A genome-wide association study for melatonin secretion

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Melatonin exerts a wide range of effects among various tissues and organs. However, there is currently no study to investigate the genetic determinants of melatonin secretion. Here, we conducted a genome-wide association study (GWAS) for melatonin secretion using morning urine 6-hydroxymelatonin sulfate-to-creatinine ratio (UMCR). We initially enrolled 5000 participants from Taiwan Biobank in this study. After excluding individuals that did not have their urine collected in the morning, those who had history of neurological or psychiatric disorder, and those who failed to pass quality control, association of single nucleotide polymorphisms with log-transformed UMCR adjusted for age, sex and principal components of ancestry were analyzed. A second model additionally adjusted for estimated glomerular filtration rate (eGFR). A total of 2373 participants underwent the genome-wide analysis. Five candidate loci associated with log UMCR (P value ranging from 6.83 × 10−7 to 3.44 × 10−6) encompassing ZFHX3, GALNT15, GALNT13, LDLRAD3 and intergenic between SEPP1 and FLJ32255 were identified. Similar results were yielded with further adjustment for eGFR. Interestingly, the identified genes are associated with circadian behavior, neuronal differentiation, motor disorders, anxiety, and neurodegenerative diseases. We conducted the first GWAS for melatonin secretion and identified five candidate genetic loci associated with melatonin level. Replication and functional studies are needed in the future.

Melatonin is a pleiotropic hormone primarily synthesized and secreted from the pineal gland. Many other tissues can also produce it, including leukocytes, bone marrow, gastrointestinal tract, neuronal cells, and gonads1-3. Melatonin regulates various physiological processes, including circadian and seasonal rhythms, energy and glucose metabolism, antioxidant effects, anti-inflammatory actions, and immune function1,3-6. There are many studies showing associations between melatonin and many disorders, including certain types of mental illness, cancer, cardiovascular disease, metabolic syndrome, type 2 diabetes, and obesity6-11. Melatonin is secreted into the circulation following a circadian rhythm with peak levels at night12. Aging was once thought to be directly associated with decreased melatonin secretion. However, there was no significant difference between circadian amplitude of the plasma melatonin between healthy elderlies and young adults13. Instead of aging, the degree of pineal calcification was associated with melatonin excretion amount14.

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Substantial evidence suggests genetic factors also play a significant role in melatonin secretion\textsuperscript{15,16}. Genome-wide association study (GWAS) has been introduced as a powerful tool to identify common genetic variants of complex diseases or quantitative traits\textsuperscript{17}. Currently, there is no published GWAS regarding melatonin levels. Here, we conducted the first GWAS of urine melatonin metabolite, 6-hydroxymelatonin sulfate (aMT6s), which surrogates the circulating melatonin level\textsuperscript{18}.

**Materials and methods**

**Study population.** Five thousand individuals aged 30–70 years old and without cancer history were enrolled from Taiwan Biobank. Biological specimens, personal and clinical information as delinked data were used in this study. Individuals with a record of neurological disorders or psychiatric illnesses were excluded from this study as these conditions may affect melatonin secretion\textsuperscript{19,20}. This study was approved by the Institutional Review Board of Chang Gung Medical Foundation and the Institutional Review Board of National Taiwan University Hospital. All subjects have provided written informed consent, and all methods were carried out in accordance with relevant guidelines and regulations.

**Urine aMT6s and creatinine measurement.** It is infeasible to draw blood samples from volunteers in the middle of the night for serum melatonin levels. Urinary aMT6s is the major metabolite of melatonin excreted from the kidneys\textsuperscript{21}. Thus, measuring morning urine aMT6s level is a practical alternative for serum melatonin level at night\textsuperscript{18}. For better correlation, spot urine aMT6s level should be creatinine-corrected to adjust the effect of variable urinary dilution\textsuperscript{22}. Urine aMT6s-to-creatinine ratio (UMCR) was calculated from urinary aMT6s divided by urine creatinine level. The concentration of aMT6s was measured in the urine of Taiwan Biobank subjects by an enzyme-linked immunosorbent assay (ELISA) kit using the manufacturer’s protocol (Human Melatonin Sulfate ELISA kit, Elab science). No significant cross-reactivity or interference between melatonin sulfate and analogs was observed. All standards via serial dilution were assayed in duplicates. The urine creatinine level was measured using a chemistry analyzer (AU5800, Beckman Coulter) with compensated Jaffe method.

**Genotyping, quality control and imputation.** Genotyping with the Axiom-Taiwan Biobank Array Plate (TWB chip; Affymetrix Inc, Santa Clara, California) was performed at the National Center for Genome Medicine of Academia Sinica\textsuperscript{23}. We use PLINK (version 1.9), an open-source whole-genome data analysis toolset, for quality control procedures\textsuperscript{24}. For SNPs with batch effect, their genotypes were set as missing. SNPs were excluded if missing genotype rate was high (>5%), minor allele frequency was low (<1%) or deviated from Hardy–Weinberg equilibrium ($P$ value < $10^{-5}$). Individuals with discordant sex (self-reported sex incongruent to genetic sex, where genetic male or female was defined by X chromosome homozygosity estimate above 0.8 or below 0.2), high missing genotyping rate (>5%), extreme heterozygosity rate (more than 5 standard deviations away from the mean) or high identity-by-descent score (≥0.1875) implying close relatedness were excluded from subsequent analyses. We computed the principal components on a linkage disequilibrium (LD)-pruned ($r^2 < 0.2$) set of autosomal variants obtained by removing high-LD regions via PLINK. Genotype imputation was carried out with SHAPEIT\textsuperscript{25} and IMPUTE2\textsuperscript{26}. We applied1000 Genomes Project Phase 3 East Asian Ancestry as the reference population. For genome annotation, Genome Reference Consortium Human Build 37 was used. Imputed SNPs with low-quality scores (info score\textsuperscript{27} lower than 0.8) were excluded. Indels were removed by using VCFtools\textsuperscript{28}.

**Statistical analyses.** Age and estimated glomerular filtration rate (eGFR) were expressed as mean and standard deviation. Urine aMT6s and UMCR were expressed as median and interquartile range. Logarithmic transformation of UMCR was done to normalize the data. GWAS analysis was carried out via PLINK v1.9 with an additive genetic model. We applied linear regression for analyzing associations between SNPs and log UMCR. Covariate adjustment in Model 1 included age, sex, and the first ten principal components of ancestry. eGFR was additionally adjusted in Model 2. We used a genome-wide significance threshold of $P < 5.0 \times 10^{-8}$\textsuperscript{29}. Since this threshold is very conservative for small sample size, we set the level for suggestive significance at $P < 5 \times 10^{-6}$\textsuperscript{30,31}. The Manhattan plot and quantile–quantile plot were generated by the qman R package\textsuperscript{32,33}. Regional association plots were made via LocusZoom\textsuperscript{34}. The proportion of phenotypic variance explained by SNP was calculated using the following items: effect size estimate of each minor allele on log UMCR, standard error of the effect size, sample size, and minor allele frequency for the SNP\textsuperscript{35}. The statistical power of this study was calculated using methods for quantitative GWAS\textsuperscript{36}.

**Bioethics statement.** This study was approved by the Institutional Review Board of Chang Gung Medical Foundation and the Institutional Review Board of National Taiwan University Hospital. All subjects have provided written informed consent and all methods were carried out in accordance with relevant guidelines and regulations.

**Results**

Five thousand subjects were enrolled from Taiwan Biobank initially. One withdrew from the study. 2361 did not have their urine collected in the morning and were excluded. 128 people had documented neurological or psychiatric illness. 137 individuals did not pass quality control procedures. After imputation and quality control, 7,897,704 autosomal SNPs remained. After log transformation of UMCR, data is still not normalized, but the shape of the histogram is better. We performed a GWAS analysis for log UMCR in the remaining 2373 subjects. The characteristics of our study population are listed in Table 1. Age is not significantly associated with
Table 1. Descriptive characteristics of study subjects. Data are mean ± SD, median (IQR) or number (%), as appropriate. Age is at specimen collection. eGFR estimated glomerular filtration rate (by modification of diet in renal disease equation), UMCR urine aMT6s/creatinine ratio.

### Characteristics

| Characteristic | Value          |
|---------------|----------------|
| Total participants, N | 2373           |
| Age, year      | 50.75 ± 10.83  |
| Males, N (%)   | 890 (37.51)    |
| eGFR, ml/min/1.73 m² | 108.20 ± 27.83 |
| Urine aMT6s, ng/ml | 20.41 (11.88–30.19) |
| UMCR, ng/mg    | 16.98 (10.32–27.33) |

Table 2. Association of genetic loci with log UMCR in a Taiwan Han Chinese population. Model 1 was adjusted for age, sex and the first ten principal components of ancestry. Model 2 was additionally adjusted for eGFR. SNP single nucleotide polymorphism, Chr chromosome. SNPs are imputed with high info score (0.831, 0.988 and 0.946 for rs142037747, rs9645614 and rs6451653, respectively).

| SNP     | Chr | Position     | Nearest gene | UMCR increasing allele | Other allele | UMCR increasing allele frequency | Model 1 | Model 2 |
|---------|-----|--------------|--------------|------------------------|-------------|---------------------------------|---------|---------|
| rs17681554 | 16  | 73,016,768   | ZFH3         | A                      | C           | 0.804                           | 6.86 × 10⁻⁷ | 6.83 × 10⁻⁷ |
| rs142037747 | 3   | 16,121,712   | GALNT13      | G                      | A           | 0.989                           | 7.82 × 10⁻⁷ | 7.73 × 10⁻⁷ |
| rs7571016   | 2   | 155,166,873  | GALNT13      | A                      | G           | 0.617                           | 1.53 × 10⁻⁴ | 1.54 × 10⁻⁴ |
| rs9645614   | 11  | 36,159,947   | LDLRAD3      | A                      | G           | 0.953                           | 2.90 × 10⁻⁶ | 2.91 × 10⁻⁶ |
| rs6451653   | 5   | 42,915,584   | SEPP1-FLJ2255 | G                     | A           | 0.869                           | 3.44 × 10⁻⁷ | 3.42 × 10⁻⁷ |

Discussion

In this first GWAS on melatonin secretion, we identified five suggestive loci associated with variation in log UMCR. rs17681554 is located within ZFH3 (Zinc Finger Homeobox 3). ZFH3 is a transcriptional regulator which contains four homeodomains and seventeen zinc fingers. During neuronal differentiation, there is a preferential expression pattern of ZFHX3 isoforms. Further studies are needed to elucidate a direct linkage between ZFH3 and melatonin.

rs142037747 and rs7571016 are located near GALNT13 (polypeptide N-acetylgalactosaminyltransferase 15) and within GALNT13 (polypeptide N-acetylgalactosaminyltransferase 13), respectively. These two polypeptide N-acetylgalactosaminyltransferases of the same family catalyze initiation of mucin-type O-linked glycosylation by adding N-acetylgalactosamine to serine or threonine residues of the polypeptide chain. Glycosylation is associated with cell adhesion, signal transduction, molecular trafficking, and differentiation in central nervous system development. Whether and how GALNT13 or GALNT13 significantly affects melatonin levels remains determined.

rs9645614 is located within LDLRAD3 (low density lipoprotein receptor class A domain containing 3). LDLRAD3 alters the proteolysis of amyloid precursor protein and increases the production of amyloid beta-peptide (Aβ). The primary pathogenesis of Alzheimer’s disease (AD) has been attributed to the extracellular aggregation of Aβ. Patients with neurodegenerative disorder such as Alzheimer’s disease exhibit reduced serum and cerebrospinal fluid melatonin levels comparing to age-matched controls.
Figure 1. Manhattan plot of the GWAS results for log UMCR. SNPs are plotted on the x axis according to their chromosome position against association with log UMCR on the y axis. The red horizontal line represents the suggestive association threshold of $P = 5.0 \times 10^{-6}$.

Figure 2. Quantile-quantile plots of log UMCR.
Figure 3. Regional association plots of log UMCR. (A) rs17681554, (B) rs142037747, (C) rs7571016, (D) rs9645614, (E) rs6451653.
Our present unbiased genetic study, revealing the LDLRAD3 variant associated with melatonin secretion from pineal gland, provides additional evidence for potential mechanistic explanation in AD patients with altered melatonin levels. rs6451653 is located between SEPP1 (selenoprotein P, or SELENOP) and pseudogene FLJ32255. SEPP1 serves as a phospholipid hydroperoxide glutathione peroxidase and thus protect the plasma membrane from oxidative damage and is expressed in all brain tissues. SEPP1 is secreted from astrocytes to neurons for prevention of oxidative damage. Several studies demonstrated that Sepp1 knockout mice displayed cerebellar ataxia, anxiety, impaired spatial memory, and widespread neurodegeneration in various studies. Also, deletion of SEPP1 in dogs resulted in central nervous system atrophy and cerebellar ataxia. It is convincing that the SEPP1 variant is associated with melatonin levels.

This study also showed borderline significance regarding the positive correlation between age and log UMCR. Since aging causes sarcopenia, the subsequently decreased creatinine excretion from urine increases the substance-to-creatinine ratio. Our results support the current concept that aging itself will not cause a decrease in melatonin secretion or excretion.

There was a concern that aMT6s excretion may be altered when renal function declines. A previous study enrolling 20 elders demonstrated that 24-h urine aMT6 was a reliable surrogate for plasma melatonin level, at least among individuals with GFR 24.6 ml/min or above. Our study confirmed that morning UMCR is not significantly correlated with eGFR, and adjusting eGFR in GWAS analysis essentially did not influence the results.

There are limitations to our study. First, it lacks replication of the result in another cohort. We searched in the UK Biobank, but melatonin as phenotype does not exist in the database. Moreover, the sample size is relatively small; thus, for the time being, these SNPs can only be seen as suggestive signals. The statistical power of this GWAS is only 56%, and therefore there are true loci that remain to be identified and validated. Also, there might be individuals receiving medications that can affect melatonin levels not documented in the Taiwan Biobank data due to the imprecise nature of the questionnaire survey. However, production or selling of melatonin pills is illegal in Taiwan, and thus this important confounding factor may not be significant in our study.

In summary, we have performed the first GWAS regarding melatonin secretion to date. This GWAS identified five highly suggestive genetic loci encompassing genes that demonstrated potential functional connectivity between the genes-associated melatonin level and circadian behavior, neuronal differentiation, cerebellar ataxia, neurodegeneration and Alzheimer's disease. Replication and functional studies of these genetic variations are warranted to understand better the regulation of melatonin secretion and related clinical disorders.

Data availability

Individual researchers may request to use the data for specific projects on a collaborative basis. Our data has been submitted to the NHGRI-EBI GWAS Catalog (accession ID: GCST90101875).

Received: 5 July 2021; Accepted: 4 May 2022
Published online: 16 May 2022

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**Acknowledgements**

This work is supported by Grants from the Ministry of Science and Technology in Taiwan (MOST 105-2314-

B-182-062, MOST 106-2314-B-182-043), the Translational Medical Research Program of Academia Sinica (ASTM-108-01-04), National Taiwan University Hospital, Yunlin Branch Intramural Grant (NTUHYL106.X003, NTUHYL.107S004, NTUHYL.111.X019, NTUHYL110.X017) and Chang Gung University, Taoyuan, Taiwan (NMRPD1F154, NMRPD1G0711, and BMRPD08). We would like to thank Taiwan Biobank for providing the biological specimens and information for our research, and the staff of the Core Lab, Department of Medical Research, National Taiwan University Hospital for technical support during the study. We would also like to thank Professor Lee-Ming Chuang at the Department of Internal Medicine, National Taiwan University Medical College for the critical review and valuable discussion about this manuscript. Lastly, we thank the Data Science Statistical Cooperation Center of Academia Sinica (AS-CFII-108-117) for statistical support.
Author contributions
P.-H.L., G.-T.C. and Y.-C.C. contributed to the experimental design. Y.-S.W., H.-C.K., Y.-C.L., Y.-S.C., Y.-Y.H., C.-H.L., W.-Y.L., J.-W.L., Chih-Neng H., J.-J.H, M.-L.H., H.-L.L. and Y.-C.C. contributed to the funding, sample procurement and data generation. G.-T.C. was responsible for data analysis; P.-H.L., Chia-Ni H., and C.-Y.S. provided statistical consultation. K.C.-W.L. wrote the revised discussion. Manuscript writing was done by G.-T.C., P.-H.L. and Y.-C.C.

Competing interests
The authors declare no competing interests.

Additional information
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