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Circadian Oscillation of Natural Antisense Transcripts Related to Human Core Clock Genes

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Abstract

Background: Circadian clocks are autonomous intracellular oscillators that synchronize metabolic and physiological processes with the external signals. So, misalignment of environmental and endogenous circadian rhythms leads to disruption of biological activities in living organisms. Noncoding transcripts including antisense RNAs are an important component of the molecular clocks. Commonly, the antisense transcripts are involved in the regulation of gene expression. *PER2AS* and *CRY1AS* are the only known Natural Antisense Transcripts (NAT) among the core clock genes, which overlap with the *PER2* and *CRY1* genes, respectively. In this study, we hypothesized that *PER2AS* and *CRY1AS* like the other clock genes, exhibit the oscillatory behavior in a 24-hour period and affect the expression of *PER2* and *CRY1*.
Methods: First, the A549 cell line was cultured under standard conditions. After horse serum shock, RNA extraction and cDNA synthesis was performed; then the expression fluctuations of *PER2AS*, *CRY1AS*, *PER2*, and *CRY1* were measured with Real-time PCR.
Results: Our result showed that *PER2AS* and *CRY1AS* had similar oscillation patterns with their sense strand during 24-hour period.
Conclusions: Therefore, we suggested that *PER2AS* and *CRY1AS* transcripts probably by preventing the interaction of miRNAs with *PER2* and *CRY1* mRNAs, influence the expression of them, positively.

Keywords: *CRY1*, *CRY1AS*, Natural Antisense Transcripts, *PER2*, *PER2AS*.

Introduction

The majority of living organisms are equipped with a time measuring system that is known as a circadian clock. The suprachiasmatic nucleus (SCN) as a master regulator of the mammalian circadian clock is responsible for synchronizing internal biological rhythms with day/night periods and controlling the peripheral clocks in the other tissues (1-4). The dysregulation of circadian rhythms is closely involved in the development of different human metabolic diseases. Furthermore, clock genes play a key role in the cell cycle and cancer-related processes like cell proliferation, apoptosis, and DNA damage response; thus, improper expression of them are associated with various cancers (5-9).

From a molecular viewpoint, the circadian clock is composed of multiple interconnected transcriptional/translational feedback loops (TTFL). the primary feedback loop contains the core clock genes (CCG) including *BMAL1*, *CLOCK*, *Period* (*PER1/2/3*) and *Cryptochrome* (*CRY1/2*) family genes (8, 10). BMAL1-CLOCK heterodimer in a positive feedback loop, activate the expression of *PER* and *CRY* genes. Consequently, PER and CRY proteins dimerize in the cytosol and after translocation to the nucleus, interfere with the BMAL1-CLOCK transcriptional activity. Eventually, by reducing the expression of *PER* and *CRY* genes, the inhibitory effect of them is eliminated from the BMAL1-CLOCK complex (6,11,12).

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Natural antisense transcripts (NAT) are single-stranded noncoding RNAs (ncRNAs) that are transcribed from the opposite strand of coding or non-coding genes. They can be cis or trans-acting and generally play a critical role in the regulation of their target gene expression, positively or negatively (13, 14). Dysfunction of some antisense RNA (asRNAs) can lead to several diseases such as diabetes, cancers, and neurological disorders (15-18). The effect of antisense transcripts on mRNA expression is based on the cellular location of sense and antisense RNA interaction. Antisense transcripts that remain in the nucleus, inhibit the translation of mRNAs, but in some cases that the sense-antisense RNA duplex form in the cytoplasm, asRNAs can increase the stability and expression of the sense strand. This is done by competing with common miRNAs and covering their binding site within target mRNAs (19). More than 70% of the human genome generates antisense transcripts. As mentioned, some of them are considered as main regulators of gene expression; thus, their disruption can cause various diseases. For instance, *MACC1-AS1* is an antisense RNA that sponges the tumor suppressor miRNAs (miR-384 and miR-145-3p) related to pleiotrophin and c-Myc proto-oncogenes; hence, the upregulation of *MACC1-AS1* leads to increased cell proliferation and invasion (20-21).

So far, two cis-acting natural antisense transcripts related to the human core clock genes called *PER2AS* and *CRY1AS* have been identified that originated from *PER2 and CRY1* genes, respectively. Recent studies in the mouse liver indicated that m*Per2as* and m*Per2* transcripts have antiphasic oscillatory patterns. Furthermore, it became clear that *mPer2as* is one of the clock controlling factors (22).

We hypothesized that antisense transcripts present in the core loop of human circadian clock may be involved in circadian clock control. Therefore, we evaluated the expression of *PER2AS* and *CRY1AS* compared to *PER2* and *CRY1* genes to find out the possible interaction between antisense and parental gene expression.

Materials and Methods

Cell culture

A549 cell line was obtained from the Pasteur Institute of Iran and was cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin, and L-glutamine (PSG). Then, cells were incubated in humidified atmosphere at 37 °C with 5% CO2.

Clock genes synchronization using horse serum shock method

In order to clock genes stimulation, approximately 2×10^5 cells/well were seeded in a 24-well plate in triplicate. After three days the cells became confluent and at this time they were exposed with serum-rich media, DMEM supplemented with 50% horse serum. After 2 hours' incubation at 37 °C, this medium was exchanged with pre-warmed serum free DMEM plus PSG. Finally, every 2 hours' cells were washed twice with ice-cold PBS and harvested in 200 µl/well Trizol (Gibco, Life technologies, Carlsbad, CA, U.S.A) at 13 time points and during a 24 hours' period (23).

RNA extraction and cDNA synthesis

Total cellular RNA was isolated by Trizol reagent (Gibco, Life technologies, Carlsbad, CA, USA) according to the manufacturer's guidance. The purity of RNA was measured at 260/280 nm with a UV spectrophotometer and the extracted RNA was stored at -70 °C. Then, 1 µg of total RNA was reverse transcribed using the cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA)*.* For this purpose, according to the manufacturer's specification, 1 µg of RNA was initially treated with DNase I enzyme, then 1 µl of random hexamer primer plus RNase-free water (up to 12 µl) was added to the mixture and placed at 65 °C for 5 min. In the next step, 5X Reaction Buffer (4 µl), RiboLock RNase Inhibitor (1 µl), 10 mM dNTP Mix (2 µl) and reverse transcriptase (M-MLV) enzyme (1 µl) were added, respectively (total volume 20 µl). The mixture was incubated for 5 min at 25 °C followed by 60 min at 42 °C and 5 min at 70 °C.

Quantitative Real-time PCR

To evaluate the expression of *PER2*, *PER2AS*, *CRY1*, *CRY1AS,* and *PSMB2* genes, qRT-PCR was performed in a volume of 20 µl using 10 µl SYBR Green 2X master mix (Yekta Tajhiz Azma, Tehran, Iran), 1 µl cDNA, and 0.5 µl of each primer. The Proteasome 20S Subunit Beta 2 (*PSMB2*) as a reference gene was used for normalization. The PCR was carried out with the following protocol: 1 cycle at 95 °C for 30 sec, followed by 40 cycles at 95 °C for 5 sec, 60 °C for 30 sec and 72 °C for 40 sec. Table 1 shows the list of primer sequences that were applied in this study. The PCR products were checked on 2% agarose gel with 100 bp DNA ladder.

Table 1. Primer's information was used in this study.

Gene		Sequence	Amplicon (bp)
PSMB2	F	ACGGCAGCAGCTAACTTCACA	108
	R	TGGCCCTTCATGCTCATCA	
PER2	F	TTGGACAGCGTCATCAGGTA	109
	R	TCCGCTTATCACTGGACCTT	
CRY1	F	TGTGATTCGTGGACAACCAG	116
	R	TAGCTGCGTCTCGTTCCTTT	
PER2AS	F	ACCACTGCTCCTTGATGC	173
	R	CACCACACTGCGAAGAAC	
CRY1AS	F	GTCCCTGCTGAAGATGAGGA	107
	R	TTCCCTCTTCCTTGTTTCCCA	

Statistical analysis

The specificity of primers was controlled by NCBI blastn tool. Relative level of gene expression was obtained from the comparative ΔΔCT Method (CT Method). Charts were drawn using the SAS JMP Statistical Discovery software (version 13.0.0). Cross-correlation function was calculated by R software (version 3.3.2). The data of common miRNAs between the sense and antisense transcripts were collected from the miRDB database and presented in Table 2.

Table 2. The common miRNAs between the overlapping sequences of *PER2AS, CRY1AS* and their sense strands.

PER2AS & PER2	***CRY1AS & CRY1***
hsa-miR-4641	hsa-miR-92a-2-5p
hsa-miR-6504-3p	hsa-miR-128-1-5p
hsa-miR-5683	hsa-miR-128-2-5p
hsa-miR-208b-5p	
hsa-miR-208a-5p	
hsa-miR-5089-5p	
hsa-miR-504-3p	
hsa-miR-10399-5p	
hsa-miR-3140-3p	
hsa-miR-744-3p	
hsa-miR-6778-3p	
hsa-miR-4775	
hsa-miR-619-5p	
hsa-miR-5089-5p	
hsa-miR-6506-5p	

Results

PER2AS and CRY1AS follow similar oscillatory pattern with target mRNA

Evaluation of *PER2AS* and *CRY1AS* expression along with *PER2* and *CRY1* genes after horse serum shock treatment over 24 hours, showed that *PER2AS* and *CRY1AS* have a circadian oscillatory pattern and this oscillation is similar to the expression pattern of their sense strand. Indeed, although the expression level of them varies at different time points but display similar oscillatory behavior with *PER2* and *CRY1* genes (Fig. 1). in addition, it seems that the circadian expression pattern of *PER2* and *CRY1* genes be in harmony with each other**.**

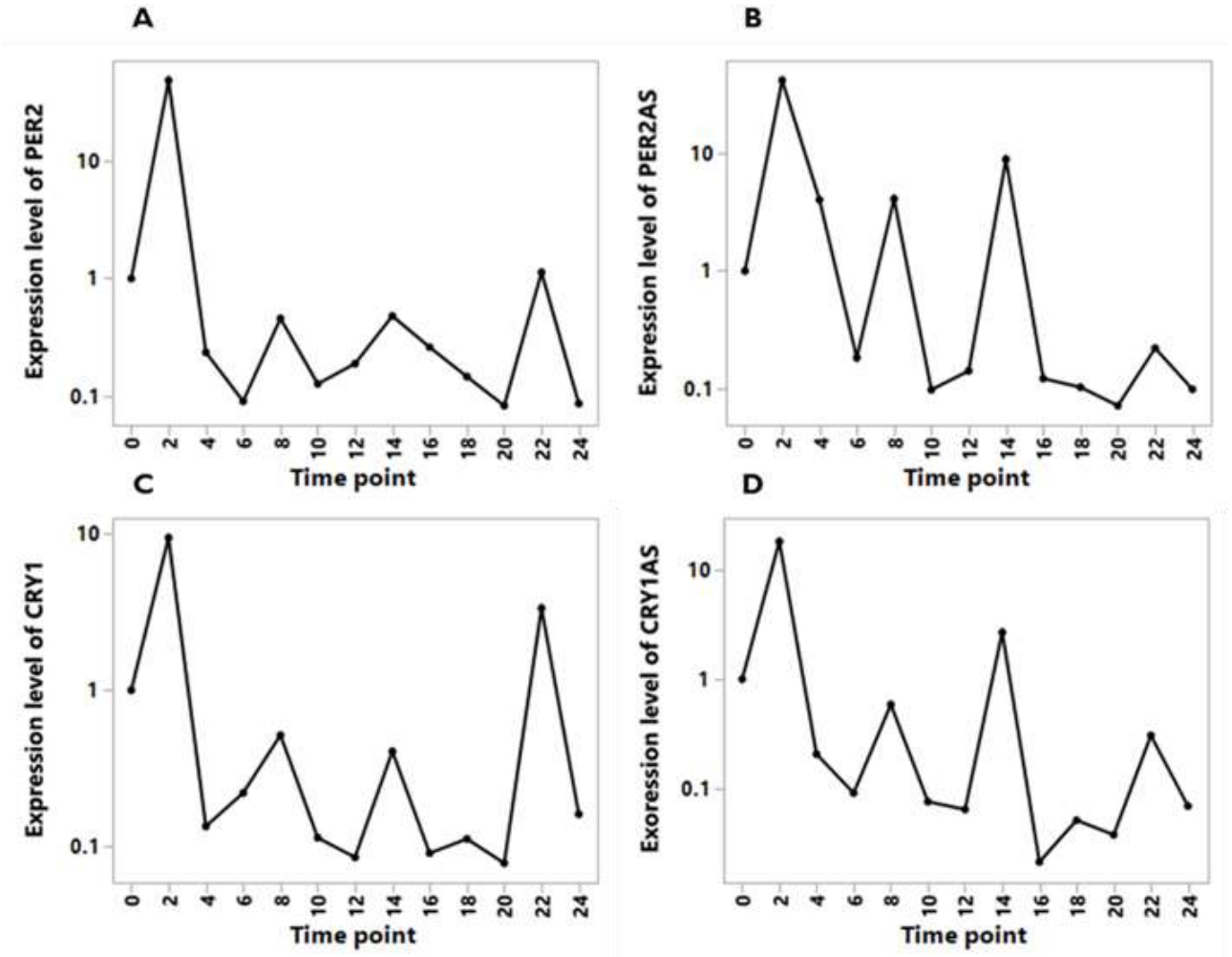

Fig. 1. oscillation pattern of the *PER2* (A), *PER2AS* (B) *CRY1* (C), *CRY1AS* (D) transcripts within 24 hrs in A549 cell line. The expression level of all transcripts following the horse serum shock was elevated relative to the control group. Similar oscillatory behavior was observed between both sense-antisense transcript pairs.

PER2AS and CRY1AS are expressed coincidently with PER2 and CRY1

Cross-correlation analysis revealed that the expression of both antisense transcripts coincides with their target mRNA without time lag. the highest correlation coefficient for *PER2AS-PER2* and *CRY1AS-CRY1* transcript pairs were 0.97 and 0.93, respectively. Also, the strongest correlation of *CRY1* and *PER2* genes was observed at lag= 0 (r= 0.94), which means, their expression was correlated to each other (Fig. 2).

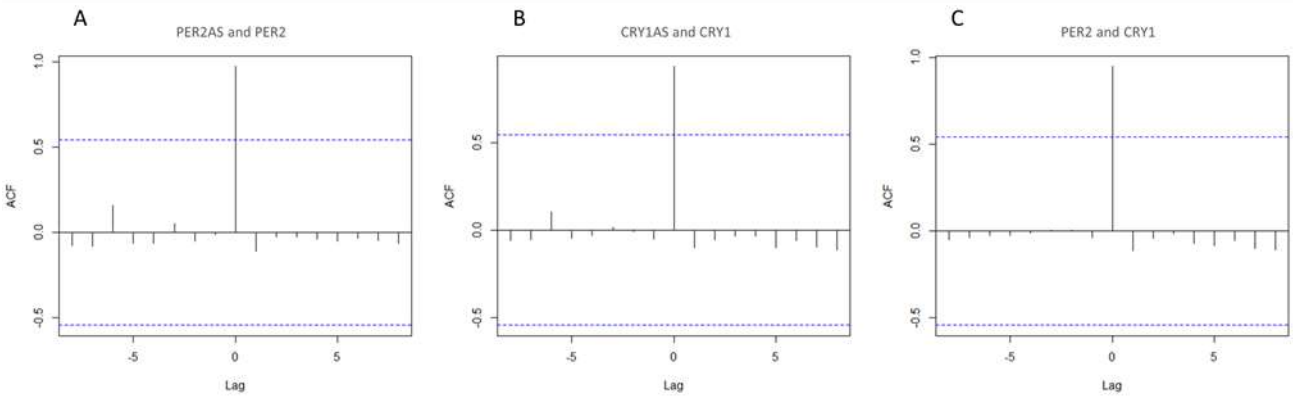

Fig. 2. Cross-correlation analysis between the expression of *PER2AS-PER2, CRY1AS-CRY1* and *PER2-CRY1* gene pairs. (A-C) there is the maximum positive correlation for all transcripts at lag= 0. Horizontal dashed line as a threshold line determines the significance level of cross correlation between 2 genes**.**

Discussion

Today, it has been revealed that some antisense transcripts play a pivotal role in cellular and molecular processes (24, 25). Based on this findings, antisense transcripts of the circadian clock are probably involved in its regulation. So, in this study, we assessed the expression oscillation of NATs along with their ancestral genes in the clock. Our results showed that *PER2AS* and *CRY1AS* as the sole antisense transcripts in the molecular clock genes, have a rhythmic expression pattern within 24 hours and the circadian fluctuation of them is similar to *PER2* and *CRY1* mRNAs, respectively. According to ccf analysis, the strongest positive correlation between the expression of these sense and antisense transcripts was observed at lag= 0. In fact, they oscillate synchronously but independently of each other. Recently, a study demonstrated that the mouse *Per2as* transcript also exhibits circadian rhythmic expression, except that its fluctuation is antiphasic with the m*Per2* mRNA. However, it was reported that the *mPer2* level enhanced dramatically following the overexpression of *mPer2as* (22). Although the majority of asRNAs interfere with the expression of their target genes, the *PER2AS-PER2* and *CRY1AS-CRY1* pairs are expressed in coordination with each other. Therefore, it can be suggested that *PER2AS* and *CRY1AS* regulate the expression of *PER2* and *CRY1* by masking the miRNAs binding site within *PER2* and *CRY1* mRNAs.

In conclusion, *PER2AS* and *CRY1AS* may have a regulatory function in the clock network and further studies are needed to be performed about their biological and pathological significance.

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The Authors declare that there is no conflict of interest.

References

1. Relógio A, Westermark PO, Wallach T, Schellenberg K, Kramer A, Herzel H. Tuning the mammalian circadian clock: robust synergy of two loops. PLoS Comput Biol. 2011;7(12):e1002309.

2. Mohamadian S, Golalipour M, Yazdani Y, Farazmandfar T, Tabarraei A, Shahbazi M. Oscillation in expression of Adenylyl Cyclase isoforms: new insight to regulation of molecular clock. Biological Rhythm Research. 2017;48(1):113-9.

3. Pickel L, Sung H-K. Feeding rhythms and the circadian regulation of metabolism. Frontiers in nutrition. 2020;7:39.

4. Guan D, Xiong Y, Trinh TM, Xiao Y, Hu W, Jiang C, et al. The hepatocyte clock and feeding control chronophysiology of multiple liver cell types. Science. 2020;369(6509):1388-1394.
5. Rijo-Ferreira F, Takahashi JS. Genomics of circadian rhythms in health and disease. Genome medicine. 2019;11(1):1-16.
6. Hernández-Rosas F, López-Rosas CA, Saavedra-Vélez MV. Disruption of the molecular circadian clock and cancer: an epigenetic link. Biochem Genet. 2020;58(1):189-209.
7. Ye Y, Xiang Y, Ozguc FM, Kim Y, Liu C-J, Park PK, et al. The genomic landscape and pharmacogenomic interactions of clock genes in cancer chronotherapy. Cell Syst. 2018;6(3):314-28.e2.
8. Chen Z, Liu P, Li C, Yongluo, Chen I, Liang W, et al. Deregulated expression of the clock genes in gliomas. Technol Cancer Res Treat. 2013;12(1):91-7.
9. Roenneberg T, Merrow M. The circadian clock and human health. Curr Biol. 2016;26(10):R432-43.
10. Pett JP, Korenčič A, Wesener F, Kramer A, Herzel H. Feedback loops of the mammalian circadian clock constitute repressilator. PLoS computational biology. 2016;12(12):e1005266.
11. Koike N, Yoo S-H, Huang H-C, Kumar V, Lee C, Kim T-K, et al. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. science. 2012;338(6105):349-54.
12. Angelousi A, Kassi E, Ansari-Nasiri N, Randeva H, Kaltsas G, Chrousos G. Clock genes and cancer development in particular in endocrine tissues. Endocr Relat Cancer. 2019;26(6):R305-R317.
13. Lindsay MA, Griffiths-Jones S, Wight M, Werner A. The functions of natural antisense transcripts. Essays Biochem. 2013;54:91-101.
14. Barman P, Reddy D, Bhaumik SR. Mechanisms of antisense transcription initiation with implications in gene expression, genomic integrity and disease pathogenesis. Non-coding RNA. 2019;5(1):11.
15. Halley P, Khorkova O, Wahlestedt C. Natural antisense transcripts as therapeutic targets. Drug Discov Today Ther Strateg. 2013;10(3):e119-e125.
16. Zhao Y, Liu Y, Lin L, Huang Q, He W, Zhang S, et al. The lncRNA MACC1-AS1 promotes gastric cancer cell metabolic plasticity via AMPK/Lin28 mediated mRNA stability of MACC1. Molecular cancer. 2018;17(1):1-16.
17. Yaghoobi H, Azizi H, Banitalebi-Dehkordi M, Rezaei FM, Arsang-Jnag S, Taheri M, et al. Beta-secretase 1 (BACE1) is down-regulated in invasive ductal carcinoma of breast. Rep Biochem Mol Biol. 2019;8(2):200-207.
18. Fattahi S, Nikbakhsh N, Taheri H, Ranaee M, Akhavan-Niaki H. RNA Sequencing of Early-Stage Gastric Adenocarcinoma Reveals Multiple Activated Pathways and Novel Long Non-Coding RNAs in Patient Tissue Samples. Rep Biochem Mol Biol. 2021;9(4):478-489.
19. Khorkova O, Myers AJ, Hsiao J, Wahlestedt C. Natural antisense transcripts. Human molecular genetics. 2014;23(R1):R54-R63.
20. Faghihi MA, Zhang M, Huang J, Modarresi F, Van der Brug MP, Nalls MA, et al. Evidence for natural antisense transcript-mediated inhibition of microRNA function. Genome biology. 2010;11(5):1-13.
21. Zhang X, Zhou Y, Chen S, Li W, Chen W, Gu W. LncRNA MACC1-AS1 sponges multiple miRNAs and RNA-binding protein PTBP1. Oncogenesis. 2019;8(12):1-13.
22. Mosig RA, Castaneda AN, Deslauriers JC, Frazier LP, He KL, Maghzian N, et al. Natural antisense transcript of Period2, Per2AS, regulates the amplitude of the mouse circadian clock. Genes & Development. 2021;35(11-12):899-913.
23. Fekry B, Ribas-Latre A, Baumgartner C, Deans JR, Kwok C, Patel P, et al. Incompatibility of the circadian protein BMAL1 and HNF4α in hepatocellular carcinoma. Nature communications. 2018;9(1):1-17.
24. Statello L, Guo C-J, Chen L-L, Huarte M. Gene regulation by long non-coding RNAs and its biological functions. Nature Reviews Molecular Cell Biology. 2021;22(2):96-118.
25. Zhao S, Zhang X, Chen S, Zhang S. Natural antisense transcripts in the biological hallmarks of cancer: powerful regulators hidden in the dark. Journal of Experimental & Clinical Cancer Research. 2020;39(1):1-18.