Research Article

Melatonergic index as a prognostic biomarker of reproductive organ cancers: correlations with metabolic parameters as well as clock genes *PER1* and *TIMELESS*

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ABSTRACT

Cancers of the reproductive organs are often hard to be detected, and patients' survival rate drops considerably even when the tumor is removed. Based on the fact that melatonin levels are significantly lower in cancer cells than that in the healthy cells, and this melatonin suppression remains during tumor progression, we have examined a simple two-gene-based melatonergic system [the indices of melatonin synthesis and metabolism (*ASMT:CYP1A1*, *ASMT:CYP1A2*, *ASMT:CYP1B1*)] as a prognostic factor for reproductive organ cancer survival rate. RNA-seq data from The Cancer Genome Atlas (TCGA) of seven types of human reproductive organ tumors (n = 3571 samples) were analyzed. By stratifying the set of index values into high *vs* low risk, we observed that patients with a high melatonergic index had improved survival rates for cervical, ovarian, and endometrial cancers. Patients at high-risk (low melatonergic index) showed a trend of diagnosis of breast, prostate, and testicular cancers at the younger age, while patients with cervical, ovarian, and endometrial cancers presented with higher tumor staging. The melatonergic indices, especially the *ASMT:CYP1B1*, positively correlated with the clock gene *PER1* while negatively correlated with the clock gene *TIMELESS* in all reproductive organ cancers. We further analyzed the correlation between the expression profiles of the melatonin-synthesizing enzyme (*ASMT* gene) with metabolic enzyme-encoding genes. Notably, *LDHA*, *PDK1*, and *PDHA1* showed a higher correlation in male and female reproductive organ tumors, while *IDH1*, *SDHB*, *GLS*, and *ATP1A1* had a positive correlation in breast, testicular, and endometrial cancers. These results have provided a comprehensive evaluation of the melatonergic system in relation to the reproductive organ tumor microenvironment and identified promising gene signatures as potential biomarkers for cancer diagnostics, prognostics, and therapeutics.

Key words: melatonin, melatonergic index, reproductive organ cancer, clock genes, tumor cell metabolism

1. INTRODUCTION

Breast, cervical, ovarian, endometrial, prostate, and testicular cancers are those most frequently diagnosed in the reproductive system (1). Female reproductive organ cancers often develop silently without apparent symptoms, resulting in high morbidity and mortality (2). Because therapeutic strategies remain improving and the early detection tools are limited for most of these malignancies, female reproductive organ cancers are often diagnosed in their later stages which considerably impact the prognosis (3). In addition, some patients will develop chemoresistance and relapse after treatments due to the original cancers transform to more aggressive malignant phenotypes (4). For males, the prostate cancer has the highest incidence and it is majorly associated with age, hormonal status, diet, and family history (5). Although testicular cancer has increased in recent decades, its risk factors remain unclear (6). Given the lack of new potential biomarkers for early diagnosis to guide a more reliable therapeutic intervention, it is important to identify this biomarker to determine whether patients are at high- or low-risk for disease progression and overall survival. Recent advances in proteomics, genomics, and computational biology have improved the diagnosis and process of drug repurposing for these cancers (7).

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine produced in a circadian manner by the pineal gland with highest levels occurring at night in response to signals originating from the central biological clock (8). Additionally, the non-circadian production of melatonin occurs in mitochondria of all cells, but seemingly less in cancerous cells (9). There is credible evidence that melatonin has oncostatic and pro-apoptotic actions in reproductive organ cancers, and these actions are impacted by the tumor microenvironments (10-16).

The melatonin synthetic pathway is highly conserved and dependent on the conversion of serotonin to N-acetylserotonin (NAS) by the arylalkylamine N-acetyltransferase (AANAT) followed NAS methylation by the acetylserotonin O-methyltransferase (ASMT) (17). In parallel, melatonin is primarily metabolized by hepatic cytochrome P450 (CYPs), especially, CYP1A1 and CYP1A2, with its conversion to 6-hydroxymelatonin (6OH-MEL). Additionally, CYP1B1 is a ubiquitously-distributed CYP which is highly expressed in extrahepatic tissues to metabolize melatonin in other tissues and organs (18). Although melatonin production and biotransformation are tissue-conserved, the ratio between the melatonin synthesis and its metabolism (ASMT:CYPs) can be used as an index of melatonin levels in the tumor microenvironment. In this context, a two-gene based melatonergic index based on gene expression has been proposed as prognostic indicator of the severity or progress of a cancer. High-grade gliomas with low *ASMT:CYP1B1* exhibit higher tumor aggressiveness and the patients have a worse prognosis (19). The melatonergic index was also described as a prognostic predictor and mutational burden indicator in 14 solid tumors (20). Due to a wide variation of components associated with the microenvironment of reproductive organ tumors, a diverse spectrum of genomic data is needed to assist in an understanding of the melatonin features of each tumor microenvironment.

Central and peripheral circadian rhythms are governed by transcriptional/translational feedback loops involving clock genes, and their misalignment and products favor the development of several cancers (21). The modern lifestyle is associated with sleep deprivation, night-shift work, altered mealtime, exposure to artificial light during darkness, and metabolic dysregulation, which cause profound changes in the circadian system (22). Circadian disruption accelerates tumor development and progression, and genetic variability represents one of the dysregulated features of clock genes (23). Interestingly, some endocrine-dependent cancers possess circadian machinery that is impacted by hormones (24). Given that circulating melatonin levels exhibit a nighttime rise and is proven to have clock-like effects in some

cancers (25), the expression profile of melatonin associated with the clock genes could be useful as a potential biomarker for prediction of tumor severity and progression.

In the vast majority of cancer cells, the pyruvate transport into mitochondria is hampered due to the inhibition of pyruvate dehydrogenase complex (PDC) by pyruvate dehydrogenase kinase (PDK), with the dominant route of pyruvate metabolism being to lactate; this is referred to as aerobic glycolysis or the Warburg effect (26-28). This metabolic feature favors the growth of cancers because of the rapid ATP synthesis. Recently-acquired evidence indicates that nocturnal rise in circulating melatonin redirects pyruvate metabolism from the lactate pathway into the mitochondria thereby recoupling it to oxidative phosphorylation (9). Consequently, cancer cells may only exhibit an energy-related cancerous phenotype during the day (29, 30). Thus, identifying day:night metabolic profile that is driven by circadian changes in melatonin concentrations is essential to determine Warburg-dependent cancer signatures such as those observed in reproductive organ cancers.

Considering that the melatoninergic system may play an important role in reproductive system cancers, we investigated the RNA-seq data in patients with various cancer types. We first characterized patients' overall survival and tumor stage according to high and low melatonergic-index levels; thereafter, we correlated the capacity of reproductive organ tumors to synthesize/metabolize melatonin with the expression of clock genes and metabolism-related enzymes. These oncosignatures may provide significant value as a prospect for a prognostic method in determining the status of reproductive organ cancers.

2. MATERIALS AND METHODS

2.1. RNA-seq transcriptome analysis of seven reproductive organ TCGA cancer types.

Prior to identifying the reproductive organ cancers, we evaluated the gene expression profile of melatonin-synthetic enzymes (*AA-NAT* and *ASMT*) across 31 types of tumors compared them with TCGA and GTEx control samples. Next, we used RNA-seq data from male and female reproductive organ cancers generated using the Illumina HiSeq 2000 RNA sequencing platform available at the TCGA portal (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) (31). Collectively, 3571 samples of seven solid cancer types and 878 normal samples (TCGA and GTEx) were used: breast invasive carcinoma (BRCA, n = 1247, normal tissue, n = 291), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n = 313, normal tissue, n = 13), ovarian serous cystadenocarcinoma (OV, n = 634, normal tissue, n = 88), prostate adenocarcinoma (PRAD, n = 568, normal tissue, n = 152), testicular germ cell tumors (TGCT, n = 156, normal tissue, n = 165), uterine corpus endometrial carcinoma (UCEC, n = 596, normal tissue, n = 91), and uterine carcinosarcoma (UCS, n = 57, normal tissue, n = 78). We determined gene expression profiling using the online tool GEPIA (Gene Expression Profiling Interactive Analysis, accessed in January 2021), available at http://gepia.cancer-pku.cn/about.html (32). The genes were considered differentially expressed genes (DEGs) if Log_2 fold-change $\geq |1| \leq |1|$, p-value ≤ 0.01, and false discovery rate (FDR) < 0.05.

Next, we individually downloaded the individual gene expression data from TCGA tumors using UCSC Xena Browser (https://xenabrowser.net/) (33); GEPIA utilizes the same information of Xena, processed from the Toil Pipeline. These data present gene-level transcription values of log2(x+1) transformed RNA-Seq by expectation maximization (RSEM) normalized count within each sample to the upper quartile of reads. The gene expression data for *ASMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1* genes were downloaded for individual samples in January 2021 (Tables S1-S7).

2.2. Generation of melatonergic indices in reproductive organ cancers.

The expression levels of *ASMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1* were used to generate a model of a two-gene based system using log2-transformed RSEM values. The melatonergic index was proposed by Kinker *et al.* (19) and was further used by Lv *et al.* (20) to predict the rates of melatonin synthesis and its metabolism. To explore the melatonergic signature across male and female reproductive organ cancer types, we calculated three independent indices for each tumor: **Index 1** ($\log_2$ *ASMT* – $\log_2$ *CYP1A1*); **Index 2** ($\log_2$ *ASMT* – $\log_2$ *CYP1A2*); **Index 3** ($\log_2$ *ASMT* – $\log_2$ *CYP1B1*). Since the different CYP isozymes preferably hydroxylates melatonin in humans, *CYP1A1*, *CYP1A2*, and *CYP1B1* gene expression were used as mentioned by Ma *et al.* (18). The *CYP2C19* gene was excluded in this study because of its minor role in melatonin hydroxylation (18). The three indices based on melatonergic systems were tested as predictive prognostic factors of survival, gene correlation analysis, and gene mutation frequency. Enrichment analysis of mutated genes was generated using the Enrichr web tool. To calculate patients' overall survival rate for each tumor type, we stratified the melatonergic index values into two subgroups (high-median values and low-median values). We further correlated high- and low-values of the melatonergic indices on clinicopathological features of each tumor sample (age at diagnosis and tumor stage). The overall survival rate (%) and gene mutation frequency (%) according to TCGA tumor datasets were determined using data from the CBioPortal for cancer genomics (v3.5.4) (https://www.cbioportal.org/).

2.3. Correlation analysis between the melatonergic index and specific tumor genes.

After identifying the expression levels of clock genes (*PER1, PER2, PER3, CRY1, CRY2, ARNTL, CLOCK,* and *TIMELESS*) in reproductive organ tumors, we selected the most representative tumor suppressor and promoter genes (*PER1* and *TIMELESS*), respectively, and correlated their expression with the melatonergic indices based on Pearson's correlation coefficient. We further analyzed the relationship between gene expressions of cell energy metabolism-related enzymes (*LDHA, PFKM, G6PD, CS, PDK1, PDHA1, PDP1, OGDH, IDH1, NOX1, SDHB, ATP1A1, CPT1A,* and *GLS*) with *ASMT* levels for each tumor type.

2.4. Statistical analysis and data representation.

Four-way ANOVA was used to compare gene expression between tumor and controls. The cutoff finder survival algorithm identified the optimal cutoffs based on the most significant data (Log-rank test). The prognostic value of the three indices was plotted using Kaplan-Meier survival curves (lower vs higher melatonergic index values). Pearson's correlation test (P-value and correlation coefficients - R^2) was used to determine the correlation of the melatonergic indices (1-3) and the selected genes expression. Spearman coefficient classified the expression correlation. Fisher's exact test was used for univariate analysis. The box plots (median+IQR) and correlation graphs were analyzed with GraphPad Prism v. 6.00 for Windows (GraphPad Software, La Jolla, California, USA). Heatmaps and PCA plots for clustering analyses were performed with mean expression transformed values by web tool ClustVis (https://biit.cs.ut.ee/clustvis/) (34). Circos plot was used to identify shared genes based on correlation coefficient between each metabolic enzyme and cancer types (http://circos.ca/).

3. RESULTS

3.1. The expressions of melatonin synthetic enzymes and metabolism-associated genes are simultaneously downregulated in human reproductive organ cancers.

We first evaluated the expression levels of *AANAT* and *ASMT* on 31 TCGA tumors types. Although *AA-NAT* expression is reduced in most tumors, its general expression levels were relatively low (Figure S1 A) compared to *ASMT*. Except for six tumors [adrenocortical carcinoma (ACC), cholangiocarcinoma (CHOL), diffuse large B-cell lymphoma (DLBC), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), and acute myeloid leukemia (LAML)], the ASMT expression is reduced in all other tumor types (Figure S1 B).

A total of 3571 tumor samples obtained from seven types of reproductive organ cancers were systematically analyzed to evaluate the melatonergic system. Overall, expressions of melatonin-synthetic enzyme genes are diminished in these tumors and vary according to the pathological stages of the disease (Figure 1 A-D).

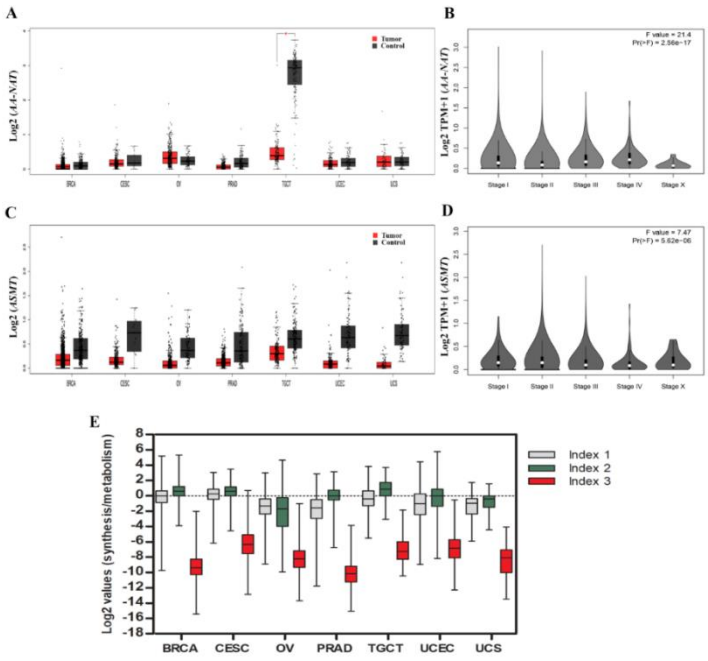

Fig. 1. The general profile of melatonin synthetic enzymes and melatonergic system in male and female reproductive organ cancers.

*A) AA-NAT expression (Log_2 TPM+1) between tumor and control samples; * P < 0.05 vs. control. B) Violin plots depicting AA-NAT expression across seven types of cancers with the pathological stages of the disease. C) ASMT expression (Log_2 TPM+1) between tumor and control samples. D) Violin plots depicting ASMT expression across seven types of cancers with the pathological stages of the disease. E) Boxplot representation of log2-RSEM normalized data of melatonin synthesis/metabolism index. The algorithm referred to index 1 (ASMT:CYP1A1), index 2 (ASMT:CYP1A2), and index 3 (ASMT:CYP1B1) was based on TCGA cancer types. BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; OV: ovarian serous cystadenocarcinoma; PRAD: prostate adenocarcinoma; TGCT: testicular germ cell tumors; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma.*

To establish the indices, log2-transformed values of *ASMT* and *CYPs* expression for every tumor type were used. Because the expression of melatonin-metabolic enzymes is tissue-specific, we provided three independent indices for metabolic rate: *ASMT:CYP1A1* (index 1), *ASMT:CYP1A2* (index 2), and *ASMT:CYP1B1* (index 3), with the latter having the lowest index (Figure 1 E). Regarding all tumors, the median value of index 1 was -0.98 (IQR from -1.61 to 0.23), index 2 was 0.0 (IQR from -1.73 to 0.86), and index 3 was -8.08 (IQR from -10.14 to -

6.35). The index 1 and 2 were positively correlated to breast ($P < 0.01$, $R^2 = 0.21$), cervical ($P < 0.01$, $R^2 = 0.3$), ovarian ($P < 0.01$, $R^2 = 0.16$), prostate ($P < 0.01$, $R^2 = 0.3$), testicular ($P < 0.01$, $R^2 = 0.26$), and uterine endometrial cancers ($P < 0.0001$, $R^2 = 0.29$), and uterine carcinosarcoma ($P < 0.01$, $R^2 = 0.1$). The index 3 and index 1/index 2 showed no correlation.

3.2. Melatonergic microenvironment as a prognostic biomarker for reproductive organ tumors.

Because melatonergic indices are relatively low and differ among seven types of reproductive organ cancers, we further analyzed individual indices by stratifying high and low values across cancers to investigate their potential association with patients' overall survival rate. Notably, patients with increased melatonin synthesis/metabolism index showed better clinical outcomes compared to patients in low-index group (Figure 2).

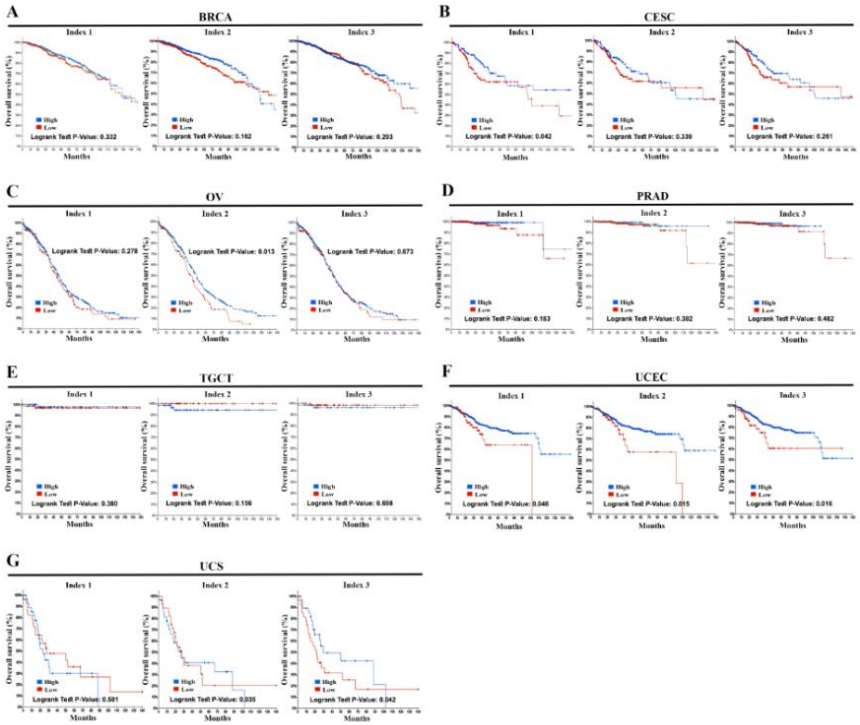

Fig. 2. The overall survival rate for patients with the reproductive organ tumors.

Kaplan-Meier analysis was based on melatonin synthesis/metabolism indices stratified by high and low values. A) BRCA, B) CESC, C) OV, D) PRAD, E) TGCT, F) UCEC, G) UCS. Log-rank test P < 0.05 indicates significant differences in clinical outcomes. BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; OV: ovarian serous cystadenocarcinoma; PRAD: prostate adenocarcinoma; TGCT: testicular germ cell tumors; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma.

The Kaplan-Meier survival analysis varied among tumors and was more discriminatory for female reproductive organ cancers (Figure 2 A-G). For Index 1, the high-value patients displayed improved survival rates for CESC and UCEC (Log-rank test $P = 0.04$, Figure 2 B, F), while marginal significance was observed in PRAD (Log-rank test $P \leq 0.18$, Figure 2 D). Regarding index 2, we observed the improved survival rate in the high-value patients with OV and UCEC (Log-rank test $P = 0.01$, Figure 2 C, F); marginally significant values

appeared in patients with BRCA (Log-rank test P ≤ 0.16, Figure 2 A). The analysis of index 3 showed that high-index values are associated with favorable prognosis in patients with UCEC (Log-rank test P = 0.01, Figure 2 F) and UCS (Log-rank test P = 0.04, Figure 2 G). This association was not observed in patients with TGCT (Figure 2 E). The analysis of patients' age at diagnosis and tumor staging (I-IV) were performed individually (Table 1).

Table 1. Diagnostic tumor age and tumor staging analysis of melatonin synthesis/metabolism based on indices 1-3 across reproductive tumors.

Tumor	Diagnostic age (mean)			Tumor staging (%)								
				Stage I		Stage II		Stage III		Stage IV		
Index 1	**High**	**Low**	**P**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**P**
BRCA	59	57	0.06	8.4	8.2	57.6	56	20.8	25.2	1.68	2.1	0.54
CESC	46	47	0.2	63.3	44.7	19.3	26.9	13.3	17.7	4.0	10.5	**<0.01**
OV	59	59.5	0.8	3.63	0.68	4.54	6.12	73.0	82.3	16.7	11.0	0.1
PRAD	62	60	**0.03**	0	0	40.2	36.5	57.8	61.8	2	1.7	0.5
TGCT	32	29	**0.05**	49.2	33.8	11.5	15.4	6.56	16.9	32.7	33.8	0.3
UCEC	63	68	**0.01**	63.0	58.6	2.17	11.5	22.6	23.0	5.1	10.4	**0.05**
UCS	69	67	0.4	44.8	32.0	6.9	10.7	27.6	42.8	20.5	14.3	0.3
Index 2												
BRCA	59	58	0.3	18.8	14.6	56.8	57.6	21.8	24	1.5	2.2	0.43
CESC	47	46	0.6	55.6	52.2	24.4	21.9	14.9	15.5	4.08	10.3	0.6
OV	58	60	0.1	3.84	0	5.38	7.33	75.0	80.6	13.5	15.5	**0.05**
PRAD	63	60	**0.01**	37.0	54.6	48	31.3	14.0	11.0	0	2.0	**<0.01**
TGCT	33	30	0.2	45.7	41.5	8.47	10.8	10.2	12.4	0	0	0.2
UCEC	63	68	0.01	63.2	57.6	9.8	7.2	21.7	28.5	5.2	7.2	0.5
UCS	68	68.5	0.5	41.4	35.6	13.8	3.57	34.5	35.7	10.3	25.0	0.9
Index 3												
BRCA	60	56	**0.01**	16.4	17.3	57.7	56.4	22.7	23.3	1.82	1.88	0.97
CESC	45	48	0.1	52.7	56.1	25.4	20.8	17.3	12.2	4.3	10.8	0.3
OV	59	59	0.8	3.61	0.68	6.1	4.06	73.8	81.7	15.0	13.5	0.5
PRAD	61	62	0.2	42.2	45.0	42.8	42.5	14.8	11.0	0	1.2	0.4
TGCT	31.5	30	0.2	49.2	39.6	4.7	14.3	9.5	12.7	0	0	0.4
UCEC	63	67	**0.04**	62.9	56.5	8.7	14.5	22.5	27.6	5.5	5.3	**0.01**
UCS	68	68.5	0.5	48.3	28.5	6.9	10.7	24.1	46.0	17	14.3	0.2

*High: high-risk values. Low: low-risk values. BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; OV: ovarian serous cystadenocarcinoma; PRAD: prostate adenocarcinoma; TGCT: testicular germ cell tumors; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma. * P < 0.05. Fisher's exact test.*

For index 1, PRAD and TGCT patients who have the low-value of melatonergic index (high risk) bear cancer at relatively young age, whereas with low-value of for melatonergic index, the patients with CESC and UCEC were at the advanced tumor staging (II-IV) at diagnosis. For index 2, PRAD and OV patients with low-value melatonergic index presented higher tumor staging. BRCA patients with low-value of the melatonergic index 3 showed younger age at diagnosis (Table 1). UCEC patients with low-value for the melatonergic index 3 had advanced tumor staging (II-III) at diagnosis.

We verified the most frequently mutated genes in reproductive organ cancers showing a low-value of the prognostic index. Specifically, CESC patients (index 1) had relatively high mutation frequency of *TTN*, *MUC4*, *MUC16*, *KMT2C*, *KMT2D*, *ADGRV1*, *CMYA5*, and *MT-ND5* genes compared to the high-value (Figure S2 A). Patients with OV and low-value (index 2) showed high mutation frequency in *USH2A*, *MUC16*, *APOB*, *RYR1*, *BRCA1*, *LRP1B*, and *RYR2* genes compared to the high value (Figure S2 B). Only the *TTN* gene had a higher mutation frequency in UCS (index 3; Figure S2 C). Finally, patients with UCEC (Figure S2 D) showed higher mutation frequency with the low values of the three indices (index 1 – *SERPINA6*, *GALNT10*, *PCDHB6*, *CACNA1G*, *MACF1*, *USH2A*, and *PIK3R1*, (index 2 – *KCNN2*, *COX5B*, *EFCC1*, *LRFN4*, *BSG*, *CHCHD6*, *LRRC61*, and *THAP4*), (index 3 – *PTEN*, *PIK3R1*, *SERPINA6*, *LOXL3*, *PKLR*, *EPHX2*, *SLC32A1*, *AKAP17A*, and *GALNT10*) compared to the controls.

3.3. Melatonergic index positively correlates with the expression of *PER1* and negatively with the *TIMELESS*.

We initially evaluated the log2-transformed expression levels of eight circadian clock genes (Figure S3). The expression of *PER1* was significantly lower (~ 2 or 3-fold lower) in all reproductive organ cancers than their control groups. *PER2* expression was lower only in TGCT and UCS, the expression of *PER3* was significantly reduced in patients with OV, TGCT, UCEC, and UCS. The expression of *CRY1* was lower in TGCT and UCEC, whereas the expression of the *CRY2* gene was profoundly reduced in patients with BRCA, CESC, OV, TGCT, UCEC, and UCS. Although *ARNTL* (*BMAL1*) expression was lower in reproductive organ cancers, the expression of the *CLOCK* gene was significantly reduced in TGCT and UCS. By contrast, *TIMELESS* gene expression was significantly higher in patients with BRCA, CESC, OV, TGCT, UCEC, and UCS than that in the controls.

We identified the most representative clock gene presenting low and high expressions in all reproductive organ cancers to investigate the association between melatonin synthesis/metabolism indices with the expression of these circadian genes. It was observed that *PER1* and *TIMELESS* genes were selectively correlated to melatonin metabolism. *PER1* expression showed a positive correlation with ASMT:CYPs (indices 1 – 3) in BRCA, CESC, PRAD, TGCT, and UCEC (Figure 3). Based on indices 2 and 3, patients with OV had a positive correlation with the *PER1* gene. For UCS, only index 3 was positively correlated with *PER1* (Figure 3). Surprisingly, *TIMELESS* expression was negatively correlated with ASMT:CYPs (indices 1 – 3) in BRCA, PRAD, TGCT, and UCEC (Figure 4). For CESC patients, melatonergic indices 2 and 3 presented an inverse correlation with the *TIMELESS* gene. Only index 3 showed a negative correlation with the *TIMELESS* gene in patients with OV. In general, *ASMT:CYP1B1* (index 3) a good correlation was found associating *PER1* and *TIMELESS* in male and female reproductive cancers.

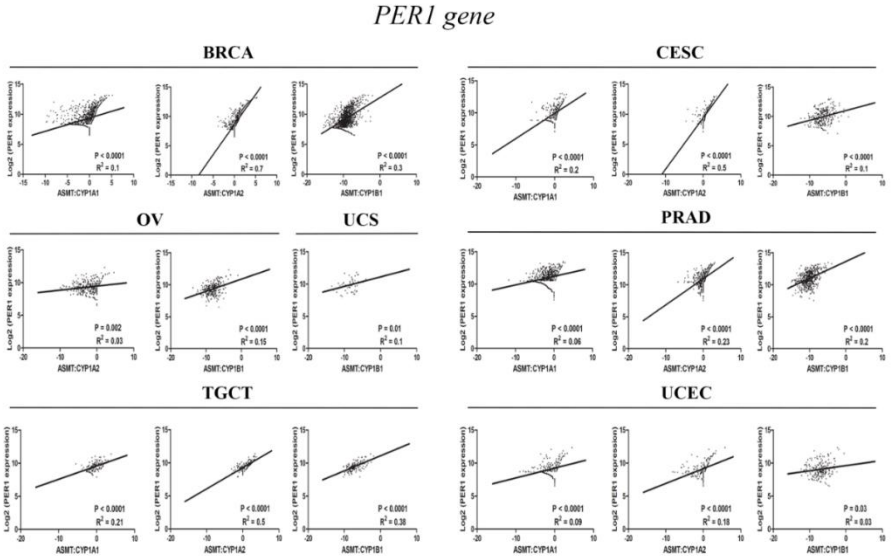

Fig. 3. Correlation between melatonergic indices (1-3) and circadian *PER1* gene expression profile in reproductive organ tumors.

Pearson's correlation coefficient was used individually for each index (ASMT:CYP1A1, ASMT:CYP1A2, and ASMT:CYP1B1). BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; OV: ovarian serous cystadenocarcinoma; PRAD: prostate adenocarcinoma; TGCT: testicular germ cell tumors; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma.

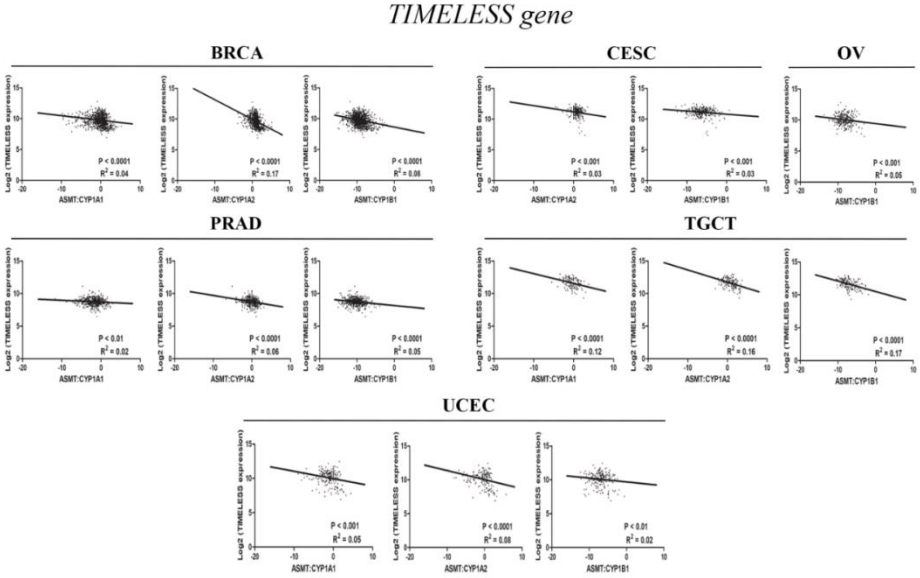

Fig. 4. Correlation between melatonergic indices (1-3) and circadian *TIMELESS* gene expression profile in reproductive organ tumors.

Pearson's correlation coefficient was used individually for each index (ASMT:CYP1A1, ASMT:CYP1A2, and ASMT:CYP1B1). BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; OV: ovarian serous cystadenocarcinoma; PRAD: prostate adenocarcinoma; TGCT: testicular germ cell tumors; UCEC: uterine corpus endometrial carcinoma.

3.4. Correlations between the expression of metabolically-active enzymes and melatonin-synthetic enzyme gene in reproductive organ cancers.

We performed an expression map of metabolic enzyme-encoding genes mainly involved in energy metabolism such as glycolysis, oxidative phosphorylation (OXPHOS), and lipid metabolism. We identified three principal clusters by tumors (PRAD, TGCT-UCS, and BRCA-CESC-OV-UCEC). The log2-normalized expression values were reduced in the majority of OXPHOS-related mitochondrial enzymes (Figure 5 A, B). The expression of the *ASMT* gene was used as parameter to evaluate the relationship between the gene expression of metabolic enzymes and melatonin synthesis. Overall, a higher correlation (rs < 0.8) was observed in male and female reproductive organ tumors for lactate dehydrogenase (*LDHA*), pyruvate dehydrogenase kinase 1 (*PDK1*), and pyruvate dehydrogenase E1 subunit alpha (*PDHA1*). Moreover, isocitrate dehydrogenase 1 (*IDH1*) showed a moderate correlation (rs < 0.4) in BRCA, TGCT, and UCEC. In contrast, succinate dehydrogenase complex subunit B (*SDHB*), ATPase Na+/K+ transporting subunit alpha 1 (*ATP1A1*), and glutaminase (*GLS*) genes had a positive correlation (rs < 0.4) with *ASMT* in BRCA, PRAD, TGCT, and UCEC (Figure 5 C). Despite the correlation, specific enzymatic activities might behave differently in a given tumor.

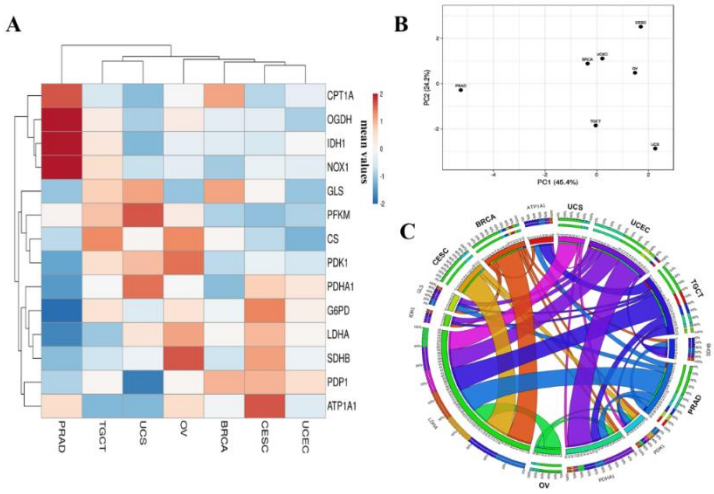

Fig. 5. Profile of metabolic enzymes correlated with cell energy and *ASMT* in reproductive organ cancers.

A) Heatmap of mean expression values (log_2-transformed data) of 14 molecules associated with different cancers. Row and columns were clustered using Euclidean distance. B) Principal component analysis (PCA) of the male and female reproductive organ cancers based on metabolic gene set (PC1 = 45.4 %, PC2 = 24.2%). C) Circus plot connecting the representative metabolic genes into specific tumor types. The correlation coefficient (metabolic gene expression related to ASMT) was corrected and used to provide shared genes. The thicker the link, the greater the interaction. LDHA: lactate dehydrogenase; PFK: phosphofructokinase; G6PD: glucose-6-phosphate dehydrogenase; CS: citrate synthase; PDK1: pyruvate dehydrogenase kinase 1; PDHA1: pyruvate dehydrogenase E1 subunit alpha 1; PDP1: pyruvate dehydrogenase phosphatase catalytic subunit 1; IDH1: isocitrate dehydrogenase (NADP(+) 1); OGDH: alpha-ketoglutarate dehydrogenase; NOX1: NADPH Oxidase 1; SDHB: succinate dehydrogenase; ATP1A1: ATPase Na+/K+ transporting subunit alpha 1; CPT1A: carnitine palmitoyltransferase 1A; GLS: glutaminase. BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; OV, ovarian serous cystadenocarcinoma; PRAD, prostate adenocarcinoma; TGCT, testicular germ cell tumors; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.

4. DISCUSSION

The knowledge of a melatonergic system based-tumor algorithm helps to guide evaluations of prognosis and disease progression. The two-gene signature model (*ASMT:CYPs* isoforms) has been systematically applied in the context of carcinogenesis and infectious diseases to identify high- from low-risk patients (19, 20, 35). Specifically, for reproductive organ tumors, we provided three melatonergic indices (1-3) which can be used to compose low or even negative values according to melatonin synthesis and metabolism by *CYPs* (*CYP1A1, CYP1A2*, and *CYP1B1*). Considering the reduced expression of *ASMT* in male and female reproductive organ tumors and the increased expression level of *CYPs*, especially the *CYP1B1*, these indices can narrow the range for evaluation of the disease progressions. Since the tumor microenvironment is closely related to melatonin levels which depend on several other factors, heterogeneous indices may be a powerful prognostic tool. We identified different index-based overall survival by dividing tumor samples of patients into two subgroups (high vs low values). Of note, index 1 was suitable to evaluate prognosis in CESC and UCEC, index 2 was for OV and UCEC, and index 3 was for UCEC and UCS. The low-value metrics are associated to the poor overall survival rate for women with reproductive organ cancer but this is not the case for prostate or testicular germ cell carcinomas. Even though the overall survival rate was not significantly influenced, the high vs low index values were present in breast cancer patients, particularly at initial stages for index 2 or long-term survival for index 3. Melatonin synthetic enzymes might respond differently at different tumor stages.

There is sufficient evidence to show that plasma melatonin concentrations are lower in patients with cervical (6.1 pg/mL in a cancer-positive group vs. 33.2 pg/mL in the control group), endometrial (60 pg/mL in a cancer-positive group vs. 120 pg/mL in the control group), and ovarian (50 pg/mL in a cancer-positive group vs. 100 pg/mL in the control group) carcinomas, and tended to further reduction with tumor progressive staging (36-38). Although melatonin level is a good parameter to evaluate tumor progression, the melatonergic index score is a better biomarker than melatonin level, especially, in early or later stages of neoplasia. Of particular interest, we noticed a higher tumor staging in patients with CESC, OV, UCEC, and PRAD at the time of diagnosis closely associated to melatonergic index. That is, a low-melatonergic index correlated with low survival rate and advanced tumor stages compared to the high-index values. Thus, this low melatonergic index could be used to predict the worse prognosis. For example, low index 1 correlated with advanced CESC stages III (25%) and IV (162%), and with advanced UCEC stages III (2%) and IV (103%). Low index 2 correlated with advanced OV stages III (7%) and IV (13%), and with advanced PRAD stage IV (0 to 2%). Low index 3 correlated with advanced UCEC stage III (18%). In addition to the prognostic importance, we also identified tumor mutational burden associated with a low-value index. Gene set enrichment analysis revealed that mutated CESC genes are involved with histone methyltransferase activity ($P < 0.0001$), NADH dehydrogenase activity ($P < 0.01$), and response to metal and calcium ion ($P < 0.03$) while mutated OV genes are related to calcium activity ($P < 0.0001$), protein kinase A binding ($P < 0.01$), and ATP binding ($P < 0.01$). The gene mutation frequency in UCEC was elevated and varied across indices. The top enrichment analysis included signaling pathways such as PTEN-dependent cell cycle arrest and apoptosis ($P < 0.0001$), eIF4E and p70 S6 kinase regulation ($P < 0.001$), autophagy ($P < 0.01$), FoxO signaling ($P < 0.01$) and focal adhesion ($P < 0.01$).

Melatonin exhibits potent oncostatic properties in reproductive organ cancers and its reduced production may predispose individuals to the development of cancer. By its antiestrogenic and antioxidant activities, melatonin can improve conventional antiestrogenic drugs' efficacy while reducing the side effects in breast cancer treatments (39). The usefulness of melatonin as a therapeutic agent for ovarian cancer is based on the fact that melatonin

regulates cell cycle, stimulates apoptosis, inhibits angiogenesis and inflammation, as well as modulates various immune cells and different cytokines/chemokines (40). Recent findings reported outstanding inhibitory actions of melatonin on cervical and endometrial cancers. In cervical cancer cells, melatonin enhances TNF-α-induced mitochondrial apoptosis via inactivation of the CaMKII/Parkin/mitophagy axis (41), and in uterine endometrial cancer cells, melatonin suppresses tumor progression by inhibiting estrogen/ubiquitin C/succinate dehydrogenase B-induced succinate accumulation (42). Melatonin's action in prostate cancer has been documented in cultured prostate cancer cells (LNCaP) and in murine model (TRAMP) (43). ERK1/2 signaling was permanently activated in the absence of melatonin and androgens, while melatonin treatment blocked the nuclear translocation of androgen receptor (AR). By changing the regulation of IGF signaling and kallikreins (KLK2 and KLK3), melatonin promoted oncostatic effects, thus prolonging the survival of TRAMP mice by 33% when administered at the initial or at advanced stages of the disease (43).

Changes in circadian clock genes are linked to cancer progression by altering clock-controlled genes (CCGs) and other tumor-related genes (44). They can participate directly or indirectly in tumor development by affecting downstream CCGs related to cell cycle, DNA damage and repair, apoptosis, and tumor immunity (45). In this context, clock genes have additional signatures that may constitute new tumor biomarkers. In most reproductive organ cancers, we observed a reduction of clock genes with tumor suppressor properties (e.g., *PER, CRY, BMAL1*) in contrast to an increased expression in the clock gene with pro-tumor activities (e.g., *TIMELESS*). The genes *PER1* and *TIMELESS* presented the highest coverage and were selected to identify potential correlations of their expression with melatonergic indices. Through this perspective, our study was the first to demonstrate a positive correlation between *PER1* with *ASMT:CYPs* ratio in patients with reproductive organ cancers. Especially, BRCA, CESC, PRAD, TGCT, and UCEC showed moderate to strong correlation with the three indices mentioned above. Otherwise, OV and UCS patients presented index-specific correlation with the PER1 gene.

The index 3 (*ASMT:CYP1B1*) showed a good correlation with *PER1* and is increased in all reproductive organ tumors; thus, it may serve as a new clinical parameter for their diagnosis. Conversely, the *TIMELESS* gene correlated with *ASMT:CYPs* in patients with reproductive organ cancers, but inversely compared to *PER1*. Again, index 3 (*ASMT:CYP1B1*) was the better index and negatively correlated with *TIMELESS*. Whether these circadian clock genes also correlate with other solid and non-solid tumors remain to be determined. Previous studies documented instability and variation in *PER1* and *TIMELESS* expression across tumors (44). Breast cancer in postmenopausal women is associated with genetic variation in the circadian gene pathway, as evidenced by an interaction between *PER1* and night-shift work (46), while overexpression of the circadian regulator *TIMELESS* correlates with genetic and epigenetic activities related to breast carcinogenesis (47). Since light-at-night is considered an important external factor which causes chaotic circadian gene oscillation (48), our attempt to correlate melatonin synthesis/metabolism with circadian genes may provide another alternative diagnostic method. More recently, RT-PCR analysis confirmed downregulation of *PER*, *CRY*, and *CLOCK* genes in cervical cancer (49), and *TIMELESS* overexpression correlated with lymph node metastasis and poor overall survival (50). Interestingly, endocrine-related cancers (e.g., breast, prostate, and ovarian cancers) are associated with altered circadian and clock genes that regulate androgen and estrogen production (23). Specifically, in endometrial cancer, downregulation of *PER1* is related to a worse prognosis, while its overexpression promoted cancer cell apoptosis (51). More recently, expression of melatonin-synthesizing enzymes and specific clock genes (*PER, CRY* and *BMAL1*) were significantly reduced and associated with worse prognosis in prostate cancer patients (52). Although *PER1* has been implicated in most endocrine neoplasms, no previous study reported its expression in testicular cancer. Overall,

our data demonstrate the melatonergic indices combined with *PER1* and *TIMELESS* genes are potential prognostic factors in reproductive tumors. Additional validation by RT-qPCR in a different cohort of patients is expected.

Cancer cell metabolism is significantly changed, and intracellular melatonin levels perhaps derived primarily from mitochondria of these cells is physiologically different in cancer cells compared to that of healthy cells (9, 10, 53). Despite the relatively low expression of enzymes involved in glycolysis, oxidative phosphorylation (OXPHOS), lipid and glutamine metabolism that were identified in reproductive cancers, we identified a correlation between different melatonergic indices with *LDHA* and OXPHOS-related mitochondrial enzymes (*PDK1*, *PDHA1*, *SDHB*, *IDH1*, *GLS*, and *ATP1A1*). The notion that the expression of melatonin-converting enzyme is correlated with the expression of these metabolic enzymes brings up a possible genetic and functional interdependence associated with these tumor types. Importantly, melatonin functions as a glycolytic agent by changing the tumor cell phenotype to a healthier phenotype (54). In this context, numerous observations point to melatonin synthesized in the mitochondria of all healthy cells but not, or at much lower levels, in cancerous cells. Mechanistically, through inhibition of HIF-1α, melatonin regulates the PDK/PDC axis allowing pyruvate to be decarboxylated to acetyl-CoA, the cofactor for the rate-limiting enzyme in melatonin synthesis, AANAT (55). Considering that melatonin redirects glucose oxidation to OXPHOS in solid tumors, the functioning of mitochondrial complexes of the electron transport chain must be readjusted to support ATP production while cytosolic lactate metabolism is significantly reduced. From this metabolic perspective, a melatonin-based index could be exploited together with these energy-related enzymes to comprehensively evaluate the metabolic state of a disease and to determine if melatonin supplementation could be administered to reverse aerobic glycolysis.

Herein, we demonstrated the likely critical prognostic value of melatonin synthesis as reflected in its metabolism to be used to detect and monitor reproductive organ cancers, especially female cancers (cervical, ovarian, endometrial cancers, and uterine carcinosarcoma). The specific ability of each tumor intracellular microenvironment to produce and accumulate melatonin may reflect overall survival risk, disease progression and relapse. More specifically, index 1 had prognostic value in CESC and UCEC, index 2 had prognostic importance in OV and UCEC, and index 3 had prognostic value in UCEC and UCS. The melatonergic indices correlated with *PER1* and *TIMELESS* clock genes, supporting a novel cooperative role as potential biomarkers for male and female reproductive organ cancers. The enhancement of melatonin machinery increased with the presence of *PER1* gene expression; however, when melatonin production is disturbed, tumors may overexpress *TIMELESS* gene. Additionally, the melatonin-based index showed a correlation with glycolysis and OXPHOS-related enzymes, thereby emphasizing its capacity to interfere with cancer cell energy production and metabolism. This study reinforces the oncosignatures of the melatonergic system and encourages its further investigation and its utilization in diagnostic oncogenesis.

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AUTHORSHIP

LGAC and RFC: conception of the idea, design of the study and drafted the manuscript; LGAC, RFC, FRFS, and DAPCZ: analysis and data interpretation. RJR: critical analysis of data and of the manuscript. All authors examined/evaluated the data and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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