Circadian Rhythm and Melatonin in Liver Carcinogenesis: Updates on Current Findings

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ABSTRACT: Liver cancer, including hepatocellular carcinoma and cholangiocarcinoma, can be devastating if not treated early. The risk factors of liver cancer include alcoholic liver disease, non-alcoholic fatty liver disease, disruption of melatonin levels, and dysregulated circadian rhythm. The circadian rhythm is a 24-hour biological clock that regulates the physiological activities at both central and peripheral levels. Its molecular mechanism exists in every cell in mammals. Disruption of the circadian rhythm has found in liver cancers as an independent risk factor. This review summarized the most recent findings about the molecular mechanisms of circadian rhythm, the crosstalk between core clock genes and melatonin, as well as the role of circadian rhythm and melatonin played in chronic liver diseases and liver cancer. Finally, we discussed the potential clinical application of circadian rhythm and melatonin for the treatment of liver cancer and discussed future perspectives of how understanding the circadian rhythm in liver cancer progression could provide new clinical applications for liver cancer treatment and diagnosis.

KEY WORDS: cholangiocarcinoma, hepatocellular carcinoma, clock genes, melatonin, circadian rhythm

ABBREVIATIONS: AANAT, aralkylamine N-acetyltransferase; **ASMT**, acetylserotonin O-methyltransferase; **BMAL1**, brain and muscle ARNT-like 1; **CCA**, cholangiocarcinoma; **CCG**, clock-controlled gene; **CLOCK**, circadian locomoter output cycles kaput; **CRY**, cryptochrome; **HBV**, hepatitis B virus; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **NAFLD**, non-alcoholic fatty liver disease; **NASH**, non-alcoholic steatohepatitis; **PER**, period; **SCN**, suprachiasmatic nucleus

I. INTRODUCTION

A. Hepatocellular Carcinoma (HCC), Cholangiocarcinoma (CCA), and Chronic Liver Diseases

Worldwide, liver cancer is the sixth most prevalent cancer diagnosis and the fourth leading cause of cancer-related death.[1] Hepatocellular carcinoma (HCC) is the dominant primary liver cancer, which is classified as the sixth most common neoplasm and the third cause of cancer-related death.[2] HCC incidence in the United States has more than doubled over the past two decades and is anticipated to continue increasing over the next 20 years due to the growing number of patients with hepatitis C virus (HCV) and/or non-alcoholic fatty liver disease (NAFLD).[3] The worldwide incidence is heterogeneous because of the variable prevalence of risk factors and risk varies according to cause, geographic area, sex, age, and degree of liver damage. Patients with cirrhosis of any cause have a risk for developing HCC,[4] and the major risk factors for HCC development are HBV/HCV infection, heavy alcohol consumption, aflatoxin B1 ingestion, tobacco smoking, and NAFLD caused by obesity and insulin resistance.[5] Aging is also a strong risk factor; incidence rates of HCC and age are directly correlated until approximately 75 years of age.[6] The prognosis for HCC is driven by the tumor stage; unfortunately, most

patients are diagnosed at an advanced stage when surgery is not an option.[7]

Cholangiocarcinoma (CCA), also known as bile duct cancer, is an epithelial cell malignancy that has an increasing mortality rate due to increased disease incidence.[8] CCA is the second most common primary liver cancer accounting for up to 20% and representing 3% of all gastrointestinal neoplasia. CCA can be classified into three types based on anatomic location: intrahepatic CCA (arising from bile ductules); perihilar CCA (also called hilar, emerges in the right and/or left hepatic duct and/or at their junction); and distal CCA (involves the common bile duct).[9] The risk factors shared by CCA subtypes are associated with chronic inflammation of the biliary epithelium and bile stasis.[10] HCV infection, obesity, NAFLD, and smoking tend to be more common risk factors in intrahepatic CCA.[11] Up to 10% of people chronically infected with *O. viverrini* and *C. sinensis* liver flukes (a common risk factor in East Asia) will develop CCA.[12] It has been reported that primary sclerosing cholangitis (PSC) is strongly associated with perihilar CCA in Europe.[13] Similar to HCC, CCA is characterized by late diagnosis and poor outcomes, being asymptomatic at early stages. Surgery with complete resection represents the only potentially curative management for CCA[14] with only a small percentage of CCA are resectable. Overall, the 5-year survival rate remains low after resection for CCA in clinical settings, reported in the range of 40–60% for intrahepatic CCA, 30–50% for perihilar CCA and 27–37% for distal CCA.[15] Therefore, it is essential to seek potential diagnostic biomarker, therapeutic targets, and understand the molecular mechanisms underlying the progression of CCA.

HCC and CCA can develop from a multistep process involving accumulation of molecular alterations, mediating both genetic and epigenetic events, which promote the formation of dysplastic nodules, which are bona fide preneoplastic lesions. The interaction between molecular and environmental events promotes the dysplastic cells to acquire more proliferative, invasive, and survival characteristics, which allows the complete transition to full-blown liver cancer.[16] Enhanced immune response, angiogenesis, and invasion/metastasis contribute to liver cancer progression, and interestingly these cellular processes can be modulated by melatonin signaling and melatonin-mediated changes in circadian rhythm.[17] In mouse models, circadian disruption augments liver cancer development and supports mechanisms regulating liver carcinogenesis.[18]

As mentioned above, the risk of developing HCC or CCA can be increased due to other liver conditions, including viral hepatitis, NAFLD, alcoholic liver disease (ALD), and PSC (specifically a risk factor for CCA).[19,20] Highlighting this, it has been reported that 60–80% of patients with chronic liver diseases report poor quality of sleep and insomnia.[21] Indeed, sleep disturbances are strongly noted in chronic liver diseases, including HCV, primary biliary cholangitis (PBC), Wilson disease and patients with cirrhosis.[22] In a small cohort, it was found that (i) chronic liver disease patients with fatigue have increased melatonin levels; (ii) fatigued patients have an altered adrenal circadian rhythm; and (iii) liver enzymes show a circadian rhythm.[23] More importantly, dysregulation of the circadian rhythm is noted in NAFLD, which is a disorder that is associated with cirrhosis and liver cancer development.[22] These associations have been demonstrated in mouse models where chronic jet lag altered the circadian oscillation of clock genes, which in turn disrupted lipid metabolism gene expression.[24] Obviously, there is an association between sleep and circadian rhythm in the severity and progression of liver disorders. One study found that non-apnea sleep disorders significantly increase the risk of developing liver cancer.[25] Similarly, disturbances in circadian rhythm may be associated with poor sleep quality in HCC patients.[26] In this review, we will describe HCC and CCA and their association with dysregulated circadian oscillations and disturbances in melatonin signaling, and briefly discuss these mechanisms in chronic liver diseases since they are precursors for liver cancer development.

B. Circadian Rhythm and Melatonin Signaling

Circadian rhythm refers to a 24-hour cycle that regulates behavioral, metabolic and physiological processes. It exists in almost every single cell in mammalian animals.[27] The mammalian circadian

system is organized in a strictly regulated hierarchical network, in which the central clock is controlled by the suprachiasmatic nucleus (SCN) in the brain and peripheral clocks. The central clock controls the systemic circadian parameters, such as temperature, melatonin release, and other systemic circadian physiological activities. Furthermore, peripheral clocks exist in the liver, heart, gastrointestinal tract, and other peripheral organs, which is under the coordination of central clock.[28,29] The entrainment of light stimulates the SCN to synchronize the metabolic activities at the organismal level, while the entrainment of food stimulates the gastrointestinal tract and liver to synchronize the clock genes at local level, which can overcome the coordination from SCN. Therefore, the coordination of external stimulus could produce a coherent rhythm across different organs, while the conflict signal from light and food may cause metabolic disorders in mammalian which are associated with disrupted circadian rhythms.[30]

As shown in Fig. 1, the molecular clock consists of genes called clock genes that form two different types of feedbacks that drive 24-hour oscillation of the biological activities in cells at the molecular level, which was shared by both the central clock (SCN) and peripheral clock (peripheral organs). During the daytime (with the stimulation of light) in SCN, the primary positive feedback starts with the transcription factor *Brain and Muscle ARNT-Like 1* (BMAL1) and *Circadian Locomoter Output Cycles Kaput* (CLOCK), which consists of basic helix-loop-helix (bHLH) elements. BMAL1 and CLOCK form a heterodimer (BMAL1:CLOCK) that translocates to the nucleus and binds to the E-box elements of the promoter region in the primary core clock genes *Period* (PER) 1/2/3 and *Cryptochrome* (CRY) 1/2, which initiates the expression of PER family and CRY family. The PER family and CRY family reaches peak level in the middle of the night. Following this transcription, the proteins PER1/2/3, Cry1/2 and casein kinase I (CKI) form complex that in turn inhibit the expression of BMAL1:CLOCK, forming a negative feedback loop.[31] Notice that, the food can also act as the external stimulus and initiate the positive feedback loop. Although circadian rhythm can be synchronized by environmental stimulus (e.g., light or food signals), it can sustain *ex vivo* or without stimulus due to the existence of the autonomous molecular circadian clock.[32–34] These processes are called transcriptional-translational feedback and are the key basis of the oscillation of clock genes and other genes under transcriptional regulation of the core clock genes.[35]

Researchers found BMAL1:CLOCK could also activate the orphan receptors retinoic acid receptor-related orphan receptors (ROR) family (α, β and γ) and REV-ERB family (α and β) in a cis-regulatory manner similar to the PER and CRY family. Furthermore, the ROR family activates the transcription of BMAL1[36] and REV-ERB family inhibits the transcription of BAML1.[37,38] In fact, both RORs and REV-ERBs can competitively bind to the elements of a target gene and promote or suppress the translation of the target gene, respectively. The RORs show differential expression in different organ systems. For example, RORα is highly expressed in the liver, skeletal muscle, lungs, kidney, and brain, RORβ is primarily found in the brain in human, and RORγ is highly expressed in the immune system. Likewise, the REV-ERBβ is highly expressed in the pineal gland, pituitary gland, thyroid, and prefrontal cortex. It is noticeable that REV-ERBs can act as a transcriptional repressor and regulate downstream target genes in a circadian fashion. Knocking out REV-ERBβ in mice caused a 30 minutes shorter period of circadian oscillations which was also more sensitive to light-induced phase shifts.[38,39]

In addition, BMAL1:CLOCK drives the rhythmic transcription of clock-controlled genes (CCGs), which in turn regulates thousands of downstream genes associated with cell cycle, apoptosis, tumor suppression, or oncogenesis. Indeed, others have revealed that about 10% of the transcripts in the liver were rhythmically expressed as demonstrated via next-generation sequencing and microarray analysis.[40,41]

The circadian rhythm is found interplaying with melatonin (N-acetyl-5-methoxytryptamine), a hormone initially found in the pineal gland, shows peak level in the dark phase and 24-hour oscillation in the blood. The synthesis of melatonin is from serotonin, controlled by two enzymes aralkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT). Melatonin *per se* showed
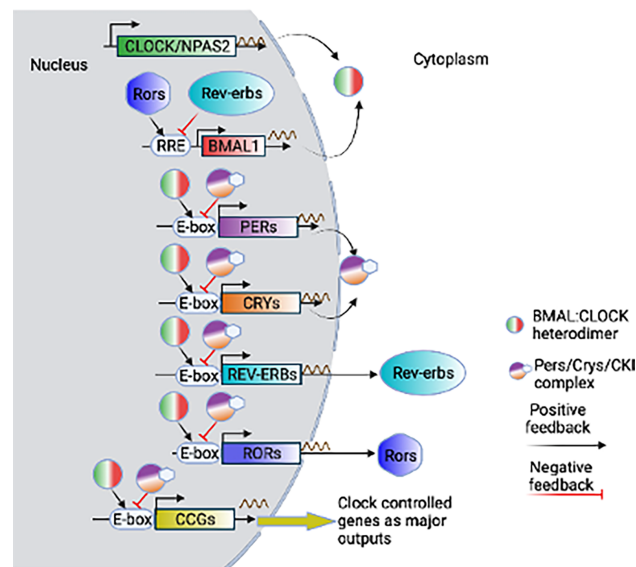
FIG. 1: Molecular clock regulation in humans. The primary positive feedback loop is initiated by the formation of heterodimer of BMAL1 and CLOCK, which acts as a transcription factor to bind to any genes that contains E-box in their promoter region, such as CRYs, PERs, RORs, REV-ERBs and CCGs. The primary negative-feedback loop is initiated by the formation of complex of CRY, PER, and CKI, which acts as an inhibitor to competitively bind to the E-box, thus inhibiting the activation of gene transcription. The other positive and negative feedback loop is involved with the RORs and REV-ERBs, respectively. The RORs and REV-ERBs bind to the RRE (ROR element) in the promoter region of BMAL1, promoting and inhibiting the transcription of BMAL1, respectively. The CCGs are representing thousands of genes that controls cell cycle, lipogenesis, apoptosis, and other essential biological processes. These genes are under the direct control of BMAL1:CLOCK. Image was made with BioRender.

multiple roles in liver cancer, such as anti-inflammation, inhibit cell cycle and proliferation, promote apoptosis, inhibit invasion, migration, angiogenesis and antioxidant effects.[42] The action of melatonin is through melatonin receptors [melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2)].[43] Many studies have shown a strong correlation between melatonin and circadian rhythm. Melatonin's circadian pattern and secretion is controlled by the central clock located at SCN of the hypothalamus.[44] In addition, melatonin could inhibit the expression of clock genes.[45] Misalignment of 24-hour melatonin production is corelated with sleep disorder and delayed sleep phase syndrome.[46] Further, melatonin could inhibit the degradation of the PER and CRY family leading to the stabilization of BMAL1 during the night.[47]

Taken together, the circadian oscillations of core clock genes and the interplay with melatonin could influence the biological activities in both central nervous system and the local peripheral organs. The crosstalk between the central clock, the peripheral clock and the melatonin regulates the progression of liver cancer.

II. CIRCADIAN RHYTHM AND LIVER DISEASES

A. Circadian Rhythm and Chronic Liver Disease

1. NAFLD

The role of NAFLD in the promotion of liver cancer is becoming an increasing concern. NAFLD can progress to non-alcoholic steatohepatitis (NASH) that is considered one of the main causes of end-stage liver disease, including cancer.[48,49] Understanding the mechanisms of NAFLD/NASH and finding effective therapeutic targets is necessary considering the global prevalence of NAFLD is ~ 24%.[48] Sleep

disorders have been associated with NAFLD,[50] and indeed obstructive sleep apnea (OSA), that can cause restlessness and sleep disturbances, is significantly correlated with steatosis, lobular inflammation and fibrosis in NAFLD patients.[51] Based on this information, changes in circadian rhythm may play a role in NAFLD severity, and in turn liver cancer progression.

At the molecular level, previous work found that the core clock gene RORα can enhance sterol 12α-hydroxylase (CYP8B1) transcription and subsequent bile acid composition changes and increased cholesterol levels[52]; however, this work was performed in the context of fasting and is not specifically related to NAFLD/NASH. CLOCK mutant ($Clk^{\Delta19/\Delta19}$) mice present with abnormal circadian rhythms, and eventually develop hepatic steatosis, hyperlipidemia, hyperglycemia and other hallmarks of metabolic syndrome.[53] Following this, another paper found unchallenged $Clk^{\Delta19/\Delta19}$ mice develop hepatic steatosis and steatohepatitis, which was accelerated in mice on a Western diet (WD) and further exacerbated in mice fed with WD and injected with lipopolysaccharide.[54] Another study has found that loss of X-box binding protein 1 (*Xbp1*) disrupts the 12-hour periodic rhythmicity, which in turn perturbs the oscillatory transcription of metabolism genes[55]; however, this study did not find changes in the 24-hour circadian rhythm of clock genes. Interestingly in a model of high sucrose diet fed mice, the amplitude of *RORγ*, *PER2*, *CRY1* and *CRY2*, and the midline estimating statistic of rhythms (mesors) of *RORγ*, *PER2* and *CRY1* were enhanced compared with control diet and high-fat diet (HFD) fed mice.[56] Interestingly, time-restricted feeding (TRF, 8–9 hours of food access during the active phase) prevented increased body weight, inflammation and metabolic disorders in multiple models of obesity, and furthermore temporary interruptions in TRF during the weekend did not disrupt the protective benefits.[57] Similarly, TRF protected mice from HFD-induced obesity, liver steatosis, inflammation and hyperinsulinemia, and these benefits were mediated by improved circadian oscillations and restoration of the circadian clock and subsequent target genes.[58] Opposite of this, one study fed mice with a high-fat high-sucrose diet at either the active phase

(night feeding, NF) or the inactive phase (day feeding, DF) to look at changes in associated damage and changes in the circadian clock.[59] It was noted that DF promoted liver steatosis via upregulation of lipogenesis genes (*Scd-1*, *Acaca*, and *Fasn*) and desynchronization of the hepatic circadian clock; these changes were not noted in NF mice.[59] These findings identify that feeding time is a significant regulator of liver damage and obesity, which is supported by other work.[60] In addition, the cannabinoid (CB)-1 receptor can promote hepatocyte lipogenesis through activation of the rhythmically expressed transcription factors SREBP-1c, ChREBP and LXR *in vitro*.[61] Importantly, it was found that histone deacetylase 3 (HDAC-3) is rhythmically expressed and corresponds with the expression of Rev-erbα (a circadian nuclear receptor), and HDAC-3 activity modulates lipid metabolism gene expression to maintain lipid homeostasis.[62] A thorough and concise review on the link between circadian rhythm and metabolism in NAFLD has been published previously.[63]

2. ALD

Alcohol consumption increases circulating levels of *PER1* and *CRY1*, along with disturbing *CRY2* oscillation patterns.[64] Other studies in mice have found that chronic alcohol consumption dysregulates circadian metabolism.[65] It was also observed that ethanol consumption altered feeding habits in mice, which may in turn perturb circadian clock gene oscillation.[66] Furthermore, this study found that alcohol altered circadian oscillations of hepatic gene transcription associated with metabolism.[65] In Drosophila models of alcohol exposure, ethanol-induced alterations in circadian rhythm are influenced by sex and age.[67,68] Similar to this, researchers have discovered that oscillations of both clock genes and CCGs are dysregulated in the livers of mice subjected to chronic ethanol feeding.[69] Importantly, this study noted that oscillations of hepatic metabolism genes are subjected to the same oscillation alterations following chronic ethanol exposure.[69] One study found the protective role of circadian rhythm in ethanol-induced liver injury.[70] Specifically, using liver-specific Bmal1 knockout (Bmal1-LKO) mice subjected to ethanol feeding had exacerbated

liver steatosis and hepatocyte apoptosis by down-regulating de novo lipogenesis and fatty acid oxidation via reduced AKT pathway and ChREBP activity, respectively.[70] This work is supported by a similar study wherein Bmal1-LKO mice have ethanol-induced hepatic clock disruption and altered rhythmic expression of lipogenesis and fatty acid oxidation genes.[71] Similarly, loss of BMAL1 exacerbated ethanol-induced liver inflammation and disrupted rhythmic expression of glycogen metabolism genes.[72] Interestingly, one study found that ethanol exposure altered hepatic oscillations of clock genes (BMAL1, CRY1, CRY2, CLOCK, PER1, and PER2) and CCGs; however, these disturbances were not noted in the SCN.[69] Further work on targeting the circadian rhythm during ALD is necessary.

3. PSC

Little work has been performed to understand changes in clock genes and circadian rhythm during PSC. It was observed an increase of CLOCK, CRY1, BMAL1, and PER1 mRNA expression in the liver of PSC patients compared with control.[73] Particularly, cholestatic rats were exposed to light or pinealectomy and had elevated expression of CLOCK, CRY1, BMA1 and PER1 compared with cholestatic rats subjected to a normal 12:12 hour light/dark cycle.[73] Opposite of this, the exposure of cholestatic rats to melatonin treatment reduced the expression of PER1, BMAL1, CLOCK and CRY1 compared with cholestatic rats receiving a normal day/night cycle.[74] These findings show a close association with melatonin signaling, circadian dysregulation and biliary and liver damage in cholestasis, including PSC.

B. Connection between Circadian Rhythm and Liver Cancer

There are a variety of epidemiological and loss-of-function studies that have demonstrated the essential function of core clock genes in reducing cancer risk, including liver cancer. Circadian rhythm disruptions such as jet lag, shifted work, sleep disorders, or shifted light stimulus were found as an independent risk factor for hormone-associated cancer types such as breast cancer and prostate cancer, as shown by epidemiological studies.[75,76] Knocking out of CRY1/CRY2 will increase the size of CCA.[77] Knocking out of PER or CRY would lead to fewer, but larger HCCs, while liver-specific knockout of BMAL1 mice developed a large number, but smaller size, HCCs.[78] Further, exposing these knockout mice to the chronic jet-lagged condition led to higher tumor incidences and faster tumorigenesis. Figures 2 and 3 described the interaction of circadian rhythm and liver cancer.

Further, studies have shown the direct role of clock genes in tumorigenesis and progression in liver cancer. The core clock gene Neuronal PAS domain protein 2 (NPAS2, analog of CLOCK) was found frequently upregulated in HCC and directly promoted cell survival of HCC via promoting proliferation and inhibiting mitochondria-dependent intrinsic apoptosis. The authors further found this oncogenic role of NPAS2 is promoted by transcriptional activation of Cell Division Cycle 25A (CDC25A).[79] Further, NPAS2 was able to increase the aerobic glycolysis and suppressed oxidative phosphorylation via hypoxia-inducible factor alpha (HIF-1α) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) axis.[80] Another study showed the expression of PER family, CRY2 and Timeless (TIM) was downregulated in HCC. Further, they found the negative correlation of PER2 and PER3 with the tumor size, and that the expression of CCG, WEE1 was downregulated in HCC, while the Cyclin B and CDC2 expressions are upregulated.[81] A loss-of-function study showed chronic jet-lagged mice showed reduced lifespan, and knocking out PER1/PER2, CRY1 and CRY2, or BMAL1 in the liver in chronic jet-lagged mice will cause spontaneous hepatocarcinogenesis, which is mostly due to the disruption of the cholesterol metabolism pathway and bile acid syntheses pathway in the liver.[78,82]

Although the core clock genes could act as oncogenes or tumor suppressors to promote or inhibit the tumor growth in liver cancer, core clock genes can be transcriptionally regulated by other oncogenes or tumor suppressors in liver cancer.[83] MYC proto-oncogene is an important regulator of cell growth and glucose metabolism,[84] and was
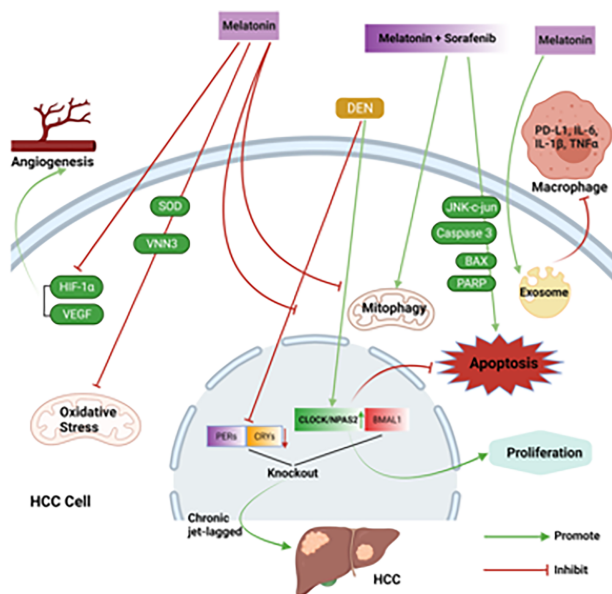
FIG. 2: Melatonin, circadian rhythm, and HCC. Melatonin and sorafenib treatment synergistically promote HCC cell line apoptosis and mitophagy. The expression of angiogenesis biomarkers and oxidative stress were inhibited upon melatonin administration in HCC cell lines. Exosomes derived from HCC with melatonin treatment could lower the PD-L1 levels and the secretion of inflammatory cytokines in macrophages. The core clock gene NPAS2 was upregulated in HCC, which promotes cell proliferation and inhibits apoptosis. Knockout PER1/PER2, CRY1 and CRY2, or BMAL1 in the liver in chronic jet-lagged mice will induce HCC. Melatonin treatment could prevent DEN induced increased expression of clock genes BMAL1, CLOCK and NPAS2, and decreased expression of CRY1 and PERs. Image was made with BioRender.
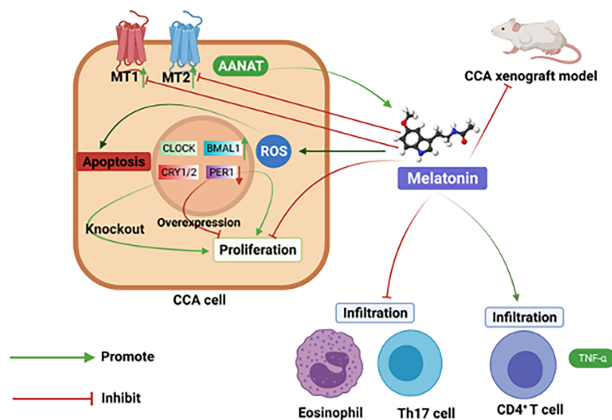

FIG. 3: Melatonin, circadian rhythm, and CCA. Overexpression of AANAT can reduce CCA cell growth. Melatonin treatment inhibits CCA growth in xenograft model and induces CCA apoptosis via activation of ROS pathways. HCC induced increased expression of MT1 and MT2 was suppress by melatonin treatment. The expression of PER1 was decrease in CCA samples, while the expression of BMAL1 was increased. Knocking out of CRY1/CRY2 can promotes CCA growth and overexpression of PER1 inhibits CCA cell growth. Eosinophil and Th17 cell infiltration were reduced in CCA treated with melatonin, while CD4+ T cell numbers and TNF-α expression was increased. Image was made with BioRender.

found to be regulated by clock genes,[85] while in turn being able to directly activate the REV-ERBs and inhibiting BMAL1 expression.[86] Expression of PER2 could be transcriptionally repressed by P53 to prevent binding to the heterodimer BAML1:-CLOCK. Further, the CCG could epigenetically be regulated by DNA methylation or non-coding RNA, which leads to the regulation of liver cancer-related pathways. miR-34a is a circadian oscillator that directly targets PER1 and inhibits its transcription.[87] A lncRNA called HULC was also shown to directly target the 5'UTR of CLOCK and promote its expression, thereby enhancing the expression of PER1 and CRY1 in HCC cell lines.[88] Circadian oscillation of histone modifications often correlated with the transcription of liver CCGs.[89]

Another way that core clock genes regulate liver cancer is through clock output genes. A bioinformatic study reveals many genes that are related to cell cycle, proliferation, apoptosis, metabolism, oncogenesis, and tumor suppression are oscillated under the control of the 24-hour molecular clock.[90,91] CK1P2 is transcriptionally regulated by REV-ERBs and RORs,[91] and WEE1 is controlled by BAML1:-CLOCK.[92] MDM2, β-catenin (oncogenes), P53 (tumor suppressor gene), Smad7, Cdk4, and Sox9 were also important and well-known CCGs that mediate tumorigenesis.[93,94] In addition, the liver, as the major center of the metabolic system, plays a central role in glucose metabolism, fatty acid metabolism, and bile acid synthesis.[95] These metabolism genes are controlled by the oscillation of the core clock genes. Therefore, disrupted circadian rhythm is also associated with metabolic disorder, especially via nutrient-responsive hormone receptors (NHRs),[32,96] which could lead to liver cancer.

Limited information about the role of circadian rhythm in CCA is present in the literatures. An early study of circadian rhythm in liver carcinogenesis (HCC/CCA mixed) found that disruptions of circadian oscillations via altered sleep patterns enhanced liver carcinogenesis in mice compared with mice with normal sleep cycles.[97] When looking at specific clock genes, Cry1$^{-/-}$ and Cry2$^{-/-}$ mice subjected to toxin-induced CCA developed more pronounced liver damage and had a higher incidence of tumorigenesis.[77] Moreover, human CCA samples have

reduced PER1, increased BMAL1, but no change in CLOCK or CRY1; loss of PER1 was noted in all CCA cell lines with varying changes of expression of other clock genes.[87] Importantly, the 24 hour oscillations of CLOCK, CRY1, PER1, and BMAL1 were all altered in human CCA cell lines, validating the fact that core clock signaling is disrupted in CCA.[87] Since PER1 loss was strong and found in all CCA cell lines, the overexpression of this clock gene was evaluated for CCA therapy. Overexpression of PER1 inhibited CCA cell growth, migration and invasion, and enhanced apoptosis both *in vitro* and *in vivo*.[87] One study found that PER1 alters the sensitivity of lung cancer cells to chemotherapeutic treatment,[98] whereas another study found that overexpression of PER1 inhibits glycolysis and cell proliferation in oral squamous cell carcinoma.[99] Few studies have been performed to evaluate circadian oscillation and CCA etiopathogenesis; therefore, this is an area of research that needs to be expanded.

III. MELATONIN, CIRCADIAN RHYTHM, AND LIVER CANCER

A. Melatonin, Circadian Rhythm, and Chronic Liver Disease

1. NAFLD

As mentioned in previous sessions, chronic liver conditions contribute to the progression of HCC and CCA. Studies have shown crosstalk of melatonin and circadian rhythm in these chronic liver diseases. Multiple studies have identified the protective role of melatonin intake in NAFLD and NASH models. Earlier studies indicated that melatonin treatment reduced hepatic steatosis and inflammation in rats fed with HFD, which was contributed to enhanced antioxidant activities.[100] One study found that melatonin administration was able to reduce body weight, hepatic steatosis, steatohepatitis and liver fibrosis in an HFD-induced model of NAFLD via reduced activation of tumor necrosis factor receptor-associated factors (TRAFs).[101] Similarly, it was observed that melatonin treatment reduced hepatic steatosis and fibrosis in an HFD-fed mouse model via enhanced mitochondrial function.[102] In hamsters

fed HFD, melatonin was shown to reduce hepatic steatosis via decreased lipogenesis pathway activity.[103] Lastly, melatonin treatment reversed hepatic steatosis, inflammation, ET stress and mitochondrial dysfunction and promoted autophagy via reduced miR-34a and sterol regulatory element-binding protein (SREBP1) expression in the presence of silent information regulator 1 (SIRT1) signaling.[104] These findings are important considering the link between melatonin, miR-34a and Per1 in the context of CCA.[87]

2. ALD

Although much work on melatonin in NAFLD/NASH has been performed, little is known about the impact of melatonin on ALD. Interestingly, circadian misalignment in night shift workers may increase the risk of developing alcohol-associated liver damage.[64] One study found that those with alcohol use disorders have severely impaired sleep along with significantly reduced serum melatonin levels.[105] Melatonin treatment has been shown to reduce alcohol-induced hepatocyte damage, inflammation and immune cell infiltration in mice.[106] Similarly, it is found that melatonin reduced lipid peroxidation in other chronic ethanol fed murine ALD models.[107]

3. PSC

Much work has been done to evaluate changes in melatonin signaling in models of PSC. It was demonstrated that melatonin was able to reduce cholangiocyte proliferation in a rodent model of cholestasis, which is important since hyperplastic growth of cholangiocytes can occur during PSC.[45] Furthermore, the effects of melatonin on cholangiocyte growth are mediated by binding to the MT1, but not the MT2, melatonin receptor.[45] These findings were confirmed in a rat model of obstructive cholestasis that was subjected to complete darkness (to increase pineal gland melatonin synthesis), wherein dark therapy reduced biliary hyperplasia, as well as liver fibrosis.[74] At last, the *Mdr2*^{−/−} mouse (murine model of PSC) have reduction of both biliary proliferation and angiogenesis after dark and/or melatonin treatment; this suggested that modulation of melatonin

therapy could be a therapeutic option for PSC.[108] To further validate these findings, one study found that chronic light exposure or pinealectomy (to reduce melatonin levels) exacerbated biliary proliferation and subsequent liver fibrosis in cholestatic rats.[73]

B. Melatonin, Circadian Rhythm, and HCC

In the liver, melatonin showed a beneficial role in HCC as anti-proliferation, antioxidant, inhibition of invasion, reducing angiogenesis and promoting apoptosis.[109] Sorafenib is the first-line chemotherapy drug to treat advanced HCC[110]; however, patients often develop chemo-resistance during the therapy leading to the failure of treatment.[111] Therefore, seeking options to increase the sensitivity to sorafenib will be important in clinical settings. Melatonin and sorafenib treatment synergistically inhibit HuH-7 cell growth via promoting the apoptosis and activation of caspase-3 and JNK-c-jun pathway.[112] Another study showed melatonin increased the sensitivity of HCC cells to the sorafenib in two different cell lines, HepG2 and HuH7. Further, melatonin was found to induce susceptibility to the sorafenib in HepG3 accompanied by enhanced PARP hydrolysis expression and BAX and mitophagy when melatonin co-administrated with sorafenib.[113] The interplay among melatonin, circadian rhythm, and CCA is shown in Fig. 2.

Resistance to apoptosis is one of the strategies for HCC progression, evidenced by the upregulation of inhibitor of apoptosis proteins (IAPs).[114] Melatonin administration could inhibit the expression of Survivin and X-linked inhibitor of apoptosis proteins (XIAP) in HCC cell lines.[114] Further, melatonin also inhibits angiogenesis, proliferation and oxidative stress. For example, the expression of angiogenesis biomarkers (VEGF and HIF-1α), cell proliferation, invasion, and migration were inhibited in HCC cell lines.[115] Another study showed that oxidative stress was inhibited upon melatonin administration in HCC cell lines as indicated by the enhanced gene expression of anti-oxidative stress biomarkers superoxide dismutase and vascular non-inflammatory molecule 3.[116] Interestingly, in HCC patients lower one-year survival following liver transplantation is associated with lower serum melatonin levels prior

to transplant.[117] Moreover, single nucleotide polymorphisms in the MT1 are associated with increased risk of HCC development.[118]

Immunotherapy has recently become one of the promising methods for treating HCC. It refers to any treatment that modulates the immunity, which could be a relatively safe option for treating patients who failed to respond to the chemotherapy drugs.[119] The PD-L1 level was found correlated with the HCC recurrence. It is noticeable that melatonin treatment-derived exosomes could lower the PD-L1 levels and the secretion of inflammatory cytokines [interleukin (IL)-6 to IL-10, IL-1β, and tumor necrosis factor alpha (TNF-α)] in macrophages when treated in HCC *in vitro* and *in vivo*.[120] Furthermore, the study found that this modulation of immunity was through inhibition of STAT2 pathway in macrophages.[120]

Conversely, melatonin was found to modulate the clock gene expression that was disrupted in an HCC rodent model. This study showed enhanced expression of core clock genes BMAL1, CLOCK, NPAS2, RORs, and Sirt1 but decreased expression of CRY1, PERs, and CK1ε in DEN-induced mice model.[121] However, the melatonin treatment could prevent this dysregulation induced by DEN via upregulation of the MT1 receptor.

C. Melatonin, Circadian Rhythm, and CCA

Considering the close association between melatonin, circadian oscillation and liver damage in different chronic liver diseases that increase the risk of CCA development, understanding disturbances in melatonin/circadian rhythm in CCA is warranted. The interplay between melatonin, circadian rhythm and the CCA is shown in Fig. 3. One study has found that melatonin serum levels, as well as biliary expression of AANAT (synthesizes melatonin) are significantly reduced in human CCA samples compared with control.[74] These findings were validated in human CCA cell lines.[74] The overexpression of AANAT in CCA cell lines were able to reduce cell growth, demonstrating an autocrine role for melatonin in modulation of CCA outcomes.[74] Furthermore, CCA growth was reduced with melatonin treatment in xenograft models.[74] In line with this,

others have demonstrated that melatonin induces CCA apoptosis via activation of reactive oxygen species pathways.[122] Another study noted that melatonin treatment reduced tumor size in hamster through increased mitochondrial antioxidant activity.[123] These findings are unsurprising considering the largely known impact on antioxidant activity of melatonin. In line with melatonin prevention of CCA progression, another study evaluated melatonin's immunomodulatory effect. Melatonin treatment reduced eosinophil and Th17 cell infiltration in CCA, and further reduced Foxp3 expression in a hamster model of CCA.[124] In this same study, melatonin enhanced CD4+ T cell numbers and TNF-α expression suggesting that melatonin may be therapeutic for CCA treatment via immunomodulatory events.[124]

Melatonin is able to signal through its receptors, MT1 and MT2. It was noted that both MT1 and MT2 expression increased in human CCA cell lines and human biopsy samples, and melatonin treatment was able to suppress the expression of these receptors.[125] However, this study did not evaluate changes in the downstream mechanisms of MT1/MT2 in CCA. Although modulation of MT1/MT2 has been noted in models of biliary hyperplasia, the impact of targeting these receptors in CCA is unknown. Considering that MT1 and MT2 can have differential effects on damage, understanding their distinct mechanisms in CCA is needed.

IV. TREATMENTS RELATED TO CIRCADIAN RHYTHM AND MELATONIN

The possible modulation of melatonin axis for cancer treatment in humans has been postulated and suggested by several experimental data. For instance, changes of urinary levels of melatonin metabolites or its peak blood levels have been associated with the risk of breast cancer.[126] On the other hand, some data suggests a possible relationship between circadian rhythm derangement (such as that occurring in night shift workers) and tumor onset, despite the findings seeming controversial.[127,128] As reported in the previous paragraphs the main primary liver cancers in humans are HCC and CCA, and both are recognized as relevant risk factors

chronic liver diseases.[129] Therefore, the attenuation or healing of underlying liver disease may possibly reduce hepatic cancer development. In this perspective a study based on 30 NASH patients treated with a 12-week course of melatonin (10 mg/day orally) are encouraging results since a significant decrease of liver enzymes was observed.[130] Possible effects of melatonin on persistent liver damage also came from a trial examining melatonin treatment (at the same dosage) in subjects with chronic statin-induced liver toxicity.[131] Again, administration of this neurohormone determined a reduction of transaminase levels of nearly one half. With regard to liver injury, another study focused on its high-dose administration (50 mg/kg body weight) in patients undergoing liver resection.[132] Results evidenced reduced levels of liver enzymes and intensive care unit (ICU) stay with the use of melatonin; however, these differences did not reach statistical significance. Clinical observations on melatonin and HCC come from two studies.[133,134] In the first, a basket trial comprising 35 patients with advanced cancer of digestive system, six patients with HCC were included. Treatment schedule included melatonin (50 mg/day) and IL-2 (3 million IU/day subcutaneously), and just one HCC patient had disease progression during treatment. More interestingly, in the same group, one subject exhibited a complete response.[134] A following study examined the possible beneficial effects of melatonin in patients undergoing TACE for HCC.[133] Melatonin therapy (20 mg/day) improved TACE efficacy and its two-year survival rate (40% melatonin vs. 26% control; $p < 0.05$). Finally, a more recent case report described the treatment with IL-2, Bacillus Calmette Guerin (BCG), and melatonin (20 mg/day) in a woman with HCC.[135] After a prolonged course of these drugs this patient exhibited vanishing of tumors and a decrease of α-fetoprotein to normal value. No other clinical attempts have been reported so far in HCC, as well as in CCA. In conclusion, several preclinical data together with the good safety of melatonin treatment may suggest its testing and possible use in liver cancers. Moreover, the most effective drugs employed for HCC systemic therapy are those (such as sorafenib, regorafenib, lenvatinib and so on) that inhibit neoangiogenic cancer processes. In this perspective, melatonin has been demonstrated to impair vascular growth in several tumor models including HCC which underscores is therapeutic potential for treating liver cancer.[136] Another approach may be represented by chronotherapy since several clock genes regulate liver biological activities including those possibly related to primary liver cancers development.[137] In a human study on 24 subjects with metabolic syndrome, the effect of a restricted food intake time (< 10 hours) to adapt to biological clock was endorsed.[138] There was a significant improvement in all major metabolic indicators (blood pressure, BMI, blood lipid levels and others) after a 12-week course of this restriction. Unfortunately, liver injury was not examined in this cohort of patients, but it is possible to speculate that this manipulation might also attenuate other metabolic syndrome complications such as NAFLD and NASH. Chrono-chemotherapy consisting in delivery of drugs according to particular biologic clock phases is also an approach under evaluation. A phase I study in the early 90s restricted traditional chemotherapy (cisplatin and oxaliplatin) according to circadian rhythm in (a 16-hour slot daily) in comparison with continuous infusion. Nine patients with HCC and two with CCA were included in the study. An improved outcome was not observed; however, administration according to circadian phases was associated to a decrease in adverse effects (neutropenia, paresthesia and vomiting) also allowing an increase of the oxaliplatin dose administered.[139]

A further experience on HCC has been published in abstract form.[140] Twenty-one patients with this primary tumor were divided in two group. All received a regimen based on the administration of liposome coated cisplatin, but one group received the drug according to daily peak of DNA methyl transferase gene expression while the remnants followed the standard time schedule. Despite the limited number of patients in the study, subjects following a chronotherapy approach had improved results (more frequent and relevant decrease of tumor size and α fetoprotein serum levels). There is no further experience published on primary liver tumors so far; however, at present level of our knowledge and despite some positive results, the chrono-chemotherapy approach has not been demonstrated to significantly improve cancer outcomes in human.[141]

V. CONCLUSION AND FUTURE PERSPECTIVES

Liver cancer is challenging to treat and highly related to circadian rhythm disturbances. Understanding circadian rhythm and its molecular mechanisms have provided strong implications for the relationship between circadian rhythm, melatonin, and liver cancer. Further, the crosstalk between core clock genes and their upstream and downstream regulators gives us a broader view of the more complicated yet profound effects of circadian clocks on liver cancer. Additionally, the increasing incidence of NAFLD globally alludes to the need to seek more connections between the nutrition homeostasis pathway and the circadian clock, which provides promising future directions to help relieve the burden of public health and eventually decrease the incidence of HCC and CCA. Indeed, the interplay between feeding time, metabolic change, gut microbiota, metabolism, and the circadian rhythm has played a pivotal role in alleviating NAFLD-related hepatic inflammation and fibrosis. Finally, the chronotherapy and circadian rhythm-based clinical application showed us great potential for the impact of circadian research in liver cancer. The circadian-related studies changed our mindset for medical practice, making both the physician consider the fact of light, feeding time and how to take advantage of circadian intervention to maximize the effects of treatment. In the future, more applications from the bench to the bedside are expected to see to improve the patient's life, and novel target or candidate pathways crosstalk with circadian regulation are expected to uncover when elucidating the tumorigenesis and progression of cancer.

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