	1 (4.0)	24 (96.0)

$T_{1-3}N_0M_0$, patients with primary tumors confined to the gut with no lymph node or distant metastases; $T_{2-3}N_1M_0$, patients with lymph node metastases but with no distant metastases; $T_{3-4}N_{1-2}M_1$, patients with distant metastases.

product was cleaved by *Bsu*RI into three fragments of 85, 48 and 26 bp, while the mutant allele produced only two fragments of 85 and 74 bp. The average percentage of patients positive for codon 12 and 13 mutation was 40 and 4%, respectively. Detailed analysis is presented in Table II. There was no significant difference in *per2* expression in patients positive for *K-ras* codon 12 mutation in cancer tissue and those without *K-ras* codon 12 mutation.

Discussion

Recent studies using *per2* gene deficient mice (10) have suggested that complex interactions between PER, several known regulators of the cell cycle and apoptosis may contribute to tumor development.

In the present study, we demonstrate for the first time the existence of a significant correlation between *per2* gene expression and tumor progress classified by the TNM staging system. A significant correlation between tumor stage and *per2* gene expression implicated the deregulation of *per2* gene expression in tumorigenesis, especially in patients with distant metastases. In these patients, *per2* gene expression was decreased in the tumor in comparison with the adjacent part of the resected gut.

Similar results were previously obtained when *per1* gene expression was measured in patients diagnosed with colon cancer (22). Several reports demonstrated deregulated expression of PER proteins in tumor tissue in comparison with the adjacent tissue of other kinds of cancer. Immunohistological analysis revealed deregulation of at least one of three PER proteins in 53 of 55 cases of breast cancer (25), and down-regulated *per1* gene expression in non-small cell lung cancer (18) and endometrial carcinoma (26). Decreased levels of *per1* gene expression have also been observed when comparing breast cancer tumor-positive and -negative patients (18).

High variability in *per* gene expression is typical in most tumor cases, and may reflect different tumor staging and grading processes, or different treatment of patients. Our results generally agree with the data indicating the deregulation of *per* gene expression in tumor compared to adjacent tissue. However, decreased expression of this gene was only observed in patients with distant metastases. It must be taken into consideration that colorectal tumors are exposed not only to systemic changes, but also to a variety of signals (molecules) coming from food and bacterial microflora, to which *per2* gene expression has previously been shown to be sensitive (27,28).

Pathways mediating *per2* gene interference with tumor development may include interaction with the proto-oncogene *c-myc*, which is involved in cell proliferation, differentiation and apoptosis. Overexpression of the *c-myc* gene has been detected in several types of human cancer, including colorectal carcinoma (29,30). Expression of *c-myc* is under circadian control (10,31), and rhythmic control of the expression of this gene has been found to be disrupted in *per2* gene mutant mice (10). Another possibility is that PER2 interacts with kinase Wee1, which regulates the activity of the cyclin-dependent kinase 1/cyclin B1 complex and can be governed by the circadian clock via the E-box (10).

It is not clear, however, whether the rhythm of PER protein, its absolute levels or both are crucial to the effect of PER2 on tumor development and growth. Timing of drug administration, sampling time and gender may all influence the effectiveness and assessment of treatment (32). *In vivo* studies have proven that, in order to suppress tumor growth, the capacity of the cyclin-dependent kinase inhibitor Seliciclib must be phase dependent. Seliciclib was most efficient when administered at a time corresponding to the time at which treatment established the proper phase relationship of the oscillating clock genes (13). Several experiments employing cell lines have demonstrated that increased *per* gene expression leads to the suppression of growth of the transformed cells (17-19), and *per2* has been suggested as a novel therapeutic strategy for the treatment of malignant tumors (20,33).

Colorectal cancer development is frequently related to the mutation of another proto-oncogen, *K-ras* (34). *K-ras* is a member of a highly conserved family of GTPases, including H-ras and N-ras, which encodes the 21-kD protein. The ras-proteins are molecular switches that bind to GTP in their active state. Normally, this binding process is strictly controlled and depends on interactions between receptor tyrosine kinases and a network of intracellular proteins. Oncogenic forms of ras-proteins are permanently activated by bound GTP. This is caused by mutations in the nucleotide binding site of these proteins and results in the prevention of the hydrolysis of GTP into GDP (35, 36). Missense mutations in codons 12 and 13 of exon 1 of the *K-ras* gene are found in 30-60% of colorectal tumors (37-39). The mutation frequency of *K-ras* in codon 12 in our cohort of patients was 40%, which corresponds with the published data (37-39). We did not find any significant correlation between mutations in exon 1 of *K-ras* and expression of the *per2* gene in the colorectal tumors analyzed.

In conclusion, our data suggest that tumor staging correlates more closely with *per2* gene expression than tumor location does in colorectal carcinoma. This finding may suggest a specific function for the *per2* gene in the process of tumorigenesis. We suggest that a proper relationship between the rhythmic expression of clock components and their absolute values may play a role in cancer development, and that their deficit or absence may negatively influence the progress of the disease.

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