Circadian clock gene *Period2* suppresses human chronic myeloid leukemia cell proliferation

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Abstract. Circadian clock genes (CCGs) are reported to serve pivotal roles in regulating the development of certain tumors, including lung cancer and colon cancer. However, their expression patterns and function in chronic myeloid leukemia (CML) remains poorly understood. The present study aimed to investigate the expression and function of circadian clock gene *Period2* (*Per2*) in human CML. *Per2* expression levels in neutrophils isolated from patients with CML and healthy donors were measured via reverse transcription-quantitative PCR. Subsequently, through lentivirus transduction, *Per2* was stably overexpressed in human CML cell line KCL22 cells, which were injected into nude mice to investigate the *in vivo* role of *Per2* by measuring CML tumor size and weight. Additionally, *Per2* expression levels in patients with acute myeloid leukemia (AML) or chronic lymphocytic leukemia (CLL) were analyzed by re-analyzing microarray data in the Gene Expression Omnibus database. *Per2* expression was significantly lower in neutrophils isolated from patients with CML compared with healthy donors, and was negatively correlated with the expression level of c-Myc. Similarly, patients with AML or CLL displayed lower *Per2* expression levels compared with healthy controls. *Per2* overexpression inhibited KCL22 cell proliferation in nude mice and *in vitro*, and induced cell cycle arrest at the G₁ phase. By contrast, the results also indicated that KCL22 cell apoptosis was not regulated by *Per2*. The present study identified *Per2* as a potential tumor suppressor in human CML.

Introduction

Circadian clock genes (CCGs) are indispensable regulators responsible for governing host rhythmic activities according to the 24-h solar cycle. Mechanistically, CCGs control circadian clock-dependent behaviors by modulating a wide range of physiological processes such as sleeping, appetite regulation, hormone secretion or cellular metabolism (1-3). To date, several CCGs have been identified, including *CLOCK*, *Bmal1*, *Period* family, *Cry1/2*, *CKIε* and *TIM* (4,5). *Period2* (*Per2*) belongs to the *Period* family and serves as a crucial regulator of the mammalian circadian clock (6). *Per2* deletion in mice causes severe arrhythmicity (7). Besides its roles in controlling the circadian rhythm, emerging evidence has demonstrated that *Per2* regulates the biological behaviors of tumor cells. For example, *Per2* induce mouse lung and breast cancer cell apoptosis (8). Moreover, *Per2* deficient mice are more prone to γ radiation-triggered tumor development (9). However, the effects of *Per2* on human cancer are not completely understood and require further investigation.

Chronic myeloid leukemia (CML) is a myeloproliferative disease that has an incidence of 1-2/100,000 individuals worldwide (10). The most common etiology of CML is fusion of the *BCR* gene with the *ABL* gene resulting from chromosome translocation (11). Identification of novel molecular targets that control the malignant behavior of CML cells may aid the diagnosis and treatment of the disease.

In the present study, we investigated the expression patterns of *Per2* in patients with CML, acute myeloid leukemia (AML) or chronic lymphocytic leukemia (CLL), as well as the role of *Per2* on CML cell function both *in vitro* and in a mouse CML model.

Materials and methods

Human specimens. Peripheral blood samples were collected from 30 patients with CML patients (21 male patients and 9 female patients; age, 19-86 years; average age, 56 years) and 30 healthy donors (18 male donors and 12 female donors, age: 21 to 77 years, 51 for average) from Yantai Yuhuangding Hospital between September 2016 and March 2018. Neutrophils were isolated from the peripheral blood samples using the Human Neutrophil Isolation kit (Tianjin Haoyang Biological Co., Ltd.). All patients provided written informed

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Abbreviations: *Per2*, *Period2*; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CCG, circadian clock gene

Key words: *Per2*, CML, cell proliferation, cell cycle

consent. The experimental protocol was approved by the Ethic Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University (approval no. 2016-185).

Cell culture. KCL22 cells (American Type Culture Collection) were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) at 37°C in a 5% CO₂ incubator.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from patient blood neutrophils, KCL22 cells and mouse tumor tissues was extracted using TRIzol (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent kit (Takara Bio, Inc.) using the following parameters: 37°C for 30 min and 85°C for 3 min. Subsequently, qPCR was performed on a ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR-Green reagent (Kangwei), according to the manufacturer's protocol. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The primer sequences used for qPCR are listed in Table I. mRNA expression levels were quantified using the 2^{-ΔΔC_q} method (12) and normalized to the internal reference gene β-actin.

Lentiviral transduction. Per2-encoding and control lentiviruses were constructed by Shanghai SunBio Biotechnology Co., Ltd. A total of 5x10⁵ KCL22 cells were seeded into 6-well plates with lentiviral particles (MOI=40) and polybrene (5 μg/ml). Following incubation for 24 h at 37°C, the culture medium was replaced with fresh RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 2 μg/ml puromycin. Cells were cultured for 15 days at 37°C to obtain stably transfected KCL22 cells and were then used for the following experiments.

Western blotting. KCL22 cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) on ice for 50 min. The amount of protein was determined using an Ads Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). A total of 20 μg/lane protein was separated via 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% fat-free milk at room temperature (18-25°C) for 90 min. Subsequently, the membranes were incubated with anti-Per2 (Abcam; ab179813; 1:1,000) or anti-GAPDH (Abcam; ab181602; 1:3,000) primary antibodies at 4°C overnight. Following primary incubation, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam; ab205718; 1:2,000) at room temperature (18-25°C) for 60 min. The bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) on a ChemiDoc machine (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 (CCK-8) assay. A total of 4x10³ KCL22 cells were seeded into 96-well plates and cultured at 37°C overnight. Subsequently, 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) was added to each well and incubated at 37°C for 3.5 h. The absorbance of each well was measured every 24 h for a total of 72 h at a wavelength of 450 nm using a microplate reader.

Lactate dehydrogenase (LDH) release assay. A total of 2x10⁴ KCL22 cells were seeded into a 96-well plate and cultured at 37°C overnight. The concentration of LDH in culture supernatant was measured using a LDH Assay kit (Abcam), according to the manufacturer's protocol. The absorbance (OD value) was read on a Microplate Reader (Promega Corporation) at the wavelength of 450 nm.

Tumor model. A total of 54 nude male mice (age, 6-8 weeks; weight, 20-25 g) were purchased from Shanghai SLAC Laboratory Animal Center) and were housed in specific pathogen free conditions. The housing conditions included a temperature of 23±2°C, 50% humidity and 12 h light/dark cycle. Mice were given food and water *ad libitum*. The mice received a subcutaneous injection of KCL22 cells (1x10⁶) in PBS into the left flank. The mice were divided into three groups: The PBS group in which mice were injected with PBS only, the Lv-scramble (scr) group in which mice were injected with KCL22 cells transduced with Lv-scr and the Lv-Per2 group in which mice were injected with KCL22 cells transduced with Lv-Per2 (n=6 mice per group). Tumor volume was measured and calculated using a caliper every 4-5 days. Tumor volume was calculated as: V = length x width²/2. On day 20-22, when the mean tumor volume in the control group reached ~1,200 mm³ (maximum tumour volume was ~1,400-1,500 mm³), mice were sacrificed by cervical dislocation. Death was verified by cessation of the heartbeat and lack of movement. Subsequently, the tumors were removed to evaluate tumor weight and gene expression. Multiple tumours were not observed in any of the mice. The animal experimental protocols were approved by the Qingdao University Ethics Committee (approval no. QD20161183).

Cell cycle detection. KCL22 cells were centrifuged at 300 x g and 4°C for 5 min, followed by permeabilization in 70% ethanol and fixation at 4°C overnight. Subsequently, cells were washed with PBS and incubated with 1 ml PBS containing 40 μl RNase and 20 μl propidium iodide (Beyotime Institute of Biotechnology) at 37°C in the dark for 10 min. Cell cycle distribution was detected by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The results were analyzed using FlowJo software (version VX1; Tree Star).

Cell apoptosis detection. KCL22 cells were washed with ice-cold PBS. Subsequently, 2x10⁵ cells were incubated in 200 μl binding buffer containing 5 μl 7-AAD and 5 μl APC-Annexin V (Biolegend Inc.) at room temperature for 10 min. Following centrifugation at 300 x g, 4°C for 5 min, cell pellets were resuspended in PBS and assessed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). The percentages of early and late apoptotic cells (Annexin V⁺, 7-AAD⁺ and Annexin V⁺7-AAD⁺) were analyzed using FlowJo software (version VX10; Tree Star).

GEO datasets. The numbers and URL links of the two Gene Expression Omnibus (GEO) datasets analyzed were: GDS2643 (ncbi.nlm.nih.gov/gds/?LinkName=geoprofiles_gds&from_uid=36949877) (13), in which gene expression profiles between chronic lymphocytic leukemia patients and healthy controls were analyzed, and GDS4407 (ncbi.nlm.nih.gov/gds/?LinkName=geoprofiles_gds&from_uid=88969842) (14),

Figure 1. Per2 expression decreased in patients with CML. Neutrophils were isolated from patients with CML and healthy controls. (A) The expression of Per2, c-Myc, cyclin D1 and Wee1. (B) The correlation between Per2 expression and c-Myc/cyclin D1/Wee1 expression was analyzed by Spearman's rank correlation test. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. CML, chronic myeloid leukemia; Per2, Period2; Wee1, WEE1 G2 checkpoint kinase.

Table I. Primer sequences used for quantitative PCR.

Gene	Sequence (5'→3')
Per2	F: TTGGACAGCGTCATCAGGTA R: TCCGCTTATCACTGGACCTT
c-Myc	F: CAACCCTTGCCGCATCCAC R: CCTCTCGTCGCAGTAGAA
Cyclin D1	F: CCCTCGGTGTCCTACTTCA R: CTCCTCGCACTTCTGTTCCT
Wee1	F: TGTGGTGGTGTGCTGCTTAT R: TTCAAAGGGAGGGTATGTCTG
β -actin	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT

Per2, Period2; Wee1, WEE1 G2 checkpoint kinase; F, forward; R, reverse.

in which gene expression profiles between acute myeloid leukemia patients and healthy controls were compared.

Statistical analysis. Data are presented as the mean $\pm$ standard deviation. All experiments were performed in triplicated. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Spearman's rank correlation test was performed to investigate the relationship between Per2 expression and c-Myc, cyclin D1 or WEE1 G2 checkpoint kinase (Wee1)

expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Per2 expression is decreased in neutrophils from patients with CML. First, the expression of Per2 in peripheral neutrophils isolated from patients with CML and healthy controls was assessed. Patients with CML displayed significantly lower Per2 expression compared with healthy controls. In terms of cell cycle-associated genes, c-Myc and cyclin D1 expression levels were significantly increased, whereas Wee1 expression was significantly decreased in patients with CML compared with healthy controls (Fig. 1A). Moreover, in patients with CML, there was a significant negative correlation between Per2 and c-Myc mRNA expression levels (Fig. 1B). However, Per2 expression was not significantly correlated with cyclin D or Wee1 expression. The results indicated that CML development might be associated with reduced Per2 expression.

Per2 overexpression suppresses CML cell proliferation in vivo and in vitro. To investigate the role of Per2 during CML tumor development, KCL22 cells that stably overexpressed Per2 were generated by lentiviral transduction [lentivirus (Lv)-Per2 KCL22]. The RT-qPCR and western blotting results indicated successful Per2 overexpression in KCL22 cells (Fig. 2A and B). Subsequently, Lv-Per2 KCL22 cells and control KCL22 cells were subcutaneously injected into nude mice. By monitoring tumor volume, the results indicated that when compared mice injected with PBS or Lv-scr KCL22 cells, Per2 overexpression significantly suppressed KCL22 cell proliferation in mice (Fig. 2C). Consistently, tumor weight was significantly

Figure 2. Per2 overexpression inhibits KCL22 cell proliferation *in vivo* and *in vitro*. Per2 overexpression was induced in KCL22 cells by lentiviral transduction. Per2 (A) mRNA and (B) protein expression levels. (C) Nude mice were subcutaneously injected with 1×10^6 KCL22 cells and tumor growth was monitored. Representative tumors on day 22 are presented (scale bar, 1 cm). (D) Tumor weight was measured on day 22. The expression of (E) Per2, (F) c-Myc, cyclin D1 and Wee1 in tumor tissues. (G) KCL22 cell proliferation was measured by performing the Cell Counting Kit-8 assay. (H) The expression of c-Myc, cyclin D1 and Wee1 in KCL22 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the Lv-Per1 group. Per2, Period2; Wee1, WEE1 G2 checkpoint kinase; Lv, lentivirus; scr, scramble; OD, optical density.

reduced in mice injected with Per2-overexpression KCL22 cells when compared mice injected with PBS or Lv-scr KCL22 cells (Fig. 2D). As expected, Per2 expression was significantly enhanced in Lv-Per2 tumors compared with controls and Lv-scr tumors (Fig. 2E). Furthermore, the expression levels of proliferative genes c-Myc and cyclin D were significantly

reduced in Lv-Per2 tumors compared with controls and Lv-scr tumors (Fig. 2F). To further investigate the role of Per2 on CML cell proliferation *in vitro*, the CCK-8 assay was performed. The results suggested that compared with controls and Lv-scr-transfected cells, Per2 overexpression significantly suppressed KCL22 cell proliferation at the 24, 48 and 72 h time

Figure 3. Per2 does not regulate human chronic myeloid leukemia cell apoptosis. KCL22 cells were transfected with Lv-scr or Lv-Per2 for 48 h. KCL22 cell apoptosis was (A) evaluated by flow cytometry and (B) quantified. (C) LDH release in the culture supernatant was measured. Per2, Period2; Lv, lentivirus; scr, scramble; LDH, lactate dehydrogenase.

points (Fig. 2G). Moreover, the levels of c-Myc, cyclin D1 and Wee1 were significantly decreased in Lv-Per2 cells compared with controls and Lv-scr cells (Fig. 2H). The results suggested that Per2 may serve as a suppressor of *in vivo* and *in vitro* CML cell proliferation.

Per2 does not regulate human CML cell apoptosis. Subsequently, whether the antiproliferative role of Per2 on KCL22 cells was associated with a proapoptotic effect of Per2 was investigated. The percentage of apoptotic cells was comparable among controls, Lv-scr and Lv-Per2 cells, which indicated that Per2 did not regulate CML cell apoptosis (Fig. 3A). Additionally, the LDH assay demonstrated that compared with controls and Lv-scr cells, Lv-Per2 cells did not display significantly altered levels of cell apoptosis (Fig. 3B). Therefore, the results suggested that the antiproliferative effect of Per2 was not due to enhanced cell apoptosis.

Per2 induces cell cycle arrest in human CML cells. Based on the aforementioned result that Per2 overexpression reduced the expression of cell cycle-related genes such as cyclin D1, it was hypothesized that Per2 regulated the cell cycle of CML cells. To investigate the hypothesis, PI staining was performed. The percentage of G₁-phase cells was significantly higher in Lv-Per2 KCL22 cells compared with controls and Lv-scr KCL22 cells. By contrast, the percentage of S/G₂-phase cells was significantly decreased in Lv-Per2 KCL22 cells compared with controls and Lv-scr KCL22 cells (Fig. 4A and B). The ratio of G₁-phase cells to S/G₂-phase cells was significantly increased by Per2 overexpression compared with controls and

Lv-scr cells (Fig. 4C). Therefore, the results suggested that Per2 induced G₁/S cell cycle arrest in human CML cells.

Per2 expression is reduced in patients with AML and CLL. Finally, Per2 expression was assessed in two other types of leukemia, AML and CLL. To assess Per2 expression in patients with AML and CLL, data mining from the Gene Expression Omnibus database was performed. Consistent with the results obtained for patients with CML, the expression level of Per2 was significantly reduced in peripheral blood mononuclear cells (PBMCs) isolated from patients with AML compared with healthy donors. Similarly, compared with healthy controls, B cells from patients with CLL displayed significantly lower Per2 expression (Fig. 5). The results indicated that Per2 may be involved in regulating multiple kinds of leukemia.

Discussion

CCGs are ubiquitously expressed in the vast majority of mammalian cells (15). In addition to the involvement in controlling circadian rhythm, increasing evidence has indicated that CCGs are involved in modulating tumor development (16,17). Moreover, abnormal Per2 expression was observed in skin, breast and gastric cancer, as well as head and neck squamous cell carcinoma (18-21). In the present study, the expression levels of Per2 were downregulated in patients with CML compared with healthy individuals, which suggested that Per2 may serve as a diagnostic marker for CML. A previous study had also reported lower expression of Per2 in PBMCs isolated from patients with CML (22); however, PBMCs also contain

Figure 4. Per2 overexpression induces G₁-phase cell cycle arrest. (A) KCL22 cell cycle distribution was assessed by propidium iodide staining followed by flow cytometry. (B) The percentage of G₁ and S/G₂-phase cells. (C) The ratio of G₁ to S/G₂-phase cells. Lv, lentivirus; scr, scramble; Per2, Period2. ***P<0.001 vs. the Lv-Per2 group.

Figure 5. Per2 expression in patients with AML and CLL. Per2 expression was analyzed using Gene Expression Omnibus microarray data (GDS2643 and GDS4407). *P<0.05 and ***P<0.001 as indicated. Per2, Period2; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia.

a large proportion of non-myeloid cells such as T and B cells. Therefore, in the present study, in order to obtain more reliable results, peripheral neutrophils were enriched instead of PBMCs to examine Per2 expression. The results also indicated that Per2 expression was negatively correlated with c-Myc expression, an oncogene whose overexpression often leads to cell hyperproliferation (23). By contrast, Per2 overexpression inhibited CML cell proliferation both *in vitro* and *in vivo*. Mechanistically, Per2 overexpression facilitated cell cycle arrest at G₁ phase. Based on the suppressive role of Per2 in CML cell proliferation, it was speculated that Per2 may serve as a detrimental factor in diseases such as granulocytopenia. Moreover, the detailed molecular mechanisms underlying the antiproliferative function of Per2 require further investigation.

In addition to the *in vitro* results, the results also suggested that compared with mice inoculated with PBS or Lv-scr-KCL22 cells, Per2 overexpression significantly reduced CML tumor growth in mice. Furthermore, Per2 overexpression reduced the expression levels of c-Myc, cyclin D1 and Wee1 in tumor tissues, compared with tumor tissues from mice inoculated with PBS or Lv-scr-KCL22 cells. The *in vivo* results suggested that targeting Per2 may serve as a potential therapeutic strategy for CML.

On the other hand, the finding that Per2 did not alter cell apoptosis was contradictory to a previous study, which reported that Per2 overexpression induced LLC mouse lung cancer cell and EMT6 mouse breast cancer cell apoptosis (5). A potential explanation for the inconsistency could be that the apoptosis-inducing role of Per2 is cell-type specific; therefore, whether Per2 affects CML cell apoptosis under certain stress conditions (such as hypoxia or chemotherapy treatment) requires further investigation. Furthermore, Per2 expression was also decreased in patients with AML and CLL, which indicated that Per2 may also have diagnostic value in other kinds of leukemia as well as in CML. However, the exact impacts of Per2 on AML and CLL require further investigation. For example, a mouse model of AML or CLL could be used to investigate whether Per2 also suppresses AML or CLL tumor growth and how Per2 alters the expression of oncogenes such as c-Myc. From a clinical view, the relationship between Per2 expression and the clinical characteristics of patients with AML or CLL, such as tumor stage or patient prognosis, requires further investigation. Moreover, the molecular mechanisms underlying the regulatory roles of Per2 on leukemia also need to be investigated in future studies. Due to the crucial function of Per2 in modulating circadian clock-dependent behaviors, it

is possible that circadian rhythmic activities may impact the development of leukemia.

Collectively, the present study identified *Per2* as a candidate tumor suppressor, which may have potential diagnostic or therapeutic values in CML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Furthermore, the GEO datasets analyzed during the current study are available at: ncbi.nlm.nih.gov/gds/?LinkName=geoprofiles_gds&from_uid=36949877 and ncbi.nlm.nih.gov/gds/?LinkName=geoprofiles_gds&from_uid=88969842.

Authors' contributions

CS conceived and designed the present study. MM and XW collected the clinical specimens. NW, MM and XW performed the experiments and analyzed the data. NW and CS drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University (approval no. 2016-185). Written informed consent was obtained from all patients. The animal experimental protocols were approved by the Qingdao University Ethics Committee (approval no. QD20161183).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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