Research Article

Melatonin and cancer: Exploring gene networks and functional categories

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ABSTRACT

While melatonin is known for its multifaceted properties and its potential to combat cancer, there has been limited exploration of the cancer-melatonin interaction at the gene network level. One of the ways to better understand the molecular mechanisms of melatonin's anti-cancer effects is to use text-mining strategies to extract relevant information that creates knowledge networks of entities and their associations. In this study, we mined gene-publication associations to search for genes most relevant to the terms of "melatonin" and "cancer". A total of 152 genes were identified and ranked, among which 15 were kinase-related and three G-protein coupled receptor genes. The hub genes (*STAT3*, *JUN*, *TP53*, *MAPK3*, *EP300*, *SRC*, *HSP90AA1*, *AKT1*, *ESR1*, and *IL6*) were involved with several pathways in cancer. After examining the melatonin-treated cancers, we mapped 25 upregulated and 51 downregulated genes; these were strongly associated with cancer hallmarks such as resisting cell death, sustaining proliferative signaling, and inducing invasion and metastasis. Upregulated genes showed molecular functions including apoptotic protease activator, caspase activator, enzyme regulator, and protein binding, whereas the downregulated genes affected protein kinase activities, transcription factor binding, protein, enzyme, DNA, and promoter bindings. By connecting gene subsets, we detected a closer relationship among breast, hepatocellular, prostate, and oral cancers, in addition to neuroblastoma and osteosarcoma in terms of changes in melatonin-related signaling pathways. TCGA data were analyzed to understand the impact of gene signatures on survival of patients, and melatonin-downregulated genes were associated with longer survival of patients with glioblastoma, bladder, breast, colon, stomach, liver, lung, and ovarian carcinomas. These results provide a global view of gene interaction networks in melatonin-treated cancers and their functional value, opening new opportunities to consider melatonin for cancer therapy.

Key words: Melatonin, cancer, target genes, regulatory network, functional enrichment, gene mining

1. INTRODUCTION

Cancer is an enormous public health problem, with an estimated 19.3 million new cases and 10.0 million cancer deaths in 2020 globally (1). Based on demographic projections, global cancer burden is expected to reach 28.4 million cases in 2040. Cancer ranks as the most important barrier to increasing life expectancy worldwide in the 21st century (2). The multifactorial etiology, genetic factors, and lifestyle conditions may further exacerbate the epidemiology of cancers (3). There are more than 150 different types of cancer and the lack of effective early-stage diagnosis hinders the cure of patients (4, 5). Cancer is considered a disease of the genes since various cellular dynamics rely on specific gene activation or repression (5). These cells are usually accompanied by evasion of apoptosis, uncontrolled cellular proliferation, cellular migration, invasion, and metastasis, and changes in energy metabolism (6). The cancer-related signaling networks are heterogeneous and provide informative data for dynamics given that the networks contain both directed and undirected interaction types (7). The identification of functional agents capable of regulating a wide range of molecules in the pathological cells would be an important therapeutic ally.

Melatonin (N-acetyl-5-methoxytryptamine), a highly pleiotropic molecule, is an anti-tumor regulator synthesized in a circadian and in a non-circadian manner (8). At the gene expression level, melatonin acts directly or indirectly on RNAs and proteins, including microRNAs, long-non-coding RNAs, Piwi-interacting RNA, DNA methyltransferases, and histone-modifying enzymes, in diverse conditions such as in cancer (8, 9). Using a meta-analysis approach, we have reported that melatonin regulates various microRNAs in breast, gastric, oral, colorectal, and prostate cancers, and glioma, and these microRNAs were potential regulators of target genes implicated in cell proliferation, differentiation, apoptosis, senescence, and autophagy (10).

Although the relationship between melatonin and cancer has been known for decades (11), the knowledge of how melatonin modulates molecular networks in different cancer types remains sparingly investigated. The rather slow progress in this area has been due to the numerous convoluted means of action of melatonin in cancer inhibition; because of this, the indolamine has been described as a selective smart killer of cancer cells (12). Melatonin has well-known actions on cancer cells such as pro-oxidative effects, regulation of estrogenic receptor expression, depletion of telomerase activity, epigenetic alterations, and disruption of energy metabolism (13-15). Additional information on the close crosstalk between genetic and epigenetic regulation of cancer by melatonin would be helpful to clarify proper molecular mechanisms that qualify the use of this molecule, along with other agents, for clinical application.

It is well documented that melatonin exerts pro-apoptotic, immunomodulatory, antiproliferative, and anti-angiogenic actions (16-18). Melatonin has long been considered a multifaceted molecule interfering with cancer hallmarks, and epidemiological studies have documented shiftwork and light-at-night as contributors associated with increased incidence of cancers (19-21). A particular interest is the fact that melatonin levels are reduced in cancer cells (22, 23), prompting us to examine whether its synthetic rate in mitochondria of cancer cells are impaired or whether the circulating melatonin is not being sufficiently taken up by these cells; the prediction of melatonin synthesis using gene expression data of cancer patients has revealed a significant decline of melatonin levels (24-26).

Considering that melatonin has functional effects on regulatory RNAs (27), we mapped gene interactions in multiple cancers and uncovered relevant gene sets that are regulated by melatonin in each cancer type. We also showed the connections of these genes with cancer hallmarks, functional enrichment, and their clinical significance.

2. MATERIALS AND METHODS

2.1. Identification of potential genes involved with melatonin and cancer.

To comprehensively identify the genes related to the terms of "cancer" and "melatonin" based on published literature, we initially used Geneshot software (28) to identify and filter the relevant genes. For better data refinement, we searched for studies using the following terms: cancer and melatonin, cancers and melatonin, tumor and melatonin, tumors and melatonin, melatonin and neoplasia, and melatonin and neoplasms. After uncovering these genes, they were ranked according to the terms into publications over the time so that any correlation between cancer and melatonin was identified. Next, we manually curated the data identified in the studies through the PubMed electronic database (http://www.ncbi.nlm.nih.gov/pubmed), linking the cancer type with the appropriate response to melatonin treatment. The inclusion criteria were: studies validated in cancer samples (*in vivo* or *in vitro*), studies with untreated tissues for comparison (control groups), and data providing statistical significance. Studies involving non-cancerous tissues/cells, reviews, studies that were not case-controlled and without complete access, and studies involving *in silico* analyses were excluded.

2.2. Protein-protein interaction (PPI) networks based on melatonin and cancer-associated genes.

Functional molecular network was generated from those genes associated with melatonin and overall cancers. The metasearch STRING database (Search Tool for Retrieval of Interacting Genes, v. 11.5) was used for creating PPI enrichment (29). The following interaction sources were set: database, text mining, experiments, and co-occurrence. We also applied the minimum interaction score of 0.900 (highest confidence), and all disconnected nodes in the network were hidden to facilitate visual analysis (P-values represented the statistical significance provided by STRING). Access in January 2023. The complex gene network interaction was used for annotation and visualization using the open-source Cytoscape platform v.3.9.1 (30) available at https://cytoscape.org/; a total of 152 nodes were integrated into a regulatory network consisting of 713 interactions (average node degree = 9.38, average clustering coefficient = 0.507). Using cytoHubba (31), the entire network was explored for important nodes and subnetworks through several topological algorithms to detect the top hub genes. These included degree, edge percolated component, maximum neighborhood component, density of maximum neighborhood component, maximal centrality and centralities based on shortest paths, such as bottleneck, EcCentricity, closeness, radiality, betweenness, and stress.

2.3. Identification of genes across melatonin-treated cancers.

We initially examined all the genes together regardless of cancer type and discriminated between those genes, which were up and downregulated by melatonin based on each cancer modality. The CancerGeneNet (32) was used to link genes that are frequently mutated in cancer-related phenotypes. This software bridges two interaction layers by connecting molecules whose activities are affected by cancer gene products to proteins that impact cancer phenotypes; this is achieved by implementing graph algorithms linking any gene of interest to the hallmarks of cancer (the score of 0.9 was defined). Next, we used melatonin-related genes to identify different ontology categories. Biological processes (Gene Ontology) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were analyzed using the EnrichR tool (available at http://amp.pharm.mssm.edu/Enrichr/), and the top 10 enriched terms were

generated according to the lowest p-value < 0.05 (Fisher exact test) provided by the EnrichR database (33, 34). To examine gene interactions that were over and under-expressed in melatonin-treated cancers, we used WebGestalt (35), available at https://www.webgestalt.org/, to translate the gene list into biological insights. First, the networks were mapped under topology-based analysis using PPI Biogrid (FDR < 0.05; Benjamini-Hochberg adjusted p-value). Next, over-representation analysis of the genes was applied to find different categories (biological process, cellular component, and molecular function).

2.4. Survival analysis.

Based on the representative individual gene set affected by melatonin, we used GEPIA2 (http://gepia2.cancer-pku.cn/#survival) (36) to determine the risk assessment and to perform overall survival of patients according to TCGA datasets [bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), stomach adenocarcinoma (STAD), glioblastoma multiforme (GBM), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), ovarian serous cystadenocarcinoma (OV)]. Using different gene signatures, we assessed individual correlations with overall survival by Cox Proportional Hazard regression, which stratified the risk groups (high-risk and low-risk), estimated by an optimization algorithm. The prognostic value of each signature predicting survival was visualized as the Kaplan–Meier method and log-rank test. Through the survival maps, we examined the survival contribution of the gene signatures influenced by melatonin in multiple cancer types using the Mantel-Cox test (FDR < 0.01).

2.5. Single-cell RNA-sequencing (scRNA-seq) analysis.

We re-analyzed publicly available scRNA-seq data to determine the transcriptional profile of 15 melatonin-related genes which showed involvement with patients' survival. Next, BRCA (GSE148673, GSE161529, and E-MTAB-8107), LUAD (GSE131907, GSE123904, HRA000154, and E-MTAB-6149), OV (E-MTAB-8107), GBM (EGAS00001002185, EGAS00001001900, and EGAS00001003845), and COAD (EGAS00001003779) scRNA-seq data were downloaded from Curated Cancer Cell Atlas (https://www.weizmann.ac.il/sites/3CA/). We selected untreated primary samples belonging to the 10X platform with count data available. The selected datasets and samples are described in the Supplementary Table 3. LIHC, BLCA, and STAD were excluded considering the lack of data availability and our inclusion criteria. The count data were processed using Seurat (v4.0.6) package (37) in RStudio software. BRCA and LUAD had more than one available dataset and were integrated using the IntegrateData function. The identities of cell types were based on expression of canonical markers.

2.6. Data representation and analysis.

To provide a reliable representation of the molecular functions into a gene set based on the GO hierarchy (FDR<0.01), we used the Biological Networks Gene Ontology tool (BiNGO) (38), visualized in Cytoscape. A hypergeometric test with False Discovery Rate (FDR) correction was applied (significance level < 0.05) when searching for overrepresented categories. Bar plots were constructed using WebGestalt software. KEGG analysis of the total gene set enrichment was created using ShinyGo 0.76.3 (39). The Circos plots were generated by connecting individual up and downregulated genes with specific hallmarks of cancer (40), considering all the cancer types (http://circos.ca/) (41). Alluvial diagrams were constructed with SankeyMATIC linking either up or downregulated genes with their particular cancer

(https://sankeymatic.com/). PCA plot and Heatmap were created using the web tools ClustVis (http://biit.cs.ut.ee/clustvis/) (42) and Morpheus (https://software.broadinstitute.org/morpheus) (43).

3. RESULTS

3.1. Identification of the genes over different cancer types correlated with melatonin.

We initially searched for genes most relevant to the publication terms of "melatonin" and "cancer" using the platform Geneshot. A total of 152 genes were identified and ranked according to the appearance and number of publications. Based on term counts, the top 10 genes were *ESR1*, *CASP3*, *TP53*, *BCL2*, *AKT1*, *IL2*, *IL6*, *INS*, *TNF*, and *HIF1A* (Table S1). Among all genes, 15 of them were kinases-related genes (*AKT1*, *ERBB2*, *MAPK3*, *EGFR*, *MTOR*, *EIF2AK3*, *SRC*, *ATM*, *CDK4*, *KDR*, *PRKCA*, *CSNK1E*, *ERN1*, *MAPK8*, and *ROCK1*) and three belonged to G-protein coupled receptors (*MTNR1A*, *MTNR1B*, and *OPN4*). Specifically, *MTNR1A* and *MTNR1B* genes encodes both melatonin receptors termed MT1 and MT2, respectively. After identifying the genes using the highest confidence of 0.9, we identified 141 connected genes (Figure 1A). This complex network was again examined and 10 hub genes (*STAT3*, score 46.0; *JUN*, score 41.0; *TP53*, score 40.0; *MAPK3*, score 39.0; *EP300*, score 39.0; *SRC*, score 39.0; *HSP90AA1*, score 35.0; *AKT1*, score 34.0; *ESR1*, score 31.0; and *IL6*, score 31.0) were detected with potential interest in the context of melatonin and cancer (Figure 1B); these genes are involved with several pathways in cancer, estrogen signaling, and immune response (false discovery rate (FDR) < 10^{-7}, combined score > 3,000), and most of them are transcription factors.

Using KEGG database, the gene network was mainly associated with pathways in cancer, lipid and atherosclerosis, PI3K-Akt signaling, viral infections, proteoglycans in cancer among others ($-Log_{10}$ FDR > 30; Figure 1C). Furthermore, the most representative GO biological processes involved with the genes associated with melatonin and cancer were cellular responses to cytokine stimuli, positive regulation of transcription, gene expression, intracellular signal transduction, metabolic processes, and regulation of apoptosis (Figure 1D, Table S2). To deeper explore this network with a pathological perspective related to cancer phenotypes, we used CancerGeneNet to develop a rational framework for the genes that are frequently mutated in cancers. Figure 1E summarizes the cellular regulatory circuits displaying membrane receptors and ligands, cytoplasmic signaling molecules, and nuclear activators (physical interactions with score 0.9).

3.2. Upregulated and downregulated genes in melatonin-treated cancers.

Among the molecules associated with melatonin and cancer, we sorted them into upregulated and downregulated genes (Table S1). A total of 25 genes were upregulated by melatonin in different cancers (P<0.05); *CASP3*, *TP53* (score 22.0), *ANXA5* (score 20.0), *CASP9*, and *CDH1* (score 17.0), which are related to apoptosis, p53 signaling, tumor suppression, were recognized as top five hub genes by degree. Figure 2A depicts the upregulated gene network connected with top ranking neighbors (*FBXO5*, *CASP10*, *XIAP*, *TRIM25*, *APP*, *RNF4*, *BCL2L1*, *ELAVL1*, *FBXW11*, and *PPP1CA*). These upregulated genes were associated with important cancer hallmarks suggesting that melatonin may reverse some of these cancer phenotypes. Of those, resisting cell death, sustaining proliferative signaling, and induction of invasion and metastasis are major hallmarks involved with these genes in overall tumors (Figure 2B). The bar charts show different categories of GO terms involved with upregulated genes (Figure 2C). The top biological processes were response to stimulus,

biological regulation, cell communication, and metabolic processing. Regarding the cellular components and molecular functions, we identified associations with the nucleus, membrane-enclosed lumen cytosol, membrane, protein-containing complex, and the main functions included protein binding, hydrolase and enzyme regulator activities, nucleic acid and ion binding, and others.

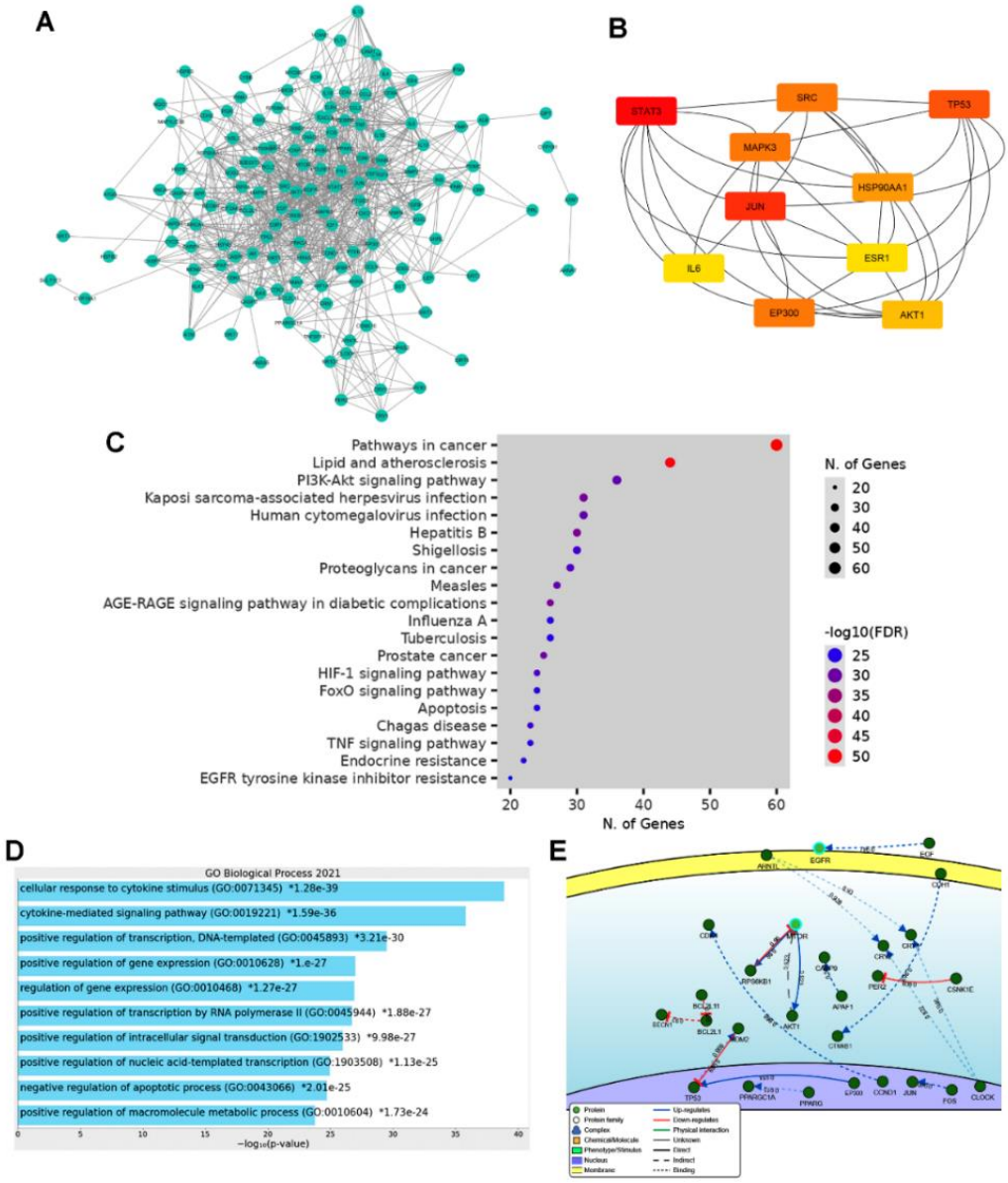

Fig. 1. Overall gene interactions and term enrichment.

A) Network interaction obtained from Geneshot list was generated in STRING and visualized in Cytoscape (PPI enrichment p-value < 1.0e-16; minimum confidence score: 0.9). B) Top 10 hub genes from the gene network were generated in Cytoscape (score > 31). C) KEGG analysis of the gene set was created in ShinyGo 0.76.3 (FDR < 0.05). D) Biological processes were based on functional enrichment of the gene set and reflect the Log_{10} p-value of the terms. E) Cellular scheme exhibiting the main molecular regulators of the gene list according to the most frequent mutations and cancer phenotypes (blue line = upregulation, red line = downregulation, dashed line = molecular binding, continuous line = direct interaction; score 0.9).

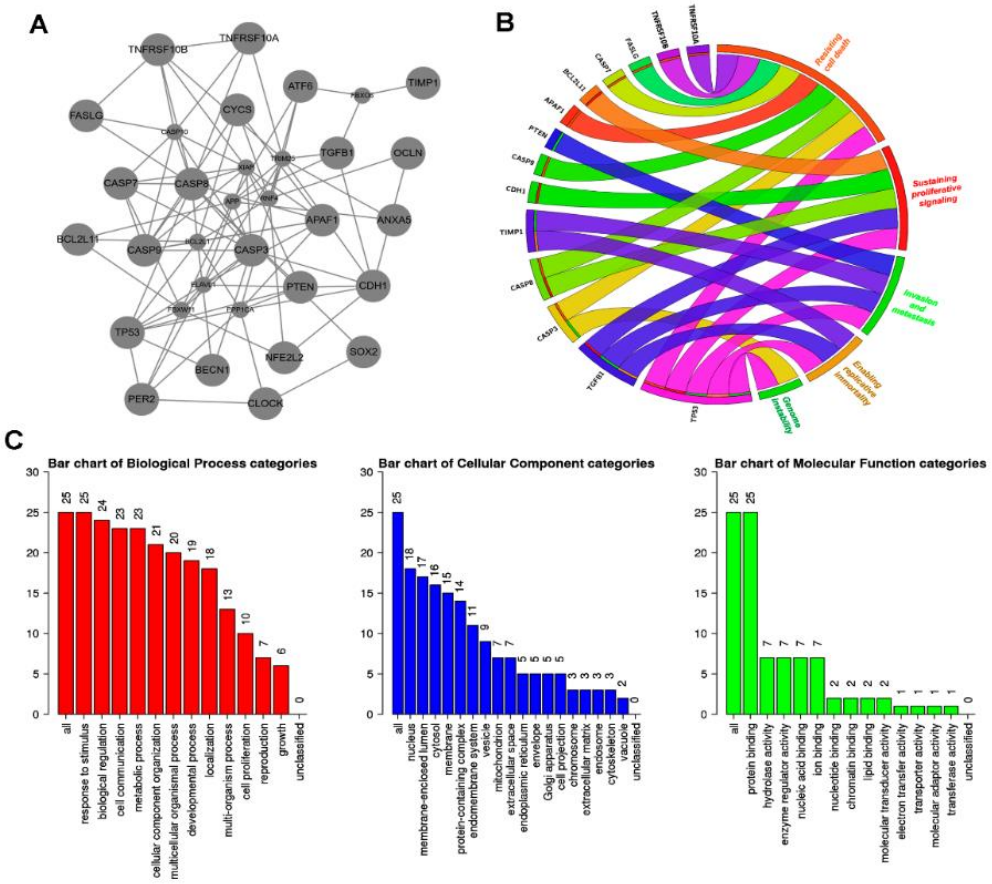

Fig. 2. Upregulated target genes in melatonin-treated cancer and related functional enrichment.

A) Network connecting the gene set (major circles = seed genes, minor circles = top ranking neighbors). B) Circos plot showing the associations of upregulated genes with cancer hallmarks; each color ribbon represents a specific target gene. C) Bar charts depicting GO categories (biological process, cellular components, and molecular functions) of the genes.

Melatonin also showed an involvement with other genes, which were downregulated in several cancer types. We detected 51 downregulated genes ($P<0.05$) and the top five hub genes were *STAT3*, *HIF1A* (score 49.0), *CTNNB1* (score 48.0), *AKT1* (score 47.0), and *ESR1* (score 46.0); these genes are mostly involved with cell survival and differentiation, angiogenesis, epithelial-mesenchymal transition, and estrogen receptor binding. As shown in Figure 3A, the downregulated genes associated with different ranking neighbors (*HRH1*, *IL6R*, *SH3GL2*, *ZBTB16*, *TP53*, *ELAVL*, *NTRK1*, *BAG6*, *COPS6*, and *UBQLN4*). The most relevant functions related to the expression of these genes were sustained proliferative signaling, invasion and metastasis, cell death resistance, and replicative immortality (Figure 3B), all of which were potentially attenuated by the melatonin-linked downregulated genes. The terms enriched by the downregulated genes were similar to those of upregulated genes and biological processes were over-represented by the response to stimuli, biological regulation, metabolic processes, and multicellular organismal processes (Figure 3C). The cellular components included associations with membrane-enclosed vesicles, nucleus, protein-containing complex, and membrane, and the top categories of molecular functions were protein binding, ion and nucleic acid binding, transferase activity, and others.

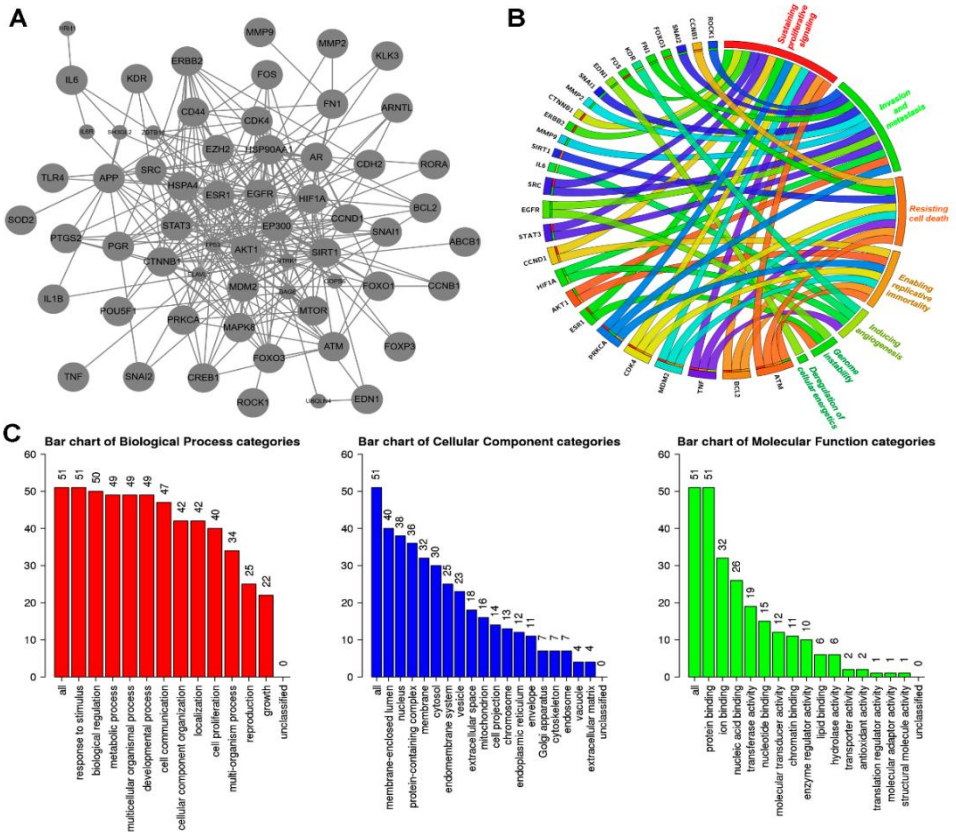

Fig. 3. **Downregulated target genes in melatonin-treated cancer and related functional enrichment**.

A) Network connecting the gene set (major circles = seed genes, minor circles = top ranking neighbors). B) Circos plot showing the associations of downregulated genes with cancer hallmarks; each color ribbon represents a specific target gene. C) Bar charts depicting GO categories (biological process, cellular components, and molecular functions) of the genes.

In addition to identifying GO terms linked to up- and downregulated genes altered by melatonin in tumors, we used BiNGO to deeply assess the overrepresentation of gene ontology categories in the biological networks (hypergeometric test, FDR < 0.01). The gene set was referred to as the molecular functions. For instance, while upregulated genes showed representative categories such as apoptotic protease activator, caspase activator activity, enzyme regulator and activator activities, protein binding, and TRAIL binding (Figure 4A), the downregulated genes were involved with protein kinase activities, transcription factor binding, protein, enzyme, DNA, and promoter bindings, transcription regulator and activator activities (Figure 4B).

3.3. Gene profile and functional enrichment are specifically associated with cancer type in response to melatonin.

We investigated the diversity of targets by which melatonin interferes with each cancer type by examining the literature; only those genes involved in at least two different cancer types were computed. From the upregulated genes, 15 out of 25 were directly associated with several cancer types. The most abundant genes regulated by melatonin in the tumors were involved with apoptosis, cell adhesion, and oncogenic signaling (*CASP3*, *CDH1*, *CYCS*, *CASP9*, and *DDIT3*), whereas the top cancer types affected by the upregulated targets were breast cancer,

colorectal cancer, and hepatocellular carcinoma (Figure 5 A). Conversely, 18 genes downregulated by melatonin interacted to a greater or lesser extent with 27 distinct cancer types. Specifically, *BCL2*, *HIF1A*, *MMP9*, *STAT3*, *SNAI1*, *CDH2*, *CCND1*, and *CDK4*, which are associated with kinase signaling, negative regulation of apoptosis, extracellular matrix remodeling, transcription regulator complex, cell migration and differentiation, were the most common genes attenuated by melatonin treatment, and breast cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, ovarian cancer, and osteosarcoma were the cancer types most influenced by these downregulated genes (Figure 5 B).

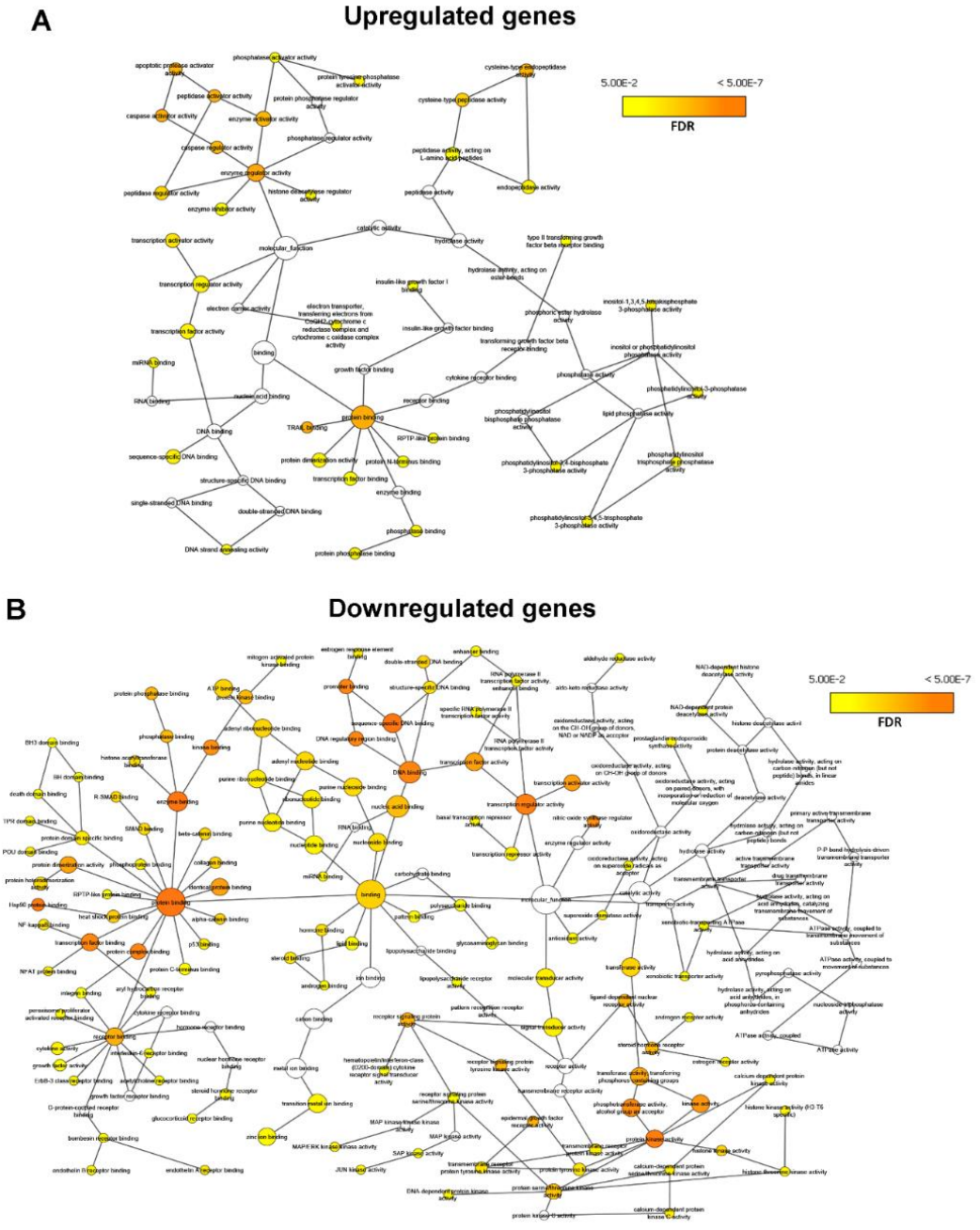

Fig. 4. The interactome of the molecular functions in response to melatonin in cancers.

BiNGO was used to overrepresent the gene set functions. A) Network generated with upregulated genes. B) Network generated with downregulated genes. The hypergeometric test corrected by Benjamini-Hochberg (FDR < 0.01).

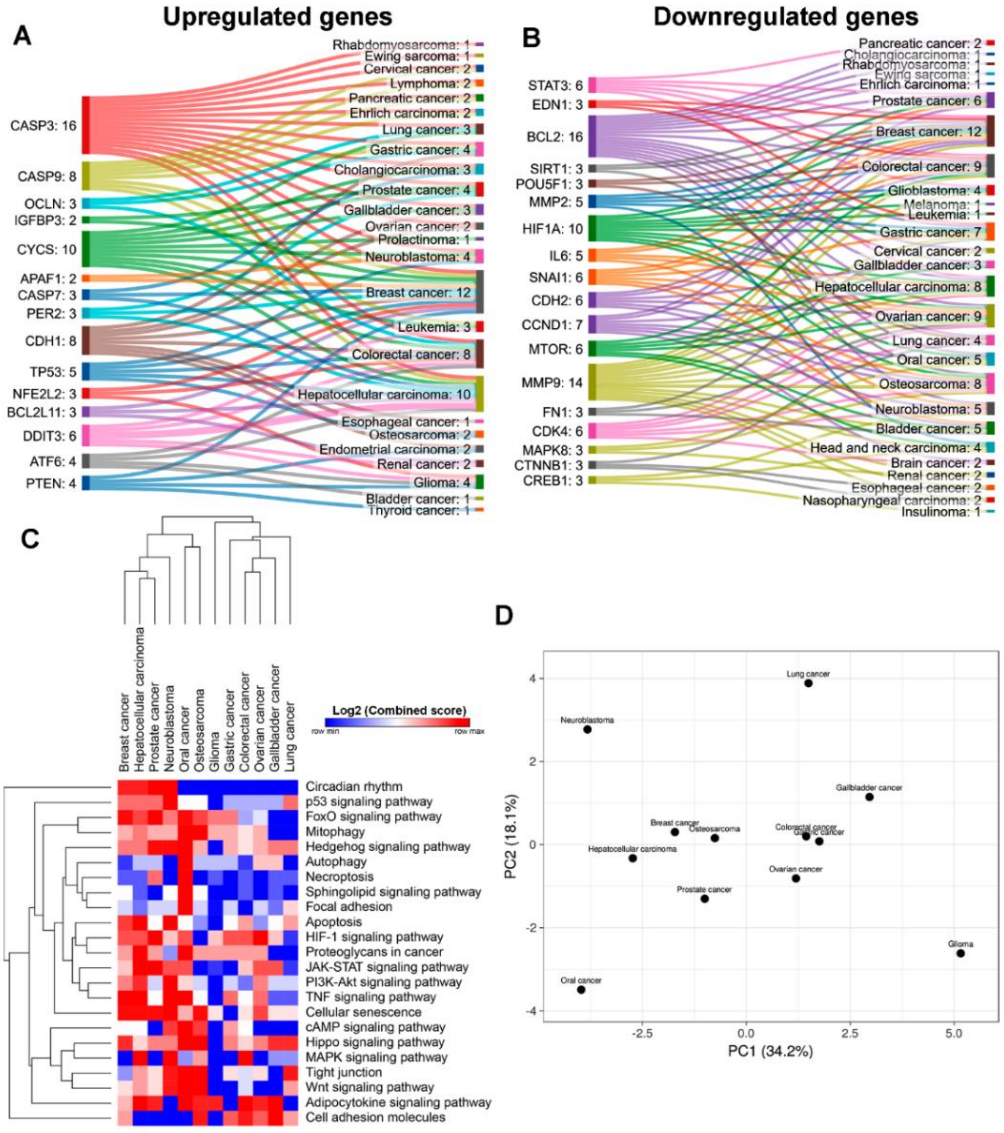

Fig. 5. Gene signatures and enrichment analysis of different cancer types treated with melatonin.

Alluvial diagrams connecting the representative upregulated (A) and downregulated (B) genes by melatonin in respective cancer types were generated using the Sankeymatic online tool (http://sankeymatic.com/). Upregulated and downregulated plots showed respectively 80 and 108 flows linking genes and cancers. C) Heatmap top-ranked categories associated with different cancers in which genes were upregulated or downregulated by melatonin, computed by the gene set enrichment analysis using EnrichR. Signaling pathways were considered for over- or under-representation (Log2 combined score, minimum and maximum values). Rows and columns were clustered using Euclidean distance. D) Principal component analysis (PCA) based on functional enrichments by target genes in different cancer types.

All over-represented genes upregulated or downregulated by melatonin ($P<0.05$) were selected for enrichment analysis using KEGG pathways (only tumors exhibiting > 5 genes affected by the indole were considered in this analysis; Figure 5 C). A total of 12 cancer types were more commonly associated with melatonin-related genes. Notably, a clear pattern of

clusterization highlighted breast cancer, hepatocellular carcinoma, prostate cancer, neuroblastoma, oral cancer, and osteosarcoma as having more changes in functional enrichment-related genes (higher combined score) compared to glioma, gastric, colorectal, ovarian, gallbladder, and lung cancers (lower combined score). The main functions in the cancer subset with higher combined scores were the p53 signaling pathway, circadian rhythms, FOXO signaling, mitophagy, the hedgehog signaling pathway, apoptosis, HIF-1 signaling, JAK-STAT signaling, TNF signaling, cellular senescence, Wnt signaling, tight junction, and the Hippo signaling pathway. Conversely, the functions in the cancer types displaying a lower combined score were mainly associated with circadian rhythms, necroptosis, and sphingolipid signaling pathway. Based on these functional mechanisms, the principal component analysis (PCA) showed that neuroblastoma, lung cancer, glioma, and oral cancer were distinguishable from each other and among all cancer types regarding melatonin's action (Figure 5 D). Next, we extrapolated these findings associating individual cancer signatures to patients' overall survival.

3.4. Melatonin alters gene signature related to a shorter overall survival of patients.

Considering that the vast majority of the molecular pathways and the gene sets regulated by the melatonin are related to cancer aggressiveness, we investigated whether the melatonin-downregulated genes indeed predict a worse prognosis for patients. To explore survival data of cancer patients, we used GEPIA2 to determine the prognostic index correlating survival curve with risk assessment. These patients were stratified into two groups (50% at high- and 50% at low-risk; median values) so that to individually evaluate gene signatures expressed in each cancer type available in the TCGA platform. The shorter survival of patients was linked to a high expression of specific genes in BLCA (*SNAI1*, *MTOR*, *CDH2*, and *FN1*; hazard ratio (HR) = 1.4, log-rank p-value = 0.027; Figure 6A), BRCA (*STAT3*, *HIF1A*, *SNAI1*, and *CDH2*; HR = 1.4, log-rank p-value = 0.037; Figure 6B), COAD (*STAT3*, *MTOR*, *SNAI1*, *POU5F1*, and *CDH2*; HR = 1.7, log-rank p-value = 0.04; Figure 6C), STAD (*BCL2*, *HIF1A*, *SNAI1*, *EDN1*, and *FN1*; HR = 1.4, log-rank p-value = 0.032; Figure 6D), GBM (*STAT3*, *HIF1A*, *MMP2*, and *MMP9*; HR = 2, log-rank p-value = 0.00033; Figure 6E), LIHC (*BCL2*, *IL6*, *STAT3*, *MMP9*, CCND1, *HIF1A*, *MTOR*, and *CDK4*; HR = 1.6, log-rank p-value = 0.0062; Figure 6F), LUAD (*CTNNB1*, *POU5F1*, and *CDK4*; HR = 1.3, log-rank p-value = 0.04; Figure 6G), and OV (*HIF1A*, *SNAI1*, and *MTOR*; HR = 1.3, log-rank p-value = 0.026; Figure 6H). Collectively, the overall survival map revealed that *FN1*, *CDH2*, *SNAI1*, *HIF1A*, *MMP2*, and *MMP9* genes were important contributors to shortening the survival of patients in most of the tumors (Figure 6 I). The gene signatures are also key candidates for disease-free survival of patients especially in BLCA, COAD, GBM, LIHC, and LUAD (Figure 6 J).

To deeper understand the gene expression signatures on specific cancer cell type, we used a curated cancer cell atlas providing collected, annotated, and analyzed cancer scRNA-seq datasets. Based on heatmap clustering, there is a clear profile of gene expression in endothelial cells of BRCA, LUAD, OV, COAD, and in microglia of GBM patients. This expression pattern was closely associated with that found in fibroblasts of BRCA, LUAD, OV, and COAD, and also with genes majorly expressed in the malignant cells of LUAD, OV, and COAD (Figure 7). More generally, *FN1* and *MMP2* genes were highly expressed in tumor-associated fibroblasts of patients with BRCA, OV, LUAD, and COAD. The genes *CTNNB1*, *EDN1*, *SNAI1*, *MTOR*, *HIF1A*, *STAT3*, and *IL6* were mostly increased in endothelial cells. Furthermore, the genes *CCND1*, *CDK4*, and *POU5F1* had increased expression in malignant cells of patients with LUAD, OV, and COAD. Specifically, BRCA patients also had high expression of *CCND1* gene in malignant cells whereas *MMP9* and *HIF1A* genes were increased in macrophages. COAD patients showed high expression of *EDN1* gene in the malignant cells,

and *MMP9*, *HIF1A*, and *IL6* in tumor-associated macrophages. OV patients showed increased expression of *FN1* and *HIF1A* genes in macrophages whereas *STAT3* was in mesothelial cells, *BCL2* in T cells, and *CTNNB1* and *EDN1* genes in endothelial cells. Finally, GBM patients showed moderate expression of *BCL2* in astrocytes, *CDK4* and *CDH2* in malignant cells, and *CTNNB1*, *HIF1A*, and *STAT3* in microglia (Figure 7). Exploring scRNA-seq datasets aid revealing upregulated targets in specific cancer cell types which are potentially affected by melatonin treatment.

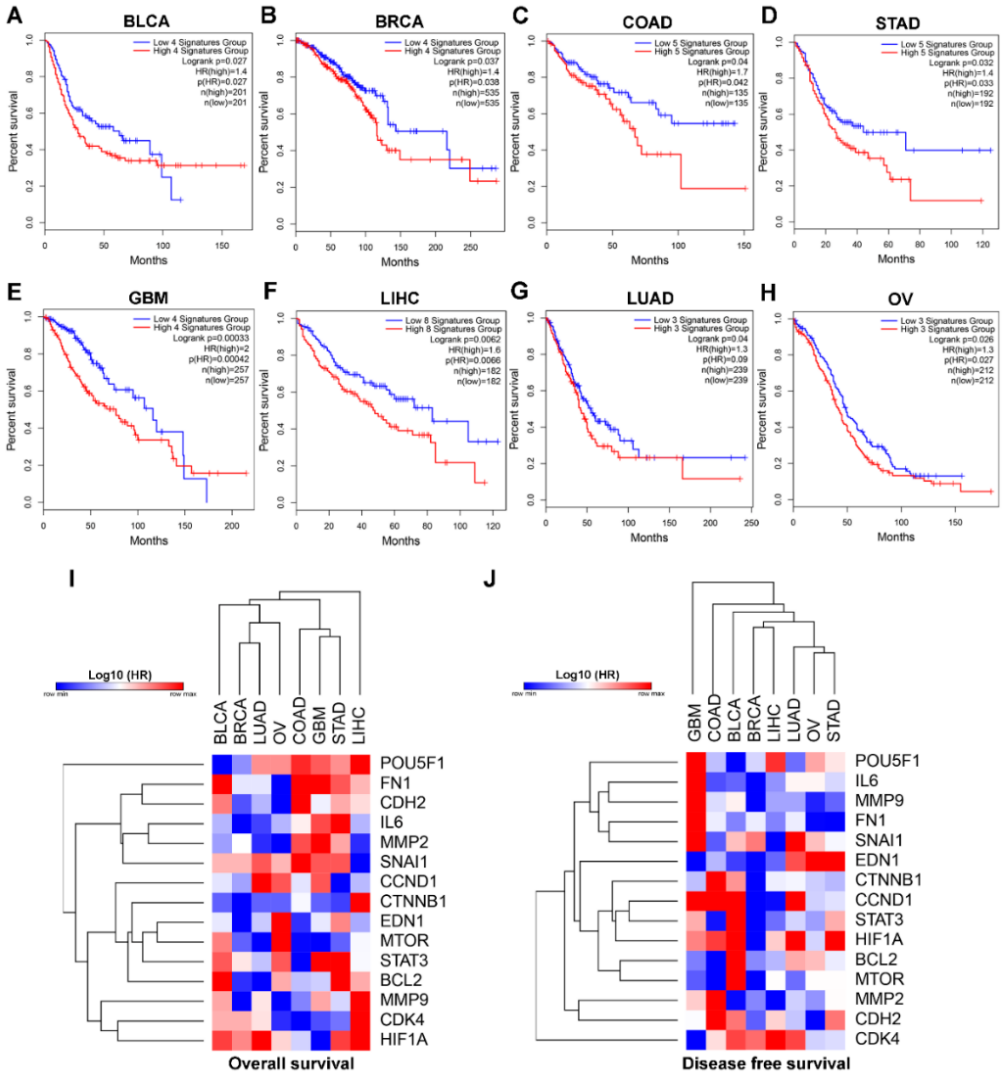

Fig. 6. The impact of gene signatures regulated by melatonin on the prognosis of cancer patients.

Overall survival curves comparing patients at high (red line) and low (blue line) risks based on overexpressed genes affected by melatonin in BLCA (A), BRCA (B), COAD (C), STAD (D), GBM (E), LIHC (F), LUAD (G), and OV (H). Kaplan–Meier curves determined by univariate Cox regression analysis show the hazard ratio (HR) corresponding to 95% confidence intervals, log-rank p-value, and the number of patients by groups. Heatmaps showing the main gene set belonging to each tumor signature relative to overall survival (I) and disease-free survival (J). Values are based on Log10 HR (+ 0.2 / - 0.2). BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; STAD, stomach adenocarcinoma; GBM, glioblastoma multiforme; LIHC, liver hepatocellular carcinoma, LUAD, lung adenocarcinoma; OV, ovarian serous cystadenocarcinoma.

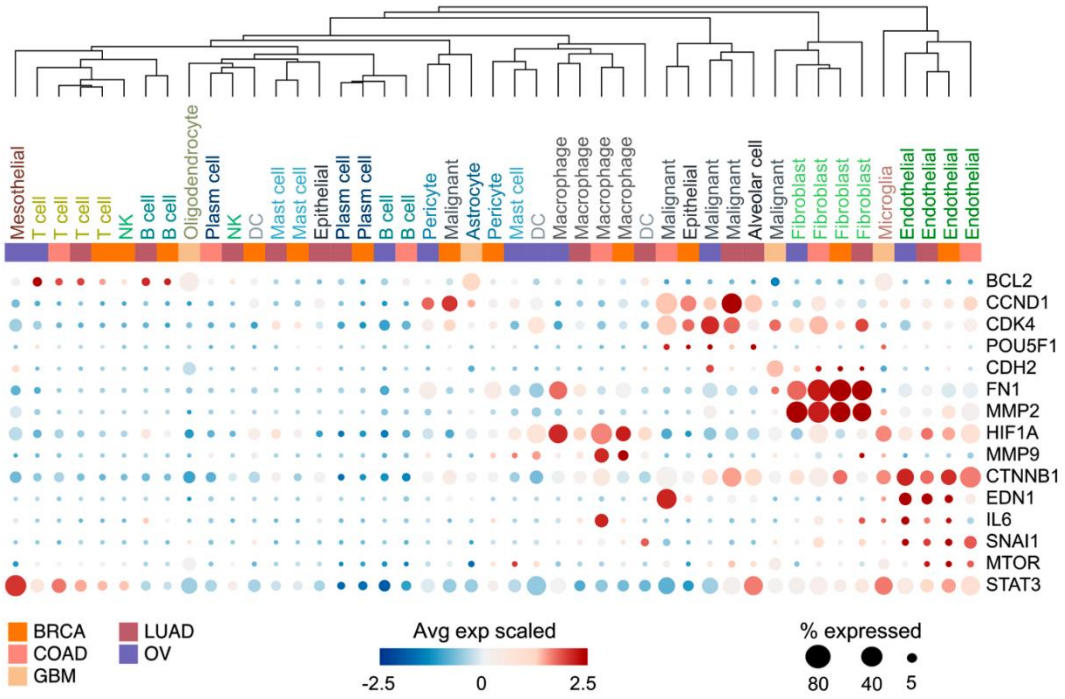

Fig. 7. Single cell RNA-seq analysis reveals a specific cell type expression profile of melatonin-related genes in BRCA, COAD, GBM, LUAD, and OV.

Unsupervised hierarchical clustering (Euclidean distance) of melatonin-related genes is presented in a heatmap according to average expression scaled (color) and percentage of cells expressing the gene (circle size). Heatmap was generated and analyzed using Morpheus online tool (https://software.broadinstitute.org/morpheus/). BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; GBM, glioblastoma multiforme; LUAD, lung adenocarcinoma; OV, ovarian serous cystadenocarcinoma.

4. DISCUSSION

The understanding of cancer molecular networks targeted by anticancer agents may help control its aggressiveness while favoring the development of novel personalized therapies (44). Here, we unveiled which genes whereby melatonin interacts with cancer and defined a core framework for specific cancer types. Through Geneshot, a search engine that ranks lists of relevant genes based on arbitrary terms, we first identified the genes involved with melatonin and cancer, and further individualized each gene set for each cancer type. A total of 141 highly connected genes (confidence score = 0.9) were found in the general cancer network, some of which only interact with melatonin at one level (*MTNR1A*, *MTNR1B*, *ASMT*, and *AANAT*), while a vast majority was composed by driver genes undergoing frequent mutations in cancer studies. As expected, these genes enriched functions are commonly observed in many cancer processes (e.g., responses to cytokine stimuli, gene expression, intracellular signal transduction, apoptotic and metabolic processes, and others). Regarding melatonin production, *ASMTL* gene has been associated with higher capacity of melatonin synthesis in females, thus enhancing protection against stressful conditions (45). We previously showed that *ASMTL* gene positively correlated with metabolism-related genes (e.g., oxidative phosphorylation) in ovarian cancer (22). The hub genes mostly affected by melatonin in cancers were S*TAT3*, *JUN*, *TP53*, *MAPK3*, *EP300*, *SRC*, *HSP90AA1*, *AKT1*, *ESR1*, and *IL6*; these molecules are involved

with IL-6/JAK/STAT3 signaling, TNF-alpha signaling via NF-kB, hypoxia, p53 pathway, and epithelial-mesenchymal transition (FDR < 0.01). Examination of melatonin's main actions in multiple cancers from a gene expression standpoint allowed for reinforcing various anticancer properties, highlighting its role as an immune enhancer, anti-inflammatory, anti-angiogenic, anti-metastatic, and pro-apoptotic agent (46). Of note, the anticancer activity of melatonin is not limited to the aforementioned mechanisms and signaling pathways, but rather represents the central elements of its gene interaction.

To better understand the involvement of melatonin-related genes with cancer hallmarks, we grouped them as upregulated and downregulated genes. Since hallmark capabilities are regulated by partially redundant signaling pathways, their value depends on the cancer's underlying molecular features (40). The genes upregulated by melatonin classified as tumor suppressor genes and transcription factors potentially alter the sustained proliferative signaling and evasion of growth suppressors. Also, genes participating in the TNF receptor pathway, apoptosis pathway, Fas signaling, caspase family, CARD family, TP53 activators and regulators involved with cell death resistance were upregulated by melatonin. Other important features counteracted by melatonin were invasion and metastasis (with the genes classified as cell-matrix adhesion and Rho signaling, proteolysis, cell motility, invasive projections, chemotaxis, cell-cell adhesion, and transcription factors), replicative immortality (genes classified as proteases and telomerase regulators), and genomic instability (genes classified as cell cycle checkpoint genes). The downregulated genes by melatonin were numerous and negatively impacted cancer cell activities. The hallmark and respective internal features associated with those genes were as follow: sustaining proliferative signaling (transcription factors, cell cycle, oncogenes, and multiple features), activating invasion and metastasis (cell-cell adhesion, Rho signaling, cell motility, Cdc42 signaling, matrix metalloproteinases, cell-matrix adhesion, invasive projections, receptor, filopodia, Rac signaling, proteolysis, and chemotaxis), resisting cell death (cell cycle pathway members, TP53 activators and cooperators, TNF superfamily, and Bcl2 family-regulated pathway), enabling replicative immortality (senescence pathway and telomerase regulation), inducing angiogenesis (growth factors and receptors, cytokines and chemokines), genome instability (DNA repair, chromatin modification molecules, and cell cycle checkpoint genes), and deregulation of cellular energetics (activator molecules). A comprehensive review documented the anticancer mechanisms of melatonin against cancer hallmarks associated with cell signaling pathways using *in vivo* and *in vitro* studies (12). These findings provide a foundation for the melatonin-mediated attenuation of cancer hallmarks at the gene expression level. This gene regulation is strongly reflected in the modulation of important molecular functions related to cancer progression as those described by BiNGO analysis.

Upon melatonin treatment, most of the upregulated genes were detected in breast and colorectal cancers, and in hepatocellular carcinoma (shared genes included *TP53, CASP3*, and *CYCS*), whereas the downregulated genes were primarily observed in breast cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, ovarian cancer, and osteosarcoma (shared genes were *BCL2* and *MMP9*). This is attributed to the high number of melatonin-related studies and a larger number of genetic signatures per cancer compared to that observed in a smaller set of tumors. Through KEGG analysis, we explored the similarities and differences among melatonin-treated cancers considering a particular gene set and terms enrichment. For instance, breast cancer, hepatocellular carcinoma, prostate cancer, neuroblastoma, oral cancer, and osteosarcoma showed similar enriched signaling pathways in contrast to glioma, gastric, colorectal, ovarian, gallbladder, and lung cancers; this information may be utilized for the planning of personalized therapies using melatonin. Melatonin has been documented to directly affect cancer cells via apoptotic, antiangiogenic, antiproliferative, and metastasis-inhibitory signaling pathways (47). As a non-toxic, potent, and synergistic therapeutic agent, melatonin

combined with conventional drugs improves cancer sensitivity and life quality of patients (48, 49). A comprehensive review involving cellular, animal, and human studies summarized the underlying molecular mechanisms, epidemiology, risk factors, and clinical significance of melatonin alone or in combination with other chemotherapies against breast, colorectal, liver, lung, prostate, skin, cervical, and ovarian cancers (47). Most of these regulatory events are dependent on gene networks explored in our current study.

Recently compiled information on the ability of melatonin to regulate diverse signaling pathways in different cancer types revealed its involvement with the PI3K-Akt-mTOR pathway, MAPK signaling, Wnt/β-catenin signaling, Notch and NF-kB signaling, JAK/STAT signaling, IGF, VEGF, FGF, and PDGF signaling pathway, and Hedgehog pathway (50). Many of these pathways and others were individually analyzed in each tumor type. The set of genes that enriched the most signaling pathways (e.g., FOXO signaling, p53 signaling, mitophagy, hedgehog pathway, HIF-1 signaling, proteoglycans in cancer, JAK/STAT signaling, PI3K-Akt and TNF signaling, cellular senescence, tight junctions, and Wnt signaling) were shared especially among breast cancer, hepatocellular carcinoma, prostate cancer, and neuroblastoma. These signaling pathways targeted by melatonin are differentially modulated in different cancer phenotypes and represent potentially important mechanisms for cancer biology and behavior. From this analysis, new therapeutic proposals for such cancers could include conventional chemotherapy or radiotherapy with melatonin. A recent study reported an important role of melatonin on the inhibition of VEGF and inactivation of HIF-1α showing that melatonin neutralizes pro-angiogenic while potentiating antiangiogenic effects induced by chemo or radiotherapeutic agents (51); this cooperative action of melatonin highlights its ability to turn cancer totally resistant to chemotherapeutics into a more sensitive tumor state. Additional studies are urgently needed to test if other regulatory factors or genes are involved with melatonin-induced chemosensitizing effects.

To verify the significance of these melatonin-related gene signatures in the context of cancer patients, we explored the melatonin-downregulated genes to uncover their impact on overall survival of patients based on the TCGA dataset. Clearly, functional gene signatures for each cancer type revealed shorter overall patient survival since the high-signature groups presented the worst prognosis; in this case, melatonin has the potential to improve patient survival by reversing the overexpression of specific cancer-associated genes. Together, we described a relevant set of genes altered by melatonin with a negative impact on overall survival of patients with bladder, breast, colorectal, gastric, lung, and ovarian cancers, in addition to glioblastoma multiforme, and hepatocellular carcinoma. Considering specific cancer types, we further identified the transcriptional profile of single cell signatures which are affected by melatonin; specific genes were similarly clustered in malignant cells, macrophages, fibroblasts, and endothelial cells. Particularly, *CCND1* and *CDK4* genes, which are cell cycle regulators, were increased in malignant cells, and *FN1* and *MMP2* genes, especially involved in cell invasion and metastasis, were highly expressed in cancer-associated fibroblasts; these molecules are targeted by melatonin and may serve as candidate biomarkers. Although mostly represented with few cells, the *CTNNB1*, *EDN1*, *IL6*, *SNAI1*, *MTOR*, and *STAT3* genes showed a similar expression profile in cancer-associated endothelial cells.

Mechanistically, up and downregulated genes mediated by melatonin may be a result of its interference in any element of gene expression machinery, potentially being modulated by non-coding RNAs and other epigenetic-related mechanisms (e.g., methylation, acetylation or LNCmRNA and miRNA regulation). The epigenetic regulation of different genes mediated by melatonin has already been documented in breast cancer (52), oral cancer (53), prostate cancer (54), malignant glioma (55), and others. Additional studies that focus on gene dynamics in the presence of melatonin will be helpful in identifying more precisely its biological mechanisms of action in different cancer phenotypes. The current data, however, can be used to guide

treatment protocols. Melatonin exerts oncostatic activities in numerous malignancies-related events with important roles in cell cycle, apoptosis, and autophagy-related processes (56); these actions are especially mediated through receptors MT1 and MT2, cytosolic binding sites including calmodulin and quinone reductase II enzyme, and, although debatable, possibly involving activation of nuclear receptors (RZR/ROR). A Pan-cancer study, involving 33 cancer types from TCGA database, identified different melatonin regulators (57); in the genomic landscape, there was heterozygous amplification of *AANAT* and *GPR50* genes, and heterozygous deletion of *PER3*, *CYP2C19*, and *MTNR1A* as the dominant alterations.

In addition to its intricate actions on molecular signatures, melatonin has numerous metabolic actions that affect the biology of tumors (58-61). The bioavailability of melatonin depends on its pharmacokinetic features, including absorption, metabolism, and elimination; these features may vary among individuals and should be kept in mind when establishing a dose protocol to support its use in cancer prevention and treatment. Because *in vivo* and *in vitro* studies have broadly detailed the mechanism of melatonin's actions on different cancer types, it is imperative to maintain the use of melatonin as an adjuvant opportunity in clinical contexts.

This study provides a new integrative view of the genes associated with melatonin into a cancer scenario. The general characterization of up and downregulated molecules in melatonin-treated cancers revealed important targets which are dysregulated in several cancer types, including rare cancers, and could be exploited to novel experiments for different cancer modalities. Melatonin is capable of influencing gene signatures associated with cancer aggressiveness, thus reflecting its anti-cancer regulatory role at both the molecular and functional levels. It should be disclosed that the number and interaction of genes for each cancer type is based on literature data and in no way does it restrict or prioritize some cancer types; as a result, we feel that melatonin has even a greater involvement in controlling the tumor gene expression. We also note that global transcriptomic analysis provides a more complete gene landscape to guide the molecular responses of melatonin in cancer studies.

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AUTHORSHIP

LGAC: conceptualization, data acquisition and curation, and drafted the manuscript. RFC, RFD, VLSC, SSC: data processing and collection and drafted the manuscript. FRFS, DACZ: collection, interpretation, and data analysis. All authors significantly contributed to the compilation of the literature and approved the final version of the manuscript.

CONFLICTS OF INTEREST

Theauthors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY

All data supporting the findings of this study are available within the paper. Data will be made available on request.

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