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Expression of circadian clock genes in leukocytes of patients with Meniere's disease

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Abstract

Objectives: The underlying etiology of Meniere's disease (MD) is not completely clear, but the precipitated triggers may alter the circadian clock in patients with MD. This study aims to survey the expression of circadian clock genes in peripheral blood (PB) leukocytes of MD patients.

Methods: We investigated the expression of nine circadian clock genes in the PB leukocytes of patients with MD and normal controls using real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Results: We observed significantly lower expression of *PER1* gene and higher expression of *CLOCK* gene in MD patients than those in normal controls ($p < 0.05$). *PER1* did not associate with the degree of dizziness handicap in the patients with MD, but a lower expression of *PER1* was significantly correlated with higher pure tone average (PTA) and speech reception threshold of the affected ear ($p < 0.05$). Patients with PTA > 30 dB had significantly lower *PER1* expression than those with PTA ≤30 dB in the affected ear ($p < 0.05$). Our qRT-PCR result was validated by fewer positively stained leukocytes for PER1 protein in the MD patients using the immunocytochemical study.

Conclusion: Our study implies the alteration of the circadian clock in patients with MD. In particular, the downregulation of *PER1* correlated with the degree of hearing loss in the affected ear. *PER1* in PB leukocytes may be a potential marker for the progression of hearing loss in MD.

KEYWORDS
circadian clock, hearing loss, Meniere's disease, PER1

Chung-Feng Hwang and Ming-Yu Yang contributed equally to this study.

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1 | INTRODUCTION

Meniere's disease (MD) is a clinical entity characterized by repeated vertigo symptoms with sensorineural hearing loss. The disease, first described by Prosper Meniere in 1861, is commonly thought of as endolymphatic hydrops in the inner ear.[1] However, to date, the exact causes of MD are generally unknown. Various theories, including infectious (viruses and syphilis), allergic, genetic, trauma, auto-immune, otoconia or otoliths, and low cerebrospinal fluid pressures, were proposed as the pathogenesis of hydrops of MD.[2,3] The natural course of vertigo and hearing loss in MD is not well understood. Although the frequency of vertigo in MD patients usually increases during the first few years and decreases later, the severe attack of dizziness may occur even 20 years after initial diagnosis.[4] The hearing of the patients with MD may worsen after the disease progression, but MD patients may have stable hearing over time.[5] In general, the outcome of MD varies between patients, possibly due to different intrinsic and environmental factors.[3,6]

Circadian rhythm is present in almost all eukaryotes with a 24-h cycle. Daily rhythmic changes are observed in several physiological processes,[7,8] which are mainly regulated by the suprachiasmatic nucleus of the anterior hypothalamus. In addition to the central pacemaker, peripheral organs including liver, heart, kidney, and peripheral blood (PB) leukocytes also contain circadian oscillators,[9] which are the networks that generate 24-h rhythms in each organ.[10] Cochlea[11,12] and inferior colliculus[13] also have the circadian clock in the experimental animal models. At least nine core circadian clock genes (*PER1, PER2, PER3, CRY1, CRY2, CLOCK, CK1ε, BMAL1,* and *TIM*) regulate central and peripheral circadian oscillators in transcriptional-translational feedback loops.[8] Disruption of circadian rhythm or altered circadian clock genes are associated with increased risks of depression,[14] diabetes,[15] cancer development,[16–18] and neurodegenerative diseases.[19,20] For example, decreased expression of several circadian clock genes has been observed in the PB leukocytes in patients with Parkinson's disease.[21,22] However, it is still unclear whether circadian disruption is linked to inner ear disorders.

Poor quality of sleep is a common complaint in patients with dizziness.[23–25] Decreased deep sleep and an elevated arousal index were observed in patients with MD.[26] Since the circadian clock plays an essential role in sleep homeostasis,[27] altered clock gene expression may associate with the sleep disturbance. However, the circadian clock pattern in patients with vestibular disorders was rarely reported. A previous study had shown that the circadian rhythmic amplitude of saliva melatonin was lower in MD patients than the age-matched controls.[28] We also observed the altered circadian clock genes expression in patients with sudden sensorineural hearing loss, especially for those with associated symptoms of vertigo.[29] These findings interest us in whether the expression of circadian clock genes was also altered in the patients with MD.

To investigate the role of the circadian clock in MD, we gathered PB from the MD patients and healthy controls and checked the circadian clock gene expression in leukocytes. In addition, we compared the expression of circadian clock genes in MD patients with different degrees of dizziness handicap and hearing loss.

2 | MATERIALS AND METHODS

2.1 | Subjects

Thirty patients (mean age: 46.27 ± 11.64 years) who came to the otolaryngology clinic in Kaohsiung Chang Gung Memorial Hospital, Taiwan, with the diagnosis of definite MD from August 2016 through October 2019 were included in the study. Definite MD was defined as two or more definitive periods of vertigo along with hearing loss, plus tinnitus or aural fullness or both according to the diagnostic criteria of 2015 Classification Committee of the Bárány Society.[30] The hearing of the MD patient was evaluated by the pure tone average (PTA) of thresholds at 500, 1000, 2000, and 3000 Hz. In some cases, the threshold at 3000 Hz was interpolated by averaging the thresholds at 2000 and 4000 Hz according to the standards of the Hearing Committee of the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS).[31] The degrees of hearing loss in the MD patients were grouped according to the AAO-HNS hearing classification system (Class A: PTA ≤30 dB, Class B: PTA > 30 dB, ≤50 dB, Class C: PTA > 50 dB).[32,33] The data of speech reception threshold (SRT) in each patient was also collected. The handicap of dizziness was evaluated by the Dizziness Handicap Inventory (DHI), which is a 25-item self-report questionnaire to quantify the dizziness-related physical, emotional, and functional impacts on daily life, and the patients were grouped according to the DHI total scores (mild: 16–34 points, moderate: 36–52 points, severe: 54+ points).[34] We excluded patients with infectious signs such as fever or local infection, previous history of autoimmune disease or head injury, imaging studies showing retrocochlear lesion, or laboratory studies showing leukocytosis, high antinuclear antibody titer, or syphilis. Patients who were diagnosed with bilateral MD or vestibular migraine, and who were using systemic steroid or hormone therapy were excluded. We also recruited 26 age- and sex-matched healthy subjects (mean age: 44.96 ± 11.9 years) who did not have hearing and vestibular disorders as normal controls. This study protocol was approved by the institutional review board (IRB) of the Chang Gung Memorial Hospital (IRB No. 104-3473B and 201801624B0) and written informed consents were obtained from the subjects before PB acquisition.

2.2 | Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of circadian clock genes

PB samples were obtained between 8:00 and 10:00 am from patients with MD and healthy controls because our previous and other studies revealed that the expression of most circadian clock genes in PB leukocytes peaked at 8:00 am.[18,35] In MD patients, PB was drawn from those who suffered from recent attacks of vertigo within 1 week to reflect the circadian clock gene expression in active disease. Each subject was informed to keep their regular diet before PB acquisition. Total RNA was extracted from the PB leukocytes using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was generated with High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacture's protocols. The design of the specific forward and reverse primers and MGB TaqMan probes for the nine circadian clock genes are as described previously.[14] Expression of human housekeeping genes, ACTB (β-actin), was used for normalizing circadian clock genes expression in qRT-PCR. All reactions were carried out in a 10-μl final volume containing 25 ng cDNA (as total input RNA), 400 nM each primer, 200 nM probe, and 5 μL 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Real-time quantitative PCR was performed in an ABI 7500 Fast Real-Time System (Applied Biosystems), and the PCR cycling parameters were set as follows: 95°C for 10 min followed by 40 cycles of PCR reactions at 95°C for 20 s and 60°C for 1 min. The expression levels of the circadian clock genes were normalized to the internal control ACTB to obtain the relative threshold cycle (ΔCt). The relative expression between MD patients and control was calculated by the comparative Ct ($\Delta\Delta Ct$) method ($\Delta\Delta Ct = [\Delta Ct(\text{MD-}ACTB) - \Delta Ct(\text{control-}ACTB)]$, fold change $= 2^{-\Delta\Delta Ct}$).

2.3 | Immunocytochemical study

Immunocytochemical staining was performed on PB total leukocyte samples from five patients with MD and healthy controls. PB leukocytes (5×10^5 cells) were cytocentrifuged onto glass slides, fixed in 1% formaldehyde/phosphate-buffered saline (PBS), and blocked for nonspecific binding with 10% bovine serum albumin/PBS. Samples were incubated with the primary polyclonal antibody against PER1 (Abcam Inc., MA) at 1:200 dilutions for 1 h and then incubated with biotinylated goat anti-rabbit antibody for 30 min. We used a horseradish peroxidase-diaminobenzidine staining kit (Abcam Inc.) to visualize the specific binding of the secondary antibody to the primary antibody. After staining, the cells were mounted, cover-slipped, and examined using a Zeiss microscope (Zeiss, Gottingen, Germany). The number of cells positively stained for PER1 were counted using the ImageJ software (NIH, Bethesda, MD), and the percentages of PER1-positive cells were quantified.

2.4 | Statistical analysis

SPSS software for Windows (PASW Statistics 18.0, SPSS, Inc., Chicago, IL) and GraphPad Prism 9.2 (GraphPad Software, La Jolla, CA) were used to analyze the results of this study. The Chi-square test was used to compare the categorical variables. The values of $-\Delta Ct$ were used for statistical analysis of gene expression. Higher $-\Delta Ct$ represented a higher expression level, and vice versa. The Student's t-test was used to detect the differences between patients with MD and healthy controls. Mann–Whitney U test was used to detect the differences in each circadian clock gene expression among patients with different degrees of dizziness handicap and hearing loss as well as to analyze the immunocytochemical study. Correlations of the expression of circadian clock genes with DHI total score and PTA or SRT of the affected ear in the MD patients were calculated by Pearson's correlation analysis. All statistical tests were two-sided and a p value of <0.05 was considered significant.

3 | RESULTS

3.1 | Characteristics of participants

The characteristics of subjects who participated in this study were listed in Table 1. There were no significant differences in age and sex distribution between MD and control groups (p > 0.05). Most patients with MD suffered from severe dizziness handicap (46.7%), followed by mild (36.7%) and moderate (16.7%) handicap. The PTA of 60% of patients with MD in our study was ≤30 dB in the affected ear, while the rest 40% of patients with MD had more than 30 dB hearing loss.

TABLE 1 Characteristics of patients with MD and controls

Parameter	MD (n = 30)	Control (n = 26)	p value
Age (year, mean ± SD)	46.27 ± 11.64	44.96 ± 11.9	0.68
Sex (n, %)			1
Male	4 (13.3%)	4 (14.3%)	
Female	26 (86.7%)	22 (85.7%)	
Dizziness handicap inventory (n, %)			
Mild (score: 16–34 points)	11 (36.7%)		
Moderate (score: 36–52 points)	5 (16.7%)		
Severe (score: 54+ points)	14 (46.7%)		
Hearing severity in affected ear (n, %)			
Class A (PTA ≤30 dB)	18 (60%)		
Class B (PTA 31–50 dB)	8 (26.7%)		
Class C (PTA >50 dB)	4 (13.3%)		

Abbreviations: MD, Meniere's disease; PTA, Pure tone average.

FIGURE 1 The expression of nine circadian clock genes in patients with MD and controls. Data are mean ± SE of $-\Delta C$. *$p < 0.05$, ***$p < 0.001$
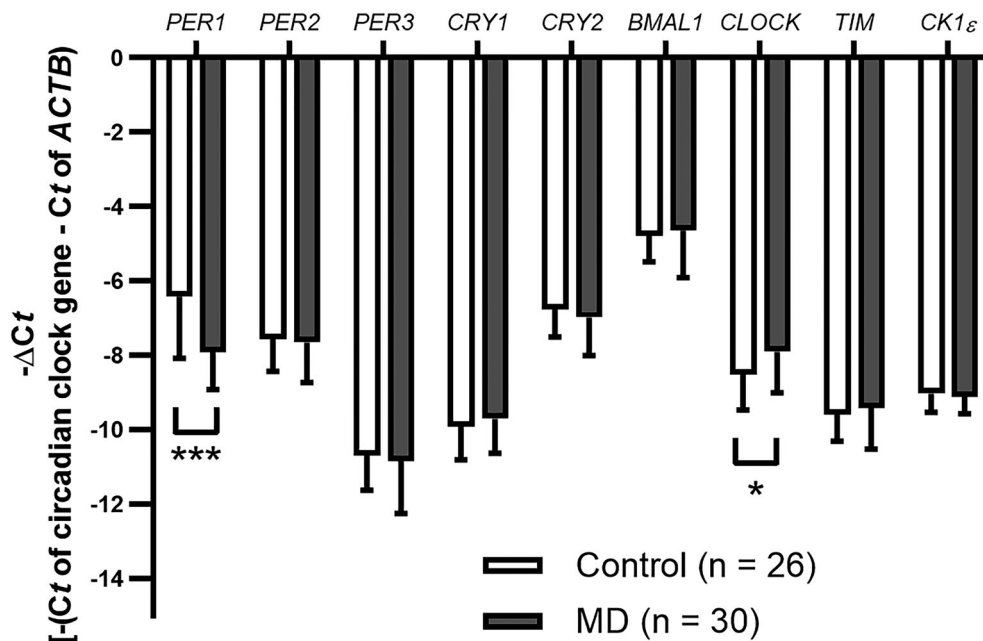
There was no significant correlation between DHI total score and PTA in the MD group ($p > 0.05$).

3.2 | Differential expression of circadian clock genes in PB leukocytes between MD and control groups

To explore whether the expression of circadian clock genes was altered in patients with MD, we used qRT-PCR to compare the expression of nine circadian clock genes (presented as $-\Delta Ct$) of patients with MD to healthy controls. As shown in Figure 1, significantly decreased expression of *PER1* ($p < 0.001$) and increased expression of *CLOCK* ($p = 0.033$) were found in PB leukocytes of patients with MD compared with controls. Further analysis of relative expression between MD and control groups by comparative Ct ($\Delta\Delta Ct$) method revealed a mean 3.45 fold decreased expression of *PER1* and an 1.12 fold increased expression of *CLOCK* in MD group compared with healthy controls.

3.3 | Expression of circadian clock genes in PB leukocytes of MD patients with different DHI total score

To evaluate the association of expression of circadian clock gene with the impact of dizziness on daily life in MD, we compared the expression of circadian clock genes between mild to moderate and severe handicap groups according to DHI total scores.[34] We found the expression of all the nine circadian clock genes was not different between mild to moderate and severe dizziness handicap groups ($p > 0.05$; Figure 2A). There was no significant correlation between
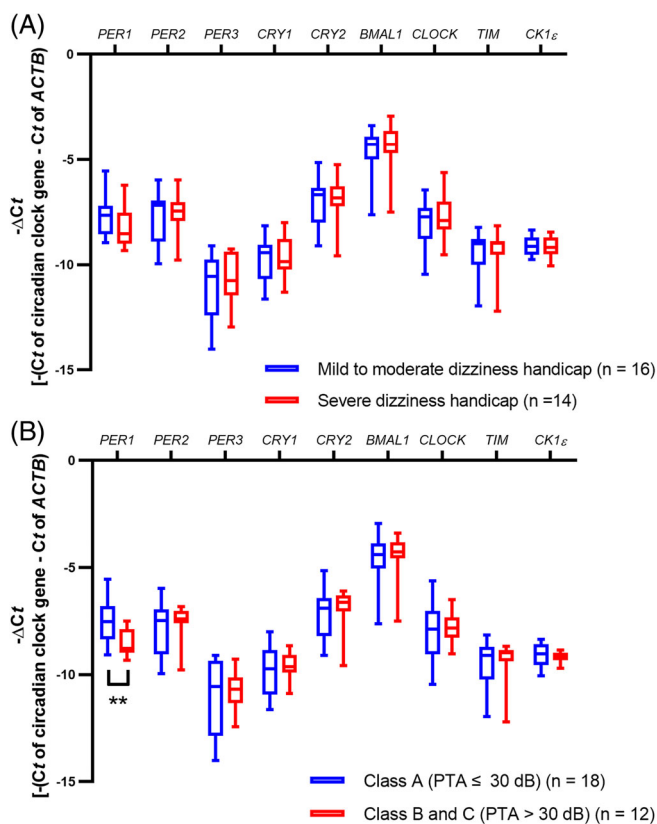

FIGURE 2 The expression of nine circadian clock genes in patients with MD with different degrees of dizziness handicap (A) and hearing loss (B). Data are median and interquartile range of $-\Delta Ct$. **$p < 0.01$

the expression of circadian clock genes and the DHI total score, as well as the physical, emotional and functional subscales scores in MD patients (all $p > 0.05$).
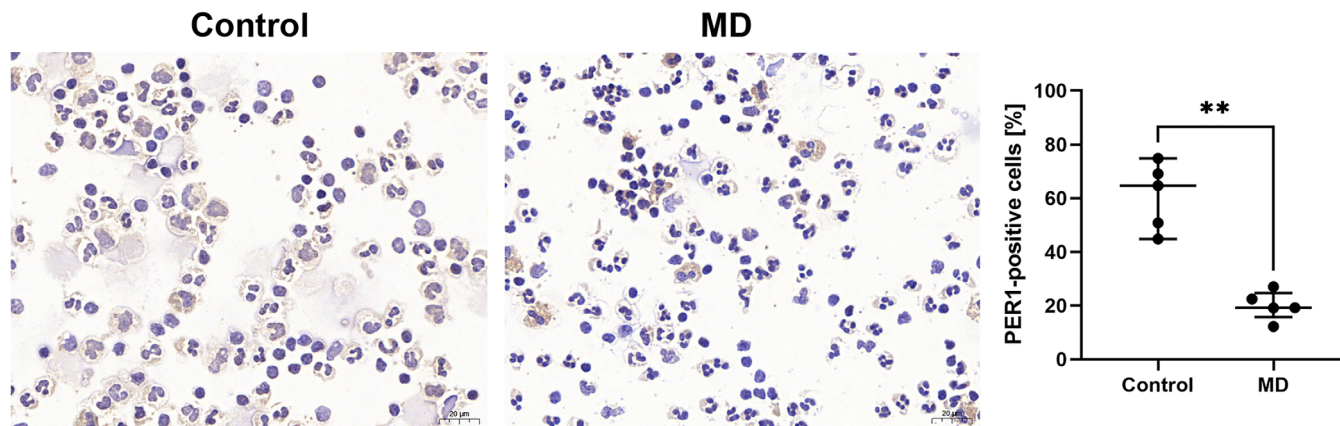
FIGURE 3 Representative image and quantification of immunocytochemical analysis for PER1 in PB leukocytes. Most cells were positively stained (brown) for PER1 in the control group, whereas only a few cells were stained for PER1 in the MD group. Data are median and interquartile range of PER1-positive cells (%). **$p < 0.01$

3.4 | Expression of circadian clock genes in PB leukocytes of MD patients with different degrees of hearing loss

We next surveyed the circadian clock gene expression in MD patients with different degrees of hearing loss in PTA of the affected ear. We observed a significantly decreased expression of *PER1* in patients with hearing loss of class B and C (PTA > 30 dB) compared with those with class A (PTA ≤30 dB; $p = 0.005$; Figure 2B). Further analysis revealed that the level of down expression in *PER1* was significantly correlated to the PTA ($r = -0.397$, $p = 0.03$) and SRT ($r = -0.371$, $p = 0.043$) of the affected ear in MD patients. In addition, *PER1* expression in the patients with stages 3 and 4 was also significantly lower than stages 1 and 2 according to the 1995 AAO-HNS staging system[36] ($p = 0.029$; Figure S1). These results imply that decreased expression of *PER1* may indicate the progression of hearing loss in MD.

3.5 | Immunocytochemical staining for PER1 in the patient with MD and control

Since gene expression of *PER1* significantly decreased in patients with MD and was associated with hearing loss, we conducted the immunocytochemical analysis of PB leukocytes from MD patients and healthy controls to validate the protein expression of the *PER1* genes. As shown in Figure 3, most PB leukocytes from the healthy control were positively stained for PER1. However, only a few cells of the patient with MD were stained by PER1 antibody. Quantification analysis revealed that more PER1-positive cells were observed in the MD group than the control group ($p = 0.008$). The immunocytochemical findings confirmed the transcript results from the qRT-PCR analysis.

4 | DISCUSSION

This is the first study to investigate the circadian clock gene expression in patients with MD. Our previous study and other report have proved the time-dependent variation pattern of circadian clock genes in PB leukocytes of healthy individuals.[18,35] As a result, we gathered the PB from each study subject only in the morning to avoid confounding circadian factors to affect gene expression. In addition, we drew the blood from MD patients who suffered from recent vertigo attacks to reflect the gene expression in active disease. In this study, we found significantly lower expression of *PER1* and higher expression of *CLOCK* transcripts in patients with MD by qRT-PCR analysis. This is in line with the antiphase feature between *PER* and *CLOCK* expression. In addition, the patients with hearing loss of PTA > 30 dB had a significantly decreased *PER1* expression compared with the group with PTA ≤30 dB. A lower expression of *PER1* was significantly correlated with higher PTA of affected ear. Our results reveal the potential association between *PER1* and clinical symptoms of MD, and imply that downregulation of *PER1* may reflect the MD hearing progression.

The attack of MD may be precipitated by several environmental factors, including the high-salt diet, alcohol, caffeine, climate change and stress.[37] These triggers may also alter the expression of circadian clock genes in MD patients. For example, a high-salt diet affected the circadian rhythm system and therefore caused sleep fragmentation.[38,39] Previous studies also proved that alcohol and caffeine could influence human circadian rhythms.[40,41] In addition, life stress could affect the hypothalamus-pituitary-adrenal axis in MD[42] and disrupt the circadian rhythm.[43] Decreased deep sleep[26] and obstructive sleep apnea[44] were also linked to MD and circadian disruption.[45] Therefore, these associations between MD triggers and circadian rhythm disruption may account for our findings of altered circadian gene expression in patients with MD during the attack. Of note, the dizziness symptom may further induce abnormal sleep duration[25] and, in turn, disrupt the circadian clock in patients with MD. In fact, previous studies had revealed that vestibular loss can disrupt the daily rhythm in rats[46] and may be associated with abnormal circadian rhythms in patients.[47]

Several reports had revealed that the handicap of dizziness patients, commonly evaluated by self-reported measures DHI, are associated with their sleep quality.[23,24] However, the sleep quality

was frequently surveyed by subjective sleeping questionnaires, and the correlations between DHI and sleep quality were only evident in vestibular migraine and benign paroxysmal positional vertigo, not in MD.[23] By objective genetic testing, our results revealed that the expression of all examined circadian clock genes did not significantly associate with DHI total score, as well as the physical, emotional and functional subscales scores in patients with MD. Our data imply that the dizziness handicap degree of MD is independent of circadian rhythm, but may be mainly influenced by the compensated and uncompensated status of vestibular dysfunction.[48]

Within the postulated mechanisms behind the formation of endolymphatic hydrops in MD, oxidative stress of the inner ear was thought of as one of the causal factors.[49–51] Overproduction of reactive oxygen species is well known as the major contributor to the damage of hair cells in acquired hearing loss.[52] It was also hypothesized that oxidative stress involved in endolymphatic hydrops contribute to the inner cellular damage and sensorineural hearing loss in the later stage of MD.[50] Dysregulated circadian rhythms may affect the cellular antioxidant system and link to oxidative stress.[53] We have recently reported that constant light dysregulated the cochlear circadian clock, augmented the production of reactive oxygen species, and increased the threshold shift of mice exposed to high-intensity noise.[12] Therefore, it was reasonable to postulate that the repeated triggers in MD disrupt the circadian clock, followed by increasing the oxidative stress in the inner ear and eventually deteriorate the hearing in MD patients.

Among the tested circadian clock genes, *PER1* is the most significantly down-regulated gene in PB leukocytes of the patients with MD, especially in whom with hearing loss of class B and C. A previous study had reported that *PER1* mRNA in human peripheral leukocytes exhibits a robust circadian expression than other circadian clock genes and thus leukocyte *PER1* could serve a molecular marker for the human circadian system.[35] As a result, the effect of possible MD triggers on the circadian clock could be obviously revealed in *PER1* expression. Notably, the lack of *PER1* deregulated cellular glutathione peroxidase-related reactive oxygen species fluctuations,[54] and may augment the oxidative stress in the hair cells. This may account for our results showing that the lower expression of *PER1* was associated with higher PTA of the affected in patients with MD. Further investigation is needed to elucidate the role of *PER1* on the auditory system in MD.

In recent years, obstructive sleep apnea (OSA) has been thought of as a risk factor for MD.[44,55] It was hypothesized that sleep apnea might lead to the insufficient supply of blood in the inner ear and might cause endolymphatic hydrops in MD.[44] Interestingly, altered expression of *PER1* was also observed in the PB leukocytes of patients with OSA.[45,56] Although we did not screen OSA in our MD patients, it is reasonable to speculate that if MD combined with OSA, OSA might be associated with the attack of MD by altering the circadian clock and *PER1*. Therefore, future subgrouping analysis to evaluate *PER1* in MD patients with and without OSA will be helpful to elucidate our speculation.

There are several limitations in our study. First, our data revealed an apparent female preponderance (about six-fold in females compared with males) in patients with MD, which is different from other reports showing only slight prevalence in females.[57] This is probably because the female patients with MD in Taiwan are usually more willing to follow audiometry in the otologic clinic and thus, definite MD could be more easily diagnosed in females. Second, the number of subjects in our study is small, which decreases the statistical power during the subgrouping analysis. The above two limitations compromised our results, and future large-scale and populational studies are needed to clarify the role of circadian clock genes in MD. Besides, since posttranscriptional and translational changes may be influenced by the periodicity of MD attacks, further design of longitudinal analysis for circadian clock genes in different periods of MD status will help to see whether the gene expression fluctuates with symptoms of MD.

Because the cochlear symptoms of MD are not necessarily related to vestibular symptoms,[58] the progression of hearing loss in patients with MD is usually hard to be predicted even under strict dietary control, lifestyle modification and medical treatment.[59] Our analysis of *PER1* expression in PB leukocytes may provide a novel way to monitor the clinical progression of MD. Further investigation to evaluate the *PER1* expression in the inner ear using MD animal models or postmortem specimens would be helpful to elucidate the possible mechanism of circadian clock alteration underlying the pathogenesis of MD.

5 | CONCLUSION

MD is an entity with variable nature course in the symptoms of vertigo and hearing loss. Our study revealed the altered expression of circadian clock genes, especially *PER1*, in PB leukocytes of the patients with MD. Although the expression of *PER1* was not associated with the DHI score, *PER1* was significantly lower in patients with PTA > 30 dB and was correlated with PTA and SRT. Our result implied that *PER1* might be a potential marker to evaluate the progression of hearing loss in patients with MD.

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SUPPORTING INFORMATION