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The expression of clock genes *cry1* and *cry2* in human colorectal cancer and tumor adjacent tissues correlates differently dependent on tumor location

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Colorectal cancer (CRC) exhibits differences in its features depending on the location of the tumor. The role of the circadian system in carcinogenesis is accepted, and many studies report different clock gene expression in tumors compared to healthy tissue. However, little attention is given to the changes in clock genes in tumors arising from various locations across the colon and rectum. The aim of our study was to investigate the expression of the clock genes *cry1* and *cry2* in human CRC tissue and tissue adjacent to colorectal tumors in a cohort of 64 patients by real time PCR. Expression of *cry1* in the entire patient cohort was higher in tumors compared to adjacent tissues in the right-sided colon but not in the left-sided colorectum. Difference in *cry1* expression between tumor and adjacent tissue in the right-sided colon was preserved in women and a trend was observed in men. Higher expression of *cry1* in the right-sided colon tumor tissue was associated with worse survival in women and the expression of *cry1* in the left-sided colorectum was significantly higher in the adjacent tissue compared to tumor in men but not in women. Expression of *cry2* was lower in the tumor than in adjacent tissue in both the right and left-sided colorectum. This trend was generally preserved, but the difference reached significance level only in the male left-sided colon, and *cry2* expression in the tumor tissue significantly correlated with location of the tumor in men with grade 2 cancer. Finally, we detected significant correlation between tumor location and *cry1* expression in the adjacent tissue and the combined results establish that tumor influence on adjacent tissue is dependent on tumor location. Changed clock gene expression should therefore be considered in specific CRC patient sub-groups.

Key words: circadian, cryptochrome, grade, gender, left-sided colorectum, right-sided colon

The circadian rhythm is an endogenous timekeeping mechanism comprising autonomous peripheral oscillators that are coordinated by a master oscillator located in the supra-chiasmatic nucleus (SCN) of the hypothalamus. The SCN generates 24 hour circadian rhythms in physiology, metabolism, and behavior. The molecular principle of this rhythm generation is based on the oscillations of clock genes that form a transcription-translation feedback loop. The positive arm of the feedback loop is formed by BMAL1 and CLOCK which belong to the family of basic helix-loop-helix transcription factors that heterodimerize, and through binding to the E-box element in the promoter region they activate the transcription of the negative elements of the loop *period* (*per1–3*) and *cryptochrome* (*cry1* and *2*) genes. CRY and PER proteins continuously accumulate in the cytoplasm and after translocation to the nucleus, they repress their own transcription [1]. Degradation of CRY and PER proteins is required for the end of repression and start of the new cycle. The process described above takes approximately 24 hours and determines the circadian period [2].

The link between the circadian system and colorectal cancer (CRC) is supported by experiments employing *per2* deficient mice. γ-irradiation accelerated tumor growth and decreased levels of apoptosis in thymocytes in *per2*$^{-/-}$ mice [3]. The circadian rhythm in expression of *per1*, *per2, dbp,* and *reverb alpha* is reduced, and the rhythm of *bmal1* is completely abolished in mice with experimentally induced CRC compared to normal colonic tissue [4]. In contrast, *cry1*$^{-/-}$ *cry2*$^{-/-}$ double knock-out or *clock*$^{-/-}$ mice are not more sensitive to γ-irradiation, and these mutants are indistinguishable from the wild-type mice [5, 6]. However, when a

mutation of *cry* genes was combined with mutation of *p53,* these mice were resistant against the early onset of cancer and had their lifespan extended approximately by 50% compared with mice solely with *p53* mutation [7]. Difference was also found in the rate of apoptosis. Ultraviolet (UV) and oxaliplatin induced apoptosis was enhanced in Ras-transformed cells isolated from *cry1/cry2/p53* homozygous mutants compared to $p53^{-/-}$ Ras-transformed cells [8].

The circadian system is also involved in DNA repair; in particular, the circadian system influences excision repair which in humans is performed by six core repair factors. One of the repair factors is *xeroderma pigmentosum* (XPA) which is directly controlled by the circadian feedback loop and exerts a daily oscillation. In the liver of $cry1/2^{-/-}$ double mutant mice, XPA is constitutively over-expressed and its daily rhythm is abolished. This change in XPA activity influences excision repair activity only marginally [9, 10].

In our study, we focused on the location of colorectal tumors. The colon exerts molecular, anatomical and bacterial changes in the proximal and distal tract and rectum, so it is not surprising that carcinomas arising from different parts of the colon show this diversity [11]. An increased immune activity in the caecum of the proximal colon in comparison to the rectum was observed in healthy probands [12]. Differences in the colon's immune system can arise from concentrations of the microbiota in the gut, which increases from proximal to distal colorectum [13]. Proximal or right-sided tumors occur more often in older patients and females and have a higher TNM stage at presentation than distal or left-sided carcinomas. In contrast to right-sided CRC, the incidence of left-sided tumors is decreasing. Epidemiological data indicates that patients with distal tumors have better overall survival. Genetic and epigenetic causes of CRC exert differences dependent on tumor location. Microsatellite instability and methylation of mismatch repair genes are typical for proximal tumors, while chromosomal instability with inactivation/deletion of tumor suppressor genes is more characteristic for distal tumors [14].

Herein, we analyze the expression pattern of *cry1* and *cry2* genes in CRC and tissue adjacent to tumors and focus on tumor location.

Patients and methods

The study involved 64 patients of both genders with previously diagnosed colorectal cancer (CRC – 38 men, 26 women) with average age 69±12 years. All patients were exposed to standard hospital practice with lights on from 6:00 a.m. to 9:00 p.m. (The First and the Fourth Surgery Departments, University Hospital, Comenius University, Bratislava). The protocol was explained and informed consent was obtained from all participants. The experimental protocol was approved by the Ethics Committee. Histopathological examinations were performed by a hospital pathologist and tissue samples were collected from the tumor and the proximal (≥10 cm above the tumor) and distal (≥2 cm below the tumor) parts of the resected colon. The surgery was conducted between 10:00 a.m. and 1:00 p.m. Tissue samples were placed in liquid nitrogen and stored at –80 °C until further processing. A detailed description of the patient cohort in this study is in Table 1.

Gene expression analysis. Total RNA was isolated from the tissue samples using RNAzol reagent (MRC, USA) according to the manufacturer´s instructions. cDNA was synthesized with the ImProm-II Reverse Transcription System (Promega, USA)II according to manufacturer´s instructions. Relative quantification of *cry1* and *cry2* was performed with the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) and the StepOne Real-Time PCR Systems (Applied Biosystems, USA). Primers for the detection of *cry1*, *cry2,* and *U6* were: *cry1* (NM_004075.4) sense 5'-CCGTCTGTTTGTGATTCGTG-3´, antisense 5´-AAGTTAGAGGCGGTTGTCCA-3´; *cry2* (NM_001127457.2) sense 5´-GGAGGCTGGTGTGGAAGTAG-3´, antisense 5´-CGTAGGTCTCGTCGTGGTTC-3´; and *U6* (NR_004394.1) sense 5´-GCTTCGGCAGCACATATACTAA-3´, antisense 5´-AAAATATGGAACGCTTCACGA-3´. Real-time PCR conditions were: activation of hot-start polymerase at 95 °C for 15 min followed by 50 cycles at 94 °C for 15s, 49 °C for 30s (52 °C for *U6*), and 72 °C for 30s. The specificity of PCR products was validated by melting curve analysis and *U6* expression was used for normalization of clock gene expression.

To evaluate gene expression in tumor and adjacent tissue (Figure 1) and survival (Figure 2), sub-sites were categorized in the right-sided colon cancer (C18.0-C18.4) and left-sided colorectal cancer (C18.5-C20).

To perform regression analysis (Figure 3), the tumor locations (C18, C19, C20) were transformed into numerical

Table 1. Clinical and pathological characteristics of patients.

Number of patients	64
Men	38
Women	26
Tumor location	
C18	36
C19	10
C20	18
Clinical stage	
I–II	32
III–IV	32
Grade	
1	12
2	49
3	3

C18 = tumors located in colon, C19 = tumors located in rectosigmoid colon, C20 = tumors located in rectum

data. Transformation was done by using an average distance of each section (colon, recto-sigmoid junction and rectum) to anus obtained from computed tomography colonography [15]. This approach has been accepted previously [16].

Statistical analysis. Paired t-test compared expression in the tumor and adjacent tissues in the right and left-sided colorectum. Survival curves were compared using the Gehan-Wilcoxon test and linear regression analysis determined the correlation between *cry1* and *cry2* gene expression and tumor location.

Results

The expression of *cry1* was significantly up-regulated in tumor tissue compared to adjacent tissue in right-sided colon tumors ($p<0.01$, paired t-test). This was not observed in left-sided colorectal tumors. Expression of *cry2* was significantly lower in tumor tissue compared to adjacent tissue in both right and left-sided colorectal tumors, and more robust down-regulation was observed in the left-sided colorectum ($p<0.001$, paired t-test, Figure 1A).

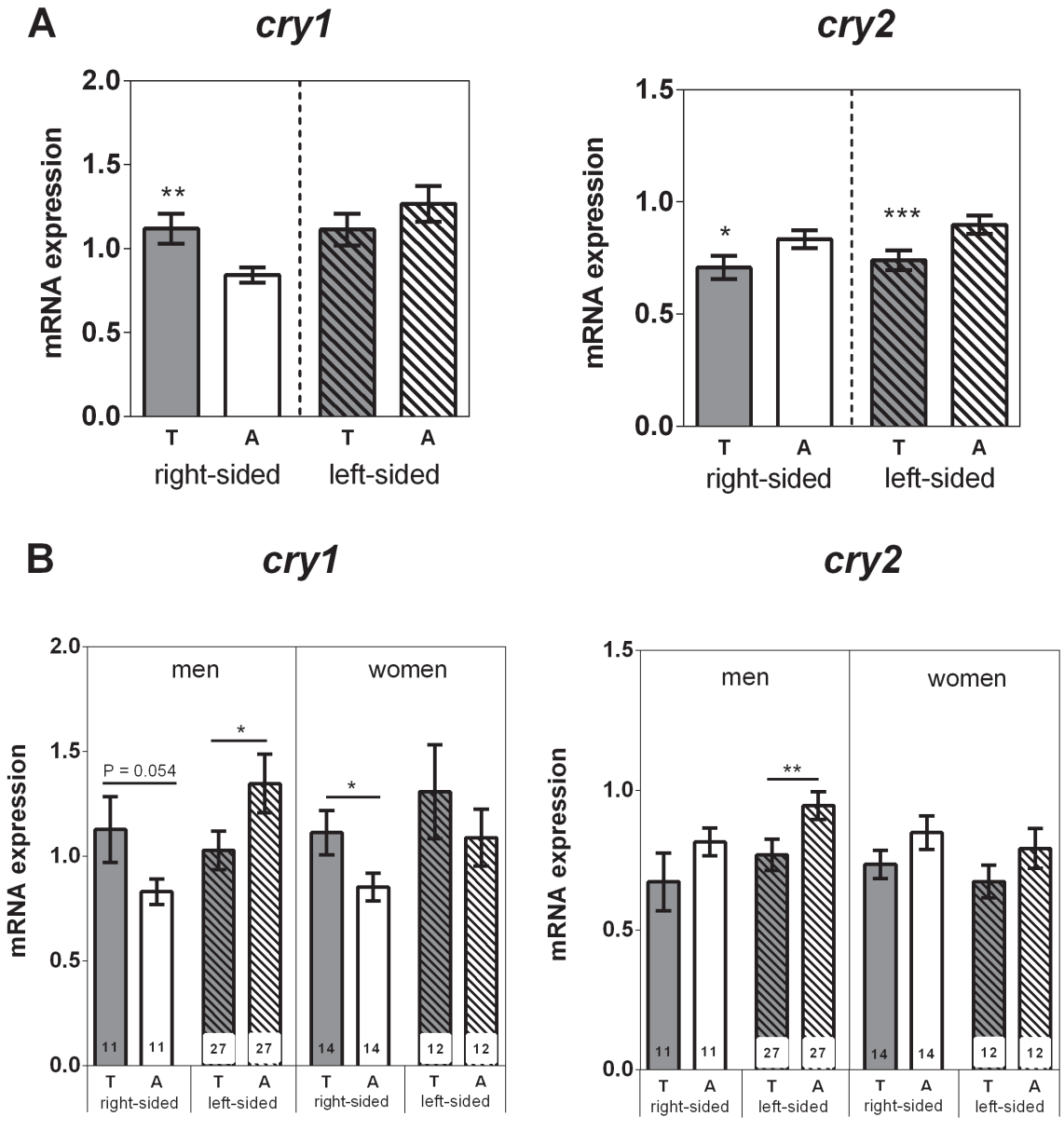

Figure 1. Expression of *cry1* and *cry2* in tumor and adjacent tissue. A) in the whole cohort of patients divided according to tumor location into right-sided tumors (C18.0-C18.4, n=25) and left-sided tumors (C18.5-C20, n=39). B) Sex-dependent expression of *cry1* and *cry2* in tumor and adjacent tissue in right-sided tumors and left-sided tumors. Numbers in columns indicate sub-group size. T – tumor tissue, A – adjacent tissue (averaged expression in the proximal and distal tissue resected during surgery); values are presented as means ± SEM; *p<0.05; **p<0.01; paired t-test.

After dividing the cohort according to gender, we detected significantly lower levels of *cry1* expression in tumor tissue compared to adjacent tissue in left-sided male tumors, but the opposite trend was observed in right-sided tumors (p=0.054, paired t-test). In women, expression of *cry1* was up-regulated in tumor tissue compared to adjacent tissue in the right-sided colon (p<0.05, paired t-test) but this was not observed in the left-sided colorectum (Figure 1B).

Expression of *cry2* was significantly lower in tumors located in the left-sided colorectum compared to adjacent tissue in men (p<0.01, paired t-test). Tumors located in the right-sided colon of men and women and left-sided colorectum of women showed the same pattern, but the difference did not reach significance (Figure 1B).

Higher expression of *cry1* in the right-sided colon tumor tissue was significantly associated with worse survival in women (p=0.045, Gehan-Wilcoxon test; Figure 2A). This association was not observed in the female cohort with tumors in left-sided colorectum (Figure 2B). In contrast, male patients did not exhibit correlation between survival and *cry* expression in right-sided or left-sided tumors (data not shown).

The cluster of male patients with grade 2 tumor (G2, moderately differentiated) exhibited significant correlation between *cry2* expression in tumor tissue and location (p=0.045; y=–56.79*x + 99.72; R=0.376; n=29; Figure 3A). This was also present in adjacent tissue (p=0.009; y=–90.58*x + 138.7; R=0.474; n=29; Figure 3B). These correlations were not observed in the women's cohort (Figure 3). There was significant correlation between *cry1* expression in adjacent tissue and tumor location in men (p=0.003; y=–36.50*x + 100.9; R=0.532; n=29) and also in women (p=0.016; y=–56.66*x + 125.1, R=0.530; n=20; Figure 3B). This correlation was not observed in the tumor tissue (Figure 3A).

Discussion

Our study revealed significant changes in *cry1* expression dependent on gender and tumor location. Expression of *cry1* was higher in tumors compared to adjacent tissue in the right-sided colon but not in the left-sided colorectum in the entire patient cohort. Difference in *cry1* expression between tumor and adjacent tissue in the right-sided colon was preserved in women, and a trend was observed in men. Higher expression of *cry1* in the right-sided colon tumor tissue was associated with worse survival in women. Expression of *cry1* in the left-sided colorectum was significantly higher in the adjacent tissue compared to tumor in men but not in women. We also detected significant correlation between tumor location and *cry1* expression in the adjacent tissue in both sub-groups of patients. These results establish that the tumor influence on adjacent tissue depends on tumor location.

Recent knowledge of CRC induced changes in *cry1* expression is inconclusive [17–19]. It is possible that changes in *cry1* expression may be linked to certain clinical and pathological features because reduced levels of *cry1* expression were observed in females, older patients (62–74 years) and in tumors located in the transverse colon [18]. Another study reports the highest expression of *cry1* in tumors located in the distal colon compared to other colon parts [17]. This supports our results, since we detected a trend of correlation of *cry1* expression in tumor tissue with tumor location in female patients; with the lowest expression in the proximal colon and the highest in the rectum. The link between *cry* expression and tumor location in individual groups of patients of the cohort may be related to the differences between the morphological and physiological states of right-sided colon and left-sided colorectal cancer tissue [14].

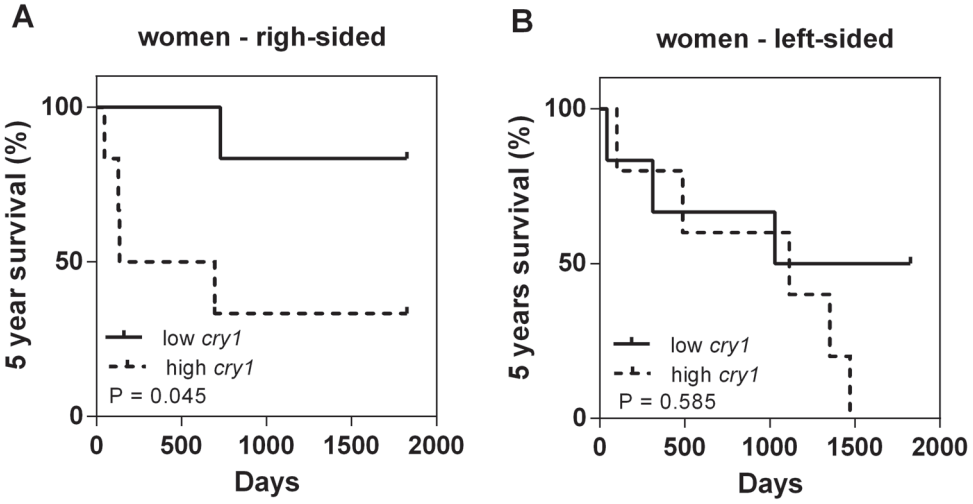

Figure 2. Five-year survival curves for women patients with tumors located in the (A) right-side of colon (n=12) and (B) left-side of colorectum (n=11) sorted according to median of *cry1* expression. Solid line indicates low *cry1* expression (≤ median of expression in the cohort,) and dotted line indicates high *cry1* expression (> median of expression in the cohort). p = level of significance (Gehan-Wilcoxon test).

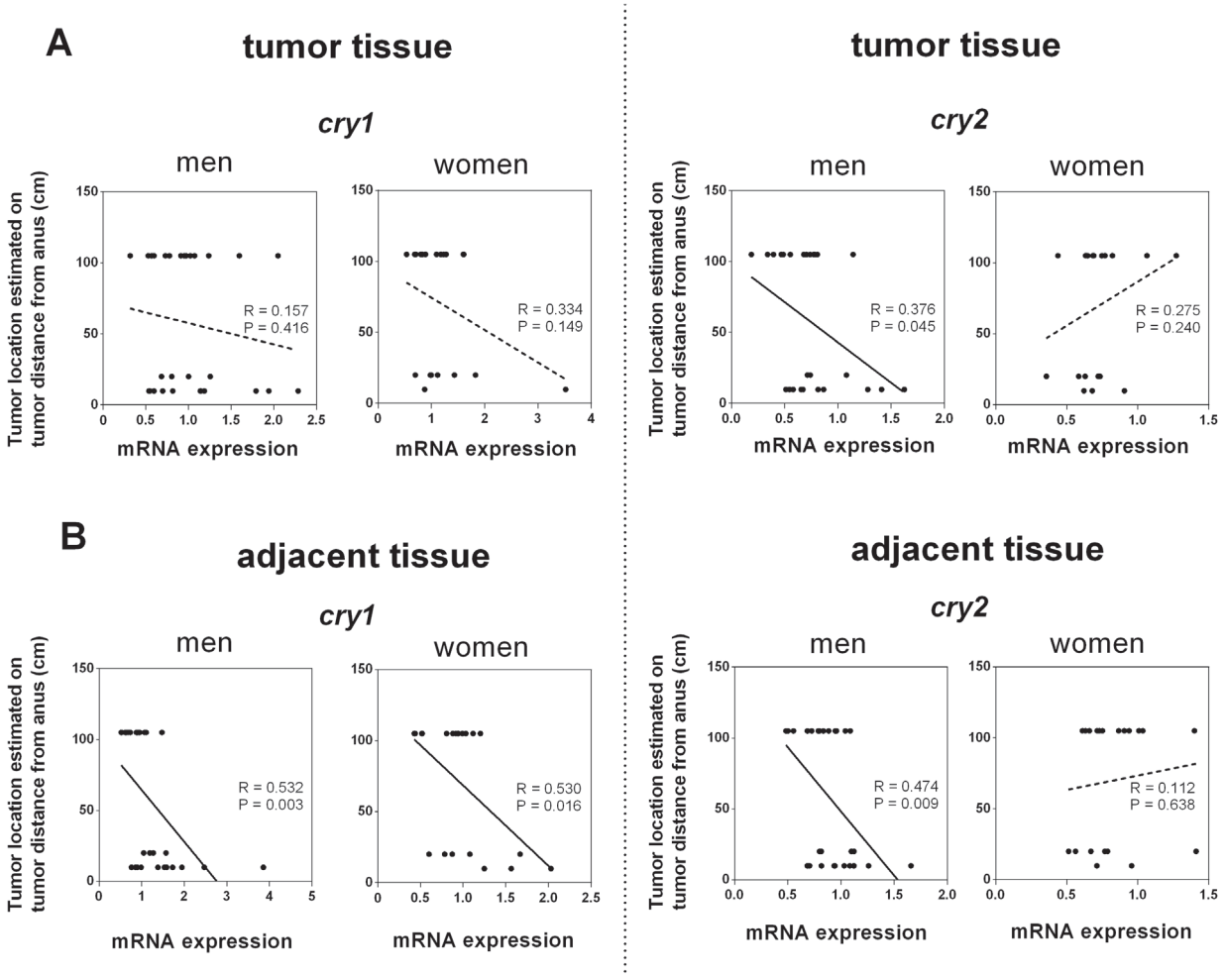

Figure 3. Correlation between tumor location and *cry1* and *cry2* expression in tumor (A) and tumor adjacent tissue (B) in patients with grade 2 (men, n=29; women, n=20). mRNA is given in relative units.

Expression of *cry2* was lower in the tumor than in adjacent tissue in both the right and left-sided colon. This trend was generally preserved in sub-groups of patients, but the difference reached level of significance only in left-sided colorectum in men. There is a substantial data supporting the assumption that expression of *cry2* in colorectal tumor tissue differs in comparison to adjacent healthy mucosa [17, 18]. Expression of *cry2* was down-regulated in colorectal tissue, similar to other genes involved in the circadian feedback loop (i.e., *per1* and *per2*) [17, 18]. This agrees with our results, and also with the significant positive correlation between *cry2* expression in tumor tissue and tumor location observed in male patients with grade 2, as in our original observation.

It is not clear if the above-mentioned gender-dependent differences are directly related to observed epidemiological changes in CRC incidence. It is accepted that proximal tumors (right-sided tumors) occur more often in females and older patients. Chemotherapy is more efficient in male patients with tumors in the proximal colon than in distal tumors, and this is not observed in women [20]. Patients with left-sided tumors benefit from anti-epidermal growth factor receptor (anti-EGFR) therapy more than patients with right-sided tumors [21]. We assume that improved knowledge of the gender, age and location-dependent spectrum of gene expression contributes to explanation of the epidemiological observation stated above.

In addition to clock gene expression, the different status of specific gene mRNA expression involved in the carcinogenesis of CRC was observed in proximal, distal and rectal cancer. Rectal cancer had higher levels of ERCC1 (DNA excision repair protein) and VEGFR (receptor for vascular endothelial growth factor) compared to proximal and distal tumors; and higher expression of thymidylate synthase (TS) compared to distal colon cancers. These findings support the above-mentioned observation that efficacy of anti-tumor treatment such as anti-angiogenic and flouropyrimidine agents may be related to tumor location [22].

Surprisingly, we also observed changes in clock gene expression in tissues adjacent to the tumors and considered healthy. There is no recent data on daily rhythms in clock gene expression in distinct parts of the human colon. Expression of *cry1* and *cry2* genes in murine proximal, middle and distal colon exhibits daily rhythm. In all examined mice gastrointestinal tract tissues, the acrophase of *cry1* expression was observed at the end of the dark part of the light/dark cycle, while *cry2* expression peaked at the beginning of the dark part of the cycle [23]. Rhythmic clock gene expression in the GIT is regulated by both central and peripheral oscillators [24]. Changes in *cry* expression along the colon are partly explained by previously reported differences between the phase of clock gene expression in the upper (duodenum) and lower (distal colon) parts of the gastro-intestinal tract [25].

Tumorigenesis attenuates the daily rhythmic pattern of clock genes in the liver [26] and colon [4]. Therefore, it is possible that changes in daily rhythm of *cry* expression in tumor tissue can influence correlation between *cry* expression and tumor location in CRC tumor tissue.

The functional relationship between changed *cry* expression and cancer promotion is not completely elucidated, but there is substantial epidemiological evidence. Expression of *cry1* significantly positively correlated with lymph node metastasis and the TNM stage of patients [19]. Similarly, patients with higher *cry1* and *cry2* expression had poorer survival rates [18] which accords with the above-mentioned observation.

At the molecular level, tumor promoting properties of *cry* genes have been demonstrated under several experimental conditions. Up-regulation of *cry1* expression promoted cancer cell proliferation and migration, while down-regulation induced inhibition of cancer cell migration [19]. The association of exogenous expression of both *cry1* and *cry2* genes was observed in several CRC cell lines with reduced apoptosis and increased proliferation [18].

The up-regulation or down-regulation mediated effects of *cry* genes on cancer proliferation and apoptosis is usually explained by the connection of the circadian system to cell cycle control. The anti-proliferation factor *wee1* is down-regulated by the clock genes PER and CRY. The daily rhythm of *wee1* expression is abolished and exerts constitutively high levels in *cry* deficient mice [27]. Moreover, deficiency of *cry1/2* protects the *p53* mutant mice against tumor development [7] and down-regulation of *per2* induces the growth of mammary tumors in mice. On the other hand, the up-regulation of *per2* increases levels of the pro-apoptotic protein *bax* and tumor suppressor protein *p53*, while its down-regulation decreases levels of the anti-apoptotic proteins *bcl-2* and *bcl-x* in murine lung and breast carcinoma [28, 29]. The tumor suppressive role of *per2* is implicated also by the significant negative correlation between *per2* expression in CRC and tumor staging [30]. Several genes involved in cell cycle control positively correlated (*hus1*, *gadd45a*, *rb1*, *cdkn2a*, *cdk5rp1*, *mre11a*, and *sumo1*) and some negatively correlated (*cdc20* and *birc5*) with *per2* expression in human CRC tissue cell cycle genes. Expression of these genes also depended on tumor staging [31].

In conclusion, we report pronounced changes in *cry1* and *cry2* expression dependent on gender and tumor location. Expression of *cry1* was higher in tumors than in adjacent tissues in the right-sided colon but not in the left-sided colorectum in the entire patient cohort. This difference was preserved in women and the trend was observed in men. Higher expression of *cry1* in the right-sided colon tumor tissue was associated with worse survival only in women. While the expression of *cry* genes in tumor-adjacent tissue exerted a pronounced trend with increasing values toward the rectum, this trend was preserved only in the male *cry2* expression when tumor tissue was analyzed. We suggest that the differences in *cry* genes expression in tumor and adjacent tissue in specific groups of patients reflect the differences in carcinogenic processes in the right- and left-sided colorectum. Finally, our results emphasize the importance of distinguishing between the right-sided and left-sided origin of the primary tumor in gene expression screening studies.

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