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Expression pattern of circadian rhythm-related genes and its potential relationship with miRNAs activity in endometrial cancer

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ABSTRACT

Objectives: The circadian clock is an autonomous oscillator that controls key aspects of cell physiology, including metabolism, transcriptional state, and cell signaling. Disturbances of circadian rhythms lead to disruption of cell and tissue homeostasis, which promotes carcinogenesis. The aim of the study was to determine the expression of circadian rhythm-related genes in endometrial cancer and to select miRNAs involved in the regulation of their expression.

Material and methods: 50 endometrial tissue samples were collected from patients who underwent hysterectomy: 40 diagnosed with endometrial cancer and 10 without cancer. Expression profile of circadian rhythm-related genes was evaluated using microarrays and validated with RT-qPCR. MicroRNA expression was assessed using microarrays. Then mirTAR tool was used to identify miRNAs involved in the expression regulation of circadian rhythm-related genes.

Results: *CLOCK* expression is disrupted in endometrial cancer, which may be due to miR-15b, miR-331-3p and miR-200a overexpression. Elevated *NPAS2* and *CSNK1D* levels may be associated with miR-432 silencing. In addition, high miR--874 and miR-200a expression may be potentially responsible for the reduction of *PER3* level.

Conclusions: Change of *CLOCK*, *CSNK1D*, *NPAS2* and *PER3* expression may suggest that circadian rhythms are disrupted in endometrial cancer. A possible mechanism of the observed changes may be related to miRNAs activity.

Key words: endometrial cancer; circadian rhythms; miRNAs; microarrays

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INTRODUCTION

Endometrial cancer (EC) is one of the most common gynecological cancers on a global scale. In 2020, there were over 400,000 new EC cases and almost 100,000 deaths due to EC worldwide [1].

Circadian rhythms and genes involved in their regulation are responsible for the cell cycle progression, proliferation and apoptosis [2]. Studies carried out so far indicate that disruption of the circadian rhythm mechanism negatively affect numerous physiological processes, which may result in uncontrolled cell division and cancer development [3]. The most important genes involved in circadian rhythm regulation include *CLOCK* (circadian locomotor output cycles kaput), *NPAS2* (neuronal PAS domain protein 2), *PER1/2/3* (period circadian clock 1/2/3), *CRY1/2/3* (cryptochrome 1/2/3), *BMAL1* (known as *ARNTL*, aryl hydrocarbon receptor nuclear translocator-like protein 1) and *CSNK1D* (casein kinase 1 delta) [4]. Genetic or functional disturbances of the circadian clock have been found in such cancers as ovarian cancer [5], breast cancer [6], prostate cancer [7].

MicroRNA (miRNA) molecules are also important in regulating circadian rhythms [9]. They belong to the group of

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small, non-coding RNAs responsible for post-transcriptional regulation of gene expression. Changes in the miRNAs level in cancer cells lead to abnormal activity of signaling pathways involved in proliferation, apoptosis and angiogenesis, and consequently to tumor progression [10].

The aim of the study was to assess the expression profile of circadian rhythm-related genes in endometrial cancer and to identify miRNAs involved in the regulation of their expression.

MATERIAL AND METHODS

This study was approved by the Bioethical Committee of the Medical University of Silesia (Sosnowiec, Poland; no. KNW/0022/KB1/130/16). Informed consent was obtained from all patients. The analysis carried out in this work complements previously published research [11].

Patients

The study included samples collected from 50 patients who underwent hysterectomy: 40 with endometrial cancer (study group) and 10 without EC (control group). Patients from the control group were qualified for the surgery due to the prolapse of the uterus. All EC patients were diagnosed with stage I endometrial cancer according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The study group was also divided according to the degree of histological differentiation: G1 (well-differentiated), 10 cases; G2 (moderately differentiated), 20 cases; G3 (poorly differentiated), 10 cases. The use of hormone replacement therapy five years before surgery, diagnosis of cancer other than endometrioid endometrial adenocarcinoma, endometrial hyperplasia with or without atypia constituted the exclusion criteria from the study group.

Endometrial samples collected during surgery were placed in sterile tubes containing RNAlater™ (Sigma-Aldrich, Saint Louis, MO, USA) and stored according to the manufacturer's instructions. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

mRNA microarrays

The expression profile of circadian rhythm-related genes was assessed in 27 samples (control, 3; G1, 7; G2, 11; G3, 6) using microarray technique (HG-U133A; Affymetrix, Santa Clara, CA, USA). All procedures were performed in accordance with the manufacturer's instructions. The methodology was described in detail in our previously published work [11]. GeneSpring GX 13.0 software (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for statistical analysis. It was performed for 133 mRNAs representing genes associated with circadian rhythms selected based on the Affymetrix NetAffx™ Analysis Center database (http://www.affymetrix.com/analysis/index.affx; accessed on 17 June 2020). ANOVA with Benjamini-Hochberg correction and Tukey's post-hoc test were performed. The microarray dataset has been deposited in the Gene Expression Omnibus (GEO) database under the accession GSE115810.

RT-qPCR

The expression profile of *NPAS2* and *CSNK1D* was evaluated in all 50 endometrial tissues samples with SensiFAST SYBR No-ROX One-Step Kit (Bioline, London, UK) and Opticon DNA Engine Sequence Detector (MJ Research Inc., Watertown, MA, USA). β-actin was used as the endogenous control. The reaction was carried out using the following primers: *NPAS2* (forward: 5′CCTCCTGTAATGGTTTTGAC 3′, reverse: 5′GAATTCCTCTAAAGGTTCGTC 3′), *CSNK1D* (forward: 5′ TTGTATCGCCACTGTATTTG 3′, reverse: 5′TGGGAAGAAGAAAGGTAGAAGTC3′), *ACTB* (forward: 5′ TCACCCACACTGTGCCCATCTACGA 3′, reverse: 5′CAGCGGAACCGCTCATTGCCAATGG 3′). The thermal profile included: reverse transcription (45°C for 10 min), polymerase activation (95°C for 2 min), 40 cycles consisting of denaturation (95°C for 5 sec), annealing (60°C for 10 sec), elongation (72°C for 5 sec). Statistical analysis was carried out with the Statistica 13.1 PL software (StatSoft, Tulsa, OK, USA). The Shapiro-Wilk test was used to evaluate normality. Normal data distribution allowed for one-way ANOVA and Tukey's post-hoc test.

miRNA analysis

MicroRNA expression profile was assessed in 11 endometrial tissue samples (control, 4; G1, 3; G2, 4). All procedures were performed in accordance with the manufacturer's instructions. The methodology was described in detail in our previously published work [11].

To predict which miRNAs could potentially participate in the expression regulation of circadian rhythm-related genes the mirTAR tool (http://mirtar.mbc.nctu.edu.tw/human/predictionIndex.php; accessed on 25 June 2020) was used.

RESULTS

Patients were characterized by age, weight and BMI, which were described as mean ± standard deviation: (C = 52.5 ± 10.48 years, 67.3 ± 13.64 kg, 25.29 ± 4.91; G1 = 67.9 ± 7.31 years, 69.9 ± 10.06 kg, 27.14 ± 3.77; G2 = 70.2 ± 12.27 years, 83.8 ± 13.89 kg, 33.69 ± 6.11, G3 = 70.8 ± 6.61 years, 78.6 ± 9.20 kg, 31.57 ± 4.22.

The one-way ANOVA with Benjamini–Hochberg correction showed that among 133 mRNAs encoding circadian rhythm-related genes, a significant change in expression was observed for 24 mRNAs compared to control ($p < 0.05$). Tukey's post-hoc test indicated that 3 mRNAs differentiate G1 endometrial cancer from control. In addition, 12 mRNAs and 5 mRNAs differentiate G2 and G3 cancer,

Table 1. The number of differentially expressed mRNAs among studied groups

Group	C	G1	G2	G3
C		3[1]	12[1]	5[1]
G1	3[1]		7[2]	9[2]
G2	12[1]	7[2]		5[3]
G3	5[1]	9[2]	5[3]	

C — control; G — endometrial cancer grade; [1]G1, G2, G3 vs C at $p < 0.05$; [2]G2, G3 vs G1 at $p < 0.05$; [3]G3 vs G2 at $p < 0.05$

Table 2. List of differentially expressed genes in endometrial cancer, determined by mRNA microarrays

ID	C	FC		
		G1 vs C	G2 vs C	G3 vs C
211426_x_at	*GNAQ*	1.5392512*	1.001996	–1.180963
210671_x_at	*MAPK8*	1.5351224*	1.6783358*	1.314751
210477_x_at	*MAPK8*	1.6583629*	1.9589097*	1.4543017*
204980_at	*CLOCK*	–1.6382707	–1.8877153*	–1.1936007
214513_s_at	*CREB1*	1.5525202	1.5872492*	–1.0408064
209967_s_at	*CREM*	–1.3894509	–1.5449941*	–1.3023547
207945_s_at	*CSNK1D*	1.4641819	2.2460847*	1.4358139
202390_s_at	*HTT*	–1.2538797	–1.8821522*	–1.3311925
204813_at	*MAPK10*	–1.012442	–2.039922*	–1.1390749
205460_at	*NPAS2*	1.5072023	1.8047009*	1.4624999
209024_s_at	*SYNCRIP*	1.9394071	3.832547*	1.2856476
201466_s_at	*JUN*	–1.4053583	–2.9399035*	–3.098988*
221045_s_at	*PER3*	–1.3402244	–2.277745*	–1.8457401*
207630_s_at	*CREM*	–1.0605571	–1.5263059	–1.7718524*
204466_s_at	*SNCA*	1.3220141	–1.8025548	–2.0455072*

C — control; FC — fold-change; G — endometrial cancer grade; ID — number of the probe *$p < 0.05$ vs C group

respectively. Within the study groups, 7 mRNAs and 9 mRNAs significantly change the expression profile in G2 and G3 cancer compared to G1 cancer. In turn, 5 mRNAs differentiate G3 cancer from G2 cancer (Tab. 1).

Further analysis of the differentiating genes showed that *GNAQ* (G protein subunit alpha q) was specific for G1 endometrial cancer, while *HTT* (huntingtin), *CLOCK* (circadian locomotor output cycles kaput), *MAPK10* (mitogen-activated protein kinase 10), *CREB1* (cAMP responsive element binding protein 1), *NPAS2* (neuronal PAS domain protein 2), *CSNK1D* (casein kinase 1 delta), *SYNCRIP* (Synaptotagmin-binding, cytoplasmic RNA-interacting protein) were specific for G2 cancer. In addition, *SNCA* (alpha-synuclein) was specific for G3 cancer. Analysis also revealed that *MAPK8* (mitogen-activated protein kinase 8) differentiates endometrial cancer from the control group regardless of its grade. Moreover, *CREM* (cAMP responsive element modulator), *JUN* (c-Jun) and *PER3* (period circadian clock 3) were common to G2 and G3 cancer. Table 2 provides details on the microarray probe number and fold-change (FC) value.

In the next step, RT-qPCR results were analyzed. The Shapiro-Wilk test showed that the obtained data was normally distributed. The expression changes of selected genes are presented as mean (M), minimum (Min), maximum (Max) and standard deviation (SD). The one-way ANOVA and Tukey's post-hoc test showed significant differences in *NPAS2* expression ($p < 0.05$; Tab. 3).

RT-qPCR analysis showed a significant increase in *NPAS2* expression in G2 EC, which validated the microarray mRNA results. Similarly, for *CSNK1D*, the direction of expression change in endometrial cancer compared to control was the same for both methods (overexpression), however, the RT-qPCR results were statistically insignificant (Tab 2, 3). Analysis of miRNA expression showed that 114 out of 1105 miRNAs, significantly change expression in EC compared to control at $p < 0.05$. Further analysis revealed that 47 miRNAs and 81 miRNAs differentiated G1 and G2 endometrial cancer, respectively. Table 4 shows a list of differentiating miRNAs when fold-change cutoff > 2 or < –2 was applied.

Table 3. Values of descriptive statistics and the result of one-way ANOVA and Tukey's post-hoc in endometrial cancer and control

Gene	Group	mRNA copies/µg total RNA				ANOVA	Post-hoc test
		M	Min	Max	SD		
NPAS2	C	4590	614	11580	2984	0.00985	G2 vs C, p = 0.008906 G2 vs G1, p = 0.020761
	G1	15946	1019	37590	13276		
	G2	23048	1747	71390	22277		
	G3	119160	19350	415300	161454		
CSNK1D	C	47042	22600	73030	16046	0.05102	NS
	G1	62181	16860	154100	39651		
	G2	49627	4111	135800	37586		
	G3	91530	16140	184100	59359		

C — control; G — endometrial cancer grade; M — mean; Max — maximum; Min — minimum; NS — not significant; SD — standard deviation

Table 4. List of miRNAs that specifically differentiate endometrial cancer from control at $p < 0.05$ and FC > 2 or < –2

Groups compared	miRNA	p value	FC
G1 vs C	miR-1909-5p	0.0073	2.17
	miR-874	0.0078	5.69
G2 vs C	miR-1826	0.0433	–2.60
	miR-1296	0.0158	–4.81
	miR-127-3p	0.0222	–7.67
	miR-134	0.0055	–9.16
	miR-483-5p	0.0441	–10.24
	miR-370	0.0444	–10.91
	miR-432	0.0159	–11.28
	miR-1246	0.0097	2.26
	miR-629	0.0270	2.39
	miR-652	0.0080	2.61
	miR-191	0.0347	3.03
	let-7a	0.0024	3.14
	miR-625	0.0235	3.21
	miR-25	0.0483	3.51
	let-7f	0.0206	3.70
	miR-15b	0.0173	3.71
	miR-200c	0.0399	4.36
	miR-181a-2-3p	0.0251	5.54
	miR-331-3p	0.0178	5.54
	miR-200a-5p	0.0336	5.63
	miR-200a	0.0229	8.32
	miR-155	0.0398	9.62
	let-7g	0.0214	13.01
	miR-183	0.0076	15.92
	miR-200b-5p	0.0067	29.14
	miR-10a	0.0027	37.20
	miR-182	0.0053	49.39
	miR-200b	0.0010	73.52

C — control; FC — fold-change; G — endometrial cancer grade

Table 5. List of genes associated with circadian rhythms and miRNAs potentially involved in the regulation of their expression, determined by microarrays and mirTAR tool

mRNA	Expression	miRNA	Expression
GNAQ	Increased	miR-183	Increased
NPAS2	Increased	miR-432	Decreased
CSNK1D	Increased	miR-483-5p	Decreased
		miR-432	Decreased
SYNCRIP	Increased	miR-432	Decreased
		miR-15b	Increased
		miR-200a	Increased
MAPK10	Decreased	miR-629	Increased
CLOCK	Decreased	miR-370	Decreased
		miR-15b	Increased
		miR-331-3p	Increased
		miR-200a	Increased
HTT	Decreased	miR-370	Decreased
		miR-191	Increased
		miR-625	Increased
		miR-15b	Increased
		miR-331-3p	Increased
		miR-874	Increased
PER3	Decreased	miR-134	Decreased
		miR-432	Decreased
		miR-874	Increased
		miR-200a	Increased

The mirTAR tool was used to assess which of the selected miRNAs potentially participate in the expression regulation of the studied genes associated with circadian rhythms that differentiate endometrial cancer from the control (Tab. 5).

Based on *in silico* analysis, there is a possibility that miRNAs are probably not involved in the regulation of *CREB1*, *CREM*, *JUN*, *MAPK8* and *SNCA* expression, however experimental data is needed to confirm this hypothesis. Decreased *MAPK10* level may be caused by increased activity of miR-629 in G2 EC. Overexpression of miR-15b, miR-331-3p and miR-200a may affect *CLOCK* level. MiR-15b, miR-331-3p, miR-191, miR-625 and miR-874 may participate in reducing *HTT* expression. In addition, high level of miR-874 and miR-200a can lead to a decrease in *PER3* expression. The results also showed that low miR-432 activity may result in overexpression of *NPAS2*, *CSNK1D* and *SYNCRIP*.

DISCUSSION

The circadian clock is an autonomous oscillator that controls key aspects of cell physiology, including metabolism, transcriptional state, chromatin modifications, and cell signaling [10]. Disturbances of circadian rhythms lead to disruption of cell and tissue homeostasis, which promotes carcinogenesis, where rapid and uncontrolled proliferation, increased metabolic demand, and resistance to apoptosis are observed [12].

The main components of the circadian clock are CLOCK and NPAS2 proteins. They participate in the cell cycle regulation, proliferation and immune functions [13]. *NPAS2*, like *CLOCK*, can form a heterodimer with *BMAL1*, which then activates the transcription of core circadian genes, including *CRY* and *PER*. Then CRY and PER proteins form a heterodimer, which regulates the activity of NPAS2 and CLOCK through negative feedback loop. CSNK1D can inhibit CRY and PER proteins [14]. In this study, we observed *NPAS2* overexpression in endometrial cancer with microarrays and RT-qPCR. Similarly, Ye et al. [15] reported increased *NPAS2* expression in hepatocellular carcinoma, squamous cell lung carcinoma and papillary renal cell carcinoma. Gao et al. [16] observed significant overexpression of *NPAS2* in lung adenocarcinoma, suggesting it as a promising diagnostic marker for this tumor. The high level of *NPAS2* revealed in our work may be the result of silencing miR-432, potentially involved in the regulation of its activity. Downregulation of miR-432 has also been reported in esophageal cancer [17], hepatocellular carcinoma [18] and cervical cancer [19]. It led to excessive

cell proliferation, invasion and migration, which promoted tumor progression [18]. On the other hand, Yi et al. [20] concluded that high *NPAS2* level is associated with better survival of breast cancer patients. A possible mechanism may be related to the regulation of *PER1* and *PER2*, overexpression of which would inhibit the growth of cancer cells. In addition, Xue et al. [21] found that reduced *NPAS2* level promotes proliferation and invasion of colorectal cancer.

In the case of *CLOCK*, Lesicka et al. [22] observed its higher level in breast cancer compared to non-tumor adjacent tissues. In turn, Cadenas et al. [23] reported that reduced *CLOCK* and *NPAS2* expression is associated with a worse prognosis in breast cancer. It was also revealed that *CLOCK* expression was decreased in ovarian cancer. In addition, it was associated with cisplatin resistance [6]. In our study, microarray analysis showed a reduction in *CLOCK* level in all three endometrial cancer grades with a significant decrease in G2 cancer compared to the control. Moreover, further analysis suggested that the *CLOCK* level may be potentially affected by miR-15b, miR-331-3p and miR-200a overexpression, however, as this is an *in silico* prediction, validation is needed. Zhang et al. [24] observed that increased miR-15b expression inhibited the proliferation of liver cancer cell line HepG2, while Chava et al. [25] suggested this miRNA as a target in neuroblastoma treatment. Interestingly, Dong et al. [26] reported different results in liver cancer patients and cell lines, indicating that miR-15b overexpression is important in cancer proliferation and invasion. The increase in miR-15b activity is also associated with the promotion of proliferation, migration and invasion of breast cancer [27], prostate cancer [28], non-small cell lung cancer [29], gastric cancer [30]. These observations suggest that mir-15b may act differently depending on the tumor type. In the case of miR-331-3p, it is reported that it acts as a tumor suppressor in colorectal cancer [31], non-small cell lung cancer [32], epithelial ovarian carcinoma [33]. In turn, Suo et al. [34] observed increased miR-200a level in ovarian cancer. Delangle et al. [35] noted in their meta-analysis increased levels of miR-15b and miR-200a in endometrial cancer, which is consistent with our suggestions. In our previous study, we also proposed that miR-15b, miR-331-3p, miR-200a can potentially affect endometrial cancer cell survival, proliferation and migration by targeting *PRKCA* [11].

CSNK1D is a member of the pleiotropic serine/threonine kinase family and participates in the regulation of the cell cycle and circadian rhythms [4, 36]. Disruption of their function leads to the occurrence of many pathologies, including cancer development [37]. Bar et al. [4] observed that a decrease in *CSNK1D* expression results in inhibition of proliferation and metastasis of triple negative breast cancer. Rosenberg et al. [38] also came to this conclusion in studies on breast cancer and indicated inhibition of *CSNK1D* as a promising strategy for cancer treatment. Mazzoldi et al. [39] reported increased CSNK1D level in ovarian cancer and suggested that its knockdown decreases cancer cell migratory capability. In our study, *CSNK1D* was overexpressed, however only the microarray results were statistically significant. Possible increase in *CSNK1D* level may be caused by the lack of regulatory effect of miR-483-5p and miR-432, possibly silenced in endometrial cancer. Studies indicate that miR-483-5p may participate in the promotion of prostate cancer [40], gastric cancer, colorectal cancer and oral cancer [41]. It has also been observed that miR-483-5p can suppress the proliferation of glioma cells [42].

In the case of other core circadian clock components, no statistically significant differences were observed in the *BMAL1*, *CRY1/2/3* and *PER1/2* expression. On the other hand, microarray analysis showed a significant decrease in *PER3* level in G2 and G3 cancers. Similarly, Tang et al. [43] reported reduced *PER3* expression in non-small cell lung cancer and described it as a promising target to control cancer progression. Hong et al. [44] noted that *PER3* was downregulated in colorectal cancer, which was due to the miR-103 activity. In this study, the reduction in *PER3* level may be related to the overexpression of miR-874 and miR-200a, however experimental data is needed to confirm this hypothesis. The recorded high level of miR-874 is consistent with previous observations in endometrial cancer [45].

The search for newer and better methods of cancer diagnosis and treatment is ongoing [46, 47]. Molecular markers can significantly help in the precise and early diagnosis of endometrial cancer. In addition, a better understanding of the mechanisms underlying EC initiation and progression could help in the development of new treatment strategies, including personalized therapies. Moreover, analysis of the relationship between mRNAs and miRNAs may reveal potential therapeutic targets [48]. It is therefore important to continue the search for markers, including miRNAs, to broaden the range of tools to fight endometrial cancer.

CONCLUSIONS

In our study, we observed higher levels of *NPAS2* and *CSNK1D* and lower *CLOCK* and *PER3* expression in endometrial cancer compared to control. The obtained results may suggest that their mutual interactions are disturbed in endometrial cancer, and there is a possibility that the responsible mechanism involves regulation by miRNAs.

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Conflicts of interest

All authors declare no conflict of interest.

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