

Disrupted Expression of Circadian Clock Genes in Patients with Bronchial Asthma

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Purpose: Circadian clock is synchronized to the 24-hour day by the daily light–dark cycle and proper function of circadian rhythm is essential for many physiological processes. Disruption of circadian rhythm can affect disease processes and influence disease severity, treatment responses, and even survivorship. In this retrospective case-controlled study, we tried to explore whether expression of circadian clock genes was disturbed in patients with bronchial asthma.

Patients and Methods: We performed real-time quantitative reverse transcriptase-polymerase chain reactions to examine the expression of the nine core circadian clock genes (*BMAL1*, *CK1ε*, *CLOCK*, *CRY1*, *CRY2*, *PER1*, *PER2*, *PER3*, and *TIM*) in total leukocytes of peripheral blood collected at chest clinics from 120 patients with asthma and 60 health individuals.

Results: Expression levels of the nine circadian clock genes were significantly different between patients and healthy individuals, but not associated with the asthma control status. We also noted the difference of *PER3* expression in asthmatic patients with and without nocturnal symptoms. In well-controlled asthmatics, expression of *BMAL1*, *CK1ε*, *CLOCK*, *CRY1*, *CRY2*, and *PER1* was significantly lower in patients with nocturnal symptoms than those without nocturnal symptoms. However, in not well-controlled asthmatics, expression of only *BMAL1*, *CK1ε*, *PER1*, and *PER2* was significantly different between patients with and without nocturnal symptoms. Binary logistic regression analysis selected *BMAL1*, *CK1ε*, *PER3*, and *TIM* as independent factors for bronchial asthma and ROC curves showed the combined expression of these four genes enhanced the capability of predicting asthma (AUC=0.924; 95% CI=0.875–0.958; *P*<0.001).

Conclusion: Our results showed altered expression of circadian clock genes in patients with bronchial asthma and down-regulated *PER3* in patients with nocturnal symptoms. Altered expression of circadian clock genes was also observed in asthmatics with or without nocturnal symptoms in well- or not well-controlled subgroups. Combined expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM* could be a potential predictor for bronchial asthma.

Keywords: bronchial asthma, nocturnal symptom, circadian clock genes, *PER3*

Introduction

Bronchial asthma is characterized by variable airflow limitation and chronic airway inflammation with a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity.¹ It is an important characteristic of asthma that the appearance of symptoms displays a circadian rhythm² and frequently exacerbates in the early morning hour at around 4:00AM, which is also an occurrence time of sudden death.³ It has been reported that about 74% of asthmatic patients are awakened by asthma symptoms at least

once per week and around 40% of patients experienced symptoms nightly.³ Nocturnal asthma is typically defined as the difference in forced expiratory volume in 1s (FEV1) and peak expiratory flow rate between daytime and night is greater than 15% in patients with asthma.⁴ This overnight decline of lung function causes a more severe form of the disease and is associated with airway inflammation and hyper-responsiveness.⁵

Circadian rhythms are intrinsic biological oscillations with an interval near 24 hours operated in mammals by the circadian clock system.⁶ Molecular circadian systems are found at the cellular level by an autoregulatory transcriptional-translational feedback loop operated with at least nine circadian clock genes including *PER1*, *PER2*, *PER3*, *CLOCK*, *CRY1*, *CRY2*, *BMAL1*, *CK1 ϵ* , and *TIM*.⁷ The circadian presentations of asthma, like airflow limitation and airway hyper-responsiveness, were associated with chronic inflammation and it may be secondary to changes of the parasympathetic nervous system and endocrine system at night.^{8,9} Circadian clock genes also regulate inflammatory responses and contribute to lung inflammation, fibrosis, immunity, and glucocorticoid response.^{10–12} Therefore, increasing evidence demonstrates the molecular circadian clock is important in the pathogenesis of asthma.

The clock in the monocyte of peripheral blood mononuclear cells (PBMC) controlled the rhythmic expression of downstream genes¹³ and influenced on innate immunity including gating of pattern-recognition receptors response, clock-controlled cytokine response and recruitment to tissues.^{13–15} Rhythmic cytokine secretion from monocytes has been reported in humans under sleep/wake and laboratory conditions.^{16,17} In addition, a rhythm of the adaptive immune response was measured as antibody production at different times of the day.¹⁸ That hints that either T or B lymphocyte response and the adaptive immune system was associating with the molecular circadian clock.^{19,20} Some asthmatic patients with nocturnal symptoms had a significantly higher number of alveolar eosinophils at 4:00AM than at 4:00PM, and the increase of alveolar eosinophils correlated with a decrease in FEV1.^{3,21} Also, the number of macrophages, neutrophils and CD4 T-cells in the bronchoalveolar lavage fluid from patients with asthma were higher at 4:00AM than at 4:00PM, and the increase of CD4 T-cells also correlated with decreased FEV1.^{3,21}

Recently, several studies found circadian clock underpins allergic reaction and possibly influences the

periodicity of allergic disease.^{22–24} The induction of asthma was associated with markedly increased inflammation in the lungs in mice lacking *Bmal1* expression in myeloid cells, and a higher numbers of eosinophils and increased IL-5 levels in the lung and serum.²⁵ Since altered circadian rhythm deregulates epithelial barrier function and immune function, the two fundamental biological aspects of allergic disease, the circadian clock is strongly related to allergic disease.

For the substantial associations of circadian presentations between asthma and allergic diseases, and for the lack of reports about expression of circadian clock genes in patients with asthma, we therefore hypothesized that the expression of circadian gene expression might be disrupted in asthmatic patients and correlated with nocturnal symptoms of asthmatic patients. To test our hypothesis, we constructed a retrospective case-controlled study focusing on expression of circadian clock genes of PBMCs in patients with bronchial asthma and healthy individuals and to explore the correlation of circadian clock gene expression between difference control status and clinical presentation in these patients. In addition, we also tried to build a prediction model for asthma by finding the expression of a specific circadian clock gene in PBMCs.

Patients and Methods

Patients, Healthy Subjects, and Samples

We conducted this retrospective case-controlled study between January 2019 and December 2019 at Kaohsiung Chang Gung Memorial Hospital (K-CGMH), Taiwan. The design of this study adhered to the principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board (IRB) of the CGMH Ethical Committee (IRB No. 201801642B0A3C501). Written informed consent was obtained from all participants.

Adult asthmatic patients were recruited from the Division of Pulmonary and Critical Care Medicine of K-CGMH and the Division of Chest Medicine of Kaohsiung Medical University Hospital (KMUH) in Taiwan from January 2013 to January 2015. The candidates had symptoms such as episodic breathlessness, wheezing, cough, and chest tightness and the diagnosis of asthma according to the Global Initiative for Asthma (GINA) guidelines, with or without reversible airway from spirometry that showed an increase in FEV1 of at least

12% and at least 200 mL from the prebronchodilator value. Those who met the previous criteria were regarded to have asthma if they had had at least one asthma exacerbation or needed use of asthma prescription in the 12 months before the clinics visit. Patients were excluded if they were physician-diagnosed with chronic airway obstruction, emphysema, tuberculosis, or cancer.

We obtained residual cDNA samples of PBMCs and collected clinical parameters and outcomes from 120 patients with asthma (60 men and 60 women, aged 25–73) and 60 age- and gender-matched healthy individuals (30 men and 30 women, aged 20–73). All the enrolled subjects were aged 18 years or older. Pregnant women, psychiatric patients, and individuals taking sleep pills were excluded. Subjects enrolled in this study did not experience shift work or jet lag 1 week before PB collection. The study participants were screened for eligibility and recruited from the pulmonary clinics and health examination center of K-CGMH and KMUH during the period of January 2013 through January 2015. Clinical characteristics including gender, age, smoking habits, body weight, body mass index (BMI), body fat, body height, waist, IgE, FEV1, PEF (peak expiratory flow rate), and asthma control test (ACT) were recorded. We defined the well-controlled and poor-controlled asthma as ACT scores equal to or more than 20 and below 20, respectively. Patients with nocturnal symptoms were identified in the third question of ACT, with a score lower than 5, which means asthma symptoms (wheezing, coughing, shortness of breath, chest tightness, or pain) have woken patients up at night or earlier than usual in the morning during the past 4 weeks.

Analysis of Expression of Circadian Clock Genes

The expression of the nine circadian clock genes and *ACTB* gene (as endogenous reference control) was analyzed using real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously described.^{26,27} The relative threshold cycle (ΔCt) of the circadian clock gene was obtained by normalizing the threshold cycle of the circadian clock gene to the *ACTB* gene of the same sample, ie, $\Delta Ct = (Ct \text{ of circadian clock gene} - Ct \text{ of } ACTB \text{ gene})$. Thus, a higher ΔCt value represents a lower expression level

and a higher $-\Delta Ct$ value represents a higher expression level.

Statistical Analysis

We used MedCalc software (MedCalc Software Ltd, Ostend, Belgium) and Graph Pad Prism 7.04 (GraphPad, San Diego, CA, USA) for all statistical analyses. The sample size was estimated by G power software ([Supplementary Figure S1](#)). Student's *t*-test was used to detect the differences between patients with bronchial asthma and healthy subjects, and Kruskal–Wallis test was used to detect the differences among participants with well- or not poor-controlled asthma and healthy subjects in each circadian clock gene expression. Post-hoc analysis was used for comparing the difference among subgroups. Mann–Whitney test was used to detect the differences between asthma patients with and without night symptoms. Receiver operating characteristic (ROC) curve was plotted and ROC area under the curve (AUC) was calculated to compare the discriminating ability of each circadian clock genes. Binary Logistic regression with multivariable analysis modelled the effects of selected independent variables on whether the expression of a specific circadian clock gene could be a predictive marker for asthma or not. The values of ΔCt or $-\Delta Ct$ were used for all statistical analysis. All tests were two-sided with statistical significance set at 0.05.

Results

Characteristics of Study Participants

The characteristics, including gender, age, smoking habits, body weight, BMI, body fat, body height, and waist of the 120 patients with bronchial asthma and 60 healthy individuals are listed in [Table 1](#). All the characteristics are not different between patients and healthy individuals. The values for IgE, FEV1, PEF, and ACT score are also listed for the patients with bronchial asthma. The characteristics of well- and not well-controlled asthmatics and asthmatics with and without night symptoms are listed in [Table 2](#).

Expression of Circadian Clock Genes in Patients with Bronchial Asthma

In order to explore whether expression of circadian clock genes altered in patients with bronchial asthma, we performed qRT-PCR to examine the expression of a panel of nine human circadian clock genes in the PBMCs. We found significant difference of expression of all the nine circadian clocks genes

Table 1 Characteristics of Study Participants

Characteristics	Healthy Individuals (n=60)	Bronchial Asthma (n=120)	P-value
Gender (Male/Female)	30/30	60/60	1.0000
Age, years	50.98±13.07	50.63±12.07	0.8586
Smoking (None/Ever-smoking)	47/13	75/45	0.4128
Body Weight, Kg	65.49±14.06	68.24±13.75	0.2108
BMI	24.34±3.00	25.74±4.53	0.1100
Body Fat, %	27.76±8.51	29.32±12.00	0.3696
Body Height, cm	164.30±9.76	162.61±7.17	0.1918
Waist, cm	82.05±10.48	86.25±16.79	0.0780
IgE, IU/mL		330.07±543.39	
FEV1, L		2.34±0.72	
PEF, mL/min	327.51±128.21	369.78±97.63	0.0658
ACT score		20.05±3.67	

Notes: Age, BMI, body weight, body height, body fat, waist, IgE, FEV1, PEF, ACT score are expressed as mean standard deviation. The P-values indicated were evaluated with Student's t-test, except for sex and smoking (Chi-square).

Abbreviations: BMI, body mass index; FEV1, forced expiratory volume in 1 second; PEF, peak expiratory flow rate; ACT, asthma control test.

between asthmatic patients and healthy individuals (Figure 1A). Except for *BMALI*, the expression of the other eight genes was down-regulated in patients with bronchial asthma.

We divided the patients into with and without controlled status, but the expression of all the nine circadian clock genes was not different between these two groups of patients (Figure 1B).

Table 2 Characteristics of Well- and Not Well-Controlled Asthmatics and Asthmatics with and without Night Symptoms

Characteristics	Well-Controlled (n=60)	Not Well-Controlled (n=60)	P-value	With Night Symptoms (n=53)	Without Night Symptoms (n=67)	P-value
Gender (Male/Female)	28/32	32/28	1.000	27/26	34/33	0.996
Age, years	51.02±11.67	50.25±12.54	0.575	49.55±12.51	51.49±11.73	0.667
Smoking (None/Ever-smoking)	43/17	33/27	0.010*	30/23	45/22	0.056
BMI	25.28±3.94	26.20±5.03	0.345	25.99±5.20	25.55±3.94	0.454
Body Fat, %	31.56±10.99	30.71±8.55	0.296	25.61±4.70	25.49±3.82	0.225
Waist, cm	86.00±10.94	89.48±13.89	0.157	87.28±12.52	87.10±11.13	0.312
IgE, IU/mL	279.59±482.72	380.56±602.33	0.307	398.12±606.30	266.56±479.04	0.282
FEV1, L	2.37±0.72	2.30±0.74	0.610	2.29±0.75	2.39±0.70	0.818
PEF, mL/min	369.13±93.71	370.43±103.51	0.400	366.08±102.90	373.49±94.22	0.545
ACT score	23.10±1.41	17.00±0.50	0.020*	18.69±3.35	21.12±3.57	0.222
Night Symptoms, n (%)	44 (73.3%)	23 (38.3%)	0.009**	53 (100%)	0 (0%)	–

Note: Age, BMI, body fat, waist, IgE, FEV1, PEF, ACT score are expressed as mean standard deviation. The P-values indicated were evaluated with Student's t-test, except for sex, smoking and night symptoms (Chi-square).

Abbreviations: BMI, body mass index; FEV1, forced expiratory volume in 1 second; PEF, peak expiratory flow rate; ACT, asthma control test.

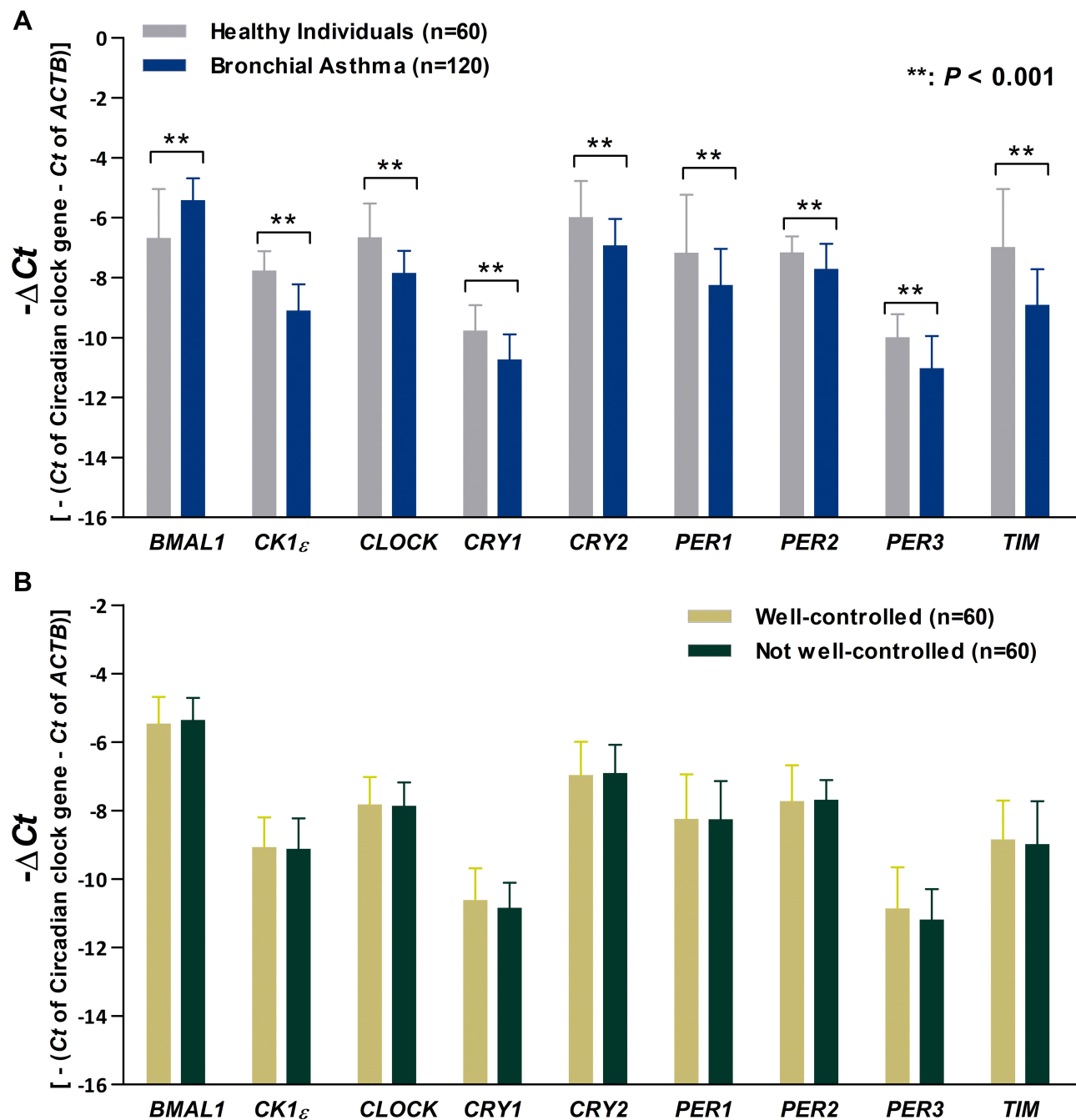


Figure 1 The expression of nine circadian clock genes in 120 patients with bronchial asthma and 60 healthy individuals as determined by real-time quantitative reverse transcriptase polymerase chain reaction. **(A)** All nine genes were significantly deregulated in patients with bronchial asthma ($P < 0.001$). **(B)** The expression of all nine circadian clock genes was not different between patients with and without controlled of bronchial asthma. Data presented are mean \pm standard deviation of $-\Delta Ct$ [-(Ct of Circadian clock gene - Ct of *ACTB* gene)]. Higher $-\Delta Ct$ represents higher expression level, and vice versa.

Expression of Circadian Clock Genes in Asthmatic Patients with and without Night Symptoms

To investigate whether the expression of circadian clock genes correlated with night symptoms, we further divided the asthmatic patients into with and without night

symptoms groups and performed a Mann-Whitney test to investigate the differential expression of circadian clock genes between these two groups. Among the nine genes, *PER3* was the only gene with lower expression in the group with night symptoms ($P = 0.0408$) (Figure 2 and Supplementary Table S1).

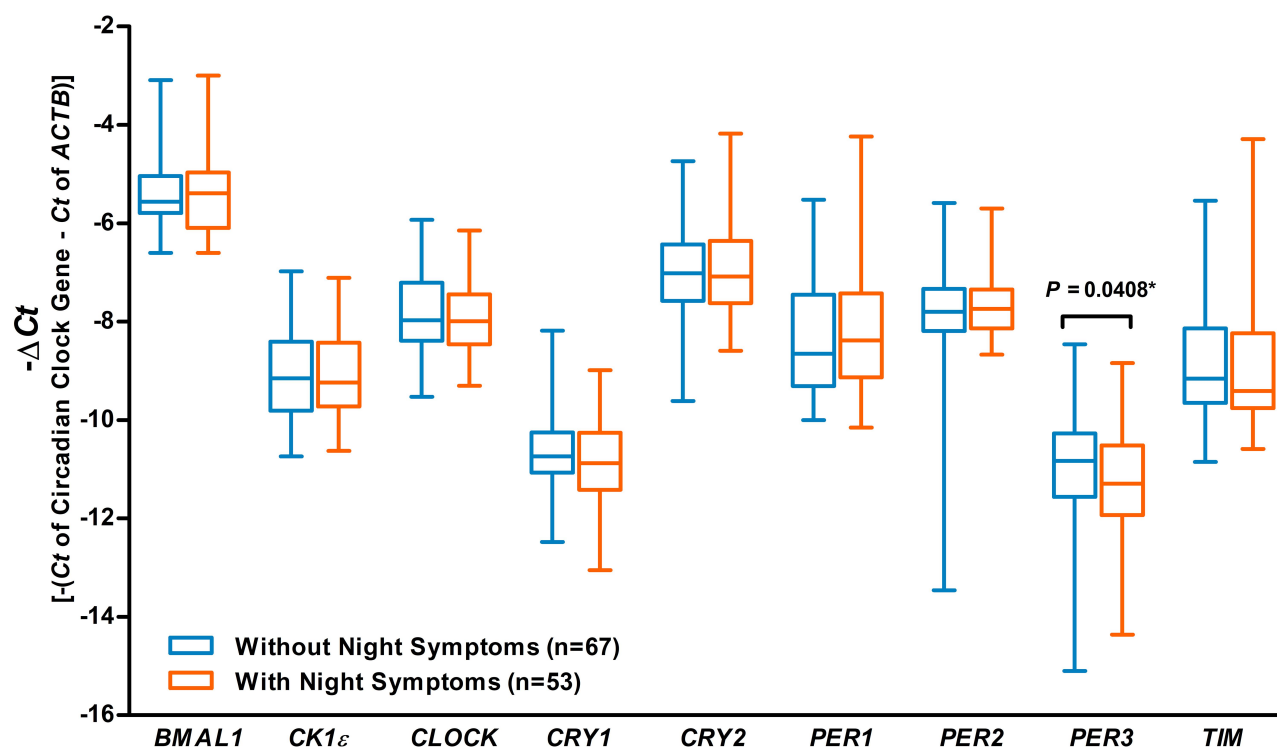


Figure 2 The expression of the nine circadian clock genes in asthmatic patients with and without night symptoms. Expression of *PER3* was significantly down-regulated in asthmatic patients with night symptoms as evaluated by Mann–Whitney test using values of $-\Delta Ct$ [$-(Ct \text{ of circadian clock gene} - Ct \text{ of ACTB gene})$]. Data presented are median and range of $-\Delta Ct$. Higher $-\Delta Ct$ represents higher expression level, and vice versa. * $P < 0.05$.

Expression of Circadian Clock Genes in Well- or Not Well-Controlled Asthmatics with and without Night Symptoms

We further divided the well-controlled asthmatics into groups of with and without night symptoms and found significant down-regulation of *BMAL1*, *CK1ε*, *CLOCK*, *CRY1*, *CRY2*, and *PER1* in the group with night symptoms (Figure 3A and Supplementary Table S2). We also divided the not well-controlled asthmatics into with and without night symptoms groups, and altered expression of *BMAL1*, *CK1ε*, *PER1*, and *PER2* was observed between these two groups (Figure 3B and Supplementary Table S2).

Predictive Ability for Bronchial Asthma Based on the Expression of Circadian Clock Genes

To investigate the discriminative performance of expression of circadian clock genes between the groups of asthma and healthy individuals, we constructed ROC curves and calculated the AUC for each gene. Among the nine circadian clock genes, binary logistic regression analysis selected *BMAL1* (Odds ratio=0.23; 95% confidence interval (CI)

=0.12–0.43; $P < 0.0001$), *CK1ε* (Odds ratio=12.34; 95% CI=3.92–38.77; $P < 0.0001$), *PER3* (Odds ratio=2.15; 95% CI=1.08–4.26; $P = 0.0293$), and *TIM* (Odds ratio=0.45; 95% CI=0.45–0.82; $P = 0.0097$) as independent factors for bronchial asthma (Table 3). Combined expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM* enhanced the prediction of bronchial asthma (Odds ratio=624.52; 95% CI=115.38–3,380.37; $P < 0.0001$) (Table 3). We also performed ROC analysis and evaluated the AUC of ROC curves to investigate whether patients with bronchial asthma could be distinguished from healthy subjects based on their expression of circadian clock genes (Figure 4). The discriminating predictive abilities of *CK1ε* and four combinations were found to be excellent (AUC=0.879 and 0.924, respectively), and predictive abilities of *BMAL1*, *PER3*, and *TIM* were acceptable (AUC range=0.683–0.798). Our results demonstrated the combined expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM* might have the potential to be a predictor for bronchial asthma.

Discussion

In this case-controlled study, we found the expression of all the nine circadian clock genes was altered when

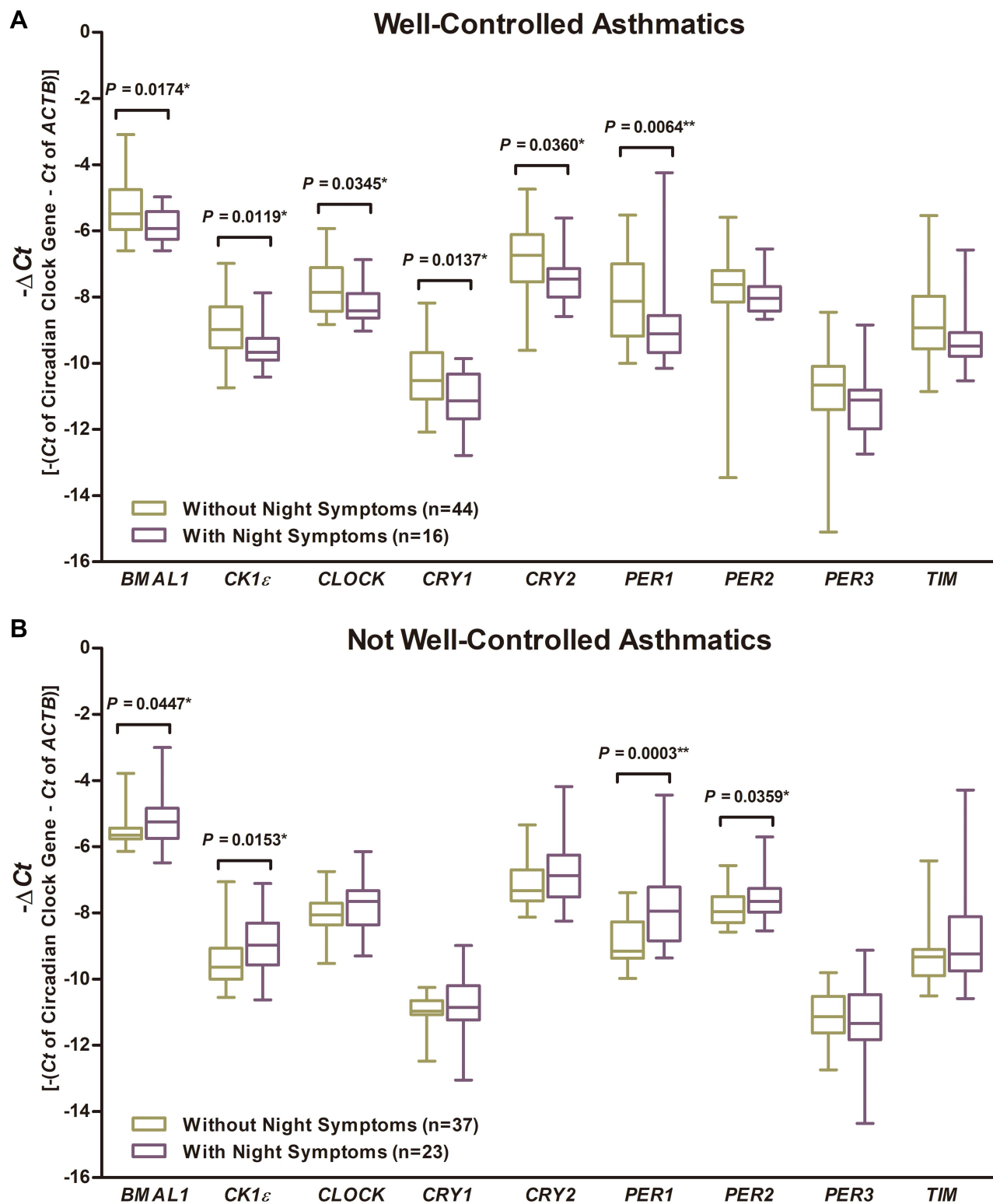


Figure 3 The expression of nine circadian clock genes in patients with and without control of bronchial asthma and with or without night symptoms. **(A)** In patients with well-controlled bronchial asthma, the expression of *BMAL1*, *CK1ε*, *CLOCK*, *CRY1*, *CRY2*, and *PER1* was down-regulated in those with night symptoms compared with those without night symptoms. **(B)** In patients with not well-controlled bronchial asthma, the expression of *BMAL1*, *CK1ε*, *PER1*, and *PER2* was significantly different in those with night symptoms compared with those without night symptoms. Data presented are median and range of $-\Delta Ct$ [-(Ct of Circadian clock gene - Ct of *ACTB* gene)]. Higher $-\Delta Ct$ represents a higher expression level, and vice versa. $^*P < 0.05$ and $^{**}P < 0.01$ were evaluated by Mann-Whitney test using values of $-\Delta Ct$.

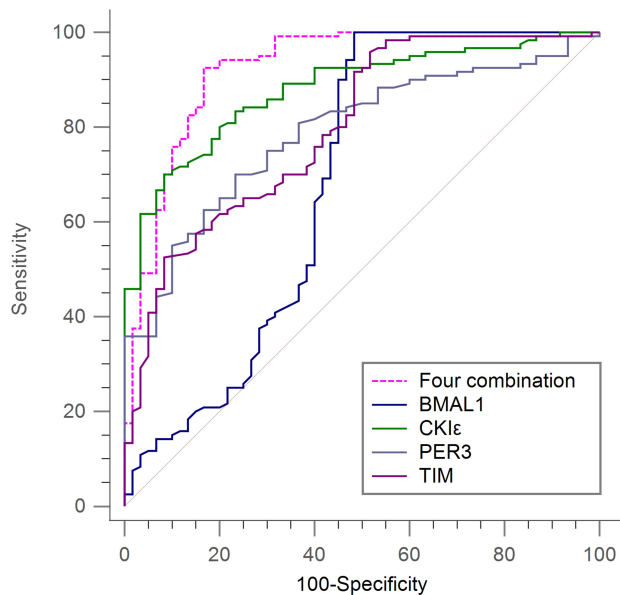
Table 3 Binary Logistic-Regression Analysis of Predictors of Bronchial Asthma by Expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM*.

Independent Factor	Coefficient	SE	Odds Ratio (95% CI)	P-value
<i>BMAL1</i>	−1.48	0.32	0.23 (0.12–0.43)	<0.0001
<i>CK1ε</i>	2.51	0.58	12.34 (3.92–38.77)	<0.0001
<i>PER3</i>	0.76	0.35	2.15 (1.08–4.26)	0.0293
<i>TIM</i>	−0.79	0.31	0.45 (0.25–0.82)	0.0097
Constant	−13.55	3.18		
Four combination			624.52 (115.38–3,380.37)	<0.0001

Note: The reference for the odds ratio is the absence of the corresponding risk factor.

Abbreviations: SE, standard error; CI, confidence interval.

compared with that in health individuals. In consistent with our finding, a recent study demonstrated significantly decreased expression of *TIM* in the blood of children with asthma as compared to the healthy children.²⁸ Other than this report on the *TIM* gene, there have so far been no



Variable	AUC	SE	95% CI
<i>BMAL1</i>	0.683	0.050	0.610 to 0.751
<i>CK1ε</i>	0.879	0.025	0.822 to 0.923
<i>PER3</i>	0.788	0.034	0.721 to 0.846
<i>TIM</i>	0.798	0.035	0.732 to 0.854
Four combination	0.924	0.023	0.875 to 0.958

Figure 4 Receiver operator curves demonstrate the predictive ability for bronchial asthma based on the expression of *BMAL1*, *CK1ε*, *PER3*, *TIM*, and four combination.

Abbreviations: AUC, area under curve; SE, standard error; CI, confidence interval.

reports to directly link the deregulated circadian clock genes and bronchial asthma in humans. Obstructive sleep apnea (OSA) has been reported to be prevalent in nocturnal asthma.²⁹ Our previous study indeed observed expressions of eight circadian clock genes (except *PER1*) at midnight were significantly downregulated in patients with severe OSA.³⁰ This may imply the disruption of circadian rhythm caused altered lung function in both asthma and OSA. Deregulated *Per2* and *Bmal1* have been associated with inflammation or exacerbated immune function and lung functions in animal models. Recently, a study had disclosed that aberrant light/dark situation aggravated virus-induced, asthma-like inflammation.³¹ *Bmal1*-deleted mice were more susceptible to bacterial infections and inflammation,^{12,31,32} and developed more extensive airway inflammation than wild-type mice.³³ Inflammatory factors could affect peripheral clock activity and the phase, period, and amplitude of expression of circadian clock genes in normal lung tissue were changed by exposure to cigarette smoke, viral or bacterial infection.^{34,35} In addition, hormonal or autonomic nerve activity synchronized peripheral clocks to the suprachiasmatic nucleus rhythm and affect the asthma activity.³⁶ The aberrant hormone secretion or autonomic nerve activity might desynchronize peripheral clock rhythm and might affect the airway activity and allergic reaction. For example, chronic stress would be relating to arrhythmicity in *Per2* expression in mice.³⁶ *Per2* gene regulates daily IL-1 β production in macrophages and IFN- γ production in NK cells, altered *Per2* gene thus leads to the impairment of immune function.³⁵ The majority of asthmatic patients have an allergy reaction and several studies showed the circadian disruption can promote allergy.^{24,37–39} The mechanism might relate to circadian gating of epithelial

barrier function and immune responses that evolved to complex environmental physical, chemical, and biological stimulants and enhance host defense after exposure of stimulants.^{24,40}

In this study, we noted a lower expression of *PER3* in asthmatic patients with night symptoms. *PER3* was thought to be less important than the other two family members, *PER1* and *PER2*, in regulating the circadian clock. However, down-regulation of *PER3* has been reported in various types of human cancers,^{26,27,41,42} and has been associated with sleep homeostasis and mental disorders in humans. Interestingly, *PER3* was also the most down-regulated gene at midnight in OSA and was selected for predicting OSA.³⁰ Hence, our finding may add evidence to strengthen the crucial role of *PER3* in fine adjustment of response in the time-keeping system in humans.

Another novel finding of this study was the combined expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM* has the potential to predict bronchial asthma. Although depending solely on the expression of circadian clock genes may not be sufficient to predict asthma, they did provide a new reference for evaluating asthma.

Although we have obtained novel and clinically relevant results that could give an insight into a more profound understanding of asthma, there are limitations of this study. First, all the PB samples were obtained in the daytime and only at one time point and therefore the oscillation of circadian clock genes may not be reflected fully. Second, we did not collect cases at different treatment times to validate whether the changes of gene expression related to the disease status of patients. In addition, this is a correlation study, although we have linked circadian clock genes to with or without control and night symptoms of patients with asthma, no mechanisms of these gene functions were investigated. Due to this study is a retrospective study, we lack detailed medication, treatment response and some data, such as fractional exhaled nitric oxide (FeNO) and allergic profile. Moreover, for the retrospective study with residual samples, we did not have samples to verify our qPCR data at the protein level using either Western blot or immunohistochemical staining.

Conclusion

This is the first study that directly links circadian clock genes to human asthma. We demonstrated expression levels of the nine core circadian clock genes were altered in patients with

bronchial asthma. We also discovered *PER3* could be a new reference for evaluating the asthmatics patients with nocturnal symptoms and the combined expression of *BMAL1*, *CK1ε*, *PER3*, and *TIM* has the potential to be a predictor for asthma. Our results may not be sufficient for clinical diagnosis of asthma presently, but they did provide new directions for future studies. Investigating the mechanisms of downregulated circadian clock genes in asthma and the molecular causal effects of circadian clock genes and asthma will also let us take a step forward to reveal the mysterious roles circadian clock genes play in asthma.

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Disclosure

The authors report no conflicts of interest in this work.

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