Arch Biol Sci. 2022;74(2):111-118 https://doi.org/10.2298/ABS220223009N

# Dysregulation of *PER3* clock gene and its only pseudogene in colorectal cancer and type 2 diabetes

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**Received**: February 23, 2022; **Revised**: March 7, 2022; **Accepted**: March 8, 2022; **Published online**: March 30, 2022

**Abstract:** The period (*PER*) family genes (*PER1*, *PER2*, and *PER3*) play a fundamental role in regulating the day/night cycle. *PER3* has a pseudogene variant, *PER3P1* or *PER4*, whose role and expression pattern is unclear in human health and diseases. This study was performed to evaluate the expression levels of normal *PER* family members and the *PER3P1* pseudogene in colorectal cancer (CRC) and type 2 diabetes (T2D). Blood samples were taken from 50 diabetic patients and analyzed using real-time PCR for quantification of *PER3* and *PER3P1* expression. Colorectal tumor tissues of 50 individuals were also used to evaluate the expression of *PER* members. All *PER* members, including *PER3P1*, were found to be downregulated in colorectal tumor samples. Blood samples collected from diabetic subjects revealed an opposite expression pattern; both *PER3* and its pseudogene were found to be upregulated when compared to the control group. Our results reveal coordination between the expression pattern of *PER3P1* and normal *PER* family genes. Based on our findings and the pathological importance of this pseudogene, it can be suggested that *PER3P1* may be one of the key regulators of the molecular clock network and *PER* family expression. This hypothesis needs to be confirmed by further studies.

**Keywords:** circadian clock; *PER3*; *PER3P1* pseudogene; colorectal cancer; type 2 diabetes

## INTRODUCTION

The circadian clock is involved in diverse biological (cell cycle, proliferation, apoptosis, DNA repair mechanisms) and physiological activities [1-4]. Widespread diseases such as diabetes and cancer have been linked to the circadian clock [5-7]. The downregulation of cryptochrome circadian regulator (*CRY*) *1* and *2*, *PER1*, *PER2, PER3,* and brain, and muscle ARNT-like 1 (*BMAL1*) genes have been reported in patients with head and neck squamous cell carcinoma (HNSCC) [8]. The mammalian master clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus and coordinates peripheral clocks in tissues through neural and hormonal signals [2,9-11]. The circadian clock consists of several transcriptional translational feedback loops. Period (*PER1*, *PER2,* and *PER3*), cryptochrome (*CRY1* and *CRY2*), *CLOCK* and *BMAL1* genes, which are present in the first loop, are regarded as core clock genes (CCG). BMAL1 and CLOCK protein products form a heterodimer complex that binds to the E-box element of *PER* and *CRY* genes and mediates their transcription. PER and CRY dimers translocate into the nucleus and suppress the activity of the BMAL1:CLOCK complex. The inhibitory effect of PER and CRY proteins is removed over time, allowing the next cycle to begin [10,12-14]. The only pseudogene discovered to be correlated with the circadian clock is *PER3P1*, which originates from the *PER3* gene. *PER3P1* has been reported to have a high degree of evolutionary conservation between humans and rhesus monkeys. As the fourth member of the *PER* family genes, it can improve our knowledge about the core clock regulation mechanism [15-17]. The expression pattern of this pseudogene and its correlation with other members of the *PER* family genes remains unclear. So far, no biological activity or pathological role has been reported for this pseudogene, and it was assumed that it does not produce an active protein with an enzymatic activity or a regulatory role.

Therefore, in the current study, we examined the changes in expression and clinical value of *PER3P1* and its parental gene (*PER3*) in colorectal cancer (CRC) and type 2 diabetes (T2D) using bioinformatics and laboratory investigations. In the context of recent findings of the key regulatory role of non-coding RNAs in gene expression and overall cell physiology, the results presented in this study indicate that *PER3P1* could be a key regulator of the *PER* family genes and should be considered for a better understanding, diagnosis and prognosis of certain pathologies that are directly or indirectly related to circadian clock disorders.

## MATERIALS AND METHODS

### Ethics statement

This research was performed in accordance with the principles of the Declaration of Helsinki. The study was conducted according to the institutional review board (IRB) standards for research and ethics approval of the Golestan University of Medical Science (Gorgan, Iran). Informed consent was received from all patients before participation in this study.

### Bioinformatics analysis

Before experimental research, an online web tool (https://tnmplot.com/analysis/) was used to compare the expression of *PER* family genes in tumor and normal colorectal tissues. This platform uses data from The Cancer Genome Atlas (TCGA), the Gene Expression Omnibus (GEO), the Genotype-Tissue Expression (GTEx), and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) databases to evaluate gene expression in normal, tumor and metastatic tissues [18]. The prognostic value of *PER1/2/3* and *PER3P1* gene expression in CRC patients was obtained from the Kaplan-Meier plotter (http://kmplot.com/analysis) database. This open-access website examines the effect of 54,675 genes on patient survival status in 26 different types of cancer [19,20]. The correlation of *PER* family members in colorectal cancer was assessed using the GEPIA database (http://gepia.cancer-pku.cn/). This online resource is based on the TCGA and GTEx projects and provides valuable information regarding gene expression profiling, correlation analysis, etc. in normal and tumor tissues [21]. Results with $P<0.05$ were considered significant.

### Patients and specimens

Fifty pairs of tumor and adjacent normal tissue samples were obtained from surgical procedures performed on 50 patients with CRC who had not undergone any chemotherapy. Specimens were quickly frozen after biopsy and stored in liquid nitrogen at -70°C. Blood samples were taken after overnight fasting from 50 T2D patients and 50 healthy individuals who served as the control group. Informed consent was obtained from all patients according to the ethical guideline of Golestan University of Medical Sciences (Gorgan, Iran). The summary of patient information is presented in Supplementary Tables S1 and S2.

### RNA extraction, cDNA preparation and real-time PCR

Total RNA from tissue and blood mononuclear cells was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco, Life Technologies, Carlsbad, CA, USA). cDNA was generated from 1 μg of purified RNA using the cDNA synthesis kit according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, USA). The expression of *PER3* and *PER3P1* genes was assessed by real-time PCR using the SYBR Green PCR Master Mix. The mRNA levels in tissue and blood specimens were normalized to *β*-actin and *GAPDH* genes, respectively. The primer sequences are presented in Supplementary Table S3. PCR was performed using the following temperature program: initial denaturation at 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 40 s. The expected PCR product sizes are presented in Supplementary Table S3. PCR products were evaluated by electrophoresis on a 2% agarose gel.

### Statistical analysis

The CT method was used to calculate the difference between mRNA expression levels. Charts were prepared using GraphPad Prism (ver. 9.3.1) and R software (ver. 4.1.2). Data normality was checked by the Shapiro-Wilk test; results with $P>0.05$ indicated that

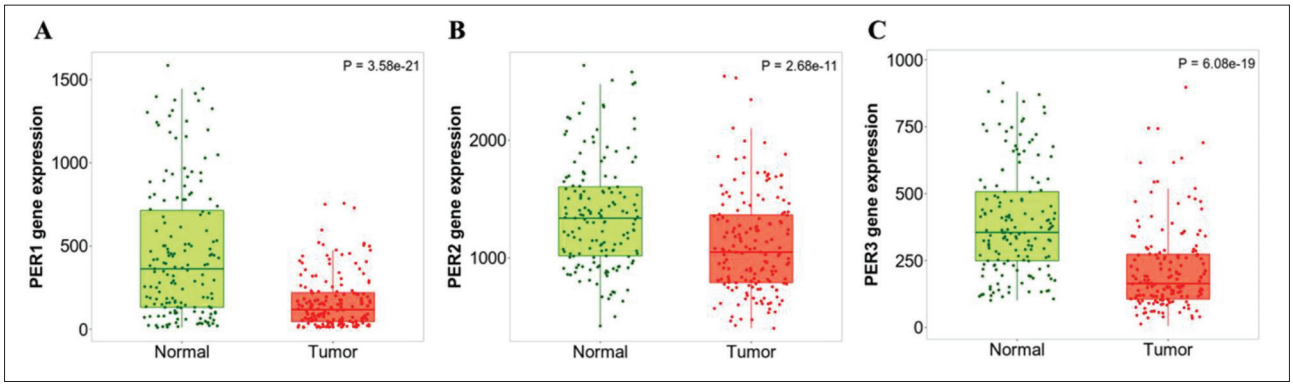


**Fig. 1.** Expression analysis of *PER1/2/3* genes in CRC by the TNMplot web tool. Changes in expression *PER1* (**A**), *PER2* (**B**) and *PER3* (**C**) based on gene chip data from the GEO database in 160 paired tumor and adjacent non-tumor colorectal tissues.

the data were normally distributed. The paired t-test was carried out to determine whether there is a significant difference in *PER1/2/3* and *PER3P1* expression in CRC tumors and adjacent normal tissues. To reveal potential differences in *PER3* and *PER3P1* expression between T2D patients and the control groups, unpaired t-test and nonparametric Mann-Whitney U tests were utilized for data with normal and non-normal distributions, respectively. Receiver operating characteristic (ROC) curve analysis was applied to evaluate the gene diagnostic value using a graphical plot. This test determines whether a shift in gene expression is of diagnostic value to segregate patients and normal individuals. Spearman's correlation test was performed to determine the correlation of variables. Results with $P<0.05$ were statistically significant.

## RESULTS

### Bioinformatics analysis of gene expression

To explore the biological role of *PER3P1* as the only known pseudogene in the circadian clock, we examined its expression pattern and its relationship with other *PER* genes, especially its parental gene (*PER3*), in CRC using the TNMplot database (https://tnmplot.com/analysis/). A significant decrease in *PER1/2/3* expression in colorectal tumor tissue compared to adjacent normal tissue was observed (Fig. 1). *PER3P1* expression data was unavailable on the website.

### Prognostic value of *PER* gene expression in CRC

Prognostic value assessments of *PER* family genes in 165 CRC individuals by the Kaplan-Meier plotter database demonstrated that CRC patients with high or low expression of *PER1* (P=0.31; Fig. 2A), *PER2*

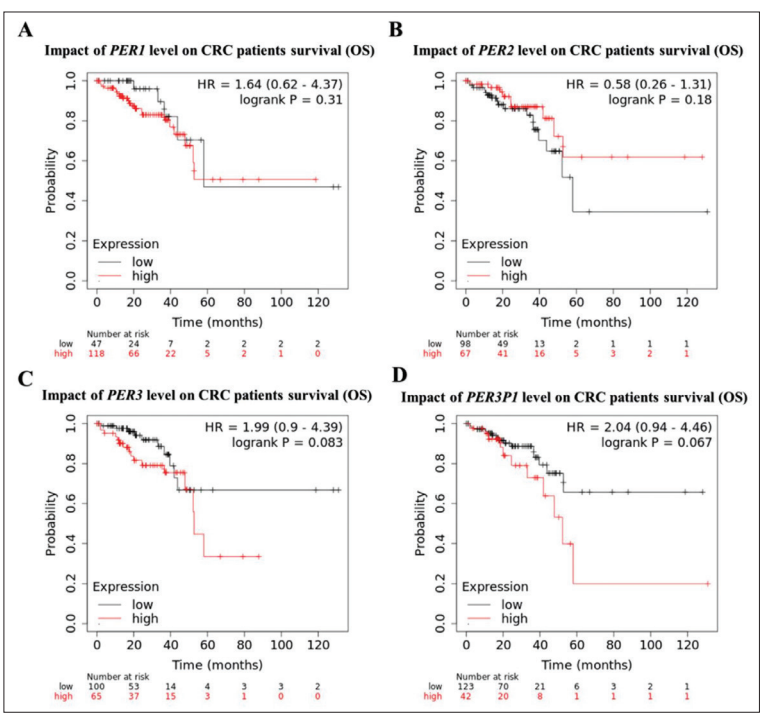


**Fig. 2.** Evaluation of the prognostic value of *PER* family genes using the Kaplan-Meier Plotter database in 165 individuals with CRC. Association of *PER1* (**A**), *PER2* (**B**), *PER3* (**C**) and *PER3P1* (**D**) levels with OS in CRC subjects.

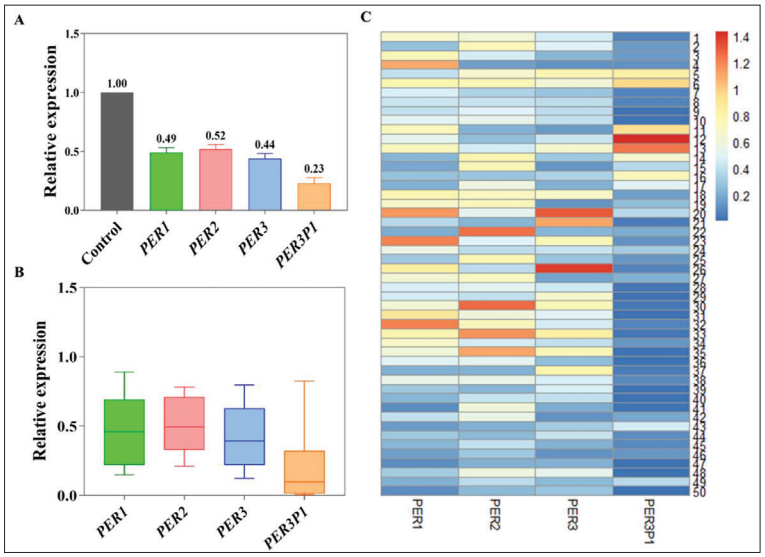


**Fig. 3.** Laboratory evaluation of *PER* family mRNA levels in CRC tissues compared to normal tissue. The expression of *PER1*, *PER2*, *PER3* and *PER3P1* genes decreased simultaneously in tumor tissue (**A**). Box plot (**B**) and heat map (**C**) display the expression level of *PER* family genes obtained by qRT-PCR in CRC patients.

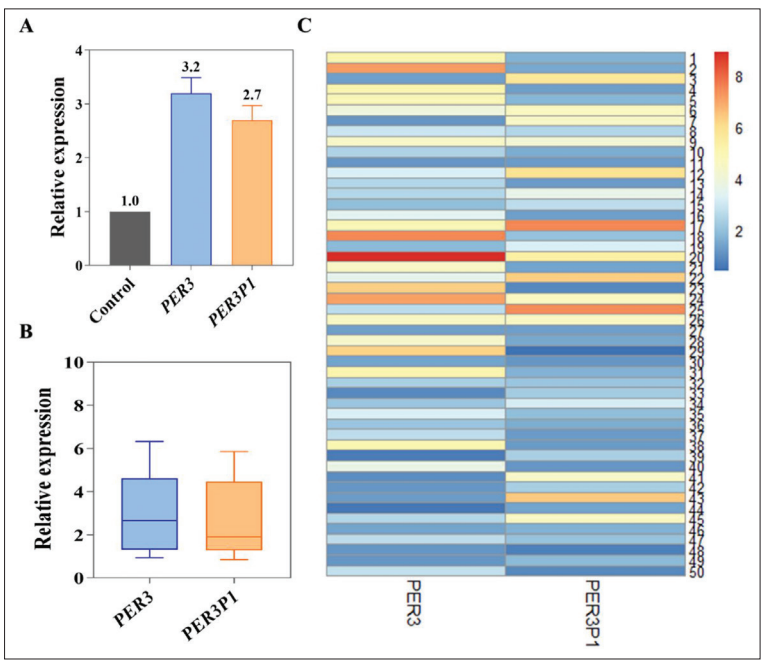


**Fig. 4.** Laboratory validation of *PER3* and *PER3P1* expression changes in T2D patients. The expression of *PER3* and *PER3P1* was significantly upregulated in T2D patients (**A**). Box plot (**B**) and heat map (**C**) display the levels of *PER*3 and *PER3P1* genes obtained by qRT-PCR in T2D patients.

(P=0.18; Fig. 2B), *PER3* (P=0.08; Fig. 2C) and *PER3P1* (P=0.06; Fig. 2D) displayed no significant difference in overall survival (OS). Further investigations revealed that changes in *PER3P1* levels were significantly associated with OS in patients with clear cell renal cell carcinoma, hepatocellular carcinoma, pancreatic ductal adenocarcinoma and gastric cancer (P<0.05; Supplementary Fig. S1). Therefore, it can be regarded as a potential prognostic biomarker in these cancers.

### *PER* family gene expression in CRC

All *PER* members exhibited an expression shift in colon cancer tumor tissues obtained from patients. The maximum downregulation rate was displayed by pseudogene *PER3P1*. The expression level of *PER3P1* in tumor tissues was calculated to be 23% as compared to the surrounding normal tissues (i.e. 77% downregulation). Compared to normal tissues, *PER1*, *PER2* and *PER3* expression levels decreased by 51%, 48%, and 56%, respectively (P<0.05) (Fig. 3A). Our findings are consistent with the information provided in the TNM plot database. The box plot and heatmap of *PER1/2/3* and *PER3P1* expression alterations in CRC patients are shown in Fig. 3B-C.

### *PER3* and *PER3P1* levels in diabetic patients

*PER3* and *PER3P1* expression patterns in diabetic patients were estimated by qRT-PCR to be completely different from those observed in colorectal tumor tissues. Compared to the control group, a 3.2-fold increase in *PER3* expression and a 2.7-fold increase in *PER3P1* expression were observed in diabetic patients (Fig. 4A). The box plot and heatmap of *PER3* and *PER3P1* expression changes in T2D patients are presented in Fig. 4B-C.

### The diagnostic utility of *PER1/2/3* and *PER3P1* expression in CRC and T2D

ROC curve analysis confirmed the diagnostic capacity of *PER* genes in patients with CRC and T2D. The proximity of the area under the ROC curve (AUC) to 1 indicates a diagnostic value with excellent accuracy [22,23]. Based on our findings, the

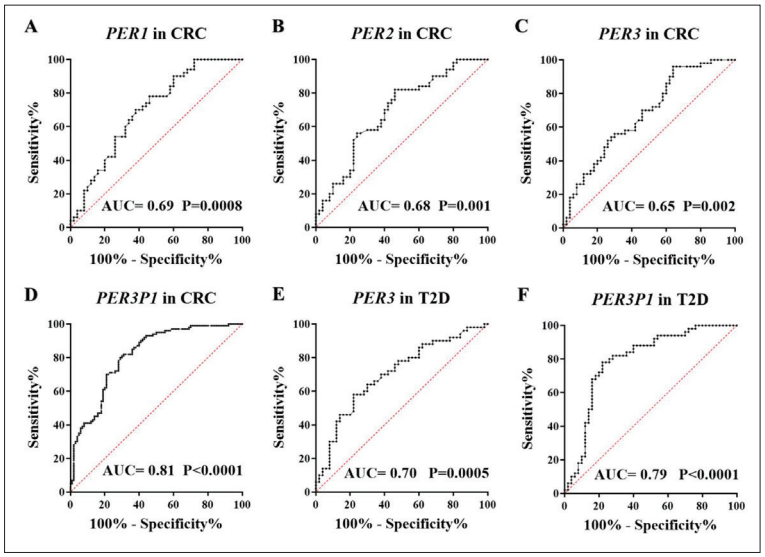


**Fig. 5.** Determination of the diagnostic value of *PER* genes using ROC curve analysis based on experimental findings. ROC curves for discriminating between the expression levels of *PER1* (**A**), *PER2* (**B**), *PER3* (**C**) and *PER3P1* (**D**) between matched CRC and normal specimens. ROC curves for discriminating between *PER3* (**E**) and *PER3P1* (**F**) expression levels in T2D patients and the control group.

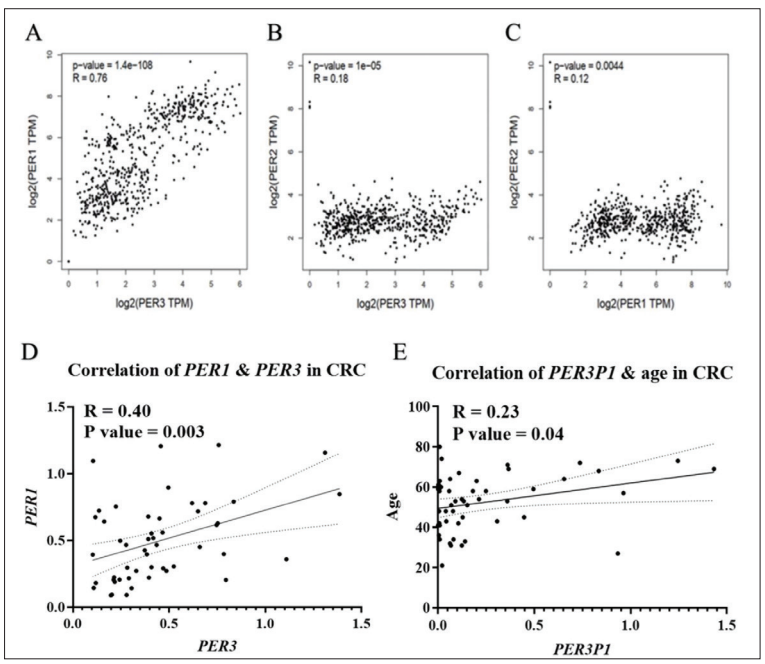


**Fig. 6.** Correlation analysis of the expression of *PER* family genes. In the GEPIA database, higher and lower expression correlation belongs to *PER3*/*PER1* (**A**) and *PER1*/*PER2* (**C**), respectively. *PER3*/*PER2* (**B**). There is a positive correlation between the changes in expression of the *PER1*/*PER3* pair in CRC patients (**D**). A positive correlation was observed between the *PER3P1* levels and the age of CRC patients (**E**).

expression of the *PER3P1* pseudogene in CRC patients (AUC=0.81, P<0.0001, 95% CI=0.7558 to 0.8732; Fig. 5D) and T2D patients (AUC=0.79, P<0.0001, 95% CI=0.7014 to 0.8858; Fig. 5F) has excellent diagnostic power. Expression evaluation of *PER1* (AUC=0.69, P=0.0008, 95% CI=0.5914 to 0.7974; Fig. 5A), *PER2* (AUC=0.68, P=0.001, 95% CI=0.5846 to 0.7922; Fig. 5B) and *PER3* genes (AUC=0.65, P=0.002, 95% CI=0.5729 to 0.7815; Fig. 5C) indicates acceptable performance as diagnostic tests in CRC patients. In addition, the results revealed that the *PER3* mRNA level creates acceptable discrimination between diabetics and normal individuals (AUC=0.7, P=0.0005, 95% CI=0.6009 to 0.8059; Fig. 5E). Our results indicate that the *PER3P1* pseudogene has the highest diagnostic value among the *PER* family genes for CRC and T2D.

## Correlation analysis

Positive correlations of *PER1*/*PER3*, *PER2*/*PER3* and *PER1*/*PER2* can be confirmed by searching the Gene Expression Profiling Interactive Analysis database (GEPIA, http://gepia.cancer-pku.cn/). *PER1*/*PER3* pairwise has the highest correlation coefficient (P<0.05) (Fig. 6A-C). Spearman correlation analysis of our findings revealed a positive correlation between *PER1* and *PER3* expression (R=0.4, P-value=0.003, 95% CI=0.1318 to 0.6177; Fig. 6D) in patients with CRC. In addition, a positive correlation was found between the age of CRC subjects and *PER3P1* pseudogene expression levels (R=0.23, P-value=0.04, 95% CI=-0.05081 to 0.4913; Fig. 6E).

## DISCUSSION

We assessed the dysregulation of *PER* family genes and their prognostic value in colorectal cancer using bioinformatics tools. Data mining revealed that the

expression of *PER1/2/3* is dramatically downregulated in patients with colon cancer. The correlation of the *PER3P1* expression level and OS of CRC patients was found by survival analysis to be not significant (P=0.06). Searching in the Kaplan-Meier Plotter database indicates that this pseudogene has a valuable prognostic power in human cancers as diverse as kidney cancer, liver, gastric and pancreatic cancers (P<0.05). These organs play a fundamental role in maintaining homeostasis and therefore in human health. Thus, a significant relationship between *PER3P1* dysregulation and the OS of cancer patients is a reflection of its biological importance in homeostasis balance.

We measured the expression of the *PER3P1* pseudogene in tumor tissues of CRC patients to determine whether there is a correlation between this pseudogene and normal *PER* family genes (*PER1*, *PER2*, *PER3*). We found that the *PER* family genes were downregulated in human CRC tissues. These findings are consistent with previous studies reporting *PER* family expression in colorectal and breast cancers [24-27]. We found that the *PER3P1* pseudogene follows the same trend and becomes downregulated in CRC tissues. We also examined the *PER3* and *PER3P1* expression levels in blood samples of diabetic patients and found that these genes are upregulated in these patients. In addition, we found that *PER3P1* possesses an excellent diagnostic capacity for distinguishing between T2D (AUC=0.79) and CRC patients (AUC=0.81). Several pseudogenes (double homeobox A pseudogene 10 (*DUXAP10*) and phosphatase and tensin homolog (*PTENP*) *1*) have been introduced as diagnostic and prognostic biomarkers in various cancers [28-30]. A recent meta-analysis study emphasized the importance of *PER1/2/3* genes as potential biomarkers in cancer prognosis. According to this report, *PER1/2/3* genes are closely correlated with the OS of cancer patients; the higher the expression of these genes, the better the prognosis expected in the patients [31]. Based on our findings, the *PER3P1* pseudogene has the potential to be employed as a diagnostic and prognostic biomarker, and even as a therapeutic target in human cancers. The circadian clock has been found to play a pivotal role in regulating systemic homeostasis [32,33]. Furthermore, an imbalance in homeostasis is associated with diseases such as diabetes and cancer [34,35]. Thus, it can be proposed that aberrant expression of *PER3P1* participates in the disruption of homeostasis.

The high evolutionary conservation of non-coding RNAs could be a reflection of their functionality [36,37]; the 92% similarity of the *PER3P1* sequence in humans and non-human primates such as rhesus monkeys increases the likelihood of its role in biological processes. According to experimental and clinical findings and evolutionary conservation, *PER3P1* may have undiscovered roles in controlling cellular events and regulating the expression of other *PER* family members. A decoy mechanism is a potential route through which this pseudogene regulates its target genes, the mechanism in which a pseudogene transcript traps common miRNAs, affecting the expression of target genes [38-40]. The *PER3P1* transcript may compete with mRNAs of normal *PER* genes for binding to common miRNAs through the construction of a competing endogenous RNA (ceRNA) network, thereby regulating their expression. However, more studies are necessary to confirm that the *PER3P1* pseudogene regulates the expression of *PER1*, *PER2* and *PER3* circadian clock genes, and to elucidate the exact mechanism by which it mediates its regulatory roles.

## CONCLUSIONS

The results of our study indicate significant co-downregulation of *PER1/2/3/PER3P1* and co-upregulation of *PER3*/*PER3P1* in CRC and T2D, respectively. Changes in *PER3P1* pseudogene expression could be a valuable diagnostic biomarker in CRC and T2D. Our results suggest that *PER3P1* may exert regulatory roles over *PER* family genes. More studies are needed to confirm that this pseudogene regulates the expression of other *PER* members.

**Funding:** No funding was received for conducting this study.

**Acknowledgments:** This study was approved by Golestan University of Medical Sciences (Gorgan, Iran), Approval Code 970724138, and Ethics Approval Code IR.GOUMS.REC.1397.077. The authors are grateful to the staff of Golestan University of Medical Sciences for their contributions and assistance.

**Author contributions:** PN-H and RN designed and coordinated the study. The experiments were performed and analyzed by PN-H, RN and SA. PN-H wrote the paper and all authors read and approved the final manuscript.

**Conflicts of interest disclosure:** The authors declare no conflicts of interest.

**Data availability:** No dataset was generated or analyzed during this study and data sharing is not applicable.

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## Supplementary Data

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Najari-Hanjani_7526_Supplementary%20Material.pdf