Downregulation of *Arntl* mRNA Expression in Women with Hypertension: A Case-Control Study

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Keywords

Essential hypertension · Aryl hydrocarbon receptor nuclear translocator like · Clock circadian regulator · Period circadian regulator 1 · Period circadian regulator 2

Abstract

Background: Previous studies have reported that disturbance of endogenous circadian rhythms enhances the chance of hypertension and suggested that circadian clock genes could have a crucial function in the onset of the disease. This case-control study was aimed to investigate the association of the mRNA expression of aryl hydrocarbon receptor nuclear translocator like (*Arntl*), clock circadian regulator (*Clock*), and period circadian regulators 1 and 2 (*Per1* and *Per2*) with hypertension and blood pressure levels. **Methods:** A total of 172 subjects were recruited in this study, including 86 hypertension and 86 nonhypertension controls. The mRNA expression levels in peripheral blood mononuclear cells were determined by real-time quantitative polymerase chain reaction. The differences in *Arntl*, *Clock*, *Per1*, and *Per2* mRNA expression were compared between the 2 groups, and the relationship between mRNA expres-

sion and cardiometabolic risk profiles was also assessed. **Results:** We found that the mRNA expression of *Arntl* was downregulated in the hypertension cases compared with controls in women (1.10 [0.66, 1.71] vs. 1.30 [0.99, 2.06], $p = 0.031$). There was a significant negative correlation between the *Arntl* mRNA expression and SBP ($r = -0.301$, $p = 0.004$) and DBP ($r = -0.222$, $p = 0.034$) in women. In men, a negative correlation between the *Per1* mRNA expression and SBP ($r = -0.247$, $p = 0.026$) was found. **Conclusions:** The *Arntl* mRNA expression may play an important role in progression of hypertension in women.

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Introduction

Hypertension is one of the most common chronic diseases with an increasing prevalence; moreover, it is a major risk factor for cardiovascular diseases, such as stroke and coronary heart disease [1]. About 9.4 million people

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die because of hypertension each year across the world [2]. The results of the 2017 Global Burden of Disease study showed that hypertension is still the first risk factor contributing to the global burden of the disease [3]. The etiology and pathogenesis of hypertension remains unclear and is generally accepted as a result of the interaction of environmental and genetic factors. Previous studies have shown that the aryl hydrocarbon receptor nuclear translocator like (*Arntl*, also known as *BMAL1*) rs6486121 and clock circadian regulator (*Clock*) T3111C were associated with susceptibility to hypertension [4, 5]. As per Kurbatova et al. [6], T3111C can affect the mRNA transcription level of *Clock*.

Clock and *Arntl* belong to the core components of circadian rhythm genes. Studies have shown that the pathological processes of many chronic diseases, such as insulin-dependent diabetes [7], obesity [8], and cardiovascular diseases [9], are accompanied by disturbances in the transcriptional activity of circadian rhythm genes. Rat experiments have shown that the blood pressure increased in TGR(mREN-2)27 rats, accompanied by a significant increase in *Arntl* expression [10]. Compared to that in Wistar rats, the expression of *Clock* increased in spontaneous hypertensive rats [11]. Another mice experiment showed a contradictory result; when a comparison was made with male wild-type mice, *Clock* knockout mice showed no variation in the blood pressure rhythm at 24 h; however, there was a drop in the blood pressure levels [12]. In animal research studies, *Clock* mutant mice showed an elevated blood pressure and the clock protein period 1 (*Per1*)-knockout female mice are protected from nondipping hypertension [13–15]. *Per1* and *Per2* are the target genes of the heterodimeric complex formed by *Clock* and *Arntl*. An increasing number of epidemiological studies have shown an association between circadian rhythm genes and hypertension [4, 5]. Li et al. [16] found that hypertensive patients with the C allele of *Clock* T3111C or the GG genotype of *Arntl* A1420G may be more likely to develop high nighttime systolic blood pressure (SBP). In metabolic syndrome patients, *Per2* was found to be linked to the risk factors [17]. rs6431590 in *Per2* was independently associated with the presence of nondippers in hypertensive subjects [18].

These results suggest that *Arntl*, *Clock*, *Per1*, and *Per2* may be involved in the development of hypertension. However, the results were mainly derived from animal models and focused on single locus variation. Therefore, the present study was designed as a case-control design to assess the relationship between *Arntl*, *Clock*, *Per1*, and *Per2* mRNA expression levels and hypertension.

Methods

Study Subjects

A total of 172 subjects with a mean age of 69.00 years (64.00–80.00 years) were included in this case-control study, including 86 controls and 86 patients with hypertension. The hypertensive subjects and the control subjects were from the health examination group from the hospital medical examination center between July 2019 and August 2019. Inclusion criteria for cases: hypertensive status, age >50 years, regularity of life, and untreated hypertension. Exclusion criteria for cases: secondary hypertension; other serious cardiovascular and cerebrovascular diseases, such as coronary heart disease, stroke, and cardiac infarction; and jet lag or night-shift in the previous 2 weeks. Inclusion criteria for the control: the mean SBP <140 mm Hg and diastolic blood pressure (DBP) <90 mm Hg in 3 blood pressure measurements and no history of hypertension, no cardio-cerebrovascular disease, residing in the same region as the patient; and age >50 years. The study procedures were approved by the Scientific Research IRB of Wannan Medical College Yijishan Hospital, and written informed consent was obtained from all the study subjects.

Physical Examinations

SBP and DBP were measured 3 times at a 1-min interval using an Omron electronic sphygmomanometer in a seated position after the subjects had rested well for 10 min after their blood was drawn, and their average value was obtained for the analysis. Hypertension was defined as SBP $\geq$ 140 mm Hg and/or DBP $\geq$ 90 mm Hg. Anthropometric variables (height and weight) were measured when the subject was wearing socks or was bare feet in the standing position using an ultrasonic height and weight meter (HGM 800A; Shengyuan Company, China). BMI was calculated as body weight in kilograms divided by the square of the body height in meters (kg/m^2). Waist circumference was measured 1 cm above the umbilicus in a horizontal plane by using a general tape.

Laboratory Measurements

Blood samples were obtained from participants at about 9:00 a.m. after 10 h of overnight fasting. The following biochemical indicators were assessed: fasting blood glucose (FBG), total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). The biochemical indicators were determined using an enzymatic method by the physical examination institution.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from PBMCs using the TRIzol reagent (Invitrogen, USA) according to the manual instructions. Quality and concentration of RNA was evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription of mRNA was synthesized using TAKARA PrimeScript RT reagent Kit (RR036A; Takara, Tokyo, Japan). The qRT-PCR reactions were performed with the Platinum SYBR Green Mix (Invitrogen, USA) and QuantStudio™ 7 Flex Real-Time system (Applied Biosystems, Waltham, MA, USA). GAPDH was regarded as the endogenous control for mRNA. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. $2^{-\Delta\Delta\text{Ct}}$ ($\Delta\Delta\text{Ct}$ case = ΔCt case – ΔCt control average value, $\Delta\Delta\text{Ct}$ control = ΔCt control – ΔCt control average value, and

Table 1. Demographic and clinical characteristics between hypertension and controls

	Control (n = 86)	Hypertension (n = 86)	$\chi^2/Z/t$	p value
Gender				
Male	41 (50.6)	40 (49.4)	0.023	0.879
Female	45 (49.5)	46 (50.5)		
Age, years	69.00 (64.00–80.00)	69.00 (64.00–79.00)	0.424	0.671
Blood pressure, mm Hg				
SBP	124.24±9.71	157.09±16.85	15.664	<0.001
DBP	70.73±7.85	82.98±9.55	9.186	<0.001
BMI, kg/m ²	23.60±3.30	24.51±3.13	1.851	0.066
Waist circumference, cm	80.14±8.97	82.73±8.58	1.938	0.054
TC, mmol/L	4.92±0.81	5.21±1.11	1.937	0.055
TG, mmol/L	1.22 (0.42–5.62)	1.37 (0.51–6.03)	1.818	0.069
LDL-C, mmol/L	2.69±0.64	2.98±0.85	2.546	0.012
HDL-C, mmol/L	1.42±0.41	1.39±0.34	0.542	0.589
FBG, mmol/L	5.31 (4.41–7.51)	5.35 (4.31–10.75)	0.879	0.379
Smoking				
Yes	18 (20.9)	35 (40.7)	7.881	0.005
No	68 (79.1)	51 (59.3)		
Drinking				
Yes	9 (10.5)	20 (23.3)	5.019	0.025
No	77 (89.5)	66 (76.7)		

Data are presented as n (%) or median (minimum–maximum) or mean ± standard deviation. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TC, total cholesterol; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, fasting blood glucose.

$\Delta Ct = Ct \text{ target gene} - Ct \text{ GAPDH}$). The primer sequences used for qPCR were as follows:

1. *Arntl*: 5'-TGCAACGCAATGTCCAGGAA-3' (forward), 5'-GGTGGCACCTCTTAATGTTTTCA-3' (reverse)
2. *Clock*: 5'-TGCGAGGAACAATAGACCCAA-3' (forward), 5'-ATGGCCTATGTGTGCGTTGTA-3' (reverse)
3. *Per1*: 5'-GCCAACCAGGAATACTACCAGC-3' (forward), 5'-GTGTGTA CT CAGACGTGATGTG-3' (reverse)
4. *Per2*: 5'-GACATGAGACCAACGAAACTGC-3' (forward), 5'-AGGCTAAAGGTATCTGGACTCTG-3' (reverse)
5. *GAPDH*: 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward), 5'-GGCTGTGTGCATACTTCTCATGG-3' (reverse)

Statistical Analysis

Continuous variables were tested for normality using the Kolmogorov-Smirnov test and expressed by means ± standard deviation if normally distributed or the median and interquartile range for the skew distributional data. The *t* test and Mann-Whitney U tests were used to test their differences, respectively. Categorical variable distributions between cases and controls were compared by the χ^2 test. Correlation analysis between *Arntl*, *Clock*, *Per1*, and *Per2* and continuous variables of cardiometabolic risk profiles was assessed by the Spearman correlation coefficient test. Association analysis in the case-control study used logistic regression, and odds ratio and its 95% confidence interval were calculated. Statistical analyses were performed using the SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). We considered results to be statistically significant when a 2-tailed *p* value of 0.05 was defined.

Table 2. Comparison of mRNA expression of *Arntl*, *Clock*, *Per1*, and *Per2* between hypertension and controls

	Controls (n = 86)	Hypertension (n = 86)	Z	p value
<i>Arntl</i>	1.05 (0.66, 1.63)	0.88 (0.55, 1.28)	1.776	0.076
<i>Clock</i>	0.99 (0.61, 1.67)	0.94 (0.52, 1.52)	0.913	0.361
<i>Per1</i>	0.98 (0.56, 1.68)	0.88 (0.51, 1.36)	1.286	0.198
<i>Per2</i>	1.08 (0.65, 1.80)	1.14 (0.51, 1.88)	0.006	0.995

Results

Clinical Characteristics of Participants

There was no significant difference in the age, sex, BMI, waist circumference, total cholesterol, triacylglycerol, high-density lipoprotein cholesterol, and fasting blood glucose between the 2 groups (*p* > 0.05). However, the levels of SBP, DBP, LDL-C, smoking, and drinking proportion were higher in the hypertension group than those in the control group (*p* < 0.05). The data are shown in Table 1.

Table 3. Gender subgroup of comparison of *Arntl*, *Clock*, *Per1*, and *Per2* mRNA expression in 2 groups

	Men				Women			
	controls (n = 41)	hypertension (n = 40)	Z	p value	controls (n = 45)	hypertension (n = 46)	Z	p value
<i>Arntl</i>	0.77 (0.53, 1.21)	0.73 (0.50, 1.09)	0.236	0.813	1.30 (0.99, 2.06)	1.10 (0.66, 1.71)	2.159	0.031
<i>Clock</i>	0.66 (0.40, 0.86)	0.62 (0.42, 0.94)	0.293	0.770	1.41 (1.02, 2.47)	1.35 (0.77, 1.96)	1.278	0.201
<i>Per1</i>	0.97 (0.53, 1.58)	0.75 (0.46, 1.30)	0.982	0.326	1.22 (0.72, 1.71)	0.97 (0.62, 1.45)	0.841	0.400
<i>Per2</i>	0.90 (0.48, 1.72)	0.90 (0.48, 1.73)	0.059	0.953	1.14 (0.79, 1.95)	1.34 (0.54, 1.92)	0.040	0.968

The mRNA Expression Levels of Arntl, Clock, Per1, and Per2 in the 2 Groups

Expressions of mRNA for *Arntl*, *Clock*, *Per1*, and *Per2* did not differ significantly between the groups ($p > 0.05$, Table 2). Subgroup analysis showed that the *Arntl* mRNA levels in PBMCs from the hypertension group were significantly lower than those from the control group in women ($p = 0.031$, Fig. 1). However, similar results were not found in men. For *Clock*, *Per1*, and *Per2* mRNA levels, no significant difference was found between the groups in women or in men. The data are presented in Table 3.

Correlation between Arntl, Clock, Per1, and Per2 mRNA Expression and Clinical Variables

In women, the *Arntl* mRNA expression was negatively correlated with SBP levels ($r = -0.301$, $p = 0.004$) and DBP levels ($r = -0.222$, $p = 0.034$); in men, *Per1* mRNA expression was negatively correlated with the SBP levels ($r = -0.247$, $p = 0.026$; Fig. 2). In addition, the *Per2* mRNA expression was positively correlated with the LDL-C levels in women ($r = 0.223$, $p = 0.033$). There were no correlations between the other clinical variables and the expression of mRNA for particular genes. The data are presented in Table 4 and online suppl. Figures 1 and 2 (see www.karger.com/doi/10.1159/000518669 for all online suppl. material).

Discussion

The circadian rhythm system is an important regulator of blood pressure circadian rhythm. The blood pressure of healthy people can reduce by 10%–20% at night and rise in the morning, which plays a crucial role in maintaining human health. Studies have shown that, compared with that at other times, during the morning blood pressure fluctuations, the risk of acute myocardial infarction is higher by 40%, that of sudden cardiac death is increased by 29%, and that of stroke is higher by 49%

Fig. 1. Downregulation of *Arntl* mRNA expression in hypertension. *Arntl* mRNA levels are lower in hypertensives than in controls ($p = 0.031$). The dots represent individual relative mRNA expression level. The longest line represents the median, and the whiskers represent the interquartile range. Gene expression was measured using RT-PCR, with values normalized to *GAPDH* mRNA expression. *Arntl*, aryl hydrocarbon receptor nuclear translocator like.

[19–21]. Recent trials have shown that loss of *Arntl*, *Clock*, *Per1*, and *Per2* function is closely associated with increased blood pressure.

In the present study, the results demonstrated a potential role of *Arntl* in blood pressure regulation. Compared to that in control women, the expression of *Arntl* was reduced in the PBMCs from women with hypertension. Moreover, correlation analysis indicated that the *Arntl* mRNA expression was negatively correlated with the SBP and DBP values. It is worth mentioning that few studies have reported the *Arntl* mRNA transcription levels. Genetic evidence has mainly focused on locus variation. Woon et al. [22] showed

that 2 *Arntl* haplotypes were associated with hypertension. Li et al. [16] found that hypertensive patients with the GG genotype of *Arntl* A1420G may be more likely to develop high nighttime SBP levels. Recent genome-wide association studies have identified an association between *Arntl* SNPs rs9633835 and hypertension [23]. Peng et al. [5] have provided strong evidence showing that rs6486121 variants in *Arntl* are associated with hypertension. These data strongly support that *Arntl* is a plausible candidate gene for the development of hypertension.

Previous studies have shown that many factors, such as age and sex, may affect the expression of rhythm genes [24, 25]. Animal experiments have demonstrated that the sex-based differences in the circadian clock gene expression could be attributed to sex hormones [26–28]. It is well known that variations exist at the molecular, cellular, and tissue levels between the sexes, contributing to the sex-specific differences in hypertension [29]. We speculated that the role of *Arntl* in blood pressure regulation may be influenced by patient sex and performed analyses stratified by sex. We further found in the present study that a sex-based difference existed in the association of *Arntl* and hypertension, which confirmed the hypothesis.

Evidence suggested that *Arntl* knockout mice showed dysfunction of endothelial nitric oxide synthase (eNOS) coupling and conversion and increased eNOS uncoupling and abnormal expression of related endothelial regulatory genes, resulting in increased peroxide and endothelial damage [30]. Shang et al. [31] reported that the sensitivity of *Arntl* mutant mice to vascular changes decreased, and the normal rhythmic expression patterns of the endothelial function regulator serine protein kinase and eNOS disappeared, leading to endothelial dysfunction. Further, knockout of mice *Arntl* led to endothelium-dependent vasodilation function reduction [14]. This process may involve the reduction of nitric oxide production, resulting in vascular endothelial function damage and vascular remodeling, eventually leading to blood pressure regulation disorder. Therefore, *Arntl* might play an important role in blood pressure regulation. The results of this study further suggest that downregulated mRNA expression of *Arntl* in the population may be related to the development of hypertension.

Per1 and *Per2* are the target genes for the formation of the heterodimers of *Clock* and *Arntl*. We did not find a significant difference in the *Per1* and *Per2* mRNA expression of hypertensive patients and controls; however, we observed a negative correlation between the expression level of *Per1* and SBP levels in men. Douma et al. [32] confirmed that under the condition of high salt intake, *Per1* gene

Fig. 2. Scatter plot of correlations between gene mRNA expression and arterial blood pressure. Correlations between *Arntl* and arterial blood pressure in women (**A**, **B**); correlations between *Per1* and systolic blood pressure in men (**C**). *Arntl*, aryl hydrocarbon receptor nuclear translocator like; *Per1*, period circadian regulator 1.

Table 4. Correlation between mRNA expression and cardiometabolic risk profiles

	<i>Arntl</i>		<i>Clock</i>		<i>Per1</i>		<i>Per2</i>	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
Men								
SBP	−0.105	0.350	−0.179	0.109	−0.247	0.026	−0.119	0.294
DBP	−0.021	0.852	−0.053	0.639	−0.194	0.083	0.044	0.700
BMI	−0.016	0.890	0.043	0.703	−0.174	0.120	0.076	0.506
WC	0.038	0.736	0.012	0.919	−0.216	0.053	0.097	0.396
TC	−0.204	0.067	−0.188	0.093	0.049	0.666	−0.059	0.603
TG	−0.062	0.581	0.000	1.000	−0.116	0.304	0.078	0.493
LDL-C	−0.101	0.370	−0.140	0.212	0.076	0.499	−0.032	0.779
HDL-C	−0.138	0.218	−0.073	0.515	0.001	0.991	−0.090	0.429
FBG	−0.090	0.425	−0.047	0.676	0.062	0.583	0.022	0.851
Women								
SBP	−0.301	0.004	−0.160	0.129	−0.164	0.121	−0.010	0.925
DBP	−0.222	0.034	−0.131	0.217	−0.132	0.211	−0.030	0.780
BMI	−0.098	0.356	0.037	0.728	−0.005	0.959	0.033	0.754
WC	−0.071	0.503	0.051	0.631	−0.025	0.812	−0.059	0.580
TC	−0.017	0.875	−0.110	0.300	0.038	0.719	0.183	0.082
TG	0.018	0.864	0.081	0.447	0.126	0.234	0.092	0.388
LDL-C	0.087	0.410	−0.073	0.493	0.106	0.318	0.223	0.033
HDL-C	−0.113	0.288	−0.101	0.341	−0.192	0.069	−0.042	0.695
FBG	−0.110	0.300	−0.109	0.302	0.033	0.758	0.002	0.984

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TC, total cholesterol; TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, fasting blood glucose.

knockout mice showed a significant increase in the mean arterial pressure, and *Per1* showed sex-based dependence in the regulation of cardiovascular rhythm. Male mice mainly showed nondipper hypertension, while a protective effect on nondipper hypertension mediated by *Per1* was observed in female mice [13]. Moreover, the dysfunction of the circadian function can induce angiotensin II-dependent hypertension in mice [33]. Research has shown that mice that lack *Per1* exhibit significantly negatively regulated expression of *Edn1* that could be a vasoconstrictor peptide and with hypertension [34, 35]. Kurbatova et al. [6] observed a temporal change of *Per1* mRNA level during the day in hypertensive patients. Marques et al. [36] revealed that *Per1* mRNA expressed in hypertensives renal medulla was higher than that in normotensives. However, human data on the role of *Per1* levels in PBMCs in the development of hypertension are lacking. Although we observed a negative correlation between *Per1* and SBP in men, the *r* value was only −0.247. Therefore, it remains unclear whether *Per1* is implicated in the pathogenesis of hypertension.

Studies have shown that compared with male wild-type mice, *Clock* knockout mice had no variety in blood pressure rhythm at 24 h, but there is a drop in blood pres-

sure levels [12]. Another mice experiment confirmed that *Clock* mutant male mice showed a decrease in blood pressure and heart rate, while also showing lower plasma aldosterone levels [37]. In contrast, Nakashima et al. [15] reported that *Clock* mutant mice showed reduced expression of ATP1B1, a gene encoding the $\beta 1$ subunit of Na^+/K^+ ATPase, causing increased blood pressure. In addition, the dysfunction of the circadian function can induce angiotensin II-dependent hypertension in mice [33]. Therefore, it is reasonable to believe that *Clock* plays an important role in the regulation of hypertension by the human kidney system. This process may involve the clock gene upregulating the expression of intercellular adhesion molecule-1, promoting the adhesion of monocytes to endothelial cells, and causing vascular inflammation [38]. In the current study, we did not observe relationships between *Clock* expression and blood pressure which may be attributable to the relatively small sample size.

Our results should be interpreted in the context of potential limitations. First, Kurbatova et al. [6] analyzed the RNA of oral epithelial cells in 34 hypertensive patients and found a general trend of downregulation of *Arntl* transcription levels and upregulation of *Per1* expression

from 9:00 a.m. to 5:00 p.m. We could not collect peripheral blood samples from participants at different time points, making it impossible to compare the relationship between rhythm changes and hypertension. Second, our study employed a case-control design; therefore, we could not determine the causal relationship between the *Arntl* mRNA expression levels and hypertension. Third, we did not collect the characteristics of patients' blood pressure, which limited the analysis of the relationship between genes and dipper/nondipper hypertension.

Conclusions

In sum, the levels of *Arntl* mRNA expression in PBMCs were significantly lower in hypertension patients than those in the control group in the female subjects. The results of our study emphasize the need for further investigation on the relationship between circadian clock genes and hypertension.

Acknowledgments

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Statement of Ethics

The study procedure was approved by the Scientific Research Institutional Review Board of Wannan Medical College Yijishan Hospital (No. 32 [2018]) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the participants.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Y.Y. and Z.F. contributed to study concept and design. Z.F., Y.C., L.Z., W.C., Y.J., and Y.Y. contributed to subjects' collection. L.Z. and Y.J. contributed to acquisition and analysis of data. The drafting and writing of the manuscript was done by Z.F. and L.Z. The revision of the manuscript was done by Y.Y. and Y.C. All authors approved the final manuscript.

Data Availability Statement

The datasets analyzed in the present study are available from the corresponding author upon reasonable request.

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