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REVIEW

Major roles of the circadian clock in cancer

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ABSTRACT Circadian rhythms are natural rhythms that widely exist in all creatures, and regulate the processes and physiological functions of various biochemical reactions. The circadian clock is critical for cancer occurrence and progression. Its function is regulated by metabolic activities, and the expression and transcription of various genes. This review summarizes the composition of the circadian clock; the biological basis for its function; its relationship with, and mechanisms in, cancer; its various functions in different cancers; the effects of anti-tumor treatment; and potential therapeutic targets. Research in this area is expected to advance understanding of circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) in tumor diseases, and contribute to the development of new anti-tumor treatment strategies.

KEYWORDS Circadian clock; BMAL1; cancer; tumor therapy; oncology

Introduction

The circadian clock regulates various physiological processes and biochemical reactions in the human body. This system exhibits diurnal variation characteristics with time, which are denoted circadian rhythms[1]. Circadian rhythms are relatively conserved in evolution. They are widespread in various organisms and have evolved 4 times with selective advantages[2]. Because of its rhythm, the system is also referred to as the circadian clock system. This system comprises the central and peripheral clocks located in the hypothalamus's anterior suprachiasmatic nucleus (SCN). The central and peripheral clocks have a synchronous circadian rhythm[3,4]. The central clock's function can be performed independently, whereas the peripheral clock is coordinated through various signaling molecules to achieve synchronization.

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Transcription-translation feedback loop (TTFL) is the molecular basis of circadian rhythms in organisms. TTFL regulates the central molecular circadian clock mechanism[1,5-7]. TTFL often plays a role in the SCN and peripheral tissues of the anterior hypothalamus of mammals. Optical signals are the main factors affecting the circadian clock. The optical signal stimulation received by optic nerve fibers generates downstream nerve or endocrine signal stimulation through the SCN, thereby synchronizing with peripheral organs[6-11].

The positive stimulus factor TTFL comprises circadian locomotor output cycles kaput (CLOCK), aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL), and its paralog NPAS2. ARNTL is also called brain and muscle ARNT-like protein 1 (BMAL1). TTFL binds the target sequence E-box (CTGCAG), thereby promoting the expression of transcription inhibitor cryptochrome (CRY1/2) and period (PER1/2/3), whereas CRY and PER serve as negative stimuli for TTFL[12-14]. Two different stimuli can form 2 complexes with opposite functions: the CLOCK-BMAL1 transcription activator and CRY-PER transcription inhibitor. Their growth and decline exhibits a clear circadian rhythm. As illustrated in **Figure 1**, the CRY-PER complex enters the nucleus and inhibits the function of the CLOCK-BMAL1 complex.

The above feedback loop is also consolidated by a secondary feedback loop. In the secondary feedback loop, the

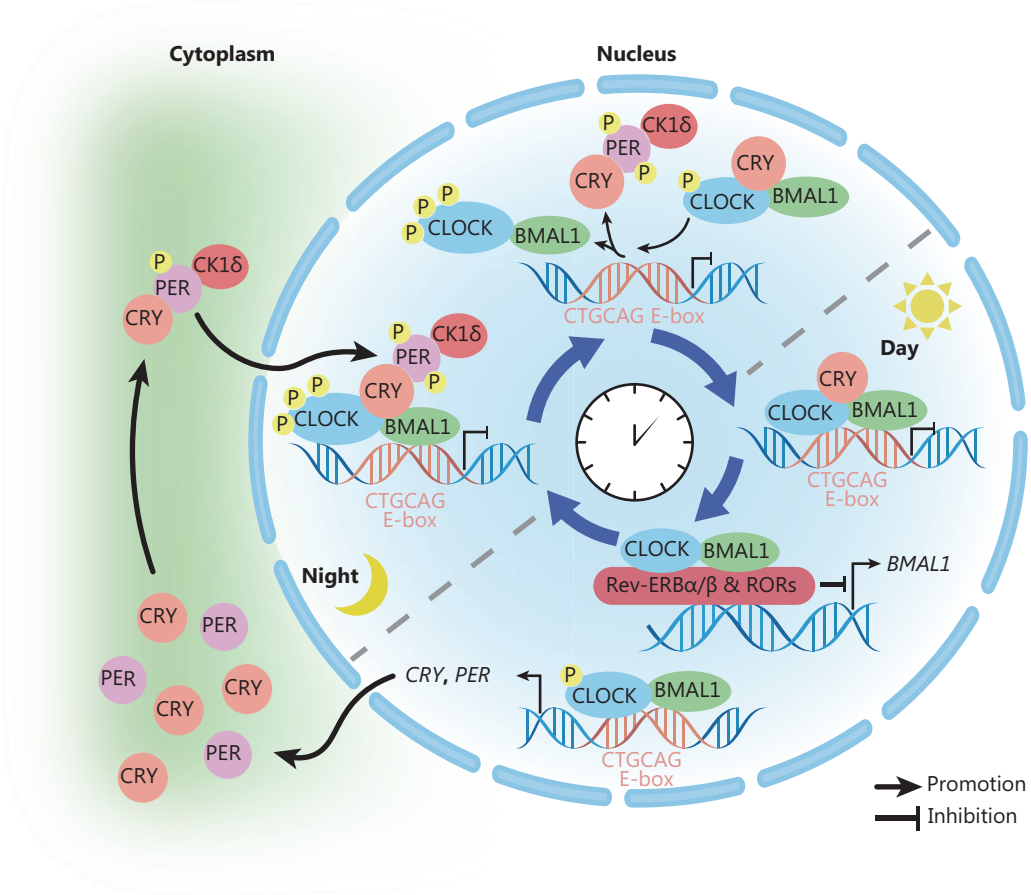

Figure 1 Molecular mechanisms of the circadian clock. The positive stimulus factor TTFL is composed of CLOCK, BMAL1, and its paralog NPAS2. This complex binds the target E-box (CTGCAG) sequence, thereby promoting the expression of transcription inhibitor CRY1/2 and PER1/2/3. CRY and PER serve as negative stimuli for TTFL. Two different stimuli form 2 complexes with opposite functions: the CLOCK-BMAL1 transcription activator and CRY-PER transcription inhibitor. Their growth and decline show a clear circadian rhythm. The CRY-PER complex enters the nucleus and inhibits the function of CLOCK-BMAL1 complex. the CLOCK-BMAL1 complex acts on the nuclear receptor Rev-ERBα/β (NR1D1/2) and RORs, and regulates their expression levels, thereby affecting BMAL1 expression and constituting the second feedback loop. Additionally, this loop is controlled by the kinase CK1δ/ε and ubiquitin ligases. TTFL, transcription-translation feedback loop; CLOCK, circadian locomotor output cycles kaput; BMAL1, brain and muscle ARNT-like protein 1; CRY, cryptochrome; PER, period; RORs, retinoic acid receptor-related orphan receptors. (In this picture, the dashed line indicates the boundary between the day and night phases, and the meaning of the remaining arrows is shown in the figure.)

CLOCK-BMAL1 complex acts on the nuclear receptor Rev-ERBα/β(NR1D1/2) and retinoic acid receptor-related orphan receptors (RORs), and regulates their expression levels, thereby affecting BMAL1 expression[1,5]. Additionally, this loop is controlled by the kinase CK1δ/ε and ubiquitin ligases[15-20]. However, this basic understanding of the molecular mechanism underlying circadian rhythm in mammals is insufficient for routine medical care. Additional information is required regarding the mechanisms.

At the beginning of the circadian clock, owing to the presence of the negative regulatory factor CRY[12-14], the CLOCK-BMAL1 complex cannot activate transcription even if it binds the E-box sequence. However, after a light period (midday of the circadian clock), CRY separates from the CLOCK-BMAL1 complex. Subsequently, because of the loss of CRY inhibition, transcription at the E-box is initiated, thereby promoting the expression of CRY and PER. Although PER continually accumulates, it cannot inhibit E-box transcription in the absence of CRY mediation. In addition, the CLOCK-BMAL1 complex acts on the nuclear receptor Rev-ERBα/β (NR1D1/2) and RORs and regulates their expression levels, thereby affecting the expression of BMAL-1[1,5]. At night, CRY and PER enter the nucleus in

the form of CRY-PER-CK1δ, and PER mediates the phosphorylation of CK1δ on CLOCK. The new CLOCK-BMAL1 complex with CRY replaces the complex that bound the E-box in the previous phase. This inhibition differs from that mediated by CRY. Finally, the CLOCK-BMAL1 complex combined with CRY binds the E-box before a new cycle begins[21].

Several studies have revealed a close relationship between circadian rhythm disorders and cancer. A meta-analysis of various human cancer transcriptome datasets has revealed the dysregulated expression of circadian genes in different tumor types[22]. First, a mutual regulatory mechanism exists between circadian and cancer genes. The genes regulated by the circadian clock include various oncogenes and tumor suppressor genes, and the core genes of the circadian clock are also regulated by oncogenes and tumor suppressor genes, which are involved in tumor onset and malignancy[23,24]. Second, the circadian clock regulates gene rhythms associated with metabolic and endocrine functions, and metabolic activity and endocrine homeostasis play important roles in tumor development[25-28]. In addition, abnormal biological rhythms promote the malignant progression of tumors by weakening the body's immunity. Immunity is an important factor restricting tumor development. Disrupting biological rhythms not only affects the innate and acquired immunity of the body, but also promotes tumor immune escape through immune checkpoints[29-32]. In summary, the circadian clock may play an important role in the entire process of tumorigenesis, and has the potential to be applied in tumor prevention, diagnosis, and treatment.

Methods

In the analysis accompanying this review, RNA-seq data (level 3) and corresponding clinical information on pan-cancer were obtained from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.com). For the predictive analysis of single genes in multiple tumors, univariate Cox regression analysis and a forest plot constructed with the "forest plot" R package revealed the *P*-values, HR, and 95% CI. Tumor mutation burden (TMB) was derived from The Immune Landscape of Cancer, published by Vesteinn Thorsson in 2018[33]. Immune-associated assessment was performed with xCell analysis. All bioinformatics analyses were performed in R software v4.0.3 for statistical analysis. If no additional explanation is provided, the rank-sum test was performed to detect the differences between the 2 groups of data, and $P < 0.05$ was considered statistically significant.

Circadian clock and cancer

Human genes are highly similar to mouse genes. Previous studies have found that nearly half the protein-coding gene expression in mice is associated with the circadian rhythm. The circadian clock controls the rhythmic expression of some genes in organisms under TTFL regulation, and these genes further regulate the expression of other genes. A total of 50%–80% of protein-coding genes in mice and humans have been found to show rhythmic oscillations[5]. The circadian clock is widely expressed in human genes and is influenced by environmental factors (light and food). When the circadian clock rhythm is perturbed by various factors, the normal biological characteristics of human organs or cells are altered. These changes may be correlated with the pathogenesis of various diseases, including some psychological diseases, endocrine diseases, and even cancer[7,10,34-39]. The physiological activities, cell metabolism, proliferation, and differentiation in the human body all show rhythmicity. Cancer cells, which abnormally proliferate in the human body, may be more susceptible to circadian clock disorders than normal cells because of their unstable characteristics.

Are circadian clock disorders associated with cancers? At present, the controversy regarding this issue has related primarily to several earlier epidemiological studies and recent studies on the clear correlation between clock disorders and cancers. Previous epidemiological investigations have revealed a lack of correlation between circadian clock disorders and the development of specific cancers. However, according to the International Agency for Research on Cancer (IARC) and other recent research, when the human circadian clock is disrupted, the likelihood of developing cancer, including lung cancer, intestinal cancer, and breast cancer, dramatically increases[5,40-46]. The above controversy may be due to the use of different research methods, or differences in sample size and representativeness. In clinical practice, the influence of circadian rhythms on human malignant tumors has been observed. Lou et al.[47] have investigated thyroid nodules in older people with varying degrees of malignancy, and concluded that poor sleep quality and biological rhythm disorders are independent risk factors. CLOCK and BMAL1 expression levels were found to be considerably higher in the malignant thyroid nodule group, whereas CRY2 expression

was significantly lower. In addition, with the popularity of next-generation sequencing, bioinformatics technology has provided further evidence. The bioinformatics analysis depicted in **Figure 2** supports a correlation between clock disorders and cancers. In different cancers, BMAL1 expression significantly differs between tumor and normal tissues. PER and CRY expression are detailed in the Supplementary Materials.

In addition to the above evidence, animal experiments have demonstrated a relationship between circadian rhythms and cancer. When CLOCK and BMAL1 mutations are artificially introduced, premature aging has been observed in mice that are not predisposed to cancer[48,49]. According to Papagiannakopoulos[50], prolonged jet lag dramatically exacerbates cancer development and progression in K-Ras and p53 deficient mice.

The mechanisms of the circadian clock in cancer progression

We investigated the mechanisms underlying the effects of the circadian clock in cancer and assessed their similarities. The mechanisms may include the following: (1) Tumor cells undergo a variety of biochemical reactions, including cell growth and senescence, cell proliferation and apoptosis, DNA damage repair process, and various metabolic proces ses[1,5,36,44,51-55]; the circadian clock may affect tumor occurrence and development by regulating these reactions[44,56]. (2) Cancer stem cells (CSCs) undergo tumorigenesis, development, and metastasis, and the circadian clock plays a crucial role in the stemness of self-renewal cancer cell subsets, such as acute myeloid leukemia (AML) and pleomorphic glioblastoma

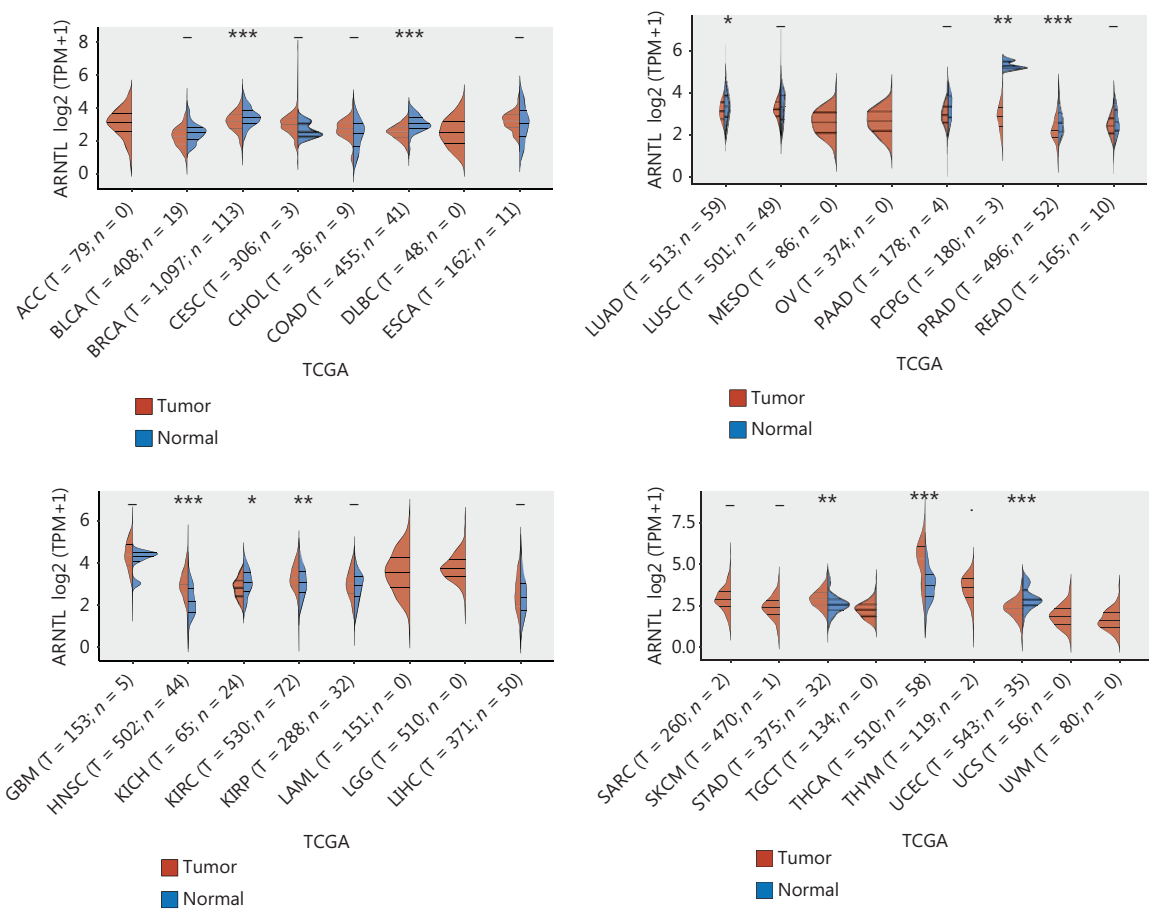

Figure 2 Continued

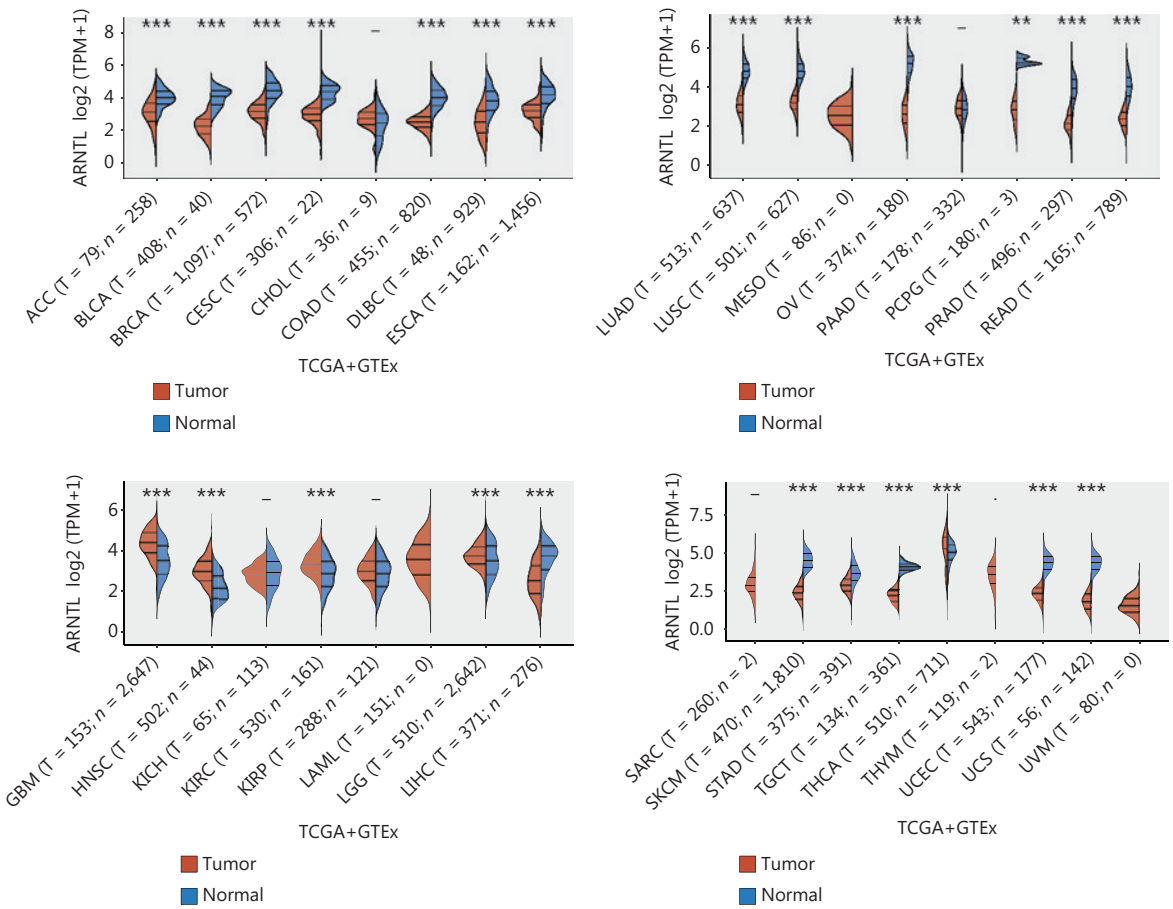

Figure 2 Expression analysis of BMAL1 in tumor tissues. The expression distribution of the BMAL1 (ARNTL) gene in tumor and normal tissues. The abscissa represents different tumor tissues, and the ordinate represents the gene expression distribution. Different colors represent different groups. *P < 0.05, **P < 0.01, ***P < 0.001, with asterisks indicating significance. The Wilcoxon test indicated that the 2 sample groups were dominant.

(GBM)[57-60]. (3) CLOCK components regulate the expression of angiogenic factors such as hypoxia-inducible factor 1α (HIF-1α), aryl hydrocarbon receptor nuclear translocator (ARNT), and vascular endothelial growth factor (VEGF) in cancer cells. Increased levels of these angiogenesis-promoting factors in the tumor microenvironment (TME) promote tumor development and metastasis[43,61-63]. (4) Previous research indicates that CLOCK regulates the inflammation mediated by myeloid cells—a crucial cancer marker[63,64]. For instance, in GBM, CLOCK changes the microglial content of GSC through transcriptional regulation of the chemokine olfactomedin-like 3[58]. The infiltration of immune cells such as macrophages and neutrophils in renal clear cell carcinoma is associated with rhythmic changes in the expression of CLOCK-associated components (BMAL1, PER, etc.)[52,65].

In addition, immune escape is an integral part of cancer progression[63]. According to Chen et al.[58], changes in $CD8^+$ T cells often affect CLOCK expression in patients with glioblastoma multiforme. In the 4T1 mouse model of breast cancer, the CLOCK component has been found to induce regular expression of Wnt family member 10A (Wnt10A) and upregulate the expression of downstream acetaldehyde dehydrogenase 3 (ALDH3A1). ALDH3A1 is a characteristic of CSCs and is associated with the degree of tumor malignancy[66,67]. Moreover, previous studies have indicated that the depletion of T cells and up-regulation of programmed death-ligand (PD-1) in patients with cancer may be associated with the widespread mutation and genomic instability of the CLOCK gene[32,52]. This evidence suggests that CLOCK gene expression in cancer cells may contribute

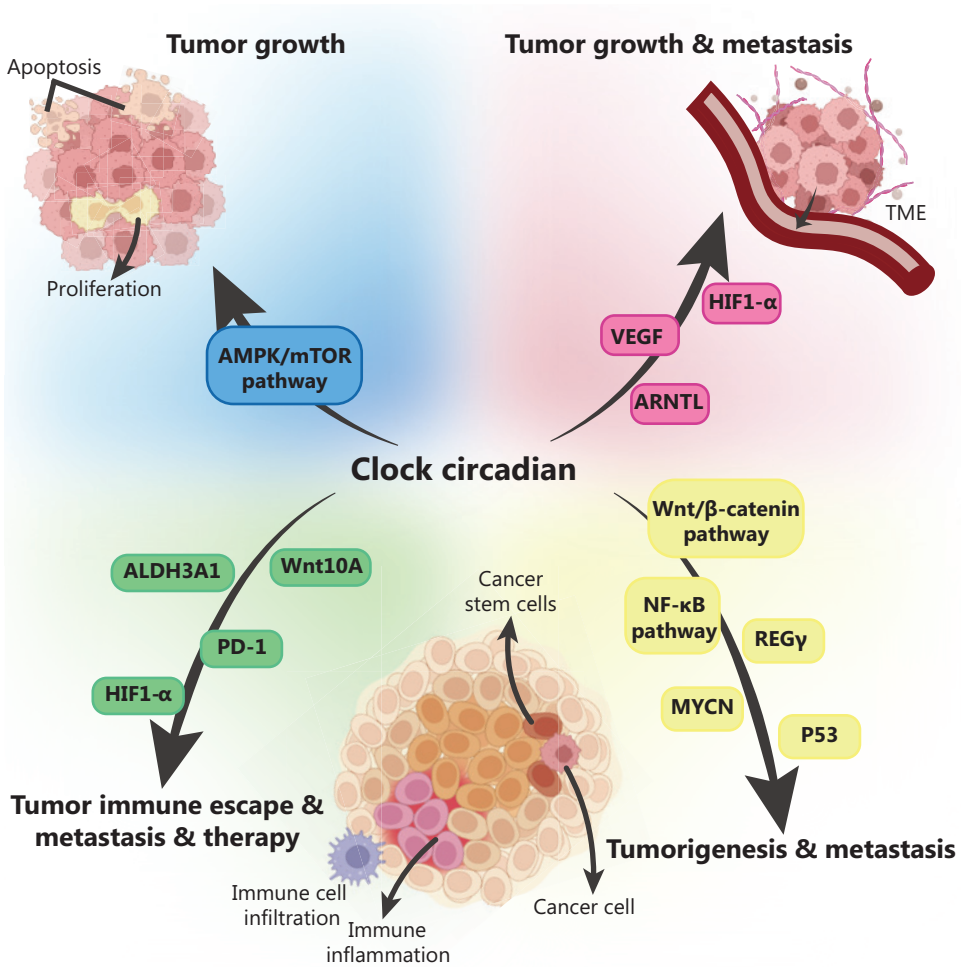

Figure 3 Mechanisms of the circadian clock in cancer. The circadian clock plays an important role in tumorigenesis, tumor growth, metastasis, tumor immune escape, and other processes by regulating various biological functions, such as apoptosis and proliferation. Among them, many signaling pathways, such as the AMPK/mTOR pathway, Wnt/ β-Catenin pathway, and NF-κ B pathway, as well as molecules, such as Hif-1α, P53, and PD-1 may play important roles.

to immune escape. **Figure 3** shows these relationships more intuitively.

CLOCK and the potential molecular mechanisms of tumorigenesis

Ghrelin

Ghrelin is a 28-amino acid peptide that acts a ligand for GHSR. It activates and promotes appetite, and affects many physiological activities, such as glucose metabolism and gastric acid secretion[68-70]. Previous studies have indicated the role of Ghrelin in tumor cells, such as those in rectal cancer, liver cancer, leukemia, endometrial cancer, and pancreatic cancer[71-75]. Regulation of the GHS-R/Ras/PI3K/Akt/mTOR pathway may be one of Ghrelin's mechanisms, particularly in colon cancer[76]. GLUT1 may be another potential target. Previous studies have confirmed that Ghrelin promotes oral cancer progression through GLUT[77]. However, Ghrelin notably has a dual role in prostate cancer, and whether it promotes tumor occurrence and development remains unknown[78,79].

According to Segers et al.[80], feeding time and BMAL1 gene expression have circadian rhythms, thus leading to periodic changes in short-chain fatty acids, and consequently affecting the release of Ghrelin. Ghrelin decreases to its lowest level within 1 hour after a meal and exhibits diurnal variation with

the circadian rhythm[81-83]. After knockout of the BMAL1 gene in mice, the circadian rhythm fluctuation of Ghrelin disappears, thus suggesting a specific relationship between BMAL1 and Ghrelin; these rhythm-associated characteristics may be associated with tumorigenesis[84].

Unfolded protein response (UPR)

The UPR is a stress response caused by protein accumulation in the endoplasmic reticulum, owing to abnormal protein folding into secondary structures. Additionally, it is a cell checkpoint pathway triggered by incorrect folding of the endoplasmic reticulum. The UPR involves Protein kinase RNA-like ER kinase (PERK), Activating Transcription Factor 6 (ATF6), and Inositol-requiring enzyme 1 (Ire1)[85].

miR-211 is a protein kinase RNA-like ER kinase-induced micro-RNA. According to Bu et al.[86], miR-211 regulates BMAL1 expression and CLOCK composition. BMAL1 inhibition impairs UPR-dependent protein synthesis and affects the adaptation of cells to cell stress caused by endoplasmic reticulum homeostasis damage. Moreover, Bu et al. have discovered that the UPR inhibits BMAL1 in Burkitt lymphoma, thereby affecting circadian rhythm and protein synthesis, and promoting tumor progression, which may be associated with the continual activation of c-myc. Knockout of PERK in c-myc-driven lymphoma has been found to lead to ER protein overload and cancer cell death[87]. Inhibition of BMAL1 by PERK-miR-211 is crucial for promoting the progression of c-myc-positive lymphoma. These results demonstrate that PERK-induced miR-211 inhibits BMAL1 expression, limits protein overload, and promotes tumor progression.

Effects on anti-tumor immunity by regulation of TAM metabolism via Hif-1α

Hif-1α is a major factor regulating glycolysis and the synthesis of metabolic gene transcription and is also crucial for the synthesis of arginase-1 (Arg-1), a urea-cycling enzyme[88-90]. Previous research has indicated that tumor-associated macrophages (TAMs) upregulate Arg-1 through Hif1-α[84], a macrophage marker. Anti-tumor immunity mediated by cytotoxic T-cell nuclear natural killer cells is an important factor affecting tumor progression. Arginine depletion in TAMs inhibits this process, which is associated with the abnormal expression of Arg-1[91,92].

We previously discovered that fluctuations in circadian rhythm regulate the inflammatory function of macrophages. Knockout of BMAL1 increases mortality in septic mice. BMAL1 controls the rhythm of monocyte recruitment, thus playing a crucial role in the immune response to pathogens[93]. Alexander et al.[94] have demonstrated that macrophages induce BMAL1 expression after stimulation (inflammation, etc.), thereby aggravating Hif-1α-dependent metabolic reprogramming and causing mitochondrial dysfunction. The balance between BMAL1 and Hif-1α affects the metabolism of macrophages and anti-tumor immunity. BMAL1 gene deletion promotes tumor growth.

Myc

Oncogenes are genes that cause malignant transformation of cells. Dysfunction in the major oncogene family Myc or abnormal proliferation often leads to the occurrence and adverse prognosis of different tumors[95-97]. Myc and its partner MAX form E-box motifs similar to those of the BMAL1 gene that control circadian rhythms. Therefore, Myc also participates in cell growth, proliferation, and death[98,99].

Previous studies have reported that Myc enhances the transcriptional activity of E-box sites and increases REV-ERBα/β expression, thus inhibiting BMAL1 function[100]. Myc expression is negatively correlated with the circadian clock[23]. As described above, PER is a negative stimulus of TTFL, and the CRY-PER complex inhibits the function of the CLOCK-BMAL1 complex. This inhibitory effect of PER has been proposed to be achieved through Myc[101-103].

According to Anastasia et al.[104], c-Myc plays a major role in CLOCK oscillators. Myc expression has a strict circadian rhythm, but its expression also responds to CLOCK function. Concurrently, PER and BMAL1 inhibit the transcription of c-Myc during tumorigenesis and development. Deletion of either of these 2 genes increases c-Myc expression, as demonstrated in a mouse lung cancer model[50].

Ras Homolog Family Member A (RhoA)

The Rho family is involved in the occurrence and development of various tumors. Its GTPase activity regulates cell transformation, division, and angiogenesis[105]. RhoA regulates the adhesion, aggregation, and morphology of various cells by controlling actin and myosin contraction[106,107]. ROCK is the downstream effector of RhoA, and cofilin (CFL)

is an actin-depolymerizing factor. Ma et al.[108] have demonstrated that CLOCK and BMAL1 regulate the dynamic conversion of F-/G-actin by controlling the RhoA-ROCK-CFL pathway, thereby promoting the proliferation and invasion of tumor cells, which may be associated with inhibition of CUL3-mediated ubiquitination and an increase in RhoA expression.

Protein kinase

Several kinases regulate Clock/Bmal and other circadian genes, such as AMP-activated protein kinase (AMPK), Akt, and mammalian rapamycin target protein (mTOR). Their roles are crucial for cancer development. Protein kinases involved in post-translational modifications play important roles in regulating the biological clock, and protein kinases may be involved in the occurrence and development of various diseases caused by biological clock disorders, including tumors. Ramanathan et al.[109] have found that inhibition of mTOR function slows circadian rhythms, thus suggesting biological regulation of mTOR in circadian rhythms. mTOR, a central regulator of multiple tumor-associated signaling pathways, plays important roles in cell proliferation, growth, differentiation, and survival. Abnormal activity of mTOR is closely associated with the occurrence, development, metastasis, and drug resistance of various malignant tumors, including lung cancer[110,111]. The PI3K/Akt/mTOR signaling pathway regulates cell growth and proliferation. Abnormal activation of this pathway is closely associated with tumor occurrence and development[112]. The AMPK/mTOR signaling pathway is involved in tumor cell proliferation, apoptosis, invasion, metastasis, and drug resistance[110]. AMPK is an important cellular energy sensor that plays a role in metabolic control and serves as a central sensor for metabolic signals[113]. On the one hand, AMPK-mediated phosphorylation of CRYs and CK1 regulates the negative feedback control of the circadian clock through proteolytic degradation, thereby shortening the rhythm cycle[114,115]. On the other hand, most early studies have suggested that AMPK itself acts as a tumor suppressor in tumors. AMPK activation is conducive to the occurrence of various malignant tumors by inhibiting the proliferation of tumor cells, and it also promotes apoptosis by inhibiting the mTOR signaling pathway[116,117]. In summary, various protein kinases and biological clocks have close and complex relationships in tumor development.

Other possible mechanisms

In addition to the potential relationship between CLOCK and tumors, various studies have suggested potential mechanisms that may have research value. For instance, in the TME in prostate cancer, if PER expression is too low, BMAL1 expression increases, thus resulting in up-regulation of β-Catenin phosphorylation and activation of the Wnt/β-Catenin pathway. PER is a negative regulator of prostate cancer stem cells and is used as a new target for prostate cancer treatment[118,119]. In other studies, some potential targets have been proposed but have not been studied, such as iron death (colon cancer), NF-κB, REGγ, MYCN (advanced neuroblastoma), and P53[120-124]. Length constraints prevent us from stating them individually herein. These mechanisms may require additional research to guide the development and exploration of related cancer treatment issues.

Effects of CLOCK components in specific cancers

In addition to the aforementioned mechanisms, CLOCK may exhibit different biological functions and characteristics in different cancers (**Figure 4**). Here, we briefly discuss the roles of CLOCK/BMAL1 in in several representative cancers.

Hepatocellular carcinoma (HCC)

HCC is one of the most common malignant tumors and the fourth leading cause of cancer-associated deaths worldwide[125]. Because of its low detection, high recurrence rates, and poor prognosis in the curable stage, the survival rate of patients is unsatisfactory despite recent progress in treatment strategies[125]. In 2012, approximately 700,000 people died from liver cancer. According to the World Health Organization, more than 1 million people will die of liver cancer in the year 2030[126,127]. The main risk factors for liver cancer include hepatitis C or hepatitis B viral infection, long-term alcohol consumption, and nonalcoholic fatty liver disease (NAFLD), including the effects of smoking, obesity, diabetes, and excessive iron load[128].

HCC, the most common type of liver tumor, is found in approximately 90% of patients with a cirrhosis history[126,129]. Previous studies have demonstrated that time-caloric restriction upregulates the expression of BMAL1, limits the transformation of liver fibrosis to cirrhosis, and further leads to HCC

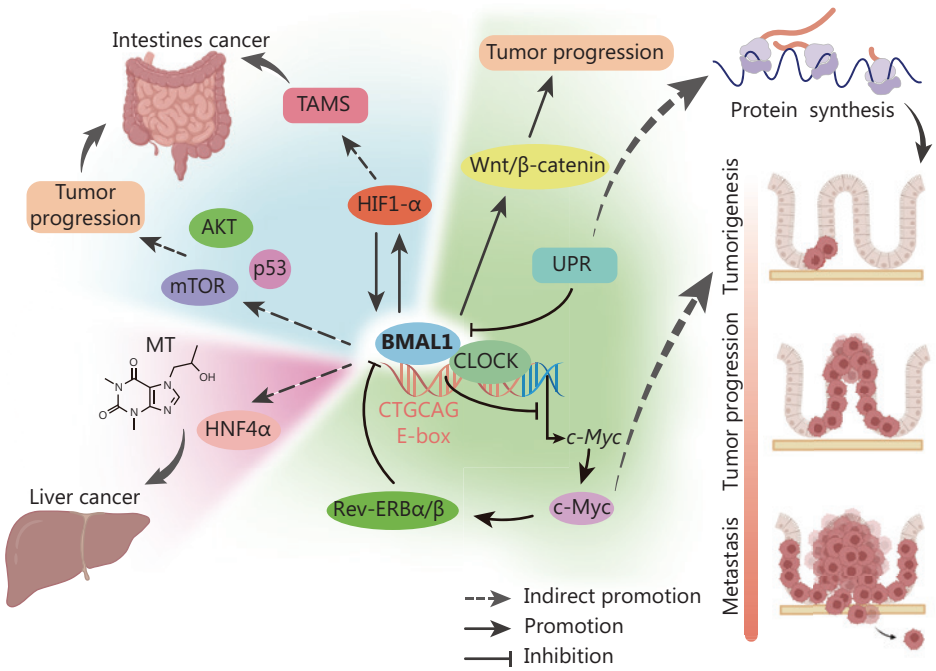

Figure 4 Functions and effects of BMAL1 in cancer. The known and potential mechanisms of BMAL1 are involved in all cancer stages, including protein synthesis, tumorigenesis, tumor progression, and metastasis. This figure was created with biorender.com.

development, as verified in a liver cancer model induced by diethylnitrosamine[130]. These findings suggest that CLOCK may influence liver cancer occurrence by affecting liver cirrhosis progression.

Metallothionein (MT) expression controlled by CLOCK is associated with various endocrine diseases, mental diseases, and tumor diseases in humans. MT expression is relatively extensive in the liver and is regulated by metal regulatory transcription factor-1 (MTF-1)[131-133]. MT expression is a prognostic indicator in patients with HCC[121]. Interactive relationships among CLOCK, MT, and HCC have been identified. Li et al.[134] have confirmed that disorders in MTs and circadian clock genes play crucial roles in human HCC, possibly because of their influence on the expression of nuclear orphan receptor factor protein (Nr1d1) and D-box binding protein (DBP). Concurrently, melatonin, a drug used to treat various diseases and inhibit tumor growth[135-137], prevents BMAL1 expression, an effect potentially associated with the upregulation of MT receptors and the decrease in Hif-1α[138].

In addition, Hepatocyte nuclear factor 4α (HNF4α) is a cytokine widely present in the nuclei of hepatocytes, which is involved in the metabolic processes of various hepatocytes[139,140]. HNF4α has various physiological functions, including inhibiting expression of multiple genes (such as cyclin D1 and CCND1) in HCC development[141-146]. HNF4α expression may be negatively correlated with tumor growth, and its overexpression may inhibit liver cancer growth. Similarly, HNF4α expression is relatively lacking in human HCC[145,147]. HNF4α has 2 different promoters, P1 and P2, producing HNF4α1/2 and HNF4α7/8, respectively. Notably, this difference also exists in colon cancer[142,148,149]. According to research by Fekry and colleagues[150], both subtypes have an apparent circadian rhythm, but P2-HNF4α directly inhibits BMAL1 expression, thereby increasing P1 isoform expression in the cytoplasm. In HNF4α-positive HCC, the increase in BMAL1 expression slows tumor growth *in vivo*, thus indicating that BMAL1 may be a potential target for reversing HNF4α-positive HCC.

Colorectal cancer (CRC)

CRC is a common malignant tumor. CRC-associated deaths account for approximately 10% of cancer-associated deaths

worldwide and are the major cause of cancer deaths[151-153]. Circadian rhythm is an important factor affecting CRC occurrence and development. CLOCK inhibits early tumorigenesis in the intestines. BMAL1 affects AKT, mTOR, and P53[154], and regulates various signal transduction pathways in intestinal stem cells (including the Hippo pathway), thus affecting CRC occurrence[155]. As described above, BMAL1 influences tumor progression by regulating the inflammatory response, in agreement with the findings of Liu et al.[156] indicating that circadian rhythm disruption leads to impaired regulatory B cell function and apoptosis of $CD4^+$ T cells in the intestinal epithelium, and subsequently enteritis progression and CRC occurrence.

Liver metastasis of CRC (CRLM) is a sign of poor prognosis in CRC, and lung and liver metastases are among the leading causes of CRC-associated deaths[157]. CLOCK expression is perturbed in CRLM model mice[158], thus suggesting the effects of CLOCK-associated components on CRLM. BMAL1 also activates Rab27a, a key molecule associated with exosome secretion, and affects CRLM by enhancing exosome secretion in CRC cells[159].

Breast cancer

Breast cancer is the second leading cause of cancer-associated deaths globally and the most common cancer in women[160,161]. In breast cancer cells, compared with normal breast cells, previous studies have found that the circadian rhythm and CLOCK gene expression show different degrees of damage[162,163], and the expression of core CLOCK genes in patients with breast cancer is altered, thus confirming the role of CLOCK in breast cancer[164,165]. BMAL1 knockout promotes breast cancer cell metastasis[166] and decreases acidosis-mediated metastasis of breast cancer by decreasing hypoxia-induced acidosis in the TME[167], thus providing a potential mechanism for preventing breast cancer metastasis.

Triple-negative breast cancer (TNBC) is a type of breast cancer with high invasiveness, poor differentiation, and ready metastasis, which accounts for about 15%–20% of all breast cancer. Owing to a lack of ER, PR, and HER-2, treatment is limited, and TNBC prognosis is poor[168]. However, studies have confirmed that acetylserotonin methyltransferase (ASMT) affects the invasiveness of TNBC cells by regulating circadian rhythms. Thus, ASMT is currently the most promising target for TNBC treatment[169]. According to recent research, BMAL1 may be directly or indirectly affected by insulin and feeding, thus resulting in changes in the characteristics of TNBC, an aspect warranting further attention[65,170].

Glioma

Glioma is a primary tumor of the central nervous system. As described earlier, in addition to the important roles of CSCs and CD8+ T cells in glioma, CLOCK alters the content of microglia in GSC through transcriptional regulation of the chemokine olfactomedin-like 3. In the tumor microenvironment of glioma, glioma-associated microglia (GAMs) are key components regulating tumor progression[171]. GAMs are divided into 2 subtypes with opposite functions: M1 and M2, which inhibit and promote tumor progression, respectively[172-174]. Exosome miRNA is an important medium for communication of various biological information inside and outside cells. Li et al.[175] have used exosome-associated technologies to confirm that M2 subtype GAMs regulate the expression of tumor-associated proteins and decrease apoptosis, thereby promoting the proliferation and metastasis of glioma, which is associated with the recruitment of miR-7239-3p and a decrease in BMAL1 expression in TAMs.

The circadian clock has a wide range of effects on specific cancers. Because of space limitations, we discuss several representative cancers. Other factors such as AML[60] and melanoma[176,177] have been associated with CLOCK. However, the specific mechanisms remain unclear or controversial, and require further investigation.

Circadian clock and cancer treatment

We have described the complicated mechanisms underlying the relationship between circadian rhythm and cancer. To some extent, these mechanisms are believed to be valuable in guiding cancer treatment. Both *in vivo* and *in vitro* experiments and statistical studies on clinical cases have shown that biological CLOCK has important research value in treating tumors or regulating the anti-tumor efficiency of radiotherapy and chemotherapy[178-181]. Several aspects are summarized below.

CLOCK affects the sensitivity of tumor cells to treatment and the efficacy of drugs

As described previously, CLOCK affects the occurrence, development, and metastasis of CRC. The standard chemotherapy

agents 5-fluorouracil and bevacizumab (Beva) are often used for metastatic CRC. Bevacizumab inhibits angiogenesis by preventing the binding of vascular endothelial growth factor A (VEGFA) to its receptor, thereby achieving antitumor therapeutic effects. However, it is effective for only some clinical patients, and its anti-angiogenic effect is adaptive[182,183], thus suggesting the possibility of drug resistance. BMAL1 upregulates the promoter activity of VEGFA and enhances its expression, as confirmed by BMAL1 inhibition[184-186]. In addition, VEGF expression in xenografts (sarcomas, melanomas, etc.) is induced by hypoxia. Circadian rhythms are inhibited by transcription of PER2 and CRY1. Therefore, ZT2 is more effective in anti-angiogenesis therapy than ZT14 (where ZT0 is bright, and ZT12 is dark)[184]. After administration of Beva and other anti-angiogenic drugs, BMAL1 expression in cancer cells increases, thus leading to the activation of VEGF expression and inducing drug resistance[187]. These results emphasize the roles of CLOCK in influencing tumor angiogenesis and anti-angiogenic drug resistance.

Epithelial-mesenchymal transition (EMT) refers to the polarization of epithelial cells to obtain characteristics of mesenchymal cells. This process enhances the migration and invasion of cells, and mediates drug resistance. EMT is the first key step in metastatic CRC development[188]. In the formation and maintenance of EMT, the downregulation of E-cadherin expression causes functional disorders of the E-cadherin/β-catenin complex, and various signaling pathways (such as TGF-β and Wnt) are involved[189,190]. A recent report[191] has indicated that BMAL1 is critical in EMT-induced CRC metastasis and drug resistance to chemotherapeutic drugs. Understanding the importance of BMAL1 in the epithelial-mesenchymal equilibrium of CRC cells will aid in studying how to reverse drug resistance to existing treatment methods. Similar findings have been reported for breast cancer and glioma.

A study on MCF10A (non-tumorigenic) and MDA-MB-231 (invasive-tumorigenic) cells with 2 different tumorigenic characteristics of human breast epithelial cells has found that knockout of BMAL1 increases sensitivity to cisplatin and doxorubicin[23]. Temozolomide (TMZ) is a DNA alkylator commonly used to treat GBM. When the effect of TMZ on GBM (including DNA damage, cell death and tumor growth inhibition) reaches the maximum daily peak of BMAL1 expression, the anti-tumor effect of TMZ has circadian regularity, which may be associated with differing sensitivity of GBM cells to TMZ, owing to their circadian regularity[192]. Similarly, in a study of GBM cells, after bortezomib treatment, cell viability has been found to change with time, and this rhythm is altered by BMAL1 knockdown[193].

In summary, the destruction of BMAL1 usually increases the sensitivity of tumor cells to chemotherapeutic drugs; however, some studies have indicated opposite results. For example, in pancreatic cancer[194], BMAL1 is targeted and inhibited by miR-135b, thus resulting in local circadian rhythm disorders. However, this destruction has been found to cause tumor resistance to chemotherapeutic drugs, in contrast to previous conclusions. Similarly, in tongue squamous cell carcinoma, BMAL1 overexpression increases sensitivity to paclitaxel, whereas decreasing the expression of BMAL1 induces drug resistance in tongue squamous cell carcinoma cells[195].

Some researchers have proposed that 1A-116 in GBM treatment, at the end of light (ZT12) administration rather than the beginning of light (ZT3) administration, prolongs survival time in mice[196]. This finding is contrary to the conclusion that at ZT2, the time of administration in CRC, anti-angiogenesis drugs have a more potent anti-tumor effect. Treatment with 1A-116 specifically inhibits the activation of Ras-related C3 botulinum toxin substrate 1 (RAC1) in GBM cells, thereby affecting the role of RAC1 in tumor progression. The mechanism of action of 1A-116 may block the binding of RAC1 to guanine exchange factor by interacting with Trp56 residues and interfering with the normal biological function of RAC1[197-200]. This contradictory phenomenon requires further research.

Beyond increasing the sensitivity of tumor cells to anti-tumor drugs, some drugs, such as doxorubicin, destroy circadian cell rhythms by inhibiting BMAL1 expression[201]. Thua, targeting BMAL1 may be a feasible strategy for improving the therapeutic effects of anti-tumor drugs. These CLOCK-mediated differences in the sensitivity of anti-cancer drugs may be used to optimize and adjust treatment regimens and medication protocols to achieve the best anti-tumor effect. However, BMAL1 has a bidirectional role: decreasing BMAL1 expression increases the sensitivity of cells to anticancer drugs, but also enhances the invasiveness of tumors and causes distant metastasis. Therefore, this conclusion should be comprehensively considered.

Potential therapeutic targets or drugs

We described some potential mechanisms through which CLOCK affects cancer progression. The components associated with these mechanisms have the potential to be used as

targets for cancer treatment. Some drugs may have anti-tumor effects by regulating circadian rhythms, as summarized below.

Ghrelin

Cachexia occurs in most cancer patients, particularly in the terminal stage of cancer and in patients with metastasis. It is a severe cancer complication that directly affects patients' tolerance to treatment, and can even cause disability or death[202-204]. More than half of all patients with cancer are affected by cachexia, and at least 20% of patients with advanced cancer die[205]. As described above, Ghrelin, an intestinal hormone, activates and promotes appetite and affects glucose metabolism, gastric acid secretion, and other physiological activities. Consequently, it is often considered to reverse cancer-associated cachexia and to be a promising treatment[206-209]. The rhythmic characteristics of Ghrelin secretion may provide a more optimized solution for future clinical applications.

CLK8

According to Doruk et al.[210] CLOCK's interaction with small molecules (CLK8) decreases the interaction between CLOCK and BMAL1 and interferes with the translocation of CLOCK into the nucleus. This decrease in CLOCK in the nucleus leads to a more stable negative arm of TTFL and enhances the role of the circadian clock. Therefore, by influencing the functions of CLOCK and BMAL1, CLK8 elicits improvements in aging, emotional disorders, and the effectiveness of cancer treatment. Another small molecule, Nobiletin, a natural polymethoxy flavonoid compound, may also enhance the CLOCK effect by increasing the PER2 level and consequently stabilizing the negative arm of TTFL.

The above small molecule substances may enhance the functions of CLOCK and BMAL1, and are the leading compounds and potential targets for preventing and treating new therapies for diseases associated with circadian rhythm changes, such as cancer.

mTOR

mTOR is a highly conserved serine/threonine-protein kinase associated with cell proliferation, protein metabolism, and autophagy[211-214]. The mTOR signaling pathway is abnormally expressed in various tumors. Although mTOR inhibitors have broad application prospects in antitumor therapy, problems remain, such as low treatment efficiency and easy recurrence.

Ramanathan et al.[109] have found that inhibition of mTOR function slows circadian rhythms, thus suggesting biological regulation of mTOR in circadian rhythms. The PTEN gene inhibits tumor growth by negatively regulating PI3K and its downstream targets (mTOR/AKT, etc.). PTEN dysfunction is observed in melanoma, breast cancer, liver cancer, and other cancers. According to previous research[215], targeting PTEN upregulates BMAL1 expression, whereas this effect is reversed by rapamycin (mTOR inhibitor). The accumulation of BMAL1 caused by PER deficiency is also eliminated by rapamycin. Thus, mTOR-Bmal may be a potential clinical target. Combined biological rhythm and anti-mTOR drugs may further improve the anti-tumor effects of traditional anti-mTOR drugs.

UPR

Metabolic disorders, viral infection, hypoxia, and tumors in the body usually cause UPR, and irreparably damaged cells may undergo apoptosis, on the basis of decreased protein synthesis. The UPR is often involved in tumor occurrence and development. As described above, it promotes the survival of tumor cells, which may be associated with the inhibition of BMAL1 expression by miR-211 induced by PERK[87,216-218]. Therefore, PERK and Ire1, as UPR transducers, are potential targets for treating tumor progression.

HSP90

The expression of some HSP90 isomers may have a circadian rhythm. Although the amplitude of this rhythm is low, it nonetheless affects the cell cycle process and may lead to time-dependent anti-tumor efficacy of some drugs. Therefore, targeting HSP90 also improves therapeutic effects[219].

MLN4924

Osteosarcoma is a malignant bone tumor that occurs primarily in adolescents. Surgery after neoadjuvant chemotherapy is a conventional treatment, and adjuvant chemotherapy (cisplatin, doxorubicin, etc.) is performed after surgery. The 5-year survival rate remains low[220,221]. Nedd8 is a ubiquitin-like molecule with a molecular weight of approximately 8 kDa whose substrate is cullin protein. Cullin protein, as

the scaffold of Cullin-Ring E3-ubiquitin ligases, participates in the degradation of cellular proteins in the ubiquitin-proteasome system. MLN4924 (Pevonedistat) is an inhibitor of Nedd8 activation enzyme, which has anti-cancer effects and inhibits the occurrence and development of tumors[222-227]. According to Zhang et al.[228], MLN4924 is an effective drug for treating osteosarcoma and has vast application prospects. RORα and BMAL1 may mediate its inhibitory effect on tumor growth.

Focus on therapeutic approaches associated with circadian-mediated immune responses

Lymphocytes often play important roles in antitumor immunity, and their biological activity is often regulated by CLOCK. Many cytokines with immune activity have a clear circadian rhythm during their production or secretion.

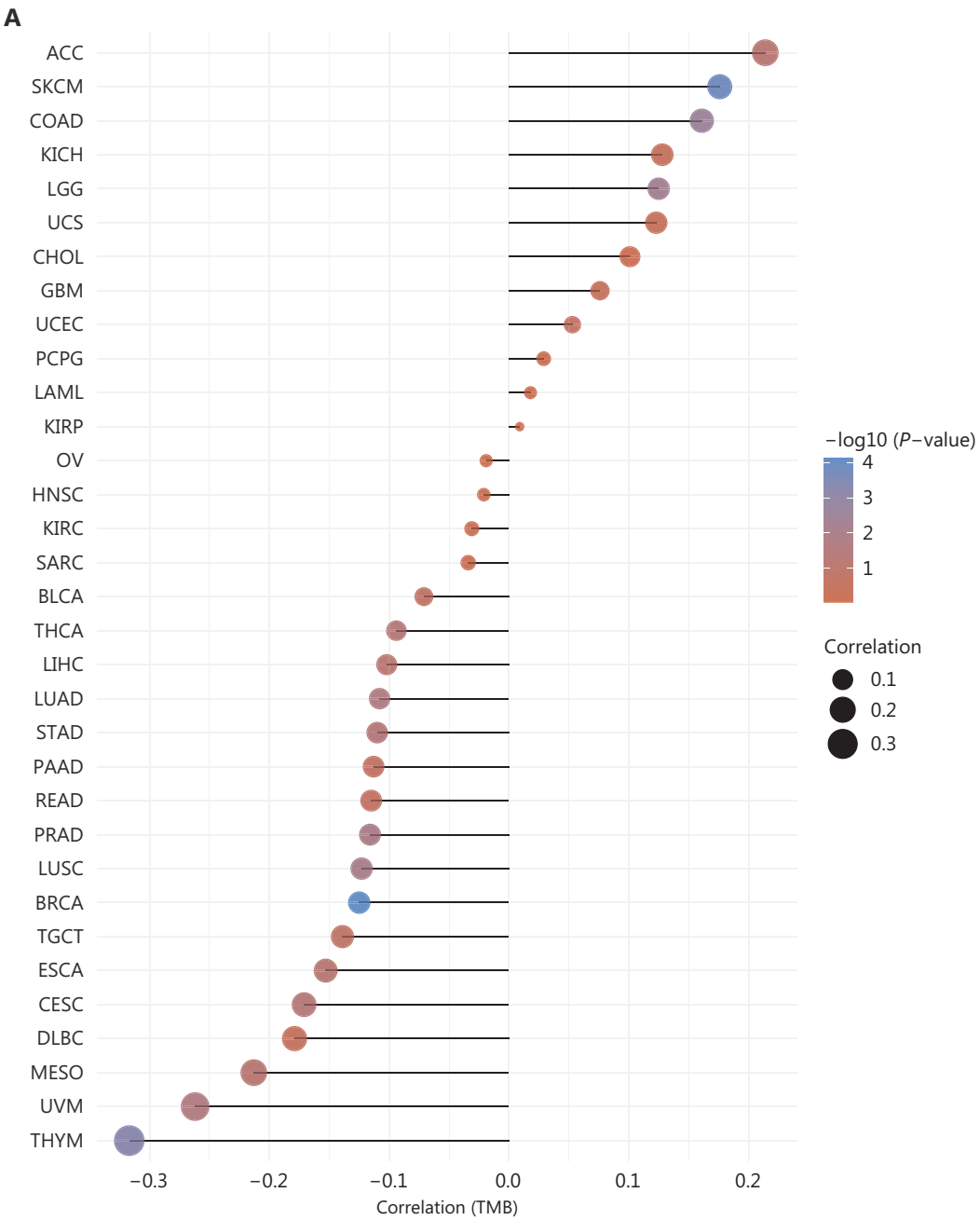

Figure 5 Continued

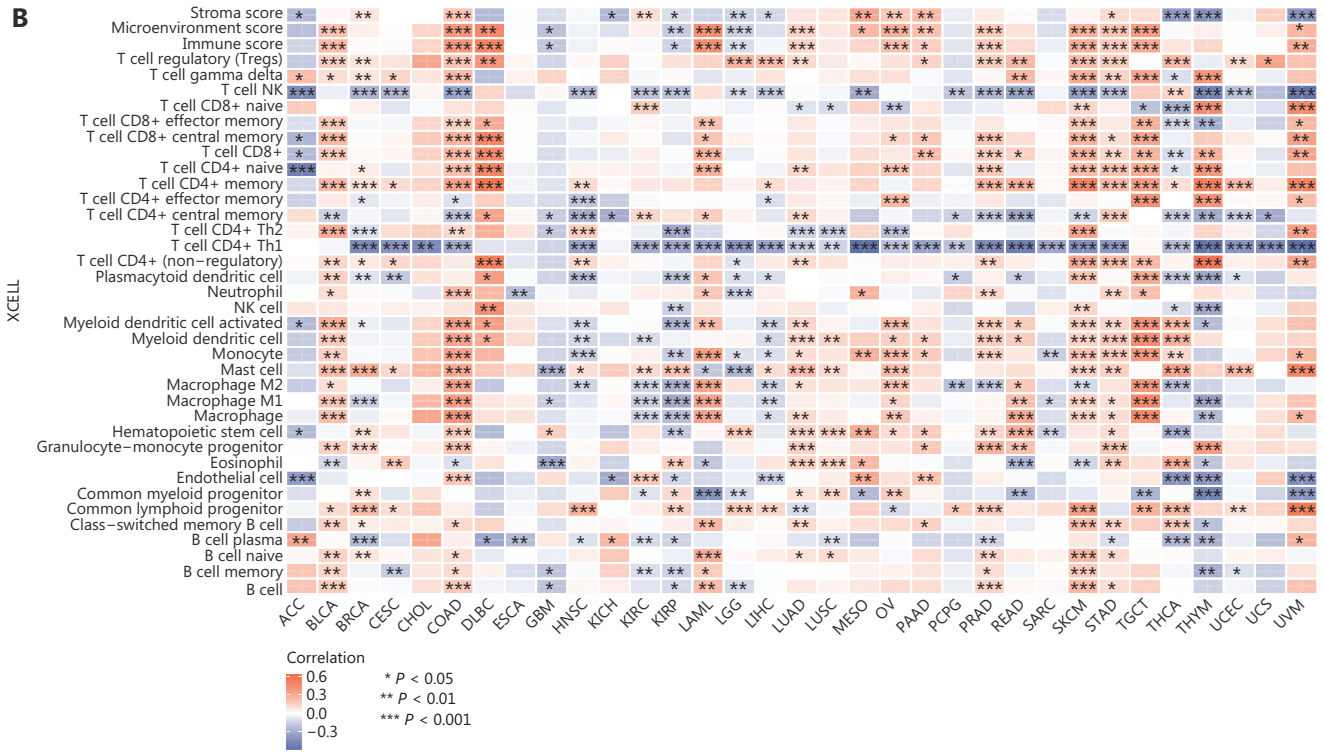

Figure 5 Immune-associated analysis and tumor mutational burden of BMAL1. (A) Tumor mutation burden: Spearman's correlation analysis of TMB and BMAL1 gene expression. The transverse coordinates represent the correlation coefficient between genes and TMB, the ordinate coordinates represent different tumors, the circular point size represents the correlation coefficient, and different colors represent *P*-value aboriginality: bluer color in the diagram indicates smaller *P*-values. (B) The xCell immune infiltration score in multiple tumor tissues, and Spearman correlation analysis heatmap of BMAL1 (ARNTL) gene expression. The abscissa represents different tumor tissues, the ordinate represents different immune infiltration scores, different colors represent the correlation coefficient, negative values represent negative correlations, and positive values represent positive correlations. Stronger correlation is indicated by deeper color. *P < 0.05, **P < 0.01, **P < 0.001, with asterisks indicating significance. The dominance of the 2 sample groups passed the Wilcoxon test.

For example, $CD4^+$ 17 helper T cells (Th17) produce interleukin-17 (IL-17), and Th17 differentiation is regulated by the transcription factor RORγT, which has a circadian rhythm[229]. RORγ activation enhances the cell differentiation and biological function of Th17 and decreases the Treg level (But Treg itself has no circadian rhythm[230]), thus inhibiting tumor growth. This antitumor effect has also been verified in animal models[231].

We also suggest that the circadian clock affects antitumor immunity by regulating immune-associated functions, such as depletion of T cells, enhancement of immune escape, and controlling the metabolic process of TAMs by affecting the function of Hif-1α. Therefore, focusing on these immune responses associated with CLOCK and BMAL1 should provide new insights into cancer treatment.

To better understand the role of circadian-mediated immunity in cancer progression, we performed a correlation analysis, and the results are depicted in **Figure 5A**. TMB reflects the density of the non-synonymous mutation distribution in protein-coding regions, usually represented by the total number of somatic tumor mutations in each MB tumor genome region. Tumors with high TMB may have more neoantigens that the immune system recognizes. As displayed in **Figure 5B**, in different cancers, BMAL1 has different or even opposite effects on the function of the immune system, in agreement with the contradictory points proposed above. Therefore, specific analyses should be performed for specific types of cancer.

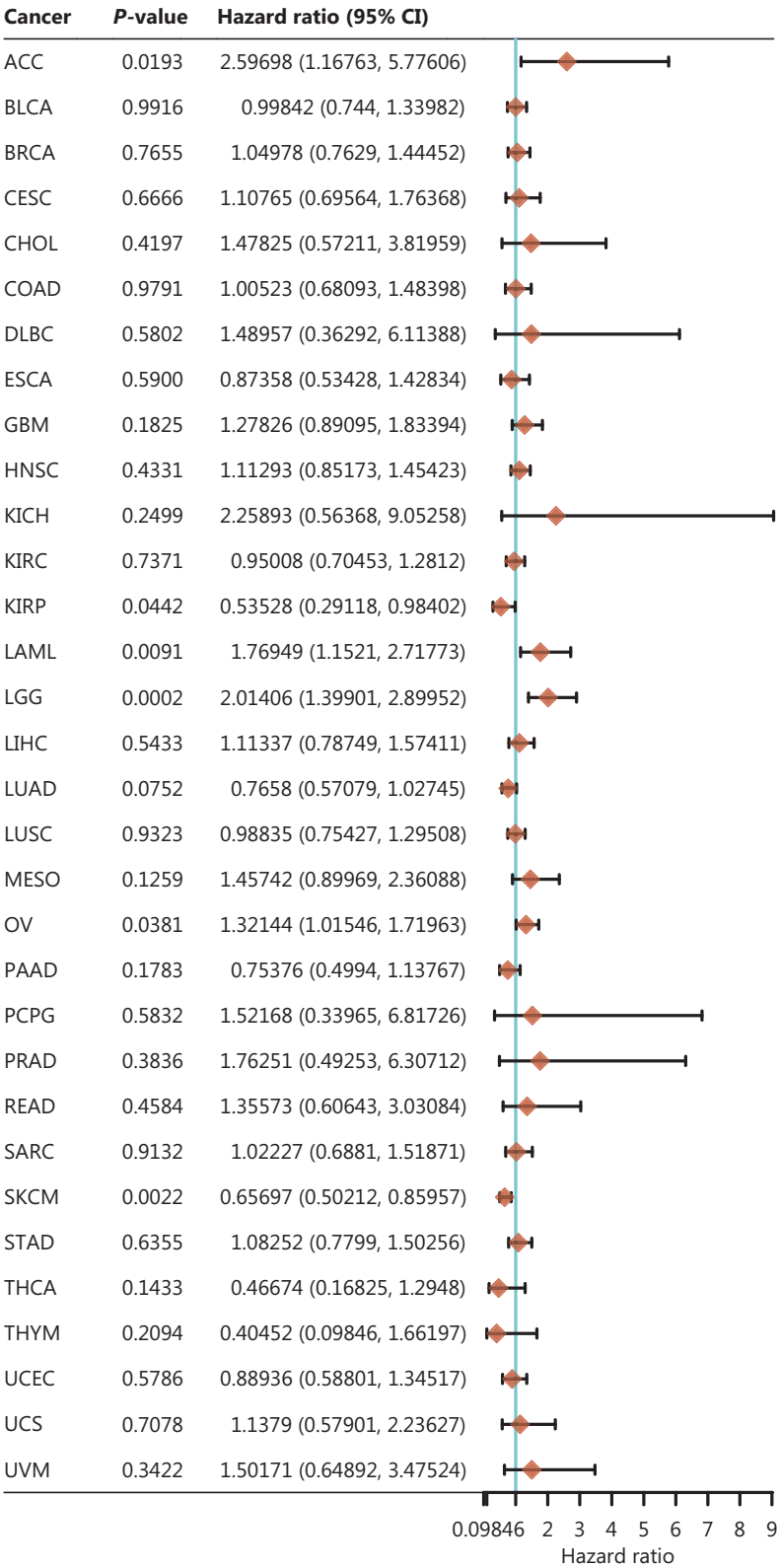

Figure 6 BMAL1 and prognosis of cancer. Forest plot: single-factor Cox analysis results for a single gene in multiple tumors, *P*-value, risk coefficient HR, and confidence interval. Abb: ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma;

DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; PEAD, rectal adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

Conclusion and perspectives

Circadian rhythms, a physiological phenomenon widely present in various organisms in nature, have attracted the attention of various scientific researchers in recent decades, and are involved in the occurrence and development of various diseases to varying degrees. This article reviewed the roles of circadian rhythms in tumor diseases, including their composition, primary mechanisms, relationships with different tumors, and potential therapeutic targets. CLOCK and BMAL1 regulate various physiological processes in tumor cells, including tumor occurrence, tumor stem cell generation, apoptosis, and the endoplasmic reticulum stress response (EIF2DNA). On the basis of TTFL and second-order rhythm, the circadian clock is usually relatively stable *in vivo*. When this rhythm is broken, the occurrence and development of tumors are affected to varying degrees. Depending on whether BMAL1 expression has a positive or negative effect on tumor growth, different characteristics are observed in different tumors. Contradictions remain in the current research on the underlying mechanisms. However, the circadian clock as a target is not affected by the lack of mechanistic uncertainty, and this field has great research value in oncology, for example to provide prognostic indicators for cancer progression (**Figure 6**). This article summarized many possible effective targets for tumor therapy and provides a reference for related research. Further modification of small-molecule compounds known to regulate the biological clock, optimization of their pharmacokinetic properties, and minimization of adverse reactions are important research directions in this field. At present, research on most small molecule regulators remains in basic experimental stages, and biological clock regulating compounds with good therapeutic effects and pharmaceutical characteristics in animal models must enter the next stage of clinical trials. In addition, owing to the rhythmic expression of circadian clock genes, the optimal administration times for clock-targeted drugs and chemoradiotherapy are considered to maximize anti-tumor effects and minimize toxic and adverse effects. For example, treating cancer by administering mTOR inhibitors as at the peak of mTOR expression improves the survival rate in mice[232,233]. These unclear mechanisms are likely to gradually be elucidated in the future, thus deepening understanding of the role of CLOCK and BMAL1, and providing new ways to treat cancer.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Feng Bi, Chenliang Zhang.
Collected the data: Chen Huang, Jian Li.
Contributed data or analysis tools: Chen Huang, Yubin Cao.
Performed the analysis: Chen Huang, Chenliang Zhang.
Wrote the paper: Chen Huang.

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