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ORIGINAL ARTICLE

Circadian Rhythm of Methylated Septin 9, Cell-Free DNA Amount and Tumor Markers in Colorectal Cancer Patients

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Abstract To determine the level of cell-free DNA (cfDNA), Septin 9 (*SEPT9)* and tumor markers (CEA, AFP, CA19–9, TPA, CA72–4). Plasma samples were collected four times a day (06:00, 12:00, 18:00, 24:00) from 9 patients with CRC (5 stage I-II, 4 stage III-IV), from one with colorectal adenoma and from one healthy control. CfDNA was isolated, quantified and bisulfite-converted. CfDNA and methylated SEPT9 were determined by RT-PCR. Plasma levels of conventional tumor markers were also measured. The lowest cfDNA concentrations were observed at 24:00 and 18:00 in stage I-III patients. In stage IV samples low cfDNA level (mean 48.2 ng/ml) were observed at several time points (6:00, 12:00, 18:00). The highest cfDNA levels were measured at 6:00 and 12:00 in CRC I-III stages and at 24:00 in stage IV samples (78.65 ng/ml). Higher in-day differences were found in stage II (43–48%) than in stage I samples (22%). Interestingly, the highest *SEPT9* methylation level was found at 24:00 in most CRC cases, in contrast to the cfDNA levels. At 24:00, all cancer and adenoma cases were positive for *SEPT9* methylation. At other time points (6:00, 12:00, 18:00) only 77.7% of CRC samples showed *SEPT9* positivity. Stage I samples were *SEPT9* positive only at 24:00. CEA and CA19–9 levels displayed correlation with the amount of cfDNA in case of late stage cases. Daytime activity can influence *SEPT9* positivity in cases with low concentration of cfDNA. Thus, it may improve screening sensitivity by collecting samples earlier in the morning.

Keywords SEPT9 · Colorectal cancer · Tumor markers · Circadian rhythm · Cell-free DNA

Introduction

Circulating cell-free DNA (cfDNA) based biomarkers are important for cancer screening, prognosis and monitoring the effect of therapies [1]. Detection of cfDNA from plasma or serum can be used as 'liquid biopsy' to provide information regarding physiological and pathological processes in the human body. Cell-free nucleic acid molecules were first observed circulation in 1948 [2]. Subsequently, the elevated concentration of cfDNA in the serum of patients with cancer compared to healthy individuals was described [3, 4]. Several publications dealt with the origin of cfDNA suggesting that only a fraction of circulating DNA is derived from tumor cells [5, 6]. CfDNA presumably originates from necrotic, apoptotic or phagocytosed cells, even in the case of cancer patients [5].

Physical activity may also contribute to an increase in the concentration of cfDNA in the blood [6, 7]. For example, elevated concentrations of cfDNA were associated with the mechanical and metabolic muscular damage resulting from physical activity, and this was attributed to oxidative stress [6–8]. Other types of non-cancer-related cfDNA release mechanisms such as NETosis (pathogen-induced cell death including the release of neutrophil extracellular traps [NETs]) can be mediated by activated immune-competent cells [9]. During pregnancy cell-free fetal DNA enters the maternal circulation due to placental apoptosis [10]. Increased cell-free fetal DNA was observed with abnormal

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placental development [11] or other complications such as preeclampsia [12].

DNase activity is also increased during physical activity and influences the cfDNA amount and the detection of disease-related biomarkers [13].

The mammalian circadian rhythm is controlled by a central 'clock' in the hypothalamic suprachiasmatic nucleus (SCN) which regulates multiple peripheral mechanisms such as metabolic homeostasis, immune response, cell proliferation, apoptosis, response to DNA damage and tumor suppression [14, 15]. Periodic changes in the number of white blood cells including monocytes, natural killer cells, and T and B lymphocytes follow a circadian rhythm [16–19]. The core circadian 'clock' regulates the nuclear transport and transcriptional activation through positive (*BMAL-1, CLOCK*) or negative (*PER1, PER2, PER3, CRY1* and *CRY2*) feedback of clock proteins [20, 21]. In bone marrow and gut epithelium the clock-controlled genes mainly play a role in proliferation. However in liver, effects are energetic and metabolic [22, 23].

The core clock SCN is also relevant in cancer progression with the circadian control of activities such as cell growth, proliferation and apoptosis [24]. Thus, for example, upset of circadian control causes elevated risk for breast cancer among women workers with rotating night shifts [25]. In addition, breast tumor progression was observed with disruption of circadian rhythm in patients with breast cancer [26].

Circadian rhythm of carcinoembryotic antigen (CEA) and alpha-fetoprotein (AFP) tumor marker levels were measured in patients with and without cancer and a circadian activity was detected. However the diurnal peak rhythmicity disappeared in cancer cases compared with non-cancer controls [27]. *TIMP-1*, a promising early detection marker for CRC showed limited circadian variation when its level was measured 7 times within 24 h [28].

Methylated Septin 9 (*SEPT9*) was identified as a candidate peripheral blood-based biomarker of CRC with 48.2–79% sensitivity and 78–98% specificity [29–37] depending on cancer stage. While showing good general sensitivity for colorectal cancer, for patients with in situ carcinomas and adenomas, its sensitivity is relatively low (30.8% and 88.2%, respectively) [38], despite the high local *SEPT9* methylation in these tissues (100% and 97.1%, respectively) [38]. To date, the circadian variation of total cfDNA and specific cfDNA markers in cancer has not been evaluated yet.

In the present study, we analyzed the circadian rhythm of cell-free DNA amount, methylated *SEPT9* and other traditionally used gastrointestinal tumor markers such as CEA, CA19–9, CA72–4, AFP and TPA. In addition, we compared the circadian status of each marker in early and advanced stages of CRC.

Materials and Methods

Patients Enrolled in the Study

Altogether 1 healthy control, 1 patient with adenoma with low-grade dysplasia (more than 1 cm in diameter and histologically tubular) and 9 patients with CRC (2 stage I, 3 stage II, 1 stage III and 3 stage IV according to the AJCC system) were prospectively enrolled in the study. Peripheral blood samples were taken using 6 ml EDTA tubes (Vacutainer, Becton Dickinson, New Jersey, USA) four times a day at 06:00, 12:00, 18:00 and 24:00. The study design was approved by the local ethics committee and government authorities (Regional and Institutional Committee of Science and Research Ethics; TUKEB Nr: 116/2008). Written informed consent was obtained from all patients. Detailed interviews for medical history and physical examinations were performed. After informed consent, plasma samples were taken from the patients. Exclusion criteria were the following: malabsorption, acute medical conditions, anemia and other malignant diseases (besides colorectal cancer). For detailed clinical and demographic data see Table 1. In the

Table 1 Clinical details of the patients

Patient code	Histological diagnosis	AJCC Stage	Gender	Age (years)
C-01	Tubular adenoma (low-grade)	-	F	60
C-02	Adenocarcinoma	II	F	72
C-03	Normal colon	-	M	65
C-04	Adenocarcinoma	II	F	73
C-05	Adenocarcinoma	III	F	74
C-06	Adenocarcinoma	I	F	79
C-07	Adenocarcinoma	IV	F	88
C-08	Adenocarcinoma	I	M	71
C-09	Adenocarcinoma	IV	M	61
C-10	Adenocarcinoma	II	M	62
C-11	Adenocarcinoma	IV	M	71

F female, *M* male

case of adenoma and tumor samples, histological diagnoses were established by expert pathologists. None of the CRC patients received chemotherapy, radiotherapy, or surgical invention prior to sampling.

DNA Extraction, Quantitative Measurement, Bisulfite Treatment, Quantitative Real-Time PCR

Plasma samples were obtained from 10 ml freshly collected blood samples. The plasma fraction was separated within 30 min of sample collection by two successive centrifugation steps each at 1350 rcf for 12 min and stored at −20° until further use.

3.5 ml of plasma was processed with the Epi *pro*Colon 2.0 Plasma Quick Kit according to the manufacturer's instructions (Epigenomics AG, Berlin, Germany). Bisulfite converted DNA from plasma samples was then analyzed using quantitative real-time PCR. A methylated *SEPT9* specific fluorescent detection probe, bisulfite converted unmethylated sequence specific blocker and primers designed in regions lacking CpG dinucleotides were used for PCR reactions (as provided by the Epi *pro*Colon PCR kit). The assay is a duplex PCR determining methylation status of *SEPT9* and total bisulfite converted DNA by amplification of the beta actin (*ACTB*) gene. Triplicate PCR reactions were performed on a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instruction.

For quantification of the DNA amount, DNA was extracted from 1 ml of plasma samples in parallel by High Pure Viral Nucleic Acid Large Volume Kit (Roche Diagnostics, Basel, Switzerland). For all patients/timepoints the concentration of plasma DNA was measured using a Qubit 1.0 fluorometer (Thermo Fischer Scientific) with High Sensitivity dsDNA reagent (Life Technologies, Carlsbad, USA).

Assessment of Tumor Markers

Quantitative determinations of tumor markers including CEA, AFP, CA19–9, CA72–4 and TPA were performed using in vitro assays in the Central Laboratory of Semmelweis University. The Liaison immunoassay evaluated CEA, CA19–9, AFP and TPA levels (DiaSorin S.p.A., Saluggia, Italy). CA19–9 cancer antigen levels were measured on the Cobas 6000 analyzer based on chemiluminescent immunoassay technology (Roche Diagnostics).

Data Analysis

For the *SEPT9* and *ACTB* duplex PCR assay, validation limits were applied according to the manufacturer's instructions. For *SEPT9* positive cases, *SEPT9* PCR CT (cycle threshold) was less than 50 and *ACTB* PCR CT was less than 33.7, while the *SEPT9* negative samples did not show *SEPT9* PCR CT and the *ACTB* PCR CT was less than 33.7. All of the amplification curves were regularly shaped; otherwise they were excluded as invalid measurements.

SEPT9 methylation was analyzed with 1/3 and 2/3 analysis rules [36]. Using the 1/3 rule, samples were declared as *SEPT9* positive if at least 1 of 3 PCR replicates had a valid SEPT9 curve. In the 2/3 analysis method, samples were called *SEPT9* positive if 2 of the 3 PCR replicates had valid SEPT9 curves.

In case of Qubit measurements, the concentrations of DNA samples (ng/mL) were calculated according to the concentration values given by the Qubit Fluorometer and the fraction of final volume and the volume of samples. Mean cfDNA values and standard deviation were calculated using Excel (Table 2).

The measurement range for tumor markers were various, 0.2–1000 ng/mL, 0.3–1000 U/mL, 0.2–1000 IU/mL, 2–400 U/L and 0.2–300 U/mL for CEA, CA19–9, AFP, TPA and CA72–4, respectively. Serum levels greater than 4.3 ng/mL, 39 U/mL, 13.6 ng/mL, 75 U/L and 8.2 U/mL were considered as the pathological range (elevated tumor markers) for CEA, CA19–9, AFP, TPA and CA72–4, respectively.

Results

Circadian Rhythm of cfDNA Concentrations

The lowest total cfDNA levels were found in normal and adenoma samples with 14.3 ± 1.27 ng/ml and 13.5 ± 0.6 ng/ml mean cfDNA amounts, respectively. Slight alterations were observed through the resting and physical activity times.

In samples from cancer patients, the mean total cfDNA 29.85 ng/ml and a slight circadian rhythm was observed. The highest cfDNA concentration was measured at midnight

Table 2 Mean cfDNA (ng/1 ml plasma) ± SD amounts in 24 h of normal, adenoma and cancer patients

Time (hh:mm)	Normal	Adenoma	Stage I CRC	Stage II CRC	Stage III CRC	Stage IV CRC
0:00	16.18	13.16	11.3 ± 1.88	20.0 ± 8.1	17.93	78.65 ± 88.66
06:00	13.74	12.86	11.14 ± 0.76	40.39 ± 28.05	34.84	48.06 ± 41.8
12:00	13.36	14.22	13.75 ± 0.9	29.42 ± 14.54	21.23	45.92 ± 55.74
18:00	13.89	13.76	10.76 ± 1.01	25.46 ± 10.69	17.98	50.76 ± 63.95

(mean 31.99 ± 31.33 ng/ml) and the lowest at 6 pm (mean 27.05 ± 16.97 ng/ml).

In stage I CRC cases, small differences were observed between the four measurements. The lowest cfDNA amount was 10.76 ± 1.01 ng/ml at 18:00 pm and a highest was 13.75 ± 0.9 ng/ml at noon. For stage II samples, the observed daily variation in total cfDNA concentration were larger (43.8–48.33%) than in stage I (22.05%). This variation increased according to the progression in cancer stages. The highest concentration was 40.39 ± 28.05 ng/ml at 6:00 am in stage II, 34.84 ng/ml at noon in stage III and 78.65 ± 88.66 ng/ml at midnight in stage IV cancers. The lowest cfDNA concentrations were measured in stage II (20 ± 8.1 ng/ml) and stage III (17.98 ng/ml) at midnight. Contrarily, the lowest cfDNA level (45.92 ± 55.7 ng/ml) was detected at noon in stage IV cancer group and the elongation was observed at midnight (Table 2).

Circadian Rhythm of SEPT9 Methylation Levels

In normal control plasma samples, *SEPT9* methylation could not be detected at any of the time points during the 24 h period. In adenoma samples, *SEPT9* positivity was observed only in the resting time of the day (at midnight and at 06:00 am). Similarly to adenoma, in stage I cancer cases, *SEPT9* positivity was detected only at midnight, however at 06:00 am, at noon and at 18:00 pm all stage I plasma samples were *SEPT9* negative. All the other stages showed *SEPT9* positivity at all time points, though the CT values varied. The methylation level of *SEPT9* was higher in the advanced tumors (stage III-IV) compared to the early stages (stage I-II) (Table 3). We observed higher CT values in stage I (39.18–39.63) and stage II (34.99–42.45) compared with stage III (27.33–29.86) stage IV (27.42–31.56) indicating higher *SEPT9* methylation levels in the later stages (Table 3).

Comparing the 1/3 and 2/3 analysis methods, differences were found in stage II cases. One of them showed positivity at all time points with 1/3 rule with the highest methylation level at midnight. However, according to the 2/3 method, this sample has *SEPT9* positivity only at 24:00, similar to stage I plasma samples. In the other stage II samples, all PCR replicates indicated methylation, except for one at 06:00 and one at 12:00. In the advanced tumors, strong *SEPT9* methylation levels were detected in all sampling time and PCR replicates.

Interestingly, the highest *SEPT9* methylation level was found at midnight in most CRC cases, such that using the 1/3 analysis method, at midnight all cancer (100%) cases were *SEPT9* positive. At other points (6:00, 12:00 and 18:00) only 77.7% of CRC samples showed *SEPT9* positivity. According to 2/3 method, the *SEPT9* positivity was the highest (77.7%) also at midnight in all CRC cases and were 66.6% in the other sampling times (Table 4).

Correlation Between Circadian Rhythm of cfDNA and SEPT9 Methylation Levels in Colorectal Cancer Cases

In order to examine the correlation between cfDNA and *SEPT9* methylation levels, CT values of PCR replicates were analyzed. In stage I and stage II cases, no correlation was found between cfDNA and *SEPT9* methylation levels, with $R^2 = 0.04$ and $R^2 = 0.0007$, respectively. Contrarily, a moderate correlation was observed in stage III ($R^2 = 0.59$) and in stage IV ($R^2 = 0.75$) samples. Thus, in resting time (at 24:00 and at 06:00) the cfDNA amounts and the *SEPT9* methylation levels were low, furthermore both parameters were increased during the physically activity period of the day (at 12:00 and at 18:00). In contrast with these findings, in stage IV, high cfDNA and *SEPT9* methylation levels were detected in the resting time, and they decreased during the daytime (Fig. 1).

Circadian Rhythm of Blood-Based Tumor Marker Levels

Each tumor marker showed different concentrations at the time points. The daily variation between stage I and stage IV cancer samples were 9–21% for CEA, 13–23% for CA19–9, 17–22% for AFP, 23–44% for TPA and 6–12% for CA72–4 markers. The highest variance was observed in the early cancer cases (stage I and II) which decreased according to the cancer progression. Elevated CEA levels were measured at every observed time only in stage III and stage IV tumors, however one stage I cancer showed close results to threshold values at every sampling time. Tumor marker CA19–9 values were within the pathologic range in all stage IV cases and interestingly in a stage II cancer, and a slight elevation was found at all analysis times. AFP was found in the normal range in all cancer cases. TPA marker showed incoherent results, since TPA was measured in pathologic range in all cases,

Table 3 Circadian rhythm of the mean SEPT9 CT values in colorectal cancer patients

Time	Stage I	Stage I	Stage II	Stage II	Stage II	Stage III	Stage IV	Stage IV	Stage IV
0:00	39.63	39.18	40.23	36.75	36.87	28.72	27.42	28.54	30.22
06:00	NA	NA	37.85	37.43	39.52	29.86	28.41	28.77	30.26
12:00	NA	NA	42.45	37.51	37.3	27.33	28.69	31.56	31.44
18:00	NA	NA	38.72	34.99	37.52	27.84	28.81	29.65	31.55

Table 4 Circadian rhythm of Septin 9 (SEPT9) positivity in patients with colorectal cancer

Time	Stage I	Stage I	Stage II	Stage II	Stage II	Stage III	Stage IV	Stage IV	Stage IV	SEPT9 positivity min. 1/3	SEPT9 positivity min. 2/3
0:00	1	1	2	3	3	3	3	3	3	100%	77.7%
06:00	0	0	1	3	2	3	3	3	3	77.7%	66.6%
12:00	0	0	1	2	3	3	3	3	3	77.7%	66.6%
18:00	0	0	1	3	3	3	3	3	3	77.7%	66.6%

except for one stage I and one stage II cancers. Finally, higher CA72–4 marker levels were detected only in two stage IV cases, however one stage IV cancer had normal CA72–4 level.

Based on these findings, in CRC cases involved in this study, only the cancer tumor markers, such as CEA and CA19–9 showed circadian cycling, thus only these two markers were involved in the correlation analysis with cfDNA amounts.

Correlation between the Circadian Rhythm of cfDNA, CEA and CA19–9 Tumor Markers Levels

In order to observe the correlation between cfDNA amount and tumor marker levels, the results of quantitative measurements were further analyzed. In case of CEA results, a weak correlation was found in stage I cancer cases, $R^2 = 0.34$ (Fig. 2), contrary to the high correlation between *SEPT9* and cfDNA concentrations. In stage II group, no correlation was observed between CEA and cfDNA levels ($R^2 = 0.02$). In advanced stages - as we found in comparison with *SEPT9* methylation – stronger correlation levels were detected with $R^2 = 0.60$ and $R^2 = 0.85$, respectively.

CA19–9 levels did not show correlation with cfDNA in stage I ($R^2 = 0.199$), II ($R^2 = 0.14$) and III ($R^2 = 0.09$). Nevertheless, in metastatic cases it showed only weak correlation ($R^2 = 0.31$) with cfDNA amounts (Fig. 2).

Discussion

The human body is physiologically synchronized on both cellular and systemic levels according to a circadian cycles,

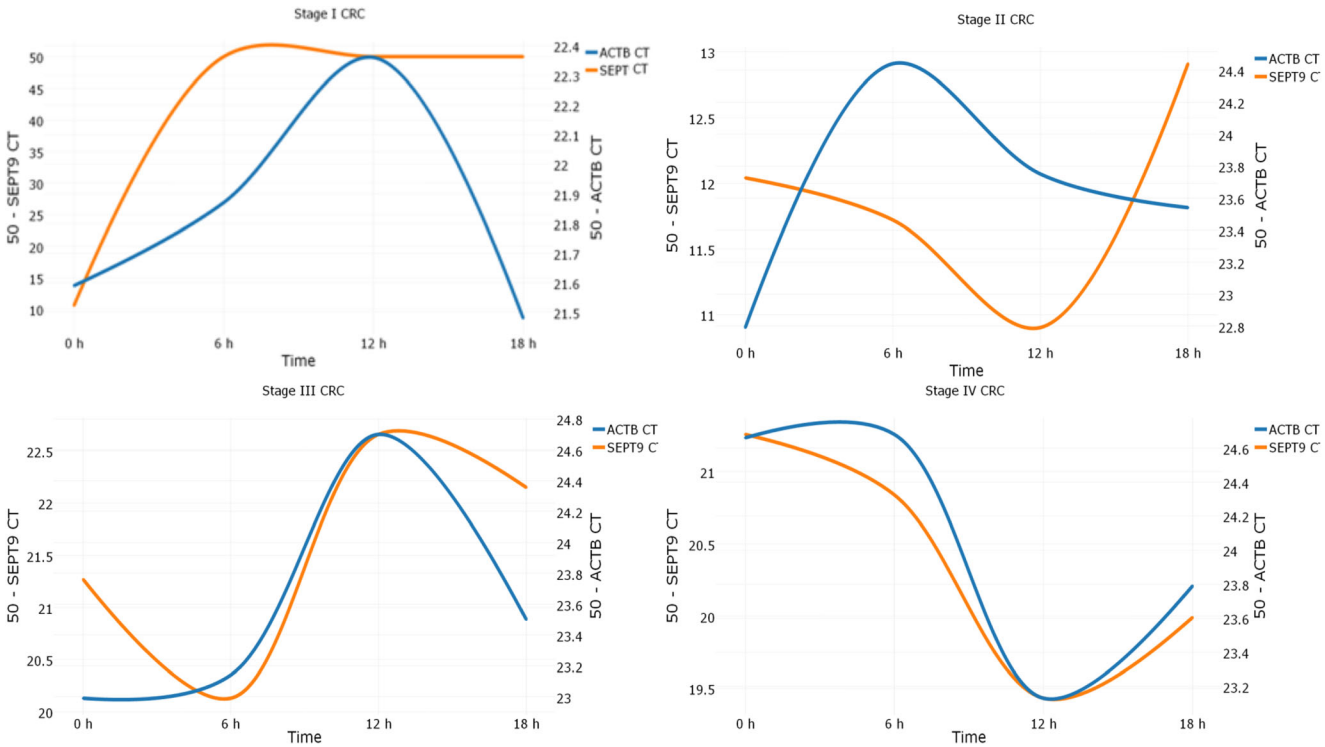

Fig. 1 Correlation between circadian rhythm of cfDNA and *SEPT9* methylation levels in colorectal cancer cases. To examine the correlation between cfDNA (ACTB - beta actin; *blue line*) and *SEPT9* (Septin 9; *orange line*) methylation levels, CT values of PCR replicates were used. In early CRC cases (stage I and stage II) no correlation was observed between cfDNA and SEPT9 methylation levels. Contrarily, a moderate correlation was observed in stage III ($R^2 = 0.59$) and in stage IV ($R^2 = 0.75$) samples

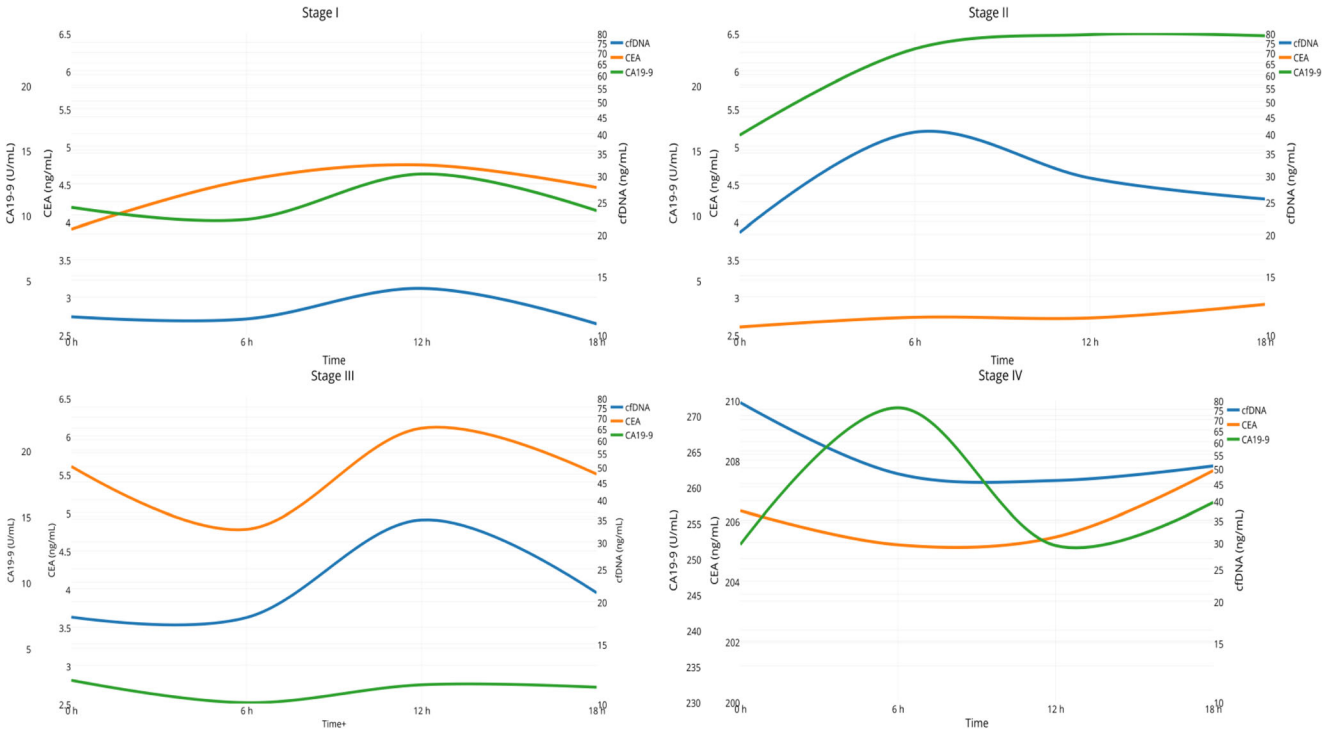

Fig. 2 Correlation between the circadian rhythm of cfDNA, CEA and CA19–9 tumor markers levels. Weak correlation was found is stage I cases between CEA (*orange line*) and cfDNA (circulating cell-free DNA; *blue line*) cases. In stage II no correlation was observed, however in advanced stages (stage III and stage IV) stronger correlation level was detected between CEA and cfDNA. CA19–9 levels (*green line*) did not show correlation with cfDNA in stage I, stage II and stage III cases. Nevertheless, in metastatic cases it showed only weak correlation with cfDNA amounts

however the circadian rhythm of cfDNA and blood-based tumor marker levels such as *SEPT9*, CEA or CA19–9 have not been described yet. In this study, we have analyzed the circadian rhythm of the above-mentioned markers in patients with CRC.

In our study, low cfDNA levels were detected in premalignant and early cancer cases. In stage IV advanced tumors, the measured cfDNA concentrations were 2–3 times higher than in early CRC cases. It may be caused by the increased rate of apoptosis, necrosis and active release of DNA, furthermore decreased DNase activity during CRC tumor progression [13, 14].

In early stage cases, lower levels of *SEPT9* methylation were observed as well. In premalignant and early CRC cases, as a result of regular daily activity, the cfDNA from non-cancer tissues may oversize the amount of *SEPT9* methylated cfDNA, thus the sensitivity of *SEPT9* methylation detection in adenoma and early cancer cases is decreased. In control cases, the lower cfDNA levels may be caused by the enhanced circulating DNase activity enhanced due to the daily physical activity [39]. Therefore, in parallel degradation of *SEPT9* may be higher as well.

In addition, the clock genes are also involved in the coordination of several cellular procedures, such as cell cycle and DNA repair [40], cell death. In our study, higher cfDNA levels were observed in the daytime period in cancer patients from stage I to stage III and elevated night time levels in stage IV patients. According to Mazzoccoli et al., significant association was found between the mRNA expression levels of some clock genes (*TIM, PER1, PER3* and *CSNKIE*) in advanced CRC stages (stage III-IV) and poorer survival [41]. *SEPT9* showed association with cfDNA level only in advanced cancer cases.

In previous studies, lower *SEPT9* methylation levels were detected in early CRC cases, with 26–84% positivity in stage I and 60–100% in stage II [30, 31, 33–36, 42]. The highest levels were reported for stage IV from 75 to 100%, where the cfDNA level was the highest. In our results we also observed an increased correlation between the cfDNA amount and *SEPT9* methylation level, suggesting that the lower *SEPT9* test sensitivity in early stages may be caused by the low cfDNA amounts. Furthermore, in advanced stages, *SEPT9* positivity was usually detected in the cases, where the highest cfDNA could be observed. In early cancer stages, the cfDNA level may have been too low for *SEPT9* detection. Specially in the active day hours, cfDNA from daily physical activity could further dilute the SEPT9 target. In these cases, only a small fraction of cfDNA would be released from the tumor. The tumor related *SEPT9* signal detection is more difficult.

Several studies showed that the robust circadian rhythm of cortisol and cytokines such as tumor necrosis factor, interleukins is disturbed in colorectal cancer [43–45]. Halberg et al. observed the circadian rhythm of CEA levels from saliva and urine in clinically healthy men [46]. Peak concentration occurred around 07:00 from salivary samples, a bit later than in case of patients with colon cancer stage II in our study. Differing from salivary results, the highest level of urinary CEA was at 15:00. We observed a similar rhythm for stages I-II as reported for salivary CEA. The diurnal peak of CEA level changes disappeared in cancer samples [28].

Our study has several limitations. The major limitation is the low sample size, but this is counterbalanced by the large number of markers measured in this work. Our finding should be validated in larger prospective data sets. Our observations might have important practical implications that should be taken into account in on-going and subsequent studies, specially for early cancer and adenoma detection collecting early morning plasma specimen for increased early cancer detection rate.

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