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Research progress on the role of circadian clock genes in the occurrence and development of hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) stands as the primary cause of cancer-related death globally, being characterized by a complex interaction of genetic, epigenetic, and environmental factors. Circadian rhythm is the internal timekeeping mechanism that aligns an organism's physiological and behavioral patterns with the 24-hour day-night cycle. The dysregulation of circadian clock genes is closely linked to the pathogenesis of HCC. The molecular mechanism of HCC is complex and multifactorial. This review reviews the expression and function of CLOCK genes including BMAL1, clock, PER2, CRY1, CRY2, NPAS2, Timeless, NR1D1, NR1D2 and CSNK1D in HCC. The review argues how dysregulation of these genes leads to abnormal cell cycle control, impaired apoptosis, and altered metabolism, which are hallmarks of HCC, and also covers epigenetic modifications and non-coding RNA that regulate the expression of genes in liver cancer circadian rhythms. This review discusses the current understanding of the role of different types of circadian rhythm genes in cancer risk and progression. And the idea of using the circadian system "chronotherapy" as a potential treatment. Further research into the underlying mechanisms of HCC circadian rhythms is warranted and may lead to the innovative approaches in cancer prevention and treatment.

Keywords: Hepatocellular carcinoma, Circadian clock gene, Regulation, Chronotherapy, Cell cycle.

1. Introduction

The International Agency for Research on Cancer (IARC) presents an overview of liver cancer and its subtypes on the basis of global, regional, and national figures in 2018. Liver cancer stands as the sixth encounter rate of all cancer forms, as well as third for the death rate worldwide [1]. In 2018, around 826,000 people had liver cancer globally, among whom 661,000 were of HCC, making it 80% of all primary liver cancers in both men and women. North Africa, East Asia, and sub-Saharan Africa are among the highest HCC proportions of total liver cancers, comprising about 94.9%, 87.2%, and 85.9%, respectively. Conversely, Northern Europe, the Caribbean, Central America, and Oceania accounted for lower HCC proportions of 35.9%, 50.5%, and 50.7% respectively [2]. Chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infections, Nonalcoholic fatty liver disease (NAFLD), aflatoxin exposure, and alcohol consumption were established as significant risk factors for HCC [3]. Also, obesity and diabetes constitute risk factors for this liver malignancy as well [4]. Cirrhosis is still considered one of the main causes of HCC, but its frequency is small and still questioned these days [5].

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The circadian rhythms' involvement in cancer is intricate and multifaceted, depending on the gene, the type of cancer, and the context of the cell. Certain experimented results indicate that some circadian genes may operate as tumor suppressors, while others suggest that they may play a role in tumor promotion and in the development of resistance to treatment. The communication of these genes, in fact, holds rhythmic coordination of the organism's physiological mechanisms and the outside environment and, in turn, the organism adapts to daily fluctuations of its surroundings. This gives clear insight into the gene functions, on account of which accurate diagnosis and treatment is possible.

2. Key circadian clock genes and their functions

Currently, there are 15 recognized circadian genes in mammals, which include: CLOCK, ARNTL/BMAL1, PER1, PER2, PER3, CRY1, CRY2, NPAS2, Timeless/TIM, NR1D1, NR1D2, CSNK1E/CKIε, and RORA, RORB, RORC [6]. The relationship between circadian rhythm genes and HCC is a significant area of study, as these genes play a crucial role in regulating cellular processes, including cell cycle progression, which can be disrupted in cancer. Within this system, the CLOCK-BMAL1 heterodimer is a crucial transcription factor that orchestrates the circadian clock's oscillation [7]. This complex activates the transcription of various downstream genes, including those encoding the PER and CRY proteins, which are involved in a negative feedback loop that regulates the clock's timing. The CLOCK-BMAL1 dimer also influences other circadian genes, such as NPAS2, which can partner with BMAL1[8].

3. Abnormal patterns of circadian clock gene expression in HCC

The CLOCK and BMAL1 genes show mammalian circadian clocks' critical components, which may be involved in HCC development and progression. The mRNA expression levels for CLOCK are significantly higher in HCC tissues than in adjacent normal liver tissues. It indicates that CLOCK may have a role in HCC, either by inducing the expression of HCC-related genes or by regulating the growth of liver cancer cells, which is consistent with other circumstantial evidence [9]. Meng Qu et al identified the cell cycle related process in HCC, which occurred by BMAL1/CLOCK targeting and were reflected in the G1 and G2/M phases of cell cycle. The diminutive gene expressions of Wee1 and Ccnb1 indicate a down-regulation in mouse BMAL1-knocked out livers compared to wild-type counterparts. In addition, p21 and Myc expressions were regulated to emphasize the disturbance in the transcriptional regulation of cell cycle genes. These findings provide evidence of the potential role of BMAL1 and CLOCK in ensuring the cell cycle is organized and regulated in HCC by modulating these checkpoint genes to affect the G2/M phase. Notably, Wee1 kinase is down-regulated in BMAL1-deficient mouse livers, which is known to enhance tumor proliferation and apoptosis in the case of liver cancer. It could be observed that Wee1 suppresses the apoptosis process and may thus contribute to the development of HCC, a phenomenon that is typical of the interdependence of communication between BMAL1 and CLOCK proteins [7]. In summary, circadian rhythm dysregulation of CLOCK and BMAL1 genes might lead to alternating cell cycles, which may initiate or promote HCC. Understanding the mechanisms is crucial for the precision therapeutics that might use the pathogenic clock to attack the main underlying cause of HCC development. These investigations provide critical perspectives on the context of circadian genes in HCC and stimulation of further basic and clinical research in this field.

The PER2 expression in 80 HCC samples was significant lower than adjacent non-cancerous liver tissues. An examination of the differences in the methylation status of the PER2 would help identify the HCC prognosis and survival rate of hypermethylated patients at different CpG sites was different. This indicates that PER2 is probably one of the players in the progression and prognosis of HCC and might even serve as a biomarker for the survival rates of HCC patients[10]. Moreover, it was found that the expression of CRY2 was reduced in 65 HCC patients' tissues with the corresponding real-time observation in 233 public databases, provided certain evidence for the significant decrease of CRY2 expression in HCC tissues in comparison with control group. This trend was corroborated in experiments with four distinct HCC cell lines, where the overexpression of CRY2 was observed to suppress the growth and invasive capabilities of the cells [11]. Furthermore, the positive expression rate

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of CRY1 in liver cancer tissues was significantly lower- by 64.58% when compared to normal liver tissues and by 23.75% when compared to paracancerous tissues [12]. In a separate cohort consisting of 185 pairs of tissue samples, CRY1 expression in liver cancer tissues was consistently found to be diminished relative to the adjacent non-cancerous tissues [13]. The diminished expression of CRY1 could potentially be linked to the presence of larger tumor diameters and incomplete tumor envelopes, suggesting a possible correlation between CRY1 expression levels and tumor aggressiveness [12]. It can be concluded as a deduction that the expressions of PER2, CRY1, and CRY2 proteins are all downregulated in the HCC cells.

The demonstrated increase of NPAS2 levels in tissue samples collected from HCC patients is obvious. The release of NPAS2 is largely responsible for the rise of the phenomenon of aerobic glycolysis, or the Warburg effect, which in cancerous cells acts as metabolic reprogramming. NPAS2 achieves this by enhancing the expression of key glycolytic genes, such as HK2 (Hexokinase 2), PKM2 (Pyruvate Kinase M2), and Glut1 (Glucose transporter). Furthermore, NPAS2 was shown to diminish PGC-1α, which is a key component of the process of mitochondria biogenesis, by the transcription process. This downregulation consequently leads to the inhibition of mitochondrial respiration, which is often replaced by glycolysis for energy production in cancer cells. Additionally, NPAS2 has been shown to directly upregulate HIF-1α (Hypoxia-inducible factor 1-alpha). This association and the function of HIF-1α as NPAS2 are transcriptional regulators of glycolytic genes and mitochondrial function regulators help cells in HCC to enhance the shift to glycolysis and probably contribute to the aggressive phenotype of these malignancies. The combination of NPAS2, HIF-1α, and metabolic pathways may help to explore the underlying mechanisms and the possible metastatic mechanisms of HCC, which may innovate the future therapy [14]. The analysis of publicly available liver cancer data indicates that the Timeless gene expression levels are elevated in HCC tissues compared to the adjacent non-cancerous tissues. Glucose uptake was terribly decreased, maintenance of normal glucose in the body also decreased and flow of lactate into cells happened too. These effects were present in the experimental group and not in promotion. Besides, a rise in the pH of the extracellular culture medium and a sudden upturn in the oxygen consumption rate of the cells were both observed, which provide clues for the changes in the metabolism. To add, the inhibition of mitochondrial oxidative phosphorylation within hepatoma cells by Timeless was also discovered, which is the vital part of respiration and energy generation within the cells. These outcomes accentuate the highly decisive role of Timeless in the changing of glucose metabolism and the consequent de-sessions of liver cancer cells. It shows glucose metabolism regulation by Timeless underlines not only the specialization of it for the growth and survival of tumors cells, but also it is possible as a therapeutic target for HCC [15]. Therefore, further investigation regarding the underlying mechanisms which inherent Timeless gene, and metabolism regulation, and development of new liver cancer treatments could be done.

The expression of NR1D1 in tumor samples was not shown to be statistically different from that in nearly identical normal tissues [16]. In addition to this, the expression of NR1D1 in HCC tissues was identified as being at much higher levels as compared to the adjacent normal liver tissues. This super irresponsibility of NR1D1 probably contributes to both the genesis and the aggressive rates of the patients with HCC but we need another ever-aware independence of biomarker to monitor the disease like HCC [17]. Further studies on the function of NR1D1 and NR1D2 in HCC will provide great insights into disease characteristics and should be considered as exploration opportunities for developing new therapies targeting these genes. CSNK1D, which codes for casein kinase I delta as its product, and this gene showed an overexpression level in HCC tissues compared with normal liver tissues. This overexpression linked to adverse outcomes in patients diagnosed with HCC, suggesting that CSNK1D may play a role in the progression of the disease and potentially serve as a prognostic indicator [18].

4. Role of circadian genes in tumor progression and metastasis

In addition, the analysis of the CLOCK gene expression in patients with HCC has shown that individuals with the lower expression of the gene CLOCK had longer overall survival and relapse-free survival rates

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than the counterparts with higher CLOCK levels. These findings imply that low expression of the CLOCK gene, which affects the dysregulation of vital cell cycle modulator genes; may tend to be positively associated with good prognosis in patients with HCC. On the contrary, reducing the expression of the CLOCK gene led to lower levels of HepG2 cell proliferation, which manifests that CLOCK may further nourish the tumorigenesis of the liver cancer cells. Therefore, it is likely that CLOCK could be one of the potential targets in the development of treatment strategies for the arresting or curtailing of tumor proliferation. It remains to be explored how exactly CLOCK operates and whether it affects the majority of cases occurring in humans [19]. Further investigation is needed to elucidate the mechanisms by which CLOCK modulates the development of HCC and subsequently checks the ability of CLOCK as a target of novel therapy. PER2 stimulates liver-carcinoma formation, thereby, the translation of PER2 can be as a liver cancer promoter directing from initiation to progression [20]. Studies revealed that when CRY2 is overexpressed, it leads to a significant decrease in the growth and cellular invasiveness of HCC cells. This suggests that CRY2 maybe a tumor-suppressor, potentially the malignant development of HCC[11].

The interplays of NPAS2 and Timeless circadian genes are deeply involved in the process of HCC development. These genes mediate cell cycle progression by promoting cell proliferation and suppressing apoptosis. Reprogramming of glucose metabolism is manipulated by the tumor cells, in order to be able to resist apoptosis and eventually promote their growth [21]. This reprogramming of metabolism comprises the enhancement of glycolysis as well as the suppression of oxidative phosphorylation, the simultaneous processes that are important for cancer cell metabolism. NPAS2 is commonly upregulate in HCC. The contribution of the heterodimer NPAS2 and BMAL1 to the development and aggressiveness of cancer cells through regulating the cell cycle is evident [22]. Nevertheless, both in vitro and in vivo provide experimental data that support the fact that the enhancement of metabolism by tumor cells of glucose metabolism through NPAS2 is the most crucial determinant of HCC growth and metastasis. This metabolic shift described as "Warburg effect". It is characterized by the preference of glycolysis as the source of energy for energy production, even under aerobic conditions. It is a hallmark of many cancer cells, NPAS2 indicates that it could be a possible therapeutic target for HCC [13]. Finding strategies that can interfere with the formation of NPAS2-BMAL1 dimer or inhibiting its influence on the downstream activation of the metabolic pathways might offer a new way for therapies against liver cancer. One of the mechanisms that disrupted apoptosis in these cells is considered to be the inhibiting of the tumor suppressor protein p53 by Timeless genes. Cancer cells can generate the necessary energy and biosynthetic precursors by regulating the metabolic pathways to promote glycolysis and regulating oxidative phosphorylation, to support their rapid growth and division [15]. This metabolic shift enables liver cancer cells to thrive in an environment that would typically trigger cell death in normal cells, which driven in part by the downregulation of p53. The mechanisms of promoting tumor growth and progression emphasizes the importance of identification and restraint the metabolic changes involved in the development of effective therapies against HCC. Therapeutics that target the metabolic regulators and pathways via p53 and other pathways could offer a new approach to restoring normal metabolic functions and thus, minimizing their capacity to proliferate continuously. Exploiting the action by invoking the role of mitochondrial metabolism is paramount, both from the p53 mutation contribution part. Therefore, further researches to figure out the connection between metabolic reprogramming and tumor suppressor proteins p53 in developing liver cancer combating approaches.

The targeted disruption or "knockdown" of the NR1D2 gene in HCC cells has been found to result in a decrease in cell proliferation and overall cell viability. The fact that the NR1D2 can be classified as an important gene for the development and maintenance of HCC cells. Moreover, the depletion of NR1D2 has been demonstrated to severely impair the abilities of HCC cells to undergo division, migrate, and invade, which are among the predominant features of the processes in the spread of cancer cells. This conclusion suggests that NR1D2 is a stimulator of cancer spreading or metastasizing, in which the cancer cells move away from the original tumor site to finally settle in other parts of the body. Furthermore, NR1D2 may have a part in the promotion of the epithelial-to-mesenchymal transition

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(EMT) that occurs during the development of HCC. EMT is the process of lining of tissues and organs, undergo genetic and phenotypic changes that enable them to take on properties of mesenchymal cells. This is one of the factors that causes the spread of cancer, as when the cells become flexible and invasive, they can be easily distributed throughout the organism. The implication of NR1D2 in EMT implies its therapeutic potential in gut-derived HCC[17]. The involvement of NR1D2 in the EMT may be developed as a way to slow down or even stop the progression of HCC, especially in regard to its metastatic. Further studies on the mechanisms featuring NR1D2 in the specified cellular processes would lead to seeking specific therapies for the patients with HCC.

Through raising protein DVL3's stability, CSNK1D is a critical regulator of various cellular processes which strengthen the Wnt/β-catenin signaling pathway. The aberrantly activated of this pathway may cause the proliferation, migration, and invasion of HCC cells, and may become one of the causes of aggressiveness and progression of liver cancer. The Wnt/β-catenin pathway is a component of the mechanisms that decide the types of cells, tissue regeneration, and homeostasis. On the other hand, its constant activation may provoke proliferative which leads to uncontrolled cell growth and the acquisition of metastatic traits in the context of cancer. The finding found that CSNK1D influences this pathway by stabilizing DVL3 highlights the complex interplay of signaling components in cancer development. The overexpression of CSNK1D upregulated spheroid formation in HCC, whereas the silencing downregulated stem cell-like properties in both cancers. CSNK1D could possibly be a regulator of an aggressive behavior in HCC cells. Through this study, we propose that targeting the CSNK1D-DVL3-Wnt/β-catenin axis as a therapeutic strategy might be a promising option for HCC. The antagonism of CSNK1D per se or its function, or the disintegration of DVL3-CSNK1D dialogues, could halt the hyperactivation of Wnt signaling, and subsequently may be against HCC progress[18]. Research targeted towards comprehending structural dynamics of CSNK1D that outperform DVL3 would be beneficial in diversifying potential treatment avenues involving this pathway in liver cancer.

However, other circadian rhythm genes, CRY1, PER2, PER3, and the ROR family members, specific roles in HCC have not been fully resolved. There was no discernable difference in the colony formation of CRY1 overexpression in two cell lines when they were compared to the control group[13]. This lack of statistical significance in the proliferation assays indicates the role of CRY1 in HCC cell growth and apoptosis is not as clearly defined as that of CRY2. Therefore, it cannot conclude CRY1 has a direct inhibitory effect on the proliferation or promotes apoptosis in HCC cells. CRY1 functions in HCC cell behavior needs more experimental evidence, as well as whether other potential impacts of CRY1 on the cells that was not captured in the previous tests. It may include extended exploration of CRY1's influence on HCC through the study of other cellular processes or different experimental variables. These would unravel the additional dimensions of these tumors beyond HCC. The precise expression levels and the functional impact of PER1 and PER3 within the context of HCC have not been definitively correlated with the disease. Further research is required to understand the potential roles and interactions of these circadian genes in the development and progression of liver cancer. The specific research needed to be completed to study the mechanisms of RORA, RORB, and RORC in HCC. These genes encode for members of the ROR family of nuclear receptors, which are known to play roles in various biological processes, including cell differentiation, circadian rhythms, and metabolism. However, ongoing research is needed to delineate the precise functions of RORA, RORB, and RORC within HCC cells. This includes examining their expression patterns, assessing how their activity might drive tumor development and spread, and how they might be used as potential therapeutic targets.

5. Challenges and Future

While advancements have been made in understanding the relationship between circadian rhythm, or "clock" genes, and liver cancer, significant challenges remain. The precise mechanisms played by the genes in the initiation and progression of HCC has not been fully understood. The expression levels of clock genes affected by myriad of causes, for example, environmental exposures, lifestyle habits, and genetic factors[23]. One major barrier in performing the study of liver cancer is the interplay of these factors. Understanding the intricate web of how these genes interact with various internal and external

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factors is essential for the progress of knowledge on this field. Future research on clinical studies by collecting and analyzing more extensive clinical data is needed to address the current challenges. Through the process of rigorous experimental validation, scientists will have the chance to discover the complex functions of clock genes in HCC and create more targeted and effective treatment strategies. The intertwined part about the role of clock genes within the tumor microenvironment, their potential use as a biomarker for early diagnosis, prognosis, or as therapeutic targets will be some of the critical areas of emphasis. Moreover, understanding how disruptions to normal circadian rhythms caused by shift work or jet lag, might influence liver cancer risk could provide new preventive measures. To conclude, although a lot of progress have been made, the full realization of these developments and the integration of the role of clock genes into HCC are still ongoing. It is needed to have a collaboration of multiple fields to overcome the current problems and explore new treatments, including genetics, molecular biology, pharmacology, and clinic oncology.

Chronotherapy is a therapeutic approach that aims to optimizes medical treatment by regulating it with the body's circadian rhythm. The method can be classified into two main types. One is the Sleep-Wake Cycle Adjustment. It is meant to modify the wake-sleep cycle of the patients, which is beneficial for managing the symptoms and improving the well-being for patients with certain diseases. By adjusting the timing of sleeping and wakefulness, this treatment provides relief for conditions that are affected by the body's circadian rhythms. The second approach is Circadian Rhythm-Tailored Treatment, which considers the situation when the individual's circadian rhythms participate in the therapeutic efficacy's improvement. It involves timing the administration of medications or therapies to coincide with the body's peak periods of receptivity, thereby potentially increasing their effectiveness and reducing the side effects[24]. Youfang Liang et al showed the data that Timeless was in stronger negative correlation with masitinib, GSK1070916, methotrexate, navitoclax, PI-103, SNX-2112, and 5-fluorouracil. The results suggest that Timeless inhibit Cluster 1 or activating Timeless may enhance the sensitivity of chemotherapy to specific anticancer drugs[25]. The role of chronotherapy in the treatment of HCC is an exciting frontier in oncology. It is a complex approach of the organism's biological patterns, thus laying the foundation for a more personalized and effective treatment based on the body's natural rhythms.

6. Conclusion

Nevertheless, the relationship between clock genes and HCC is still a new research field, and clock genes regulating circadian rhythms have been found to be dysregulated in HCC, promoting the occurrence and progression of tumors. There still have some limitations of the current understanding of this relationship. The circadian system is very complex, which incorporates a plethora of feedback loops and interactions within a variety of cellular pathways. It is difficult to separate individual clock genes' independent functions since clock genes are involved in very complicated network interactions occurring in HCC development. Genetic alterations in clock genes may differ significantly among HCC patients, which may lead to the difference in study results and the development of individualized therapies. Although some clock genes already as biomarkers for HCC, there is a lack of fully validated biomarkers that can aid in early diagnosis, prognosis, and response to treatment. While clock genes represent potential therapeutic targets, but how to effectively regulate these genes without adverse effects is a major challenge[25]. Chronotherapy is a treatment approach based on circadian rhythms, offers the prospect of a new personalized approach to increase the efficacy of existing treatments by managing the body's circadian rhythm and overcoming the challenges associated with individual differences in circadian rhythms. Besides, it has been proposed as a way of optimizing cancer treatment, which improves the outcomes of patients. Huge achievements are made in understanding the role of clock genes in HCC, but the challenges and questions that needed to overcome still remain. Future research directions will be focused on the precise molecular mechanisms via which these genes exert HCC, effecting circadian rhythm-based treatments to improve the treatment outcomes of HCC, exploring molecular chain links involved in circadian rhythm, and overcoming hurdles associated with individual differences in circadian rhythms.

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