Research Article

Blood melatonin level can serve as a potential biomarker for prostate and hepatocellular carcinomas

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ABSTRACT

Many systemic functions display circadian rhythms driven by an endogenous mechanism that is regulated by circadian-related genes and these gene expressions control a central clock in the brain and subordinate clocks in peripheral tissues. However, modern life has introduced environmental factors that often interfere with natural circadian rhythms. Importantly, circadian disruption has been identified as an independent risk factor of cancers. Melatonin is a major circadian rhythm regulator. In cancer patients, the rhythm of melatonin is often disrupted and its level is also reduced. These changes of melatonin impair its antioxidant and circadian regulatory functions on cells and tissues making them more susceptible to mutations and cancer initiation. In this context, the objectives of this study are to evaluate the sleep quality and blood levels of melatonin in patients with either prostate cancer (PC) or hepatocarcinoma (HCC) with the intent of using its levels as a potential biomarker of the cancers. The study involved 20 PC and 18 HCC patients, and 26 healthy volunteers. All blood samples were collected in the early morning, at 07:00 hours. Comparative sleep quality between PC, HCC patients and control subjects was acessed with a questionnaire, and melatonin and vitamin D were measured using conventional assays. The results revealed that patients with the worse sleep quality also had lower values of melatonin and vitamin D compared to control subjects. Notably, expression of melatonin-synthesizing enzymes and specific clock genes (PER, CRY and BMAL1) were significantly reduced and associated with worse prognosis in PC and HCC patients. These findings are consistent with the results of previous studies and suggest that disruption of the circadian rhythms, associated with changes in the light:dark cycle, has consequences for the maintenance of systemic health. We suggest that supplementation of melatonin and vitamin D may represent the important therapeutic strategies for patients with solid tumors for the purpose of improving their sleep quality and recuperative capacity.

key words: Indoleamine, solid tumors, circadian rhythm, vitamin D, sleep quality

1. INTRODUCTION

Currently, cancer is one of the leading causes related to morbidity and mortality globally, whether in developed countries or in emerging economies. Despite the high incidence rate, much of its etiology remains largely unknown as relevant factors associated with age, ethnic origin and family history being recognized as risk factors (1). Since the late 1980s, evidence has emerged linking circadian dysregulation with the pathogenesis of cancer. In 2007, the International Agency for Research on Cancer, IARC, classified "shift work that leads to an interruption of the circadian cycle" as probably carcinogenic to humans (2). Since then, several studies have evaluated the effects of various circadian disruptors, such as night work, light at night (LAN), sleep-related patterns (e.g., insufficient and mistimed sleep), and circadian genes, with the risk of developing cancer (3, 4).

Melatonin is the major product of the pineal gland which is released primarily during darkness and is closely associated with regulation of circadian cycles. Its production follows a rhythmic pattern dependent on the prevailing light:dark cycle. Therefore, serum levels of melatonin are often elevated during the night-time, compared to its low circulating levels during the day-time (5). Like melatonin, vitamin D is also related to carcinogenesis, but the production of vitamin D occurs mainly during the day in response to solar ultraviolet-B (UVB) radiation. Studies have shown that cancer mortality rate is inversely correlated with the concentration of vitamin D in plasma. Overall, vitamin D may inhibit cell proliferation while facilitating cell differentiation and apoptosis in various neoplasms (6). In this context, it is suggested that exposure to light at night is an important factor in carcinogenesis, considering that sunlight during the day allows the production of vitamin D and its absence at night allows the secretion of melatonin, both essential molecules involved in tumor suppression. It is also proposed that the administration of these compounds, together with other treatments, can be important therapeutic supplements to control cancer progression and aggressiveness (7, 8).

In recent years, The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects produced RNA-Seq data for tens of thousands of cancer and non-cancer samples, providing an unprecedented opportunity for many related fields including cancer biology. Moreover, GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool to deliver fast and customizable functionalities based on TCGA and GTEx data, provides key interactive and customizable functions including differential expression analysis, profiling plotting, correlation analysis, similar gene detection and dimensionality reduction analysis (9).

With this background, the objectives of this study were: (1) to evaluate sleep quality in PC and HCC patients compared to control subjects; (2) to quantify the blood levels of melatonin in patients newly diagnosed with solid tumors, including PC and HCC, and in healthy individuals, to identify the differences and to elicit melatonin levels as a potential biomarker; (3) to validate the data obtained in this study, through search in open database, seeking to find transcripts related pathways of melatonin production and then, to identify the possible involvement of melatonin level with those transcripts, which are strongly related to the circadian cycle and to melatonin response. Additionally, we sought to verify the correlation between serum vitamin D and melatonin levels in PC patients.

2. MATERIAL AND METHODS

2.1. Patient selection.

This is a cross-sectional study, approved by the Research Ethics Committee of the Faculty of Medicine of São José do Rio Preto (FAMERP), carried out from August 2017 to August 2018. The approved protocol numbers are 67710417.7.0000.5415 and 67706517.4.0000.5415.

In the PC arm, the study involved two groups, totaling 32 participants. Group I was composed of 20 patients treated at the Cancer Institute of São José do Rio Preto - SP. Only patients over 50 years old, with positive results for PC, and who had not undergone any previous treatment were included in the study. From this group, the serum levels of melatonin, vitamin D, and prostate specific antigen (PSA) (which was in the medical record) were obtained for each sample, together with the tumor classification according to the Gleason Score, which varied from 2 to 10 (score 2 is associated with a better prognosis and a score 10 is associated with a worse prognosis). Group II consisted of 12 healthy volunteers with no history of PC, with the same age range and characteristics of patients in the group I. From this group, the serum levels of melatonin and vitamin D were obtained for each sample. In addition, a questionnaire was applied to both experimental groups (I and II), assessing different criteria: sleep pattern, sleep hours by night, use of hypnotic medications, room illumination, and night-work routine. Only patients under regular day: night conditions and without consumption of sleep-inducing agent were considered. Clinical and surgical evaluations were under the responsibility of the medical team participating in the project.

For the HCC arm, a total of 32 participants were also selected, divided into two groups, composed of 18 patients with newly-diagnosed HCC and without previous treatment, referred by the Base Hospital of São José do Rio Preto/SP and 14 healthy individuals, with no history of liver problems (control group). For both groups, individuals of the same age range were included and immunosuppressed individuals were excluded. HCC patients were between 52 and 71 years old (12 men and 6 women). The control subjects were between 47 and 79 years old (10 men and 4 women).

2.2. Collection of samples.

All blood samples were collected early morning at 7:00 a.m. by trained professionals using venipuncture from patients with PC and HCC, at the Cancer Institute (ICA) of São José do Rio Preto-SP. All individuals were fasted before sample collection. Similarly, the samples of fasted control individuals were collected at the same time and using the same procedures. The objectives of the research were carefully explained to all participants and all of them signed the “Free and Informed Consent Form”. After blood collection, the samples were centrifuged at the Cancer Molecular Research Laboratory (LIMC), and the serum was separated and placed into conical tubes. They were rapidly frozen in a freezer -20 ºC for further analysis of the compounds.

2.4. Quantification of melatonin.

For melatonin quantification, a highly sensitive and specific ELISA test was used. The measurements were performed at the Cancer Molecular Research Laboratory of the Medical School of São José do Rio Preto (FAMERP). All samples and standards were extracted according to the manufacturer. Initially, the extraction columns were washed with methanol and distilled water. Subsequently, the columns were inserted into properly identified test tubes. The serum samples (0.5 mL) and the standards, diluted in 0.5 mL of double distilled water, were applied to the columns followed by centrifugation. Subsequently, the columns were washed with methanol and the obtained extract was evaporated using an evaporative centrifuge. After evaporation, the samples were reconstituted with 150 µL of double distilled water. In the microtiter plate, 50 µL of each extracted sample was added to the respective wells combined with 50 µL of melatonin biotin and 50 µL of melatonin antiserum. The plate was incubated for 14-20 h at 4 °C on an orbital shaker. Next, the incubation solution was discarded, and plate was washed 3 times with washing buffer and incubated with 150 µL of conjugated enzyme at room

temperature for 120 min. Again, the plate was washed and 200 µL of PNPP substrate solution was added over 40 min. Subsequently, 50 µL of PNPP stop solution was added to the plate. Optical density (O.D.) was measured at 450 nm in a microplate reader and O.D. four logistic parameters (4-PL) were determined through the adjustment curve.

2.5. Quantification of Vitamin D.

The quantification of vitamin D was performed at the Laboratory of Clinical Analysis and Pathological Anatomy (LABORCLIN), São José do Rio Preto, by electrochemiluminescence on the Roche/Hitachi cobas e601 analyzer and the values were considered normal between 30 and 60 ng/mL.

2.6. Data extraction from patients with PC and HCC.

We used publicly RNA-seq datasets from patients with prostate cancer and hepatocellular cancer available in The Cancer Genome Atlas (TCGA) database (10) and data from control prostate and liver tissue available at the GTEx portal (release V8; https://www.gtexportal.org/) (11) to identify possible associations with melatonin synthesis and related clock machinery. To provide differentially expressed genes (DEGs) we used Gene Expression Profiling interactive analysis (GEPIA) (9) available at http://gepia.cancer-pku.cn/. Next, we explored separately upregulated and downregulated target genes using EnrichR webtool to verify ontologies and pathways associated with these tumors (12). Then, DEGs matching with melatonin synthesis and clock genes were visualized and constructed using Morpheus (https://software.broadinstitute.org/morpheus) (13). The functional protein-protein association network was constructed from those genes related to melatonin. The metasearch STRING database (14) was used for mapping PPI enrichment after setting the following sources: text mining, databases, experiments, and co-occurrence (highly confidence score of 0.900). All disconnected nodes were excluded from the network. The PPI enrichment P-values represented the statistical significance provided by STRING.

2.7. Statistical analysis.

The data were subjected to statistical analysis using the GraphPad Prism 5.0 software. Melatonin levels were performed in duplicate between the different groups using the Mann-Whitney test. Kaplan-Meier curves of overall survival were generated based on the prognosis of patients with high and low melatonin levels. For the univariate analysis of clinicopathological data, we performed Chi-squared test. All results were expressed as mean ± standard error (S.E.M.) and a p value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Melatonin levels and sleep quality is compromised in patients with PC.

Regarding the sleep quality in the PC group, 0 (0%) patients were classified as “excellent”, 6 (30%) as “good”, 7 (35%) as “regular” and 7 (35%) as “bad” in terms of sleep quality. Considering the sleep quality in the control subjects, 2 (16.67%) patients were classified as “excellent”, 8 (66.67%) as “good”, 2 (16.66%) as “regular” and 0 (0%) patients were classified as “bad” having inferior sleep quality (Figure 1 A and B).

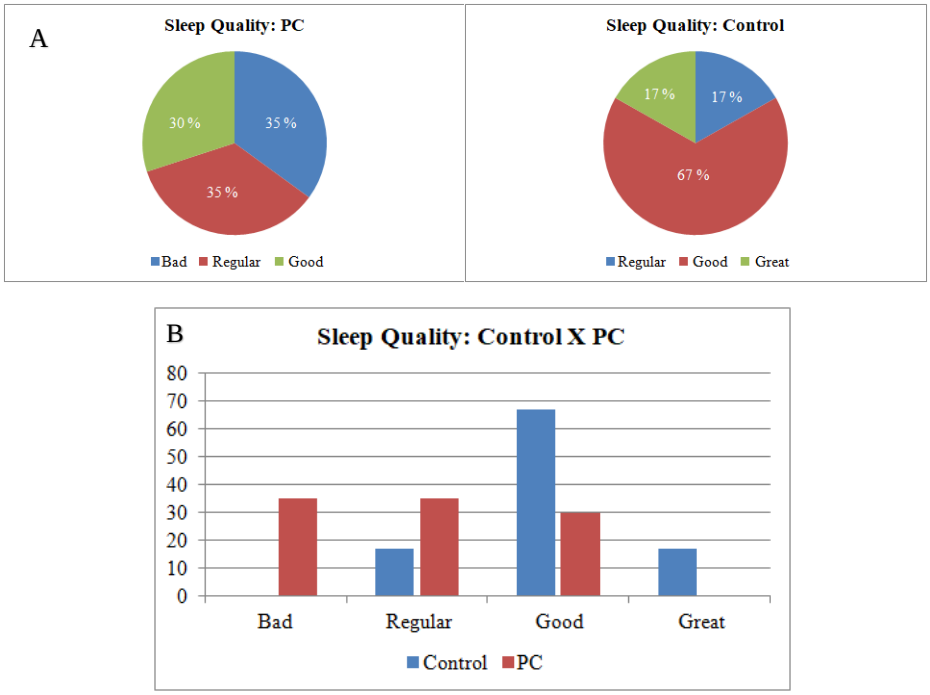

Fig. 1. Sleep quality in PC patients and healthy control individuals.

A) Evaluation of sleep quality in PC group (20 PC patients) and in control group (12 healthy volunteers) distributed in bad, good, regular and great sleep categories. B) Comparative sleep quality between PC patients and control patients based on the questionnaire (in percentage).

The values of melatonin concentrations in the PC and Control groups were obtained, with an average of 165.6 pg/mL and 316.9 pg/mL, respectively. The Pearson's correlation coefficient obtained was $R^2 = 0.9588$. There was a significant decrease (p=0.0029) in serum melatonin levels in PC patients, when compared to the control group (Supplementary Tables 1 and 2; Figure 2 A). Patients with low melatonin levels had higher Gleason Score of 9 (4+5) and 8 (4+4). After stratifying PC patients into high vs low melatonin levels, the results showed that patients with lower melatonin levels had a significant reduction in overall survival (Figure 2 B).

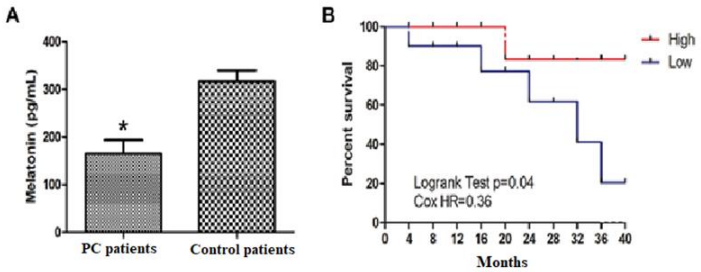

Fig. 2. Melatonin concentration (pg/mL) and patients' survival.

*A) Serum levels of melatonin were quantified in PC samples of patients (group I) and in control group (group II). Mann-Whitney test. * P < 0.05. B) Kaplan-Meier curves of survival analysis considering PC patients with high and low melatonin levels.*

After measuring vitamin D by electrochemiluminescence, statistical analysis was performed using PC samples. By distributing the samples according to the concentration of vitamin D, it was observed that in 8 of 20 patients (40%), the levels were considered below normal.

Importantly, we observed that the average of vitamin D levels obtained for this PC group was 34.45 ng/mL, which represents a value close to the lower acceptable limit. In addition, no relationship was observed between melatonin and vitamin D levels in patients with PC (Supplementary Table 1; Figure 3).

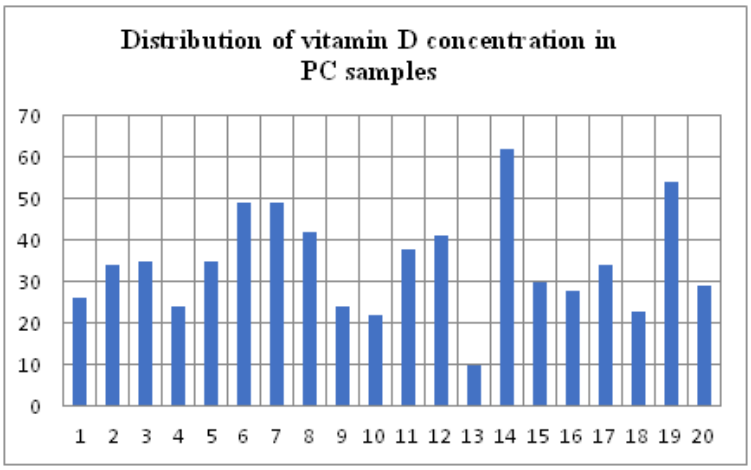

Fig. 3. Distribution of vitamin D concentration in ng/mL (vertical axis) among patients with PC (horizontal axis).

The 30-60 ng/mL interval indicates reference values used in the study.

3.2. Melatonin synthesizing-enzymes and clock genes are affected in PC patients.

After examining RNA-Seq data from PC patients, we identified 8,491 DEGs (Log2 FC ≤ |0.5| ≥ |0.5| and false discovery rate (FDR) < 0.05), in which 5,903 genes were downregulated and 2,588 were upregulated (Supplementary Table 3). Based on Reactome, the top enrichment downregulated genes (*COL, LOXL, LAM, ITGA, ITGB, HSPG, and VTN*) were mostly involved in extracellular matrix organization, elastic fiber production, collagen biosynthesis and modifying enzymes, integrin cell interactions, and assembly of collagen fibrils (Figure 4 A). Among the upregulated genes, the Ribosomal Protein Large subunit (RPL) gene family was mainly involved with translation and elongation events during protein synthesis on the ribosome (Figure 4 B). Next, we search for targets associated with melatonin synthesis and available circadian clock genes and, notably, *ASMT, NAT, CRY2, RORA, SIRT7, ROR2, PER1, PER2*, and *PER3* were significantly down-represented in PC patients (Figure 4 C). The unique upregulated target was *RORC* which is profoundly related to poor overall survival in cancer patients. The PPI analysis further revealed that these downregulated molecules showed a high interaction regarding the *PER1, 2, and 3, CRY2, RORA* and *RORC* and were especially involved in biological processes such as circadian regulation of gene expression, histone H3 deacetylation, nuclear receptor binding, and coenzyme binding (Figure 4 D). We also interrogated the drug signature database (DSigDB) relating melatonin in this gene set and this showed a very low representation (p = 0.61, combined score 0.47).

To provide clinicopathological correlation between PC samples and melatonin synthesis, we assessed the expression levels (Log2 RSEM-transformed values) of the melatonin-synthesizing enzyme ASMT (high-values versus low-values). While high-value ASMT levels were associated with reduced mutational burden and prior cancer diagnosis, no significant variation was observed over histologic type and tumor staging (Table 1).

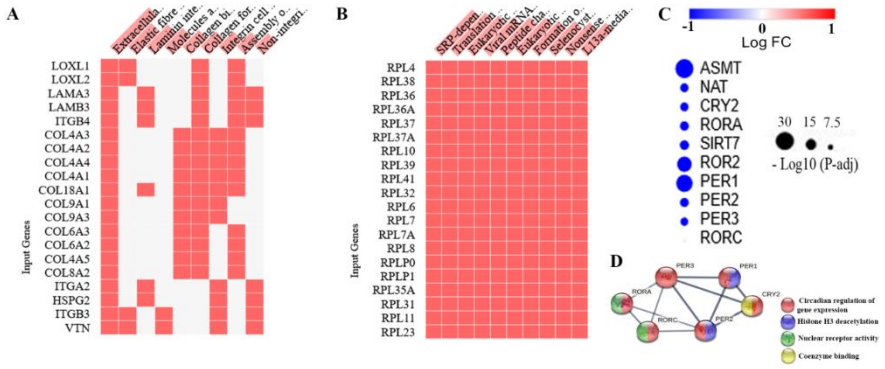

Fig. 4. Differentially expressed genes in PC patients and their relationship with melatonin and clock genes.

A) Downregulated genes in Reactome database according to combined score; B) Upregulated genes in Reactome database according to combined score; C) Functional downregulated and upregulated targets. The color of the circles in the scatter plot indicates the fold-change, while the circle size reflects its Log_{10} p-value. D) Network interaction between the potential genes regulated by melatonin in prostate cancer (PPI enrichment p-value < 1.0e-16; minimum confidence score: 0.7).

Table 1. Mutation burden, cancer diagnosis, tumor subtype and staging according to melatonin-synthesizing enzyme in PC patients.

Prostate adenocarcinoma			
Variables	**High-values**	**Low-values**	***P-value***
Mutation burden *	0.83 (0.04-1.6)	0.90 (0.08-1.7)	0.03
Prior cancer diagnosis (%) **	4.35	7.0	0.05
Histologic type (%) **			0.90
Acinar type	97.2	96.7	
Other subtypes	2.8	3.3	
Tumor stage (AJCC) (%) **			0.19
T1	-	-	
T2	-	-	
T2a	2.01	3.33	
T2b	1.2	2.92	
T2c	34.5	32.5	
T3a	29.3	35.4	
T3b	30.5	25.0	
T4	2.4	1.3	

*High-values: high-risk values based on ASMT. Low-values: low-risk values based on ASMT. P < 0.05 was considered statistically significant. * Kruskal Wallis test. ** Chi-squared test. ASMT: acetylserotonin O-methyltransferase. AJCC: American Joint Committee on Cancer Tumor Stage Code.*

3.3 Melatonin levels and sleep quality is compromised in patients with HCC.

Regarding the sleep quality, 62.5% of the HCC patients were classified as "good" and 37.5% were classified as "regular". However, in the control group, 28.6% of the individuals were classified as "great", 57.1% as "good" and 14.3% as "regular" (Figure 5 A and B).

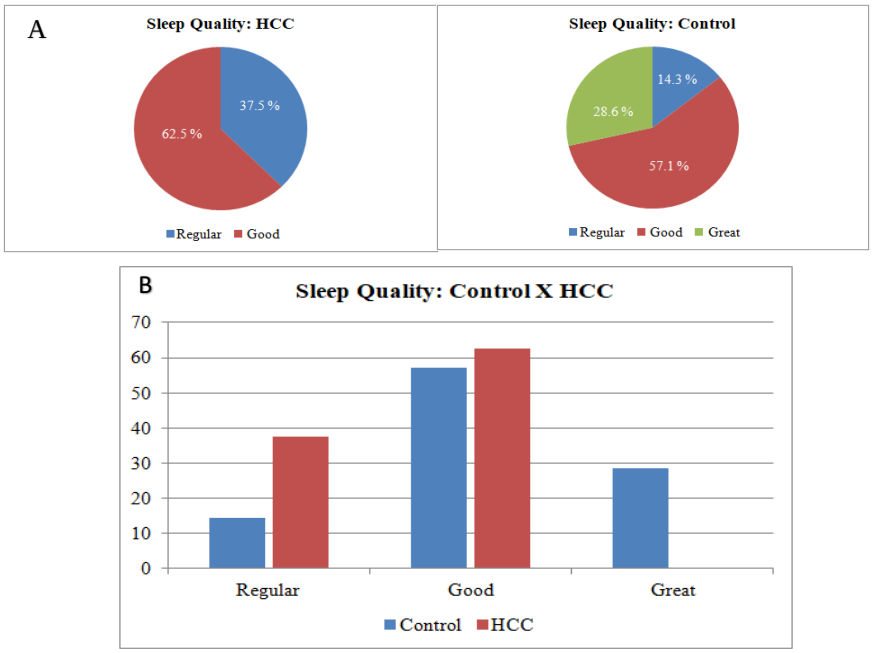

Fig. 5. Sleep quality in HCC patients and healthy individuals.

A) Evaluation of sleep quality in HCC group (20 patients) and in control group (12 healthy volunteers) distributed in regular, good and great sleep. B) Comparative sleep quality between HCC patients and control patients regarding the questionnaire (in percentage).

3.4. Melatonin levels is significantly reduced in patients with HCC.

HCC patients were between 52 and 71 years old (12 men and 6 women). Control subjects were between 47 and 79 years old (10 men and 6 women). Melatonin levels were assessed for all these participants. The average of melatonin concentration in the HCC patients was 89.14 pg/mL melatonin compared to 246.5 pg/mL in control group ($p < 0.01$) (Supplementary Tables 4 and 5; Figure 6 A). By stratifying HCC patients into high vs low melatonin levels, we noted that patients with lower melatonin levels showed a significant reduction in overall survival (Figure 6 B).

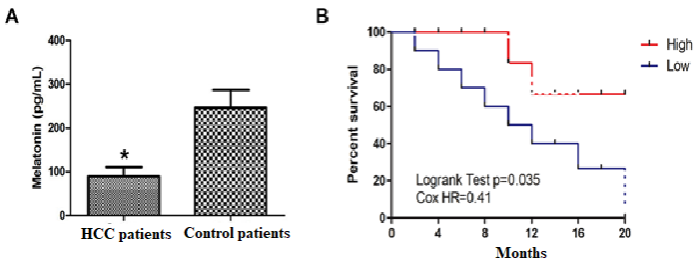

Fig. 6. Melatonin concentration (pg/mL) and patients' survival.

*A) Serum levels of melatonin were quantified in HCC samples of patients and in control samples. Mann-Whitney test. * $P < 0.05$. B) Kaplan-Meier curves of survival analysis considering HCC patients with high and low melatonin levels.*

3.5. Melatonin synthesizing-enzymes and clock genes are differentially regulated in hepatocellular cancer patients.

To describe the relationship of melatonin and circadian clock genes in HCC patients, we examined the RNA-Seq data available at TCGA database. From this analysis, we identified 7,002 DEGs (Log2 FC ≤ |0.5| ≥ |0.5| and false discovery rate (FDR) < 0.05), in which 1,871 genes were downregulated and 5,131 were upregulated (Supplementary Table 6). The top enrichment downregulated genes (*IGLV, IGLK, IGKC, IGLC, MASP, MBL, IGHG, FCN, and CYP*) were mostly involved in xenobiotics metabolism, complement cascade, oxidation, scavenging of heme and response to metal ions, metallothionein binding metals, synthesis of hydroxyeicosatetraenoic acids, and uptake of ligands by scavenger receptors (Figure 7 A). Similar to PC samples, the most representative upregulated genes were the Ribosomal Protein Large subunit (RPL) gene family mainly involved with translation and elongation events during protein synthesis on the ribosome (Figure 7 B). After searching for targets associated with melatonin synthesis and available circadian clock machinery, we detected a downregulation of *ASMT, NAT1, NAT2, PER1, ARNTL, and RORA* genes, whereas *NAT6, TIMELESS, and SIRT6* were showed to be upregulated in HCC samples (Figure 7 C). The PPI analysis was generated using these representative molecules and showed a high interaction regarding the *NAT1, NAT2, PER1, ARNTL, RORA, and TIMELESS* genes. They were mainly involved in biological processes such as circadian regulation of gene expression, regulation of double-strand break repair, N-acetyltransferase activity, and regulation of response to stress (Figure 7 D). The drug signature database (DSigDB) related melatonin in this gene set with a significant representation (p = 0.008, combined score 6.88).

Clinicopathological analyzes between HCC samples and melatonin synthesis (high-values versus low-values of *ASMT*) revealed significant correlation by gender, histologic type, and tumor staging. Lower values of melatonin-synthesizing enzyme were associated with higher tumor stages (Table 2).

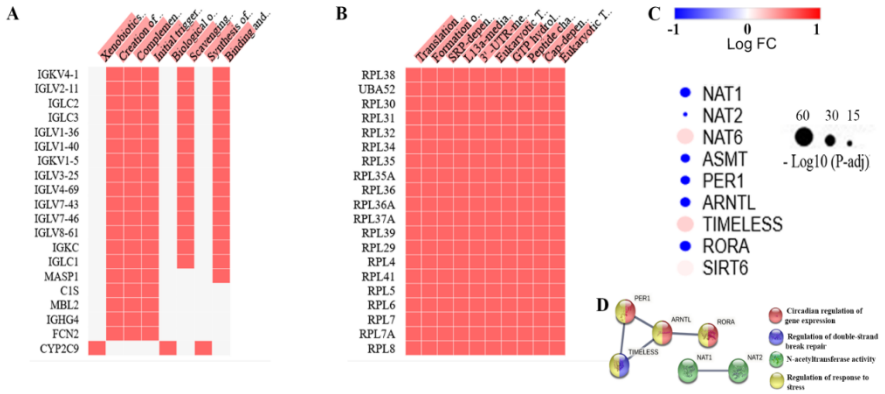

Fig. 7. Differentially expressed genes in HCC patients and their relationship with melatonin and clock genes.

A) Downregulated genes in Reactome database according to combined score; B) Upregulated genes in Reactome database according to combined score; C) Functional downregulated and upregulated targets. The color of the circles in the scatter plot indicates the fold-change, while the circle size reflects its Log$_{10}$ p-value. D) Network interaction between the melatonin-related genes in HCC cancer samples (PPI enrichment p-value < 3.33e-16; minimum confidence score: 0.7).

Table 2. Tumor diagnosis by gender, tumor subtype and staging according to melatonin-synthesizing enzyme in HCC patients.

Hepatocellular carcinoma			
Variables	**High-values**	**Low-values**	***P-value***
Sex			0.03
Male tumor	57.3	70.4	
Female tumor	42.7	29.5	
Histologic type (%)			0.05
Hepatocellular carcinoma	93.1	98.0	
Mixed carcinoma	6.9	1	
Fibrolamellar carcinoma	-	1	
Tumor stage (AJCC) (%)			< 0.05
T1	50	48.7	
T2	29.2	23.8	
T2a	-	0.35	
T2b	1.2	-	
T3	13.4	11.7	
T3a	3.66	9	
T3b	-	2.1	
T4	1.2	4.2	

High-values: high-risk values, Low-values: low-risk values both based on ASMT. P < 0.05 was considered statistically significant. Chi-squared test. ASMT: acetylserotonin O-methyltransferase. AJCC: American Joint Committee on Cancer Tumor Stage Code.

3.6. Low expression of melatonin-synthesizing enzymes is correlated with poor overall survival in prostate and hepatocellular cancer.

To further correlate how melatonin-synthesizing enzymes are effectively associated with overall survival in cancer patients, we stratified patients possessing low and high NAT and ASMT expressions using GEPIA tool according to Cox regression analysis. By interacting PC and HCC patients together, we identified that low NAT1 expression (Logrank p=0.021; Figure 8A) and low ASMT expression (Logrank p=0.012; Figure 8B) were correlated with a reduced overall survival by these patients.

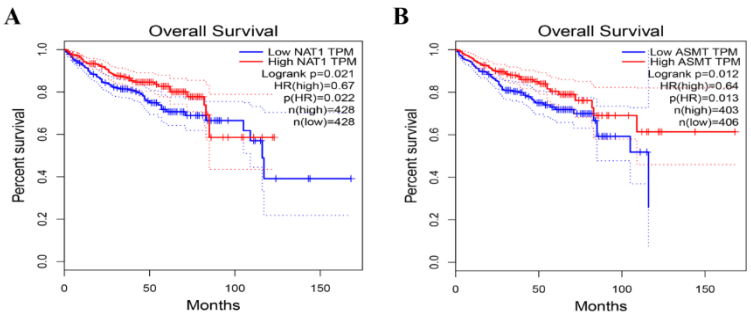

Fig. 8. Survival analysis curves considering low and high melatonin-synthesizing enzymes.

A) Overall survival in PC and HCC patient samples was calculated using the TCGA dataset, which stratified patients with high- or low-NAT1 expression (red and blue lines, respectively). B) Overall survival in PC and HCC patient samples was calculated using the TCGA dataset, which stratified patients with high- or low-ASMT expression (red and blue lines, respectively). The analyses were provided by GEPIA. Median Cut-off values of 50% (high and low) and harzard ratio (HR) with 95% confidence intervals based on Cox PH model were used. The univariate Cox regression analyses were performed on each survival Kaplan-Meier curves.

4. DISCUSSION

The circadian system in humans is complex, starts in the eye and terminates in the pineal gland, as a result of melatonin synthesis and secretion. Circadian rhythms are dependent on internal clock located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus, but also peripheral clocks have been identified in numerous tissues such as cerebral cortices, liver, kidney, heart, skin, and the retina. The SCN subsequently synchronizes peripheral clocks, thus aligning the entire circadian machinery to the external light-dark cycle (8).

Similarly, the multitude of processes governing cancer initiation, promotion, and progression, including invasion and metastasis, are temporally modulated by the host's circadian rhythmic outputs from the central circadian pacemaker in the SCN (15). At the same time, common molecular clock genes and their protein products in the master clock are expressed in both normal and cancerous peripheral tissues; they are termed peripheral oscillators being the genes and proteins expressed and regulated in a circadian manner (16).

Both vitamin D and melatonin are individually essential for the maintenance of cellular physiology; their levels of synthesis act contrarily according to day and night cycles. Vitamin D is synthesized in the skin after exposure to solar UV radiation, whereas melatonin synthesis occurs primarily at night in the pineal gland (17).

Low serum vitamin D levels are associated with sleep quality and circadian rhythms. Because sleep controls neural circuits related to the SCN, a high expression of vitamin D receptors (VDRs) is observed in these areas. These areas are known to be composed of pacemaker cells playing an important role in the first stage of sleep and in maintaining sleep. Vitamin D has been extensively reported to bind in several neural areas associated with sleep regulation in the brainstem, such as the anterior and posterior hypothalamus, substantia nigra, midbrain central grey matter, raphe nuclei, and in the reticularis pontisoralis and caudalis nuclei. Furthermore, through actions on tryptophan hydroxylases (TPH)-2 enzymes, which expresses vitamin D response element (VDRE), vitamin D may regulate tryptophan conversion into 5-hydroxytryptophan, which is metabolized to serotonin, the precursor of melatonin (18). Therefore, it is plausible that there may be a role of vitamin D in regulating sleep and circadian rhythms. Recently, Jung *et al.* (19) examined the characteristics of circadian rhythms in a mouse model of vitamin D deficiency and documented an impaired sleep-wake control with reduction in circadian rhythmicity via circadian output signals. Also, Masood *et al*. (20) demonstrated a marked circadian variation for 25-OHD in healthy volunteers and diabetic patients. Gutierrez-Monreal *et al*. (21) determined whether 1α,25-$(OH)_2$D3 could alter the expression of circadian genes in adipose-derived stem cells (ADSCs). The results showed that 1α,25-(OH)2D3 synchronized circadian clock gene expression in ADSCs, suggesting an important role of 1α,25-$(OH)_2$D3 in the molecular clock regulation. From these statements, authors believe that vitamin D may serve as biomarker in addition to melatonin, and depending on their levels, circadian disruption may occur in patients with prostate and hepatic cancers.

There is growing evidence that suggests an association between vitamin D deficiency and sleep disorders so that it might relate to the maintenance of the sleep-wake cycle. Han *et al*. (22) examined the possible association between serum vitamin D levels and the presence of sleep disturbance in patients subjected to hemodialysis when compared with healthy control subjects. Sleep quality was measured using the Pittsburgh Sleep Quality Index (PSQI). Patients with sleep disturbance showed lower levels of 25(OH)D as compared to those without sleep disturbance. Jung *et al*. (23) investigated serum vitamin D levels in fixed day indoor workers in the manufacturing industry and analyzed the relationship of vitamin D deficiency with sleep quality. A significant correlation was observed between serum vitamin D deficiency and poor sleep quality. Golan *et al*. (24) hypothesized that vitamin D and melatonin may have correlated influences in patients with multiple sclerosis (MS). After three months supplementation, 25-OH-D levels increased and nighttime melatonin secretion decreased significantly in the high-dose group, but not in the low-dose group. Importantly, after one year, a decrease in 25-OH-D levels, accompanied by a nighttime increase in urinary 6-sulfatoxymelatonin (aMT6s) were observed in the high-dose group. Finally, they concluded that melatonin secretion is negatively correlated with changes in serum 25-OH-D in IFN-β-treated patients with MS. These findings suggest that melatonin should be considered as a potential mediator of vitamin D neuro-immunomodulatory effects in patients with MS.

According to Petrou *et al*. (25), vitamin D supplementation in conjunction with standard therapies (e.g., chemotherapy, radiation therapy) may confer clinical benefits by decreasing serum prostatic specific antigen (PSA) levels and VDRs expression. Fleet *at al*. (26) tested whether long-life modification of vitamin D signaling can alter the progression of early prostate carcinogenesis in a model of high-grade prostatic intraepithelial neoplasia (HGPIN) similar to humans. They reinforced the hypothesis that the loss of vitamin D signaling accelerates the early stages of prostate carcinogenesis and suggest that higher vitamin D dietary requirements may be needed to sustain prostate health.

Preclinical research indicates that the active metabolite of vitamin D (calcitriol) or vitamin D analogues might have potential as anticancer agents because their administration has antiproliferative effects while activating apoptotic pathways and inhibiting angiogenesis. Therefore, they support the development of 1α,25(OH)$_2$ D3 and vitamin D analogues as chemopreventive and therapeutic anticancer agents (2, 27). Also, results from clinical and preclinical studies show that high doses of calcitriol can be administered safely alone or in combination with other agents to elicit or enhance anti-tumor activity (28).

More importantly, there is highly reliable evidence that melatonin alleviates cancer in the initiation, progression and metastasis phases (29). Based on previous data, lower levels of melatonin are related to the increased risk of developing cancer and these data corroborate the current findings showing a lower level of melatonin in patients with PC and HCC, when compared to control individuals (3, 30, 31).

Recently, De Castro *et al*. (32) measured blood melatonin and its metabolites in patients with breast cancer and compared them to those in healthy women. Melatonin levels were statistically higher in the control group compared to women with cancer ($p \leq 0.05$). In addition, melatonin levels were also significantly lower in women with cancer and metastasis when compared to women with cancer, but without widespread metastasis ($p < 0.05$).

Considering prostate cancer, Sigurdardottir *et al*. (33) investigated the prospective association between early morning urinary aMT6s and the subsequent risk for prostate cancer, under the assumption that men with lower levels of aMT6s have an increased risk of advanced cancer. It was concluded that men with morning levels of aMT6s below normal rate had a four times greater risk for advanced disease, when compared to men with levels above normal (34).

It is well known that regulation of the multiple functions of melatonin in cells is mediated by binding to its MT1 and MT2 receptors in the plasma membrane or by acting through receptor

independent manner (e.g., intracellular mitochondrial actions). Melatonin can be directly taken up by prostatic tumor cells through the GLUT1 transporter; the influx of melatonin appears to compete with glucose uptake (35). More recently, PEPT1/2 transporters were first localized in the mitochondrial membrane of androgen-independent PC3 cells, and mainly the PEPT1 was proven to facilitate the transport of melatonin into mitochondria (36). Additionally, Fang *et al*. (37) confirmed that VDR is a novel melatonin-binding nuclear receptor, and melatonin indirectly regulates Runx2 when it directly binds to the C-terminal binding domain (LBD) and the N-terminal binding domain (DBD) of the VDR, respectively. Moreover, melatonin's antioxidant effects including its actions via MT3 (quinone reductase 2, NQO2) and its direct free radical scavenging actions have been described (38). By its lipophilic nature, melatonin easily crosses the cell, nuclear, and even mitochondrial membranes to bind the cytosolic, nuclear, and mitochondrial proteins to finally orchestrate a variety of non-receptor mediated effects (39).

In the liver tissue, antioxidant properties of melatonin have protective functions in the hepatobiliary system against oxidative stress (40). A possible mechanism by which melatonin can contribute to the prevention of HCC relates to its ability to directly neutralize the hydroxyl radical (OH•), a highly toxic free radical (17). A single melatonin molecule is capable of removing up to ten ROS, while classic antioxidants remove only one of these reactive species (41). Although the detoxification of OH• is an important antioxidant achievement, the actions of melatonin on redox system are vast and complex. Melatonin also neutralizes hydrogen peroxide (H_2O_2) and other oxidants, including singlet oxygen ($^1O_2$), nitric oxide (NO) and the product of superoxide radical (O_2•-) interaction with NO, that is, the peroxynitrite anion (ONOO-) and/or its metabolites. In addition, it also has the hability to increase the activities of the main antioxidant enzymes, through its specific receptors (42).

Melatonin exerts protective effects by inhibiting oxidative stress, reducing inflammatory signaling, modulating autophagy flux, and controlling hepatocyte apoptosis and epithelial cell injury. Therefore, it attenuates the activation of HSCs and the proliferation of fibrogenic effector cells, ultimately reducing ECM deposition. Potentially, re-establishment of the light/dark cycle and the circadian rhythm may increase the endogenous melatonin level while improving the therapeutic effects of the indoleamine in reversing the progression of fibrosis (43).

The transcriptions of melatonin-synthesizing enzymes-encoding genes and clock genes were found to be downregulated in patients with PC and HCC. This may probably reflect a profound circadian rupture of physiological functions and a relevant interference in signaling pathways associated with clock-controlled genes, promoting an irregular production of melatonin and favoring cancer development. The downregulation of genes with tumor suppressor functions (e.g., PER, CRY, and BMAL1) can be directly related to tumor development in patients. On the other hand, some protooncogenes demonstrated to be upregulated and, consequently, stimulate tumor development. The expression of ASMT and NAT enzymes are relatively low in PC and HCC patients revealing an important prognostic factor to be considered. Melatonin seems to be capable of directly altering the expression of clock genes to affect cancer development, and depending on cancer cell type, melatonin might up or downregulate specific clock genes to control cell cycle, survival and repair mechanisms (44).

Collectively, our results revealed that patients with the worst sleep quality presented also lower values of melatonin and vitamin D when compared to control subjects. Furthermore, a reduced expression in melatonin-synthesizing enzymes and in clock genes with tumor suppressor functions may correlated with worse prognosis in PC and HCC patients. Such findings corroborate previous data and suggest that disruption of the circadian rhythms, associated with changes in the day and night cycle, have consequences for the maintenance of

organic health. We also suggest that melatonin supplementation may represent an important therapeutic strategy for patients with solid tumors such as PC and HCC in addition to potentially improving their sleep quality.

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AUTHORSHIP

DAPCZ and MGMP conceived and designed the manuscript. RJBN, LSS, FLCF e RS collected and analyzed the data. TBC e AAN performed statistical analysis and acquired the data. DAPCZ and LGAC analyzed and interpreted the data. RJR critically revised the manuscript. All authors significantly contributed to compilation of the literature and approved the final version of the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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