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Pan-cancer Genomic Analyses Reveal Prognostic and Immunogenic Features of the Tumor Melatonergic Microenvironment Across 14 Solid Cancer Types

Running Title: Pan-cancer genomic analyses of melatonergic microenvironment

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Abstract

We performed comprehensive genomic analyses of the melatonergic system within the tumor microenvironment and their clinical relevance across a broad spectrum of solid tumors. RNA-seq data from The Cancer Genome Atlas (TCGA) of 14 solid tumors representing 6,658 human samples were analyzed. The tumor melatonergic system was characterised by the rates of melatonin synthesis and metabolism using a two-gene expression model (melatonin synthesis/metabolism index). We calculated three indexes according to different melatonin metabolism isoenzymes (Index-I [*ASMT:CYP1A1*], Index-II [*ASMT:CYP1A2*], and Index-III [*ASMT:CYP1B1*]). Samples of each cancer type were classified into two subgroups (high *vs.* low) based on median values. Clinical outcomes, mutational burden, and neoepitope abundance were analyzed and compared. We found that the ability of the tumor microenvironment to synthesize and accumulate melatonin varied across cancer types and negatively correlated with tumor burden. Kaplan-Meier survival analyses and multivariable modeling showed that the three indexes played different roles across different cancers, and harbored prognostic values in breast cancer (adjusted hazard ratio $[AHR]_{index\text{-}II}$ = 0.65 [0.44–0.97]; P = 0.03), cervical cancer ($AHR_{index\text{-}I}$ = 0.62 [0.39–0.98]; P = 0.04), lung squamous cell carcinoma ($AHR_{index\text{-}III}$ = 0.75 [0.56–0.99]; P = 0.04), melanoma ($AHR_{index\text{-}I}$ = 0.74 [0.55–0.98]; P = 0.04), and stomach adenocarcinoma ($AHR_{index\text{-}III}$ = 0.68 [0.41–0.94]; P = 0.02). We further investigated its clinical relevance with tumor immunogenic features (mutational burden and neoantigen abundance), which may predict immunotherapy benefits. We observed significant negative correlations with mutational burden in the majority of tumors (P < 0.05), except cervical cancer, pancreatic adenocarcinoma, and thyroid carcinoma. Our study provides a systematic overview of the oncostatic values of the melatonergic system, and highlights the utilization of this simple and promising gene signature as a prognosticator and potential predictor of response to immunotherapy.

Key words: melatonergic system, tumor microenvironment, molecular marker, pan-cancer analyses, prognosis, mutational burden, neoantigen abundance

Introduction

Melatonin is a functionally pleiotropic molecular that is secreted primarily by the pineal gland in response to darkness. It provides time-of-day information to the organism, and ensures the synchronization of circadian and seasonal rhythms [1]. The natural synthesis of this agent involves a variety of processes [2]. Acetylserotonin O-methyltransferase (ASMT) is the final enzyme of the biosynthetic pathway, and has been reported to play a rate-limiting role in melatonin synthesis [3]. In human, melatonin is metabolized by the hepatic cytochromes (primarily CYP1A1, CYP1A2) into 6-hydroxymelatonin (6OH-MEL) [4]. Another major metabolic enzyme is CYP1B1, which has a ubiquitous extrahepatic distribution, and has been shown to be expressed at high levels in tissues such as intestine and cerebral cortex [4]. The biosynthetic and metabolic processes influence the level of circulating melatonin. It is well recognized that circulating melatonin exerts a broad range of oncostatic effects through both receptor-dependent and independent pathways [5]. The interaction of tumor cells, circulating melatonin and the associated receptors, as well as the surrounding blood vessels, fibroblasts, immune cells, extracellular matrix, and signaling molecules constitute the tumor melatonergic microenvironment [5-7].

The complex interplay between solid tumors and host melatonergic microenvironment has been studied in several cancers. For example, melatonin contributes to the crosstalk between cell-cell and cell-matrix adhesion by reducing the expression of $\alpha_v\beta_3$ integrin, which limits glioma cell migration into surrounding stroma [8]. Additionally, melatonin participates in the reduction of surrounding fibroblasts and endothelial cells by downregulating antiadipogenic cytokine expression in breast cancer [9]. However, the majority of these studies were performed on tumor cells or animal models; little has been done to observe the effects

of the melatonergic system in patients with malignant diseases. The resulting lack of data has hampered translational research on the anti-tumor properties of melatonin and further investigation of its therapeutic potentials. The wide impact and clinical relevance of the tumor melatonergic microenvironment make it critical to develop a more thorough understanding of this domain.

Recent years have witnessed the advent of next-generation sequencing (NGS) and large-scale genomics, which has enabled oncological research to move beyond single gene analysis to the integrated investigation of large-volume sequencing data [10]. For example, through The Cancer Genome Atlas (TCGA) project [11], genomic data of a wide spectrum of cancer types have become available, which greatly deepened our understanding of the genomic features of human cancer. While the tumor melatonin synthesis/metabolism system is thought to differ across varied cancer types, comprehensive genomic analysis of the tumor melatonergic microenvironment (e.g., gene expression of melatonin biosynthesis and metabolism, antitumor effects of melatonin, and their interactions with the immune system) has not been adequately explored.

Based on the above data, we conducted a pan-cancer genomic analysis of the tumor melatonergic microenvironment across a broad spectrum of solid tumors, using large-scale RNA-sequencing (RNA-seq) data of TCGA tumor samples. The melatonergic microenvironment is defined by the ratio of two-gene expression (biosynthesis gene expression [*ASMT*] divided by gene expression of the melatonin metabolic enzymes [*CYP1A1/ CYP1A2/ CYP1B1*]). This is a simple and accessible predictive model to characterise the ability of the tumor microenvironment to synthesize and metabolize melatonin, according to a previously reported study [12]. The aims of this study were to: (i) characterise the tumor melatonergic microenvironment across different cancer types; (ii) assess the prognostic values of melatonin synthesis/metabolism indexes across varied tumors; and (iii) evaluate the associations between melatonin synthesis/metabolism subgroups and immunogenic

features (e.g., mutational burden and neoantigen abundance), which have been identified as valid biomarkers for predicting response to immune checkpoint inhibitor treatment [13, 14].

Materials and Methods

Dataset and tumor types

The dataset used consisted of RNA-seq data from TCGA tumor samples (data accessed at cBioPortal for Cancer Genomics in June 2018, http://www.cbioportal.org/) [15, 16]. All samples were assayed by RNA-seq, as described by the TCGA Research Network [17]. Gene expression values were represented as RNA-Seq by Expectation Maximization (RSEM) data normalized within each sample to the upper quartile of total reads [18]. The degree of mutational burden and presence of neoantigen were adopted to assess the immunogenic features of the tumors [13, 14]. The mutational burden was calculated according to the method described by Ock et al. [19]; the mutational burden was measured by the total number of somatic mutations, including non-synonymous mutations, insertion-deletion mutations, and silent mutations, while germline mutations without somatic mutations were excluded. Neoantigen abundance was calculated according to a previous study by Rooney et al. [20]; if the mutation was predicted to produce a "binder" neopeptide with affinity <500 nM, and if the corresponding gene was expressed greater than 10 TPM (evaluated based on median expression in the given tumor type rather than the specific sample), the mutation was designated as putatively antigenic. In this study, data of mutational burden and neoantigen abundance were referenced from these two studies; detailed methods are described in published works [19, 20]. Colon and rectal adenocarcinoma and pancreatic adenocarcinoma were excluded from analyses as the neoantigen number was only available for three samples. Clinical and pathological information were obtained from the cBioPortal for Cancer Genomics.

Collectively, samples of 14 solid cancer types (N = 6,658) were investigated in the final analysis, including: bladder urothelial carcinoma (BLCA, n = 406), breast cancer (BRCA, n = 1,098), cervical cancer (CESC, n = 306), colon and rectal adenocarcinoma (COAD, n = 376), head and neck squamous cell carcinoma (HNSC, n = 520), kidney clear cell carcinoma (KIRC, n = 534), liver hepatocellular carcinoma (LIHC, n = 359), lung adenocarcinoma (LUAD, n = 508), lung squamous cell carcinoma (LUSC, n = 495), pancreatic adenocarcinoma (PAAD, n = 179), prostate adenocarcinoma (PRAD, n = 498), skin cutaneous melanoma (SKCM, n = 463), stomach adenocarcinoma (STAD, n = 407), and thyroid carcinoma (THCA, n = 509).

Predictive model of the melatonergic microenvironment

Gene expression of *ASMT, CYP1A1, CYP1A2*, and *CYP1B1* were measured using log 2-transformed values in RSEM. As per the study of Kinker et al. [12], the tumor melatonergic microenvironment was measured by the rates of melatonin synthesis and metabolism. To characterise the melatonergic microenvironment across different tumor types in more detail, we calculated the following three indexes according to different melatonin metabolism isoenzymes: Index-I (*ASMT:CYP1A1*) = $\log_2$ [*ASMT*]–$\log_2$ [*CYP1A1*]; Index-II (*ASMT:CYP1A2*) = $\log_2$ [*ASMT*]–$\log_2$ [*CYP1A2*]; Index-III (*ASMT:CYP1B1*) = $\log_2$ [*ASMT*]–$\log_2$ [*CYP1B1*]. The gene expressions of CYP1A1/CYP1A2/CYP1B1 were chosen here as they have been shown to play major roles in melatonin metabolism in humans according to published evidence [4]. To further explore the relationships between melatonergic microenvironment, patient prognosis, mutational burden, and neoantigen abundance, we classified the melatonergic system into high versus low subgroups by the median value for each cancer type.

Gene set enrichment analysis

To understand the differences in biological functions and pathways between

subgroups, gene set enrichment analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp, accessed at June, 2018) was performed on low- versus high-index subgroups [21]. We employed the Molecular Signatures Database (MSigDB) H (hallmark gene sets), C2 (curated gene sets), and C5 (GO gene sets) collection of chemical and genetic perturbations (n = 20,253 gene sets). Calculations were repeated 1,000 times for each analysis according to the default weighted enrichment statistical method. GSEA results were shown using normalized enrichment scores, accounting for the size and degree to which a gene set is overrepresented at the top or bottom of the ranked list of genes (nominal *P*-value < 0.05 and FDR ≤ 0.25).

Statistical analyses

Associations between melatonin synthesis/metabolism subgroups and categorical variables (e.g., sex, race, and disease stage) were analyzed using the χ^2-test (Fisher's exact test or Pearson's χ^2-test where appropriate), and the Mann-Whitney U test or Kruskal-Wallis test for continuous variables (e.g., age, number of mutations, and neoantigens). Correlations between gene expression were evaluated using the Spearman correlation test; the Spearman coefficient was considered to indicate poor correlation if < 0.2, moderate if < 0.4, relatively strong if < 0.6, strong if < 0.8, and very strong if > 0.8. The prognostic significance of the indexes were estimated using Kaplan–Meier survival curves, and compared by log-rank test. Cox proportional hazards model was used to calculate the adjusted hazard ratios (AHRs) and corresponding 95% confidence intervals (CIs), incorporating age, sex, race, and disease stage for adjustment. All statistical analyses were performed with SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) and R version 3.4.4 (http://www.r-project.org). Statistical significance was set at two-sided $P < 0.05$.

Results

Characteristics of the melatonergic microenvironment across 14 cancer types

A total of 6,658 tumor samples from 14 TCGA cancer types were included in the final analysis. The baseline characteristics are summarized in **sTable 1**. **Figure 1** showed the log_2-transformed values of *ASMT/CYP1A1/CYP1A2/CYP1B1* expression according to cancer types. The expression of *CYP1A1* and *CYP1A2* were significantly and positively correlated ($R^2_{overall}$ = 0.62; Pearson correlation), with moderate to strong correlations for each cancer type (**sFigure 1**), while the expression of *CYP1B1* and *CYP1A1/CYP1A2* did not show strong correlations (R^2 < 0.40 for all; Pearson correlation).

Given that the gene expression of metabolism isoenzymes were different in each cancer type, we comprehensively calculated three synthesis/metabolism indexes (Index-I [*ASMT:CYP1A1*], Index-II [*ASMT:CYP1A2*], and Index-III [*ASMT:CYP1B1*]; **Figure 1**). Overall, the median value of index-I, II, III was -0.36 (interquartile range [IQR], -1.99 to 0.50), 0.00 (IQR, -0.49 to 0.93), and -8.32 (IQR, -9.78 to -6.55), respectively. Each cancer type demonstrated heterogeneous distributions of the three indexes. We then divided the samples into two subgroups (high *vs.* low) by the median values of each cancer type. The demographic and clinical features of the TCGA patients were summarized according to synthesis/metabolism subgroups in **sTable 2**. Consistent with previous observations [12, 22, 23], elderly patients tended to have lower melatonin synthesis/metabolism index, with significant differences observed in BRCA, LIHC, LUAD, LUSC, PRAD, and SKCM; the high-index subgroup also tended to have a lower proportion of patients with late-stage disease, with significant differences observed in CESC, COAD, HNCS, KIRC, SKCM, and THCA.

Favourable prognoses were correlated with the high melatonin synthesis/metabolism index subgroup

We investigated the prognostic association of the tumor melatonergic microenvironment with clinical outcomes. As the distributions of the three indexes differed significantly among 14 cancer types, we then evaluated whether they played different prognostic roles across varied cancers. **Figure 2** summarized the results of Kaplan–Meier survival analyses. On the whole, almost all patients in the high synthesis/metabolism index subgroup had relatively favourable clinical outcomes, compared to patients in the low-index subgroup; while for each cancer type, the three indexes harboured different prognostic values. Specifically, for index-I, the high-index subgroup showed significantly improved survival among patients with CESC (P = 0.01) and PAAD (P = 0.04), while marginal significance among patients with SKCM (P = 0.09) and STAD (P = 0.08) was also observed. In terms of index-II, only patients with BRCA and STAD showed marginally significantly ($P \leq 0.10$) improved survival in the high-index subgroups. Regarding index-III, BLCA (P = 0.02), COAD (P = 0.03), KIRC (P = 0.03), and STAD (P = 0.03) showed favourable prognosis among patients with a high synthesis/metabolism index.

Next, we performed multivariable analysis to explore whether melatonin synthesis/metabolism index was an independent prognostic factor for survival outcomes, incorporating clinically relevant covariates for adjustment. The results of multivariable modeling largely supported the findings seen in the univariate analysis. Interestingly, the three indexes all remained significant prognostic factors for STAD ($AHR_{index\text{-}I}$ = 0.71; 95% CI = 0.52–0.97, P = 0.03; $AHR_{index\text{-}II}$ = 0.69; 95% CI = 0.49–0.97, P = 0.03; $AHR_{index\text{-}III}$ = 0.68; 95% CI = 0.41–0.94; P = 0.02; **Figure 3**). Index-I was an independent and favourable prognosticator for patients with CESC (AHR = 0.62; 95% CI = 0.39–0.98; P = 0.04) and SKCM (AHR = 0.74; 95% CI = 0.55–0.98; P = 0.04). The high index-II subgroup had significantly better survival among patients with BRCA (AHR = 0.65; 95% CI = 0.44–0.97; P = 0.03), and Index-III remained significant for LUSC (AHR = 0.75; 95% CI = 0.56–0.99; P = 0.04). PRAD and THCA were excluded from multivariable analyses due to the

limited number of events. Cox proportional hazards analyses of other clinical-relevant covariates for adjustment were shown in **sTable 3**.

High mutational burden was associated with the low melatonin synthesis/metabolism index subgroup

Previous studies have reported a physiological link between the pineal gland and immune system [24, 25]. Additionally, emerging evidence has shown that the degree of mutational burden and presence of neoantigen reflects tumor immunogenic features, and can predict favourable responses to immune blockade therapy (e.g., anti-PD-1/PD-L1 treatment) [13, 14]. We therefore further compared melatonin synthesis/metabolism subgroups according to the mutational burden and neoantigen abundance. We observed a tendency toward a negative correlation between the total number of somatic mutations (**Figure 4**), as well as the number of neoantigens (**Figure 5**) with the synthesis/metabolism index. Based on cancer types, the low-index subgroup had both a significantly higher number of mutations and neoantigens among patients with HNSC (index-I), KIRC (index-I), PRAD (index-I & II), and STAD (index-III), compared to patients with a high synthesis/metabolism index ($P < 0.05$ for all). Additionally, patients with BRCA (index-II), COAD (index-I), KIRC (index-I), LIHC (index-I), LUSC (index-III), PAAD (index-III) and SKCM (index-I) had a significantly higher number of somatic mutations in the low-index subgroup ($P < 0.05$ for all, **Figure 4 B, D, F, G, I**). BLCA (index-II) and KIRC (index-III) tumors with low melatonin synthesis/metabolism index harboured a higher number of neoantigens ($P < 0.05$ for all, **Figure 5 A, E**). No significant differences were observed regarding the numbers of mutations or neoantigens for CESC, or THCA.

Gene set enrichment analyses of melatonin synthesis/metabolism subgroups

We then performed GSEA to better understand how the melatonergic microenvironment functioned through potential biological pathways. We chose BRCA

(index-II), LUSC (index-III), SKCM (index-I), and STAD (index-III) as study models, as the melatonergic microenvironment influenced both prognosis and immunogenic features in these four tumors. **sFigure 2** and **sTable 4-7** illustrated the gene sets enriched in high and low melatonin synthesis/metabolism subgroups. Generally, gene sets related to hypoxia, inflammation, proliferation, metastasis, and DNA damage were enriched in the low-index subgroup, indicating that melatonin may play an anti-tumor role in cancer development and progression.

Summary of the clinical implications of melatonergic microenvironment classification

Figure 6 summarized the biological and clinical relevance of tumor melatonergic microenvironment classification. Taken together, our data demonstrated that among patients with BRCA, LUSC, SKCM, and STAD, decreased melatonin synthesis/metabolism indexes, which characterised the rates of circulating melatonin synthesis and metabolism in tumor microenvironment, was associated with reduced survival, as well as higher levels of somatic mutations and neoantigens, which indicated favourable responses to immunotherapy. For CESC tumors, melatonergic microenvironment is only correlated with prognosis; while in HNSC, KIRC, and PRAD, patients with decreased melatonin are more likely to benefit from immune checkpoint inhibitor treatment.

Discussion

Here we present several key aspects of the tumor melatonergic microenvironment based on *ASMT* and *CYP1A1/1A2/1B1* mRNA expression, determined from RNA-seq data across large-scale TCGA solid tumor samples. Initially, we applied a two-gene expression signature (melatonin synthesis/metabolism index) to characterise the melatonergic system across 14 solid tumors. Having noted that gene expression of

melatonin metabolism isoenzymes differed in each cancer type, we further used three indexes (Index-I [*ASMT:CYP1A1*], Index-II [*ASMT:CYP1A2*], and Index-III [ASMT:CYP1B1]) to comprehensively analyze the melatonergic system and compare their roles. We found that the ability of the tumor microenvironment to synthesize and accumulate melatonin was heterogeneous and negatively correlates with tumor burden, while this ability deceased with age. Secondly, we divided the samples into two subgroups (high *vs.* low) based on the median of index values, and investigated their roles in predicting survival outcomes. We show that the three indexes played different roles across varied cancers, and harbored prognostic value in BRCA, CESC, LUSC, SKCM, and STAD. Next, we determined the clinical relevance of the three indexes with tumor immunogenic features and potential predictive values in selecting patients that may be more responsive to immunotherapies, in light of its negative correlations with mutational burden (number of somatic mutations and/or neoantigens) in BLCA, BRCA, COAD, HNSC, KIRC, LIHC, LUSC, PRCA, SKCM, and STAD. To our knowledge, this is the first comprehensive genomic investigation of the tumor melatonergic microenvironment across a large spectrum of solid tumors, which provides a general overview of the oncostatic value of the melatonergic system.

In this study, a simple and readily-adapted gene expression signature was applied to characterise the tumor melatonergic microenvironment. Additionally, we used three indexes based on the melatonin metabolism isoenzymes, which provided a comprehensive and applicable model for clinical utilization. We further identified the differential values of the three indexes across varied cancer types, suggesting that the melatonergic synthesis and metabolism system may work through different isoenzymes and pathways in each tumor. Further experimental studies are needed to reveal the underlying mechanisms. The classification of the tumor melatonergic microenvironment according to this model may be a useful tool for risk stratification, and will hopefully aid in the design of future experimental and clinical studies. Specifically, we found that higher content of melatonin in the tumor

microenvironment was associated with less aggressive stage classification and favourable prognosis, which suggested that the melatonergic microenvironment may influence tumor carcinogenesis, prevent the formation of aggressive phenotypes, and therefore result in a decreased risk of death. This is consistent with previous observations that melatonin reduces the susceptibility of gastric mucosal cells to dietary carcinogens through enhanced DNA repair capacity [26], and inhibits cancer cell proliferation by decreasing DNA synthesis [27] or promoting cell differentiation [28]. Additionally, we demonstrated that the melatonin synthesis/metabolism index remained an independent prognosticator after including disease stage for adjustment in multivariable models, which implied that melatonin may influence prognosis through other biological mechanisms in addition to tumor carcinogenesis and proliferation. For instance, it was noted that melatonin impeded the epithelial-mesenchymal transition (EMT) process and cancer cell dissemination through interference with NF-κB signaling [29]. Melatonin also promotes cancer cell apoptosis by inducing cell cycle arrest [30]. Another phenomenon worth noting is that *CYP1B1* overexpression itself could enhance proliferation, migration, and invasion of tumor cells in prostate cancer and kidney cancer [31, 32]. Therefore, inferior survival in the low index-III subgroup might also be attributable to overexpression of *CYP1B1* in some cancer types; further experimental studies are needed to reveal the underlying mechanism in different cancer types in this note. The valid associations between melatonin and risk of death have facilitated randomized controlled clinical trials (RCTs) investigating the therapeutic roles of melatonin in improving survival outcomes and tumor responses. Lissoni et al. indicated that melatonin significantly improved tumor regression rate and 5-year overall survival in non-small cell lung cancer patients concomitantly treated with melatonin and chemotherapy [33]. A meta-analysis systematically analyzed eight published RCTs, and showed that melatonin dramatically improved tumor remission and 1-year survival rate, as well as decreased the incidence of treatment-related toxicity [34]. While the above results are

inspiring, published RCTs only refer to a limited spectrum of tumors (glioma [35], COAD [36], and LUSC [33, 37]). Based on our findings, we propose the conduction of more collaborative international, multi-center, large-scale RCTs in a variety of cancer types, especially in patients with BRCA, CESC, LUSC, SKCM, and STAD.

In addition to its prognostic implication, the tumor melatonergic environment also distinguished patients with distinct immunogenic features. Our findings indicated that patients with a low melatonin synthesis/metabolism index tended to harbour a higher number of somatic mutations and/or neoantigens, and would be potentially more likely to benefit from immunotherapies. Somatic genome instability is a hallmark of cancer genomes, and a highly complex mutation landscape has been reported to originate from distinct DNA damage and repair processes [38]. Previous research has also demonstrated that somatic mutations can subsequently generate neoantigens [13, 14], which in turn could be recognized by the immune system, triggering an anticancer immune response and therefore likely to be associated with favourable responses to immunotherapy [39]. In this vein, melatonin in the tumor microenvironment could significantly limit the extent of DNA damage and enhance DNA repair processes [40], which potentially explained the lower mutational burden and neoantigen abundance in the high-index subgroup. A considerable number of published studies have explored the immune-neuroendocrine role of melatonin [41, 42], while less work has been done to evaluate its predictive value for the immune response in solid tumors. The analysis of associations between the melatonergic microenvironment and tumor immunogenic features represents a novel aspect of melatonin research, and highlights the importance of future investigations on the immunotherapeutic role of melatonin across diverse tumor types.

Among the solid tumors investigated in this study, several cancer types (e.g., LUAD, PAAD, and THCA) were found to neither correlate with clinical prognosis nor the number of mutations and neoantigens. The failure to detect associations in TCGA data may be due to lack of adequate follow-up time, limited event rates, and

biased population distribution, among other considerations. Additionally, it also suggests that our two-gene predictive model alone is not sufficient to stratify those groups of individuals; integration of other melatonergic molecular biomarkers (e.g., expression of melatonin receptors [43]) are needed in future investigations. Nevertheless, although melatonin synthesis/metabolism index alone could not substitute for traditional parameters to predict survival outcome, it may facilitate the establishment of optimal prediction models when incorporated with other clinicopathological factors. Importantly, this simple model can be readily adapted to PCR-based analysis of formalin-fixed paraffin-embedded (FFPE) clinical specimens, which is extremely efficient and cost-effective.

The main limitation of this study is that the ability of these indexes to predict the response to immunotherapy requires further validation in cancer patients treated with immune checkpoint inhibitors. Future studies are needed to address this limitation. Nevertheless, our findings are important and provide new insights into the melatonergic microenvironment of solid tumors. Secondly, we only adopted the gene expression of three enzymes involved in melatonin metabolism (*CYP1A1, CYP1A2,* and *CYP1B1*) to establish the two-gene indexes. Future studies that comprehensively investigate and compare different metabolic enzymes and their combinations are warranted. Thirdly, the evidence that the two-gene index could represent circulating melatonin in the tumor microenvironment was derived from previous research in glioma [12]. However, given the distinct contexts of different tumors, future experiments are needed to verify the associations between circulating melatonin and the two-gene indexes across different cancer types.

Conclusion

In conclusion, we comprehensively characterised the melatonergic microenvironment across 14 solid tumors using RNA-seq data from TCGA. Our findings revealed that the capacity of the tumor microenvironment to synthesize and accumulate melatonin

can distinguish patients with different risks. Additionally, it correlated with tumor immunogenic features (mutational burden and neoantigen abundance) and served as a potential predictive marker in selecting patients responsive to immunotherapies. Our study provides a systematic overview of the oncostatic values of the melatonergic system, and highlights the utilization of a simple and promising two-gene signature for clinical practice. Going forward, it lays groundwork for the design of future experimental and clinical studies.

Author contributions

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Conflict of interest

The authors have no actual or potential conflicts of interest to declare.

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Figure Legends

Figure 1. Distributions of gene expression of *ASMT, CYP1A1, CYP1A2, CYP1B1*, and melatonin synthesis/metabolism index-I/II/III across 14 TCGA solid tumors. (A) Boxplot distributions of log_2-transformed values of *ASMT, CYP1A1, CYP1A2,* and *CYP1B1* according to TCGA cancer types. (B) Boxplot distributions of log_2-transformed values of melatonin synthesis/metabolism index (Index-I [*ASMT:CYP1A1*], Index-II [*ASMT:CYP1A2*], Index-III [*ASMT:CYP1B1*]), according to TCGA cancer types. The dashed lines indicate the median values of all tumor samples.

Figure 2. Kaplan-Meier plots of overall survival for patients in different melatonin synthesis/metabolism subgroups across 14 TCGA solid tumors. (A) BLCA, (B) BRCA, (C) CESC, (D) COAD, (E) HNSC, (F) KIRC, (G) LIHC, (H) LUAD, (I) LUSC, (J) PRAD, (K) PRAD, (L) SKCM, (M) STAD, (N) THCA. $P < 0.05$ represents significant differences in survival outcomes.

Figure 3. Cox proportional hazards analyses of melatonin synthesis/metabolism (A) index-I, (B) index-II, and (C) index-III across 14 TCGA solid tumors.

Note: age (continuous variable), sex (Male vs. Female), race (White vs. Asian vs. Black vs. American Indian vs. Others), disease stage (stage III-IV vs. stage I-II) were

included in the multivariable model for adjustment. PRAD and THCA were excluded from the analyses due to the limited number of events.

Figure 4. Mutational burdens by different melatonin synthesis/metabolism subgroups across TCGA solid tumors. Boxplot distributions of $\log_2$-transformed values of the number of somatic mutations between subgroups of melatonin synthesis/metabolism index I-III, according to TCGA cancer types (A–N). The number of mutations differed significantly in index-I subgroups for COAD, HNSC, KIRC, LIHC, PRAD, and SKCM; in index-II subgroups for BRCA and PRAD; and in index-III subgroups for LUSC, PAAD, and STAD. *P* values are calculated by Wilcoxon rank-sum test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 5. Neoantigen abundance by different melatonin synthesis/metabolism subgroups across TCGA solid tumors. Boxplot distributions of $\log_2$-transformed values of the number of neoantigens between subgroups of melatonin synthesis/metabolism index I-III, according to TCGA cancer types (A–I). The number of neoantigens differed significantly in index-I subgroups for HNSC, KIRC, and PRAD; in index-II subgroups for BLCA and PRAD; and in index-III subgroups for KIRC and STAD. *P* values are calculated by Wilcoxon rank-sum test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). COAD and PAAD are excluded from the analysis due to the limited number of patients with neoantigen data.

Figure 6. Biological and clinical relevance of the tumor melatonergic microenvironment classification based on a two-gene mRNA expression model. The tumor melatonergic microenvironment is categorized into high versus low subgroups based on median values of melatonin synthesis/metabolism index. The high-index subgroup is characterized by higher content of circulating melatonin, less aggressive tumor biological behaviors (e.g., cell proliferation, metastasis), favourable

prognosis, etc., while the low-index subgroup harbours a higher number of somatic mutations and neoantigens, and would potentially be more likely to respond to immune checkpoint inhibitor treatment. The proposed classification is simple and applicable, and would help tailor optimal therapeutic strategies for solid tumors.

Supplementary Figure Legends

sFigure 1. Correlations between gene expression of three melatonin metabolism enzymes (*CYP1A1, CYP1A2,* and *CYP1B1*). The gene expression of *CYP1A1* is significantly correlated with that of *CYP1A2* across all TCGA cancer types. R^2 and *P* values are calculated by the Spearman correlation test. Red font for the Spearman coefficient indicates strong or very strong correlation, yellow font indicates relatively strong correlation, orange font indicates moderate correlation, and black font indicates poor correlation.

sFigure 2. Gene Set Enrichment Analysis (GSEA) of melatonergic microenvironment classification. GSEA between the high-index subgroup and low-index subgroup in (A) BRCA ($N_{low\text{-}index}$ = 551, $N_{high\text{-}index}$ = 547), (B) LUSC ($N_{low\text{-}index}$ = 251, $N_{high\text{-}index}$ = 244), (C) SKCM ($N_{low\text{-}index}$ = 232, $N_{high\text{-}index}$ = 231), and (D) STAD ($N_{low\text{-}index}$ = 260, $N_{high\text{-}index}$ = 203).

Supplementary Tables

sTable 1. Baseline characteristics of TCGA samples across 14 cancer types

sTable 2. Associations between the melatonin synthesis/metabolism index and clinicopathological features across 14 TCGA solid tumors

sTable 3. Cox proportional hazards analyses of clinically relevant covariates across 14 solid tumors for overall survival

sTable 4-1. GSEA results showing pathways enriched in low ASMT/CYP1B1 index-II group of breast cancer

sTable 4-2. GSEA results showing pathways enriched in high ASMT/CYP1B1 index-II group of breast cancer

(nominal *P*-value < 0.05 and FDR < 0.05 were set here for selection)

sTable 5-1. GSEA results showing pathways enriched in low ASMT/CYP1B1 index-III group of squamous cell lung cancer

sTable 5-2. GSEA results showing pathways enriched in high ASMT/CYP1B1 index-III group of squamous cell lung cancer

(nominal *P*-value < 0.05 and FDR < 0.05 were set here for selection)

sTable 6-1. GSEA results showing pathways enriched in low ASMT/CYP1B1 index-I group of melanoma

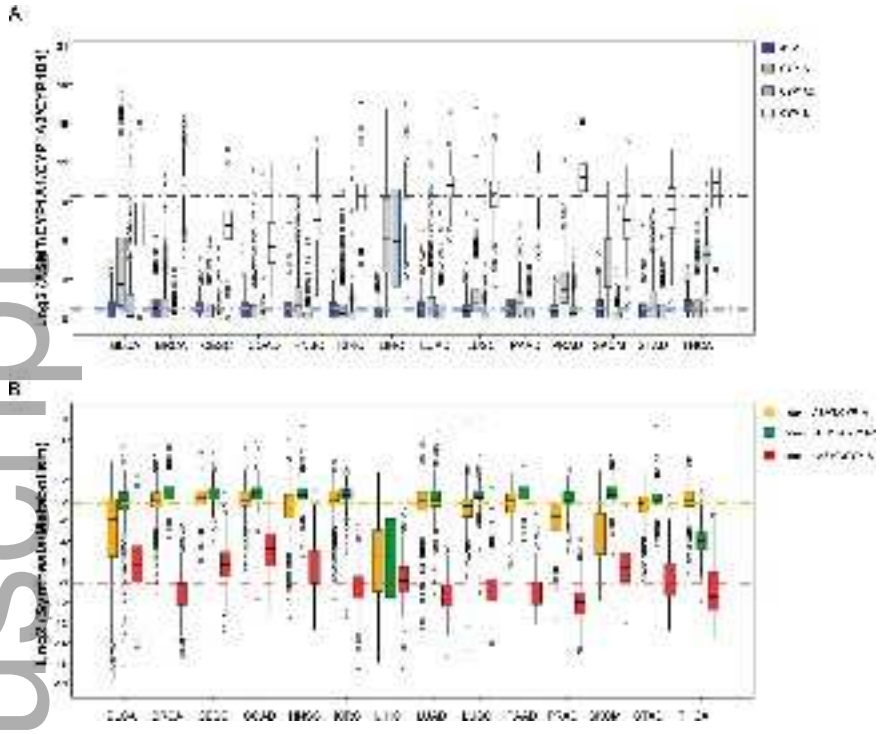
sTable 6-2. GSEA results showing pathways enriched in high ASMT/CYP1B1 index-I group of melanoma

(nominal *P*-value < 0.05 and FDR < 0.05 were set here for selection)

sTable 7-1. GSEA results showing pathways enriched in low ASMT/CYP1B1 index-III group of gastric cancer

sTable 7-2. GSEA results showing pathways enriched in high ASMT/CYP1B1 index-III group of gastric cancer

(nominal *P*-value < 0.05 and FDR < 0.05 were set here for selection)



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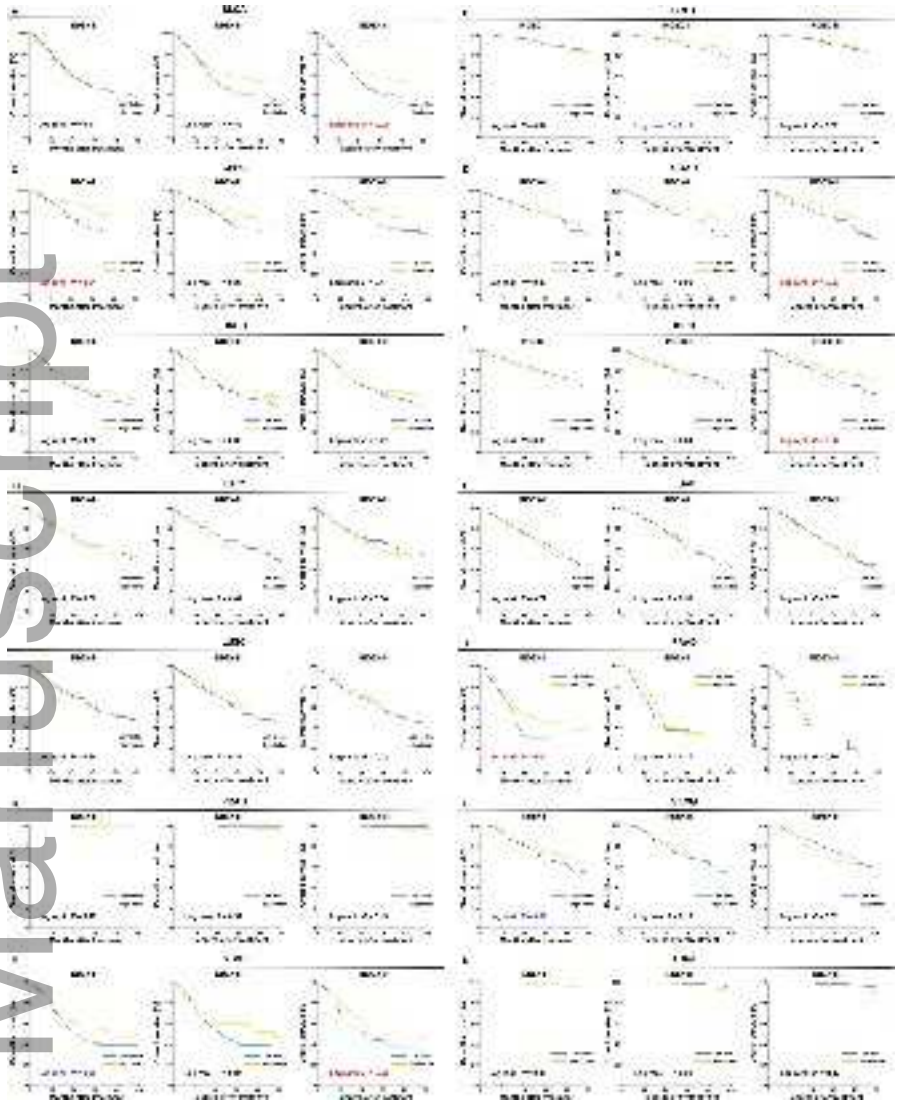

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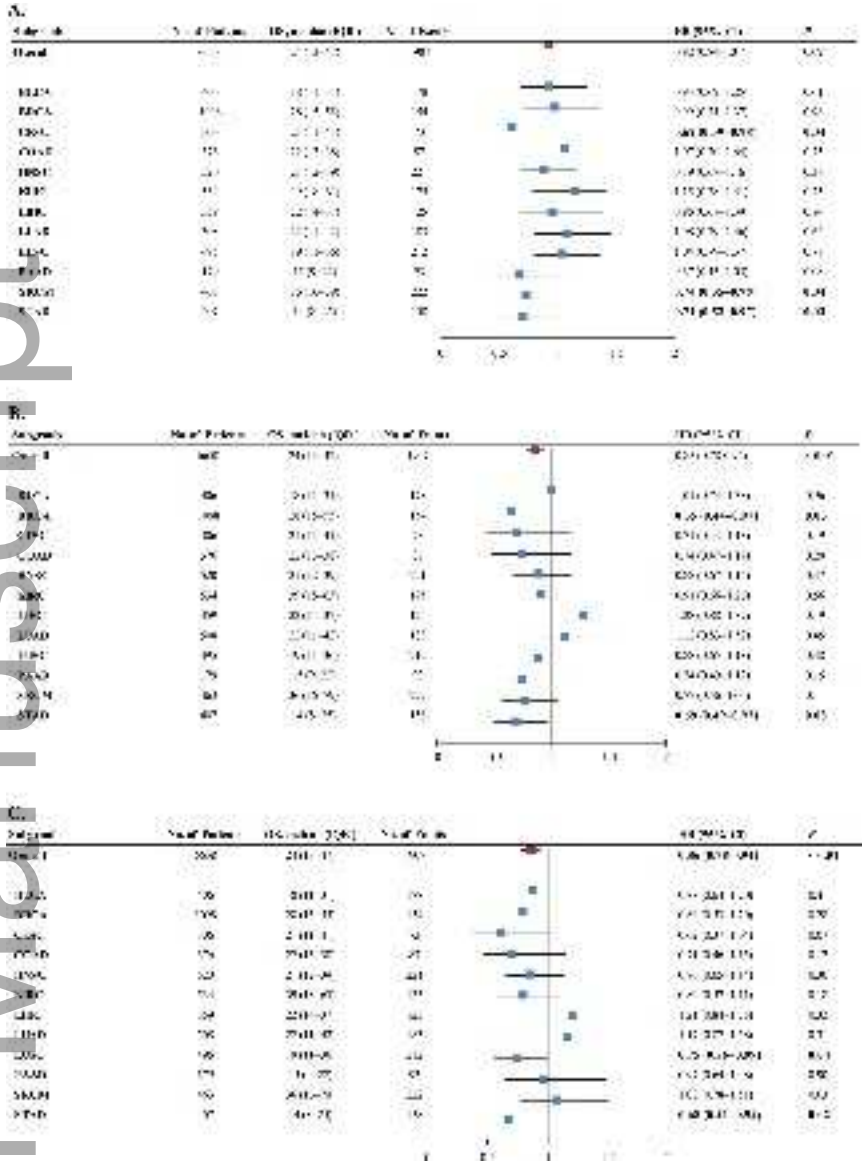

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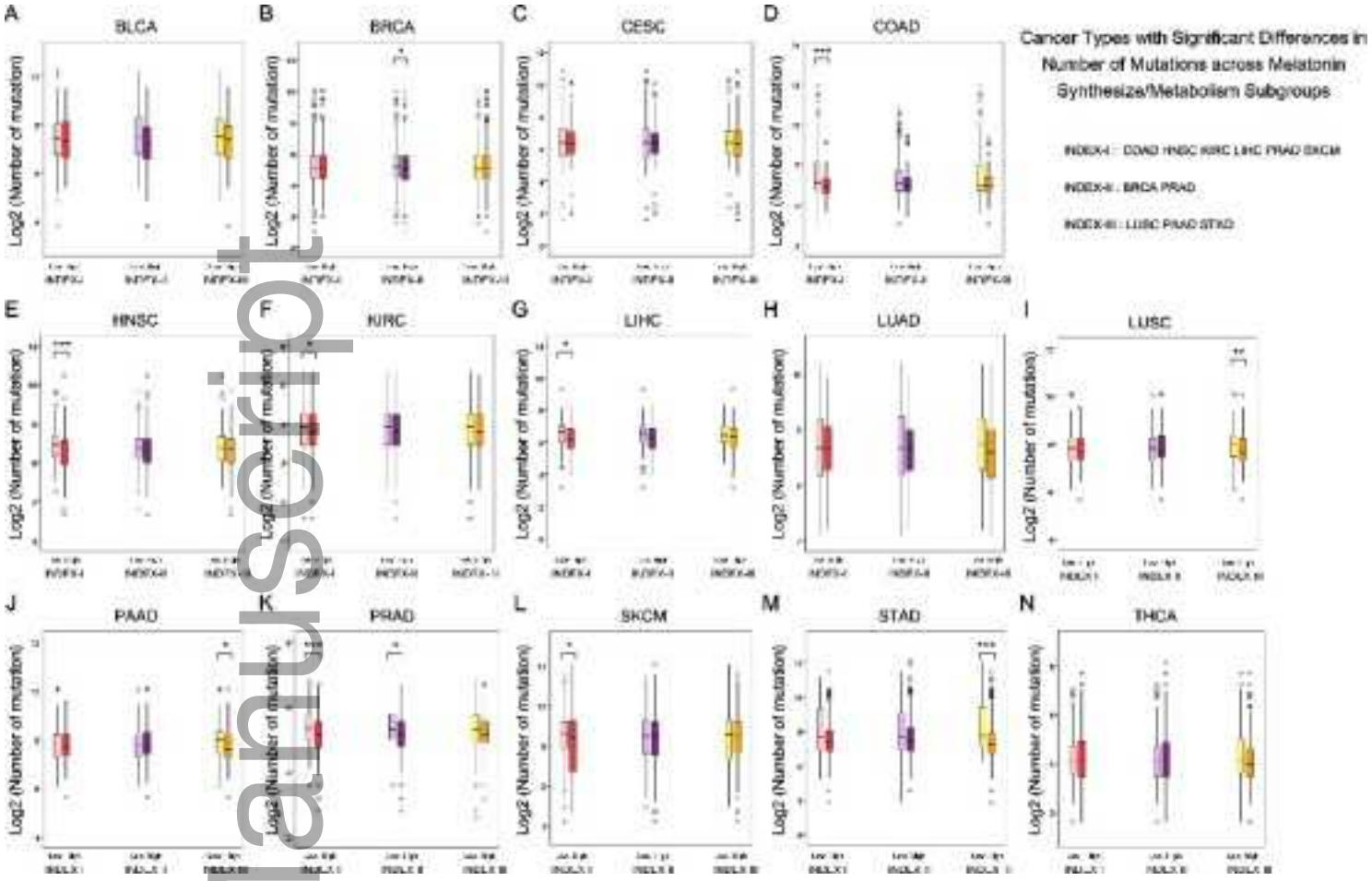

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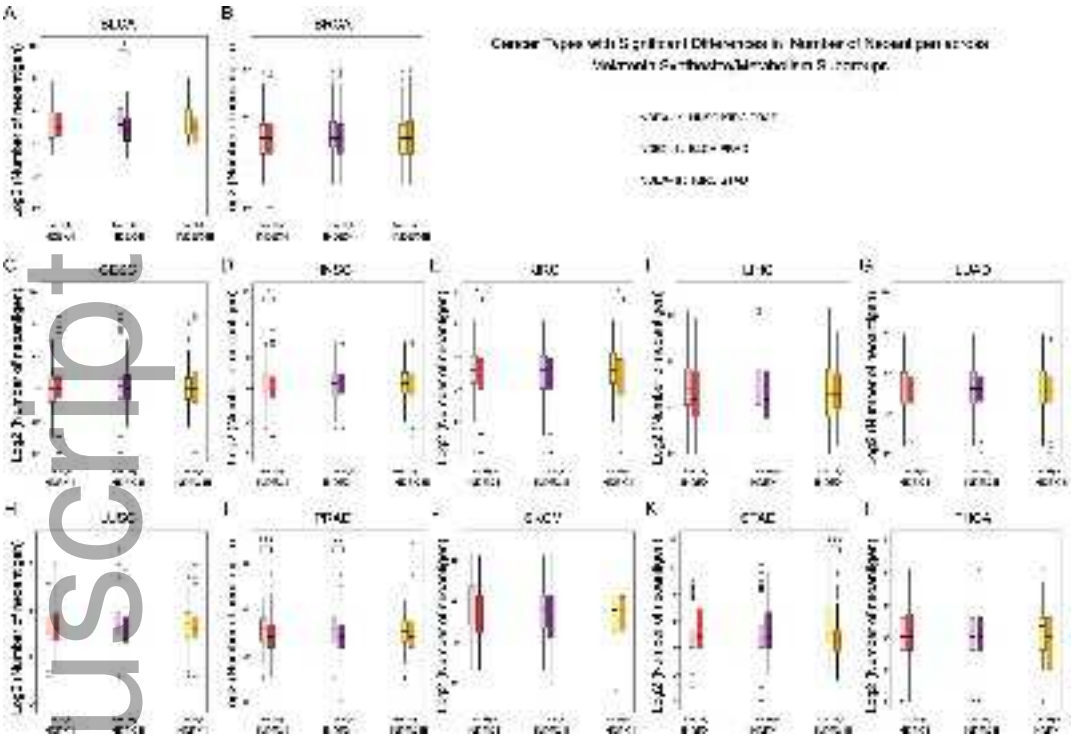

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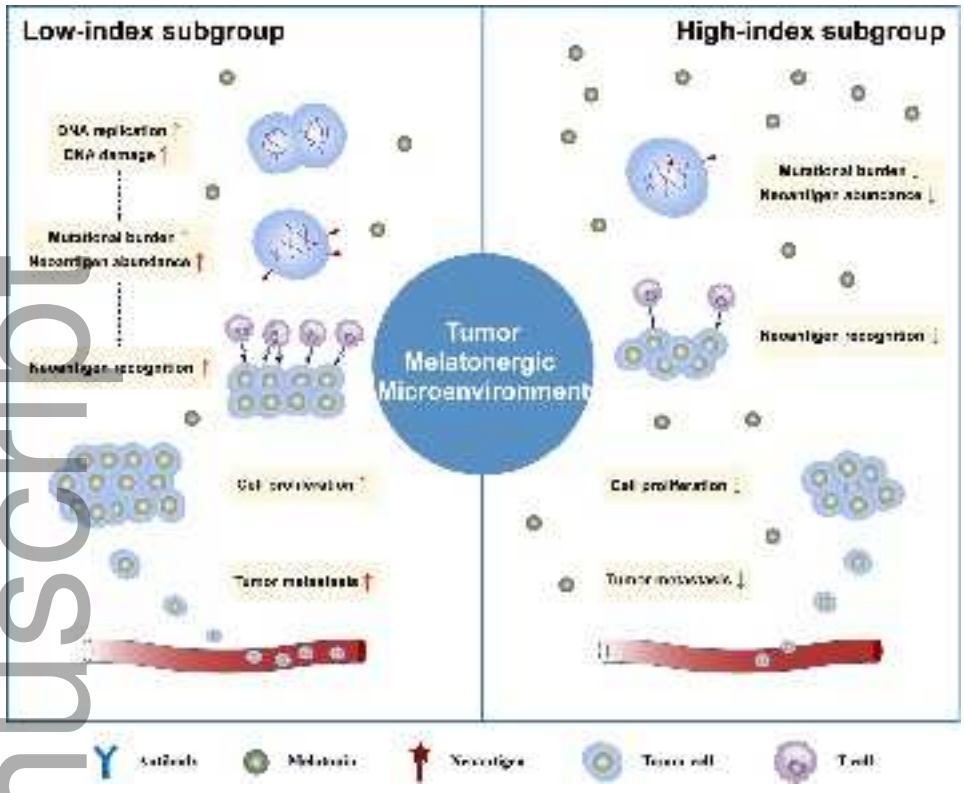

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