Research Article

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Association of circadian genes with HCC

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Bioinformatic analysis of genetic changes CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 proteins in HCC patients

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Abstract

Background and Aim: Genes related to the circadian rhythm control various biological processes. The aim of this study was to comprehensively investigate the mutational and mRNA profile of core circadian rhythm genes in hepatocellular cancer (HCC) samples.

Materials and Methods: In this study, the gene profile of a total of 369 patients with HCC was examined over the data obtained from the cancer genome atlas database through-cBioPortal. The effects of mutations on protein were examined by scoring the Polymorphism Phenotyping v2, Mutation Assessor, and SIFT-databases. While the association of genes with other genes was determined with the GeneMANIA-database, the association of expression levels in the genes with overall survival (OS) was evaluated with the Kaplan–Meier Plot database.

Results: As a result of the analyses, there were a total of 25 mutations. Decreased expression levels of PER1 (1.3e-05), PER3 (p=0.046), and CRY2 (p=1.8e-06) genes were found statistically associated with shorter OS. It was also found that increased expression levels of the PER2 (p=0.045) gene were associated with longer OS, and increased expression levels of the NPAS2 (p=9e-04) gene were associated with shorter OS.

Conclusion: In particular, changes in the PER1, PER2, CRY2, and NPAS2 genes may provide possible molecular targets in chemotherapy and immunotherapy for HCC patients.

Keywords: Circadian rhythm genes; CRY2; hepatocellular cancer; NPAS2; PER1; PER2

Introduction

Hepatocellular carcinoma is the sixth most common type of cancer worldwide and the third highest cause of cancer-related death.[1] Hepatocellular cancer (HCC) accounts for 85–90% of all primary liver cancers with increasing incidence and mortality.[2–4] Prevalence, late-stage detection, and its aggressive nature resulting in a median overall survival (OS) of and lt; 6 months.[5] In the current situation, early-stage liver resection and liver transplantation are merely curative treatment modalities for HCC. Moreover, other treatment modalities such as radiotherapy and chemotherapy are unsatisfactory due to limitations in understanding its pathogenic mechanism.[6] At present, targeted drugs, including sorafenib and regorafenib, have been able to improve the prognosis of patients with HCC. However, several problems continue to occur in these treatment protocols, including drug resistance, serious side effects, and other problems.[7] Therefore, it is important to elucidate the molecular mechanisms and identify new therapeutic targets for HCC.[8] Recent studies suggest that it is more effective to adjust cancer treatment protocols according to the circadian clock.[9] The molecular circadian clock is the one that coordinates the internal time with the external environment. It is an evolutionarily conserved system.[10,11] In mammals, the circadian rhythm can be synchronized with the 24-h environmental cycle created by the earth's rotation.[7] The circadian clock is important for the maintenance of cellular homeostasis and the management of many biological activities and has close relation with molecular events associated with tumorigenesis, called chronotherapy.[9] The evidence indicates that approximately 10% of the human genome is affected by circadian clock genes.[7] Analysis of these core circadian genes could lead to the discovery of biomarkers that indicate disease onset.[3] Liver diseases and hepatocarcinogenesis may interact directly or indirectly with the circadian clock. Although the relationship between circadian disorder and the progression of hepatocellular carcinoma is not fully understood; yet, it may be involved in the development of malignant tumors, stimulation of liver carcinogenesis, and tumor initiation.[7,10,12]

In this study, in light of the above information, data such as possible changes in circadian clock genes, genome profiles, mutation types, and survival analysis in patients with HCC were examined in detail by means of bioinformatics web tools.

Materials and Methods

Establishment of the Working Group

HCC dataset (n=369) was obtained from the cBioPortal database. Raw data are accessed through cBioPortal. cBioPortal is an open-access bioinformatics tool that provides data from the cancer genome atlas (TCGA). Data were downloaded on January 24, 2023. The flowchart of the experimental design is shown in Figure 1.

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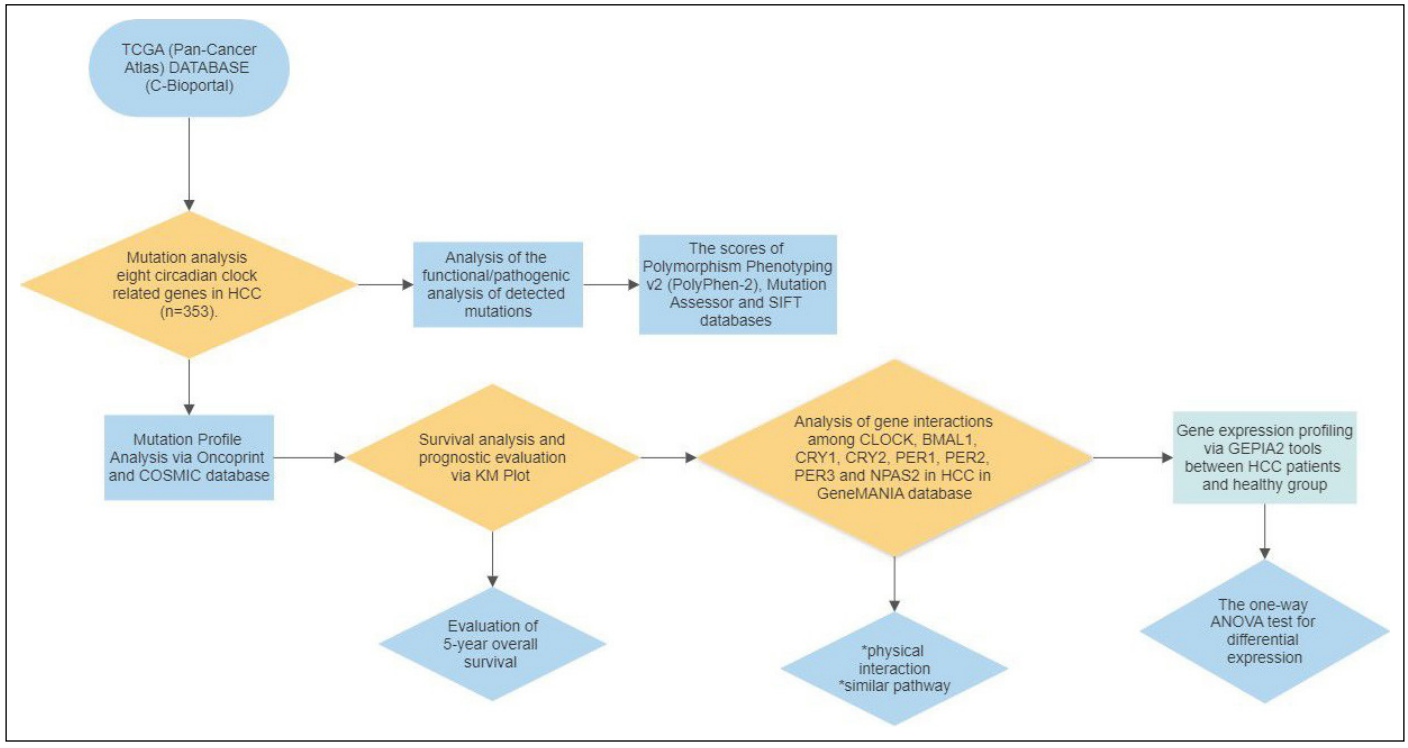

Figure 1. Flowchart of the experimental design.

Mutation Profile Analysis

cBioPortal web tool was used for mutation profile analysis. With OncoPrint, an interface option provided with this web tool, changes in the CLOCK, BMAL1 (ANRTL), CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes were monitored in patients with HCC. Amino acid position and localization, nucleotide change, cancer subtype, cancer stage, histological grade, and coexpression levels of selected genes were also evaluated using the cBioportal web tool. In addition, confirmation of somatic mutation was determined using the COSMIC (https://cancer.sanger.ac.uk/cosmic) database.

Survival Analysis and Prognostic Evaluation of CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 Genes in Patients with HCC

KM Plot (https://kmplot.com/analysis/), an online database of gene expression and clinical data, can be used to analyze associations between gene expressions and cancer survival rates. Among the types of cancer that can be analyzed is HCC.[13] We used this tool to understand the prognostic values of the expression levels of the circadian rhythm-related CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes in HCC patients. We also analyzed the number of patients, median values of mRNA expressions, 95% confidence intervals, hazard ratios, p-values, and other relevant information, as well as OS values of patients with HCC under the expressions of associated genes. Resources for databases include GEO, EGA, and TCGA.

Analysis of Gene Interactions Among CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 in HCC

GeneMANIA (https://genemania.org/) was used to understand the relationships of the studied genes, both among themselves and with other genes, and to establish a network.[14] GeneMANIA finds other genes associated with a set of input genes using a huge set of functional association data. Association data include protein and genetic interactions, pathways, coexpression, colocalization, and protein domain similarity.

Gene Expression Profiling Interactive Analysis Dataset Analysis 2 (GEPIA2)

GEPIA2 (http://gepia2.cancer-pku.cn/#index), an upgraded version of GEPIA, is a web-based data platform that can be used to compare tumor tissues and normal tissues. In addition to functions such as differential expression analyses, spectrogram plotting, correlation analyses, patient survival analyses, and similar gene detections found in its old version GEPIA, new functions such as survival maps, isoform usage profiling, loaded expression data comparisons, and cancer subtype classifiers are also available. It also offers customized analyzes where users can upload their own RNA-seq data and compare it to TCGA and GTEx samples while upgrading some of the original legacy functionality.[15]

Analysis of the Functional/Pathogenic Analysis of Detected Mutations

The scores of Polymorphism Phenotyping v2 (PolyPhen-2), Mutation Assessor, and SIFT databases were used to determine the possible pathogenicity and clinical effects of mutations detected in CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes. PolyPhen-2 is an online available bioinformatics tool that helps predict the possible effects of mutations on the stability and function of proteins using structural and comparative evolutionary analyzes of amino acid positions of possible mutations and SNPs. Based on a combination of all these features, the program estimates the probability of a mis-

sense mutation causing damage to the protein and provides the user with a score [probably damaging (score >0.9), possibly damaging (0.5< score ≤0.9), and benign (score ≤0.5) or unknown].[16] Mutation Assessor (http://mutationassessor.org/r3/) is a bioinformatics tool that uses a segmented multiple sequence alignment to reflect functional specificity and generates conservation scores for each column to represent the functional impact of a malformed variable. It gives the scoring results as low, medium, high, and neutral. The SIFT algorithm (https://sift.bii.a-star.edu.sg/) is a bioinformatics tool that predicts whether an amino acid affects its position and protein function based on sequence homology and physical properties of amino acids. The program classifies an amino acid change as either tolerable or harmful based on protein function. SIFT gives predictive results for missense variants as damaging (score and lt; 0.05) and tolerable (score ≥0.05).[16]

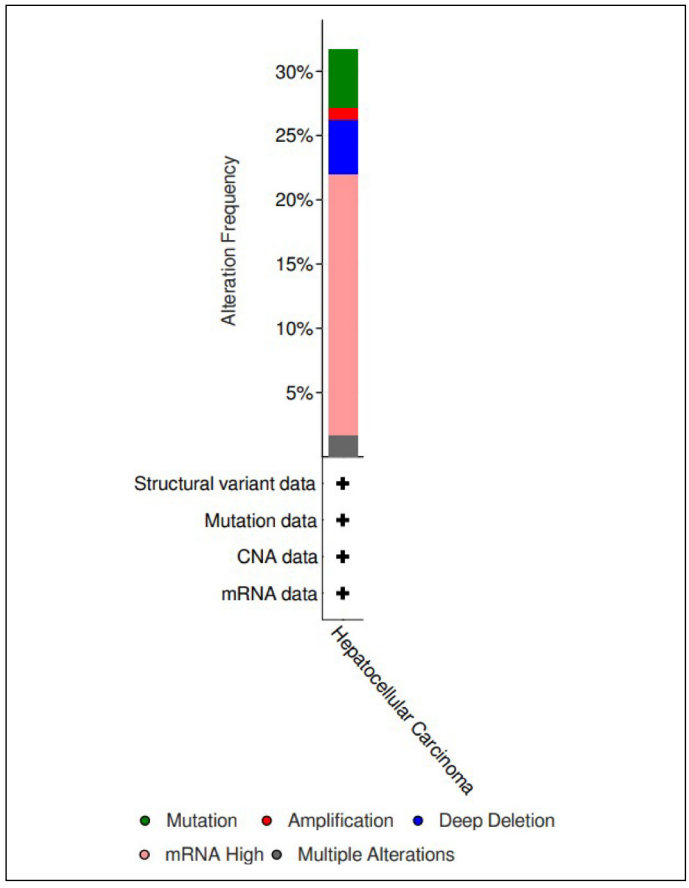

Figure 2a. Summary of changes in CLOCK, BMAL1 (ARNTL) CRY1, CRY2, PER1, PER2, PER3, and NPAS2 in hepatocellular cancer patients.

Statistical Analysis

We used the TCGA Pan-Cancer Atlas, a dataset from cBioPortal, to obtain patient raw data and to statistically evaluate the range of mutations in the CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes and the coexpression levels of the present genes. Other statistical analyses used in the evaluation of study data were performed on the GEPIA2 database. The one-way analysis of variance test was used to measure differential expression (HCC patients [n=369] and healthy control [n=160]). Since the variances are homogeneous (equal variances assumed), the post hoc Tukey test was preferred.

For the survival analysis, a KM plotter was applied, with all default settings and recurrence-free survival was preferred, with the auto-best cutoff values and J-best probe set. All possible cutoff values between the lower and upper quartiles were determined, and the best-presenting threshold was subsequently used as the cutoff. A log-rank $p<0.05$ was considered statistically significant.

Results

Mutation Profile Analysis Results

The cBioPortal web tool was used to analyze changes in CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 proteins in HCC patients. Among 369 cases, 25 cases (11%) of HCC patients had genetic changes in CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 (Fig. 2a, b). There was no significantly mutual exclusivity and co-occurrence among CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 ($p>0.05$). TCGA dataset illustrated that among circadian rhythm-related factors, mutation rates were highest in PER3 and NPAS2 (7%), followed by PER2 (6%), PER1 (5%), CRY1(%5), and CRY2 (%5) and mutation rates were lowest in CLOCK (4%). No mutation was found in the BMAL1 gene in HCC samples. The types of mutations encountered in HCC in the CLOCK, BMAL1, CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes are shown in Table 1. While the most common type of change encountered in gene mutations was SNP/missense mutation (20 mutations, 80%), 1 splice (4%), 3 FS deletions (12%), and 1 IF deletion (4%) were found. The number of patients with mutation was determined as 20 men (80%), and 5 women (20%). No germ cell-derived mutations were detected among the mutations examined (except for unknown mutations). Except for mutations of unknown somatic or germ cell origin, the others were found to be of confirmed somatic origin. In the presence of missense mutations detected in the examined genes, phenotypic changes occur in the protein depending on the missense mutation type (conservative-non-conservative) due to the formation of a codon encoding a different amino acid on the mRNA as a result of base pair change, and its function may or may not be impaired. In addition, the summary of histologic grade, lymph node stage, tumor stage, and race category in HCC patients with the mutation are shown in Figure 3.

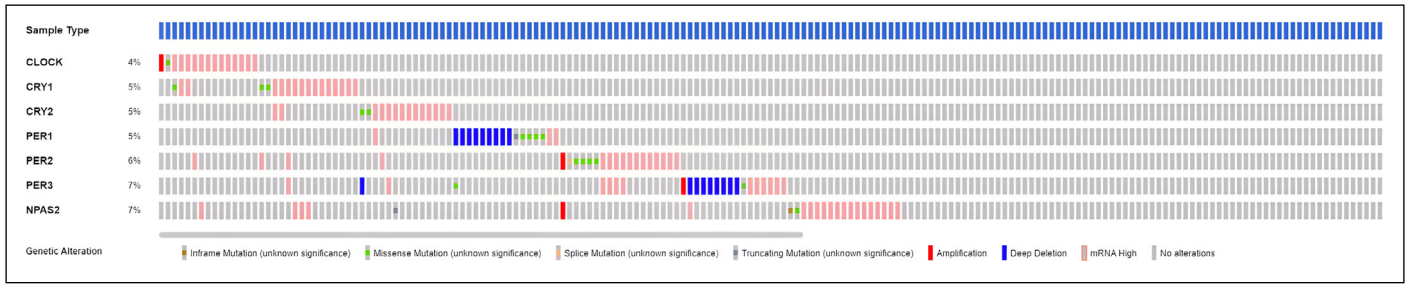

Figure 2b. Summary of OncoPrint query changes to CLOCK, BMAL1 (ARNTL) CRY1, CRY2, PER1, PER2, PER3, and NPAS2.

Table 1. Mutation profiles of CLOCK, BMAL1 (ARNTL) CRY1, CRY2, PER1, PER2, PER3, and NPAS2

No	Gen	Nt Change	AA position	Variation type	Mutation assesor score	SIFT score score	PolPhen-2
M-1	CLOCK	c.206A>T	***D69V***	MS	2.60 (medium)	0.00 (delet.)	0.74 (Poss. D.)
M-2	CRY1	c.1130G>C	***G377A***	MS	4.20 (High)	0.00 (delet.)	1.0 (Prob. D.)
M-3	CRY1	c.1715A>C	***D572A***	MS	1.10 (low)	0.36 (TLC)	0.00 (benign)
M-4	CRY1	c.130T>C	***S44P***	MS	1.95 (medium)	0.06 (tolerated)	0.03 (benign)
M-5	CRY2	c.575G>T	***R192L***	MS	2.03 (medium)	0.02 (delet.)	0.21 (benign)
M-6	CRY2	c.821A>T	**Y274F**	MS	-0.04 (neutral)	1 (tolerated)	0.00 (benign)
M-7	PER1	c.438G>C	***K146N***	MS	2.77 (medium)	0.00 (delet.)	0.98 (Prob. D.)
M-8	PER1	c.2962_2998del	***Q988Lfs*122***	**FS del**	UN	UN	UN
M-9	PER1	c.2195del	***G732Efs*12***	**FS del**	UN	UN	UN
M-10	PER1	c.1030A>G	***I344V***	MS	0.07 (neutral)	1 (tolerated)	0.65 (Poss. D.)
M-11	PER1	c.1976C>T	***T659I***	MS	2.02 (medium)	0.02 (delet.)	0.05 (benign)
M-12	PER1	c.2512A>G	***K838E***	MS	1.94 (low)	0.03 (delet.)	0.35 (benign)
M-13	PER1	c.1825G>T	***A609S***	MS	0.68 (neutral)	0.38 (tolerated)	0.01 (benign)
M-14	PER2	c.317A>T	***D106V***	MS	2.44 (medium)	0.00 (delet.)	0.40 (benign)
M-15	PER2	c.421G>T	***A141S***	MS	3.46 (medium)	0.00 (delet.)	1.0 (Prob. D.)
M-16	PER2	c.2951G>T	***R984L***	MS	3.27 (medium)	0.00 (delet.)	1.0 (Prob. D.)
M-17	PER2	c.1628-1G>A	***X543_splice***	**Splice**	UN	UN	UN
M-18	PER2	c.2767G>A	***A923T***	MS	1.49 (low)	0.48 (tolerated)	0.01 (benign)
M-19	PER3	c.2468G>A	***G823D***	MS	1.50 (low)	0.20 (tolerated)	0.04 (benign)
M-20	PER3	c.598C>T	***R200W***	MS	0.00 (neutral)	0.03 (delet.)	0.82 (Prob. D.)
M-21	PER3	c.2305C>T	***P769S***	MS	2.08 (medium)	0.06 (tolerated)	0.92 (Prob. D.)
M-22	NPAS2	c.362del	***P121Rfs*10***	**FS del**	UN	UN	UN
M-23	NPAS2	c.1139A>T	***K380M***	MS	3.09 (medium)	0.00 (delet.)	0.97 (Prob. D.)
M-24	NPAS2	c.1396C>G	***P466A***	MS	1.01 (low)	0.25 (tolerated)	0.01 (benign)
M-25	NPAS2	c.662_670del	***G221_E223del***	**IF del**	UN	UN	UN

Delet: Deleterious; Med: Medium; Poss. D: Possibyl Damage; Prob. D: Probably Damage; TLC: Tolerated low confidence; MS: Missense mutation; FS del: Frameshift deletion; IFdel: In-frame deletion; UN: Unknown.

The CLOCK gene contains the PAS-A (between amino acids 110–176) and PAS-B (between 274 and 379 amino acids) domains belonging to the transcription factor family and the basic helix-loop-helix (bHLH) (between amino acids 35–83) domain. One SNP/missense mutation was detected in the bHLH region of the CLOCK gene. This is specifically the region where DNA binds for transcription initiation, and the mutation in question may possibly cause abnormalities in transcription initiation.

CRY1 and CRY2 genes contain DNA photolyase (between amino acids 5–159 and amino acids 45–198, respectively) domain and FAD-binding domain of DNA photolyase (between amino acids 212–488 and amino acids 252–528, respectively). One SNP/missense mutation was detected in both regions of the CRY1 and CRY2 genes. Apart from the CRY1 gene in these regions, 1 missense mutation was detected in exon-12.

The PER1, PER2, and PER3 genes contain the PAS (amino acid range 360–461, 333–434, and 274–376, respectively) domain and the Period C (amino acid range, 1032–1214, 1046–1234, and 991–1115) domain. Although no direct mutations involving these regions were found, 5 missense mutations and 2 FS deletion mutations were found in the exons of the PER1 gene outside of these regions. Especially since FS mutations occur before the Period C domain, they may cause changes in the expression of this region. Period C domain, like PAS domains, is a 100% conservative splice region in the evolutionary process.

There were 4 missenses and 1 splice in the PER2 gene and 3 SNP/missense mutations in the PER3 gene. Just like the CLOCK gene, the NPAS2 gene contains a bHLH region (between 10 and 57 amino acids), PAS-A (between 85 and 156 amino acids), and PAS-B (between amino acids 149–352) domains. One FS deletion was detected on the PAS-A domain. The PAS domain is a 100% conserved splice region in the evolutionary process. Mutations occurring in these regions are likely to cause abnormalities in the expression of genes. This is because frameshift mutations result in an abnormal or defective protein product containing an inappropriate amino acid sequence. Depending on the location of the mutation, such proteins may be completely new or unusable. Frameshift mutation can also result in a stop codon. This formation of an early stop codon in mRNA may terminate the translation process and result in the formation of a short-length polypeptide, that is, truncated protein. Apart from this, in exon-8, one IF deletion was detected while two SNP missense mutations were found.

Survival Analysis and Prognostic Evaluation Results of CLOCK, BMAL1 (ARNTL), CRY1, CRY2, PER1, PER2, PER3, and NPAS2 Genes in Patients with HCC

We used the Kaplan–Meier Plot (https://kmplot.com/analysis/) analysis to assess 5-year OS. Data on the OS assessment are shown in Figure 4. When OS results were evaluated, decreased

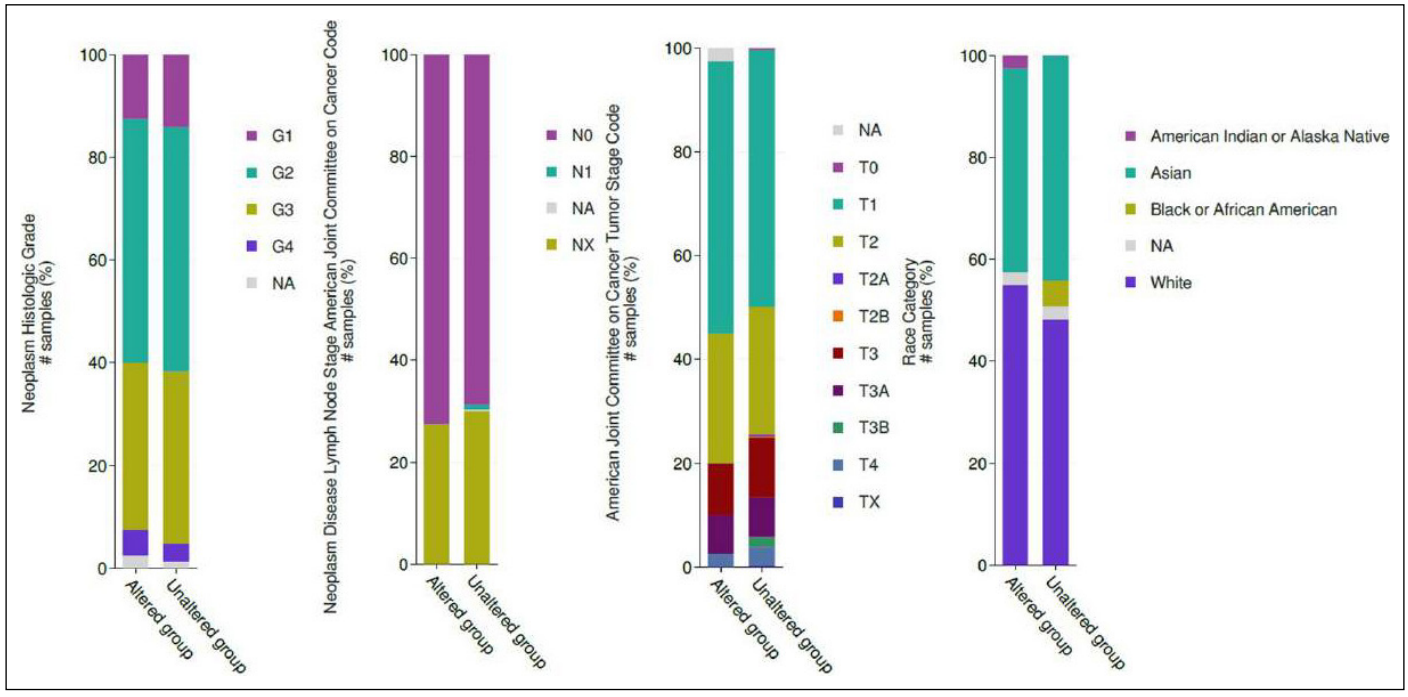

Figure 3. Summary of histologic grade, lymph node stage, tumor stage, and race category in hepatocellular cancer patients with mutation.

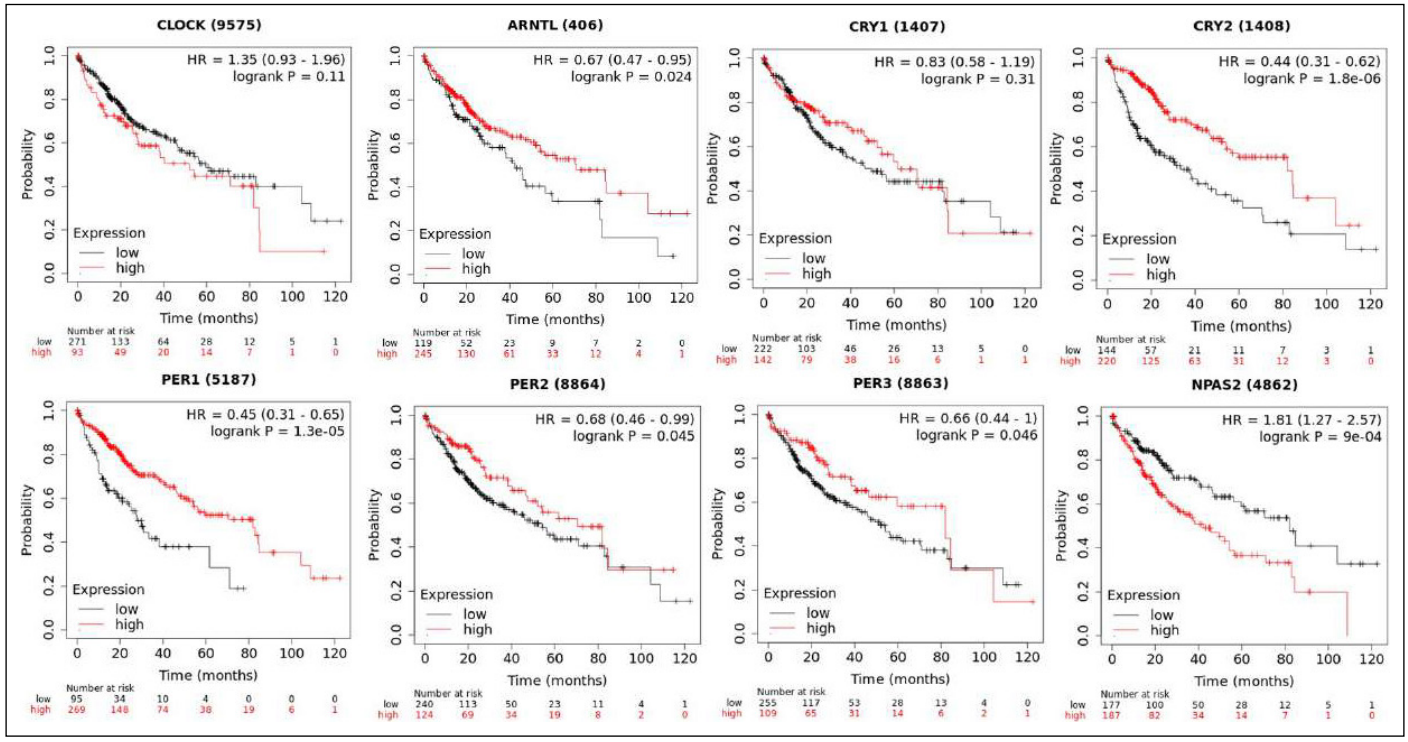

Figure 4. Different expressions of CLOCK BMAL1, PER1, PER2, PER3,CRY1, CRY2, and NPAS2 in hepatocellular carcinoma (HCC) patients in the overall survival curve (using the Kaplan–Meier plotter). The red line represents the survival rate curve of patients with HCC who expressed the gene, and the black line represents the survival rate curve of HCC patients who did not express the gene.

expression levels of BMAL1 (p=0.024), PER1 (p=1.3e-0.5), PER3 (p=0.046), and CRY2 (p=1.8e-06) genes were found to be statistically associated with shorter OS. In addition, increased expression levels of the PER2 (p=0.045) gene were associated with longer OS while increased expression levels of the NPAS2 (p=9e-04) gene were associated with shorter OS. No significant correlation was found between CLOCK, CRY1 expression levels, and OS in HCC patients (p>0.05).

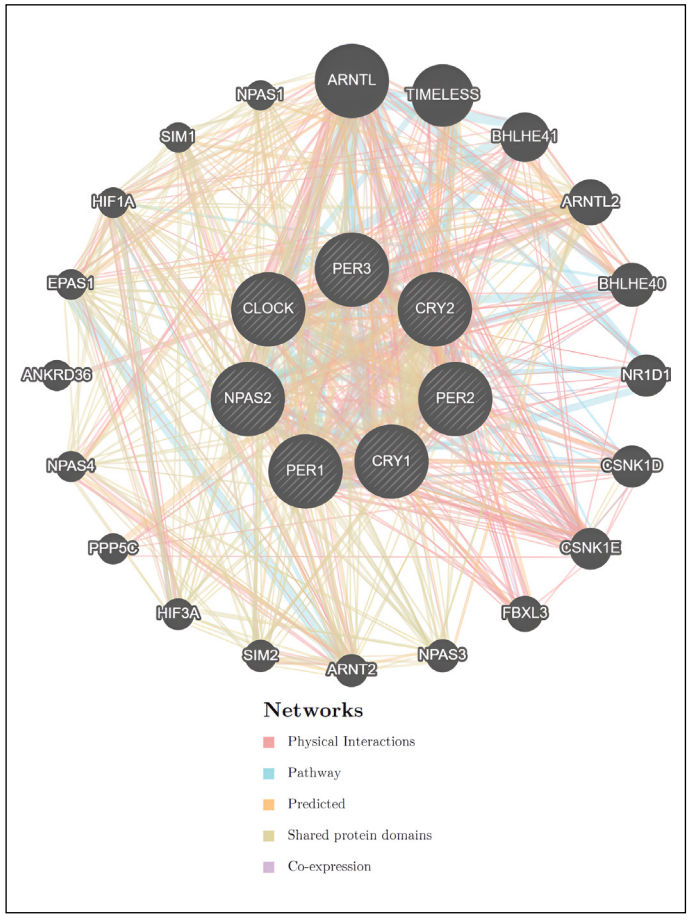

Figure 5. Gene interactions among CLOCK BMAL1, PER1, PER2, PER3,CRY1, CRY2, and NPAS2 in hepatocellular carcinoma. The color of the connecting line represents the type of interaction.

Results of Gene Interaction Analysis Among CLOCK, BMAL1 (ARNTL), CRY1, CRY2, PER1, PER2, PER3, and NPAS2 in HCC

We used the GenMANIA software program to examine gene-gene interactions (GGI). The data we obtained as a result of our analysis are presented visually in Figure 4. GeneMANIA was used to construct a GGI network consisting of CLOCK, BMAL1 (ARNTL) CRY1, CRY2, PER1, PER2, PER3, and NPAS2 and to analyze functions that may be related to networks of these genes. They were all surrounded by 18 different nodes, representing genes that may have physical interactions, coexpressions, predictions, colocalizations, pathways, and gene interactions. Especially when examined in terms of interaction density, BHLHE41 and BHLHE40 genes and NPAS2 and CLOCK genes use common pathways, and these two genes have physical interactions with CLOCK, NPAS2, CRY2, PER2, and CRY1 genes. The CLOCK and NPAS2 genes contain HLH regions; however, common protein domains do not exist. The NR1D1 gene and the CLOCK, NPAS2, and PER1 genes use the same pathways, and there is a physical interaction between the CLOCK, NPAS2, CRY1, and PER2 genes. Casein kinase 1 (CSNK1D) and Casein kinase 1 epsilon genes use common pathways with CLOCK and NPAS2 genes and have physical interactions with all examined genes. The F-box/LRR-repeat protein 3 gene, on the other hand, may interact physically with all genes except the PER3 gene (Fig. 5).

Gene Expression Profiling Interactive Analysis Results

In patients with HCC (n=369) and healthy participants (n=160), CLOCK, BMAL1 (ARNTL), CRY1, CRY2, PER1, PER2, PER3, and NPAS2 genes were found in GEPIA2 (http://gepia2.cancer-pku.cn/) #index) database, it was seen that there was no statistical difference between the expression levels of the examined genes in tumor tissue and normal tissue (Fig. 6) ($p>0.05$).

Analysis of the functional/Pathogenic Analysis of Detected Mutations Results

The scores of PolyPhen-2, Mutation Assessor, and SIFT databases were used to examine the reflections of the effects of mutations in the analyzed genes. Information on mutations as a result of this scoring is given in Table 2. According to the analysis and scoring results, the mutation occurring in the CRY1 (p.G377A) gene was determined as the mutation that caused the most changes in protein structure and function and was the most pathogenic SNP/missense.

Discussion

In this study; the circadian clock genes CLOCK, BMAL1 PER1, PER2, PER3, CRY1, CRY2, and NPAS2 were examined in terms of possible changes in genome size, interactions with variety of proteins, changes of its function, and its survival in a total of 369 HCC samples. A total of 25 mutations were found in 8 genes in a total of 369 samples, and 80% of these mutations consist of SNP/missense mutations. The data obtained show that especially some missense mutations are in the splice regions that are perfectly conserved in the evolutionary process, and besides that, there are also splice, IF deletions, and FS deletions, although rare. Previous studies have shown that an imbalance of factors related to the circadian rhythm can lead to the formation of cancer. In fact, disruption in circadian rhythm has been identified as a carcinogen.[17–19] Disruption of the circadian rhythm plays a key role in tumorigenesis and facilitates the formation of cancer features. Contrariwise, oncogenic processes directly weaken circadian rhythms.[18] The available evidence strongly supports the idea that the circadian clock controls the physiological homeostasis of the liver and plays an important role in hepatocarcinogenesis.[1]

The transcription factor BMAL1 (ARNTL) is an essential component of the mammalian circadian clock; in its absence, circadian behaviors are disrupted. BMAL1 function is essential for daily molecular oscillations, particularly in the liver.[20] Mutations in the bHLH, PAS-A, and PAS-B domains of BMAL1, which are 100% conserved in the evolutionary process and are very important for transcription, affect the circadian cycle.[21] In the analysis, no mutations were found in the BMAL1 gene neither in the aforementioned regions nor in the exon regions of HCC samples. In addition, although mRNA expression levels in HCC samples seem to be downregulated, there was no statistical difference found between mRNA expression levels when compared to normal samples. According to the survival analysis results, decreased BMAL1 mRNA expression levels in the group with HCC were associated with shorter OS.

CLOCK contains the p.D69V SNP/missense mutation in the bHLH region, which is an E-box binding region. At the molecular level, the circadian mechanism consists of a positive loop of bHLH transcription factors CLOCK and ARNTL/BMAL1. Transcription begins after CLOCK: BMAL1 forms a dimer structure and binds to the E-box region.[22] Therefore, such an increase in transcription caused by a muta-

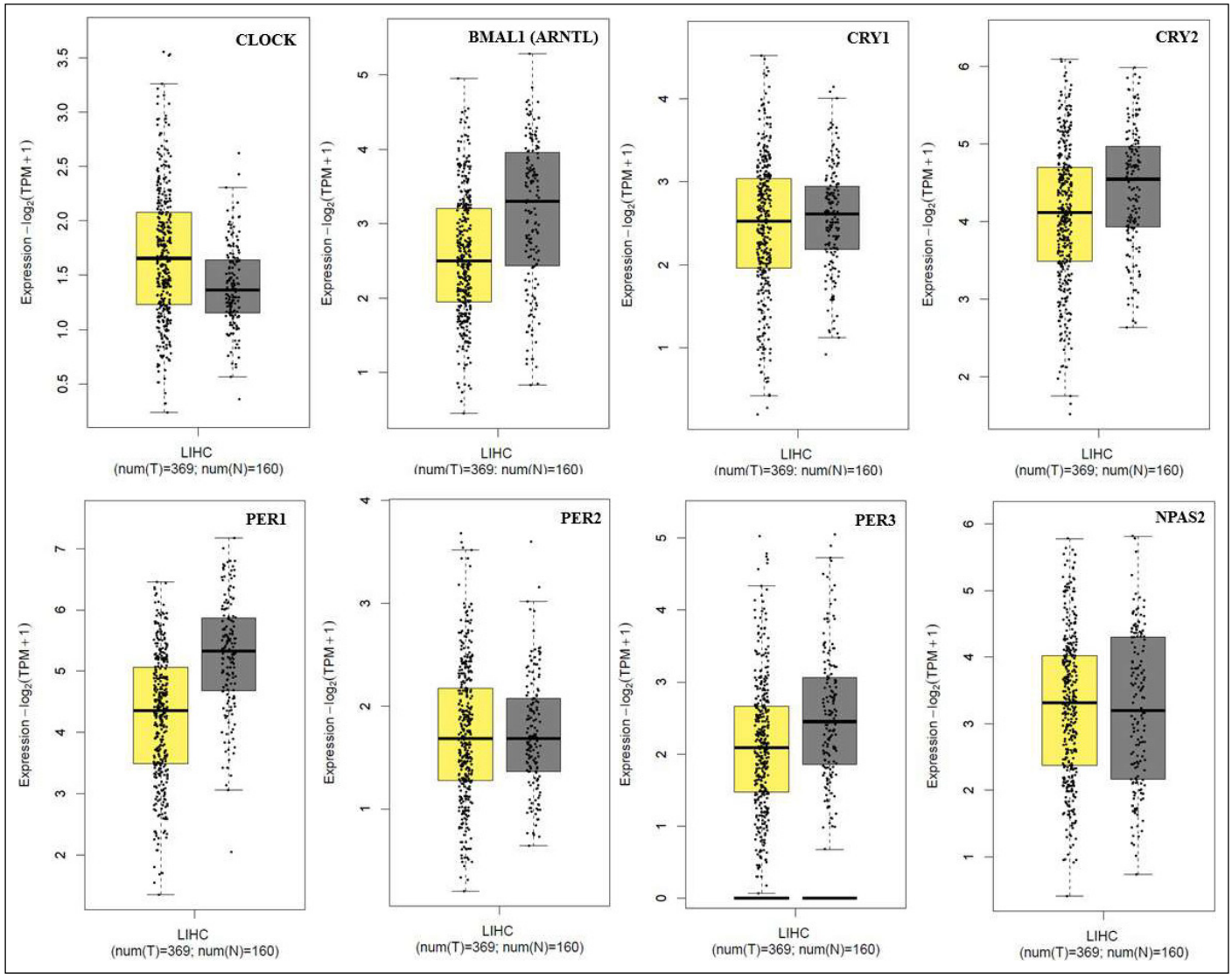

Figure 6. mRNA expressions of CLOCK BMAL1, PER1, PER2, PER3,CRY1, CRY2 and NPAS2 in hepatocellular cancer (yellow) and normal liver tissues (gray).

tion in the E-box binding region could play an active role in cell proliferation and cancer formation.[21–23] In the HCC samples examined, merely the above-mentioned mutation was found in the CLOCK gene.

According to the scoring results obtained from three different sources at the point of the effect of the mutation on the functional state of the protein, the effect of the effect in the SIFT algorithm on the function of the protein appears to be quite effective, while it has been concluded that there is a moderate effect in the other scoring results. Although CLOCK gene mRNA expression levels seemed to be relatively increased in HCC samples when compared to healthy samples, this result was not statistically meaningful. In addition, increased expression levels of the CLOCK gene were not found to be significantly associated with OS. Although the localization of the mutation in the CLOCK gene seems to be important, its effect on the clinical reflection is controversial in light of these results.

PER and CRY genes are important negative regulators of circadian rhythms.[24] When the PER and CRY proteins reach a certain concentration in the cytoplasm, they are transferred to the nucleus and act on the BMAL1:CLOCK or BMAL1:NPAS2 dimers.[25] The PER family (PER1, PER2, and PER3) has two splice regions that are 100% conserved in the evolutionary process. The first of these, the PAS domain, is the ligand-binding region, and this region acts as a sensor for oxygen and light in signal transmission. The second is the Period protein 2/3C-terminal region. No mutations were found in these regions of PER1, PER2, and PER3 genes in the patient group with HCC. However, two important FS deletion mutations of exon-17 and exon-19 regions were detected in PER1 (G732Efs*12 and Q988Lfs*122, respectively). While the exon-17 region contains five residue phosphorylation sites in post-translational modification, the exon-19 region contains two different residual phosphorylation sites and one residual methylation site. In addition, these regions are followed by the period protein 2/3C-terminal region. As a result of cascading FS deletion mutations, the formation of the termination code and the formation of a dysfunctional polypeptide chain are likely. The p.X543_splice mutation has been detected in the PER2 gene. As a result of such a mutation, during the

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Table 2. Circadian gene mutation analysis

No	Gen	Nt change	Somatic status	Variation type	Localization	AA position	Type of cancer	Sub.	G	Neoplasm disease stage AJC	NHG	DA	OS (m)
M-1	CLOCK	c.206A>T	Confirmed somatic	Missense mutation	Exon-5	***D69V***	HCC	LIHC	M	STAGE II	G2	44	25.2
M-2	CRY1	c.1130G>C	Confirmed somatic	Missense mutation	Exon-7	***G377A***	HCC	LIHC	M	STAGE I	G1	65	73.8*
M-3	CRY1	c.1715A>C	Confirmed somatic	Missense mutation	Exon-12	***D572A***	HCC	LIHC	M	STAGE IIIA	G3	73	27.9*
M-4	CRY1	c.130T>C	Confirmed somatic	Missense mutation	Exon-1	***S44P***	HCC	LIHC	F	STAGE I	G2	38	12.0
M-5	CRY2	c.575G>T	Confirmed somatic	Missense mutation	Exon-4	***R192L***	HCC	LIHC	M	STAGE I	G3	43	46.8*
M-6	CRY2	c.821A>T	Confirmed somatic	Missense mutation	Exon-6	***Y274F***	HCC	LIHC	M	STAGE I	G2	70	0.3*
M-7	PER1	c.438G>C	Confirmed somatic	Missense mutation	Exon-4	***K146N***	HCC	LIHC	M	STAGE IIIA	G2	74	4.6
M-8	PER1	c.2962_2998del	UN	FS del	Exon-19	***Q988Lfs*122***	HCC	LIHC	M	STAGE IIIA	G2	74	4.6
M-9	PER1	c.2195del	UN	FS del	Exon-17	***G732Efs*12***	HCC	LIHC	M	STAGE IIIA	G2	74	4.6
M-10	PER1	c.1030A>G	Confirmed somatic	Missense mutation	Exon-8	***I344V***	HCC	LIHC	M	STAGE I	G2	76	9.9*
M-11	PER1	c.1976C>T	Confirmed somatic	Missense mutation	Exon-16	***T659I***	HCC	LIHC	M	STAGE I	G3	65	75.6*
M-12	PER1	c.2512A>G	Confirmed somatic	Missense mutation	Exon-19	***K838E***	HCC	LIHC	M	STAGE II	G2	67	26.8*
M-13	PER1	c.1825G>T	Confirmed somatic	Missense mutation	Exon-15	***A609S***	HCC	LIHC	M	STAGE I	G3	53	13.9
M-14	PER2	c.317A>T	Confirmed somatic	Missense mutation	Exon-4	***D106V***	HCC	LIHC	F	STAGE II	G2	58	13.5*
M-15	PER2	c.421G>T	Confirmed somatic	Missense mutation	Exon-4	***A141S***	HCC	LIHC	F	STAGE IIIA	G3	39	11.3
M-16	PER2	c.2951G>T	Confirmed somatic	Missense mutation	Exon-19	***R984L***	HCC	LIHC	M	STAGE IIIA	G2	73	11.9*
M-17	PER2	c.1628-1G>A	UN	Splice	NA	***X543_splice***	HCC	LIHC	M	STAGE II	G2	48	4.2
M-18	PER2	c.2767G>A	Confirmed somatic	Missense mutation	Exon-19	***A923T***	Hepatocholangio carcinoma (mixed)	LIHC	M	STAGE I	G1	64	27.2*
M-19	PER3	c.2468G>A	Confirmed somatic	Missense mutation	Exon-17	***G823D***	HCC	LIHC	M	STAGE II	G3	24	0*
M-20	PER3	c.598C>T	Confirmed somatic	Missense mutation	Exon-5	***R200W***	HCC	LIHC	M	STAGE II	G2	66	8.6
M-21	PER3	c.2305C>T	Confirmed somatic	Missense mutation	Exon-17	***P769S***	HCC	LIHC	M	STAGE II	G2	66	8.6
M-22	NPAS2	c.362del	UN	FS del	Exon-5	***P121Rfs*10***	HCC	LIHC	F	STAGE I	G2	72	66.3*
M-23	NPAS2	c.1139A>T	Confirmed somatic	Missense mutation	Exon-12	***K380M***	HCC	LIHC	M	STAGE II	G3	24	19.7*
M-24	NPAS2	c.1396C>G	Confirmed somatic	Missense mutation	Exon-15	***P466A G221_E223del***	HCC	LIHC	M	STAGE II	G3	24	19.7*
M-25	NPAS2	c.662_670del	UN	IF del	Exon-8		HCC	LIHC	F	STAGE I	G3	56	29.5

*: They are patients who are still alive. Delet: Deleterious; Med: Medium; Poss. D: Possibyl Damage; Prob. D: Probably Damage; TLC: Tolerated low confidence; Sub.: Subtype; G: Gender; M: Male; F: Female; AJC: American joint committee; NHG: Neoplasm histologic grade; DA: Diagnosis age; OS (m): Overall survival (months); FS del: Frameshift deletion; IFdel: In-frame deletion; UN: Unknown.

splicing process, an exon that is responsible for coding the protein on the gene can be removed, so a shorter polypeptide structure can be formed and disorders in the protein's function may occur. It can be said that the effect of other missense mutations observed in PER genes on the function of the protein was examined and a moderate effect may occur according to the scoring results obtained from different sources. When PER1 and PER3 gene mRNA expression levels were compared with healthy samples, it was found that the PER2 gene mRNA levels were relatively increased in HCC patient samples, but these changes were not statistically significant. As a result of the survival analysis, decreased PER1 and PER3 expression levels were found to be associated with shorter OS, while increased PER2 expression levels were associated with longer OS. Although the mRNA expression levels of genes are not meaningful when compared with the healthy population, we can say that serious mutations in the PER1 (G732Efs*12 and Q988Lfs*122) gene may cause shorter OS. On the contrary, we can state that a mutation in the PER2 (p.X543_splice) gene may cause longer OS and can be considered as a positive prognostic factor.

Missense mutations were detected in 3 different exon regions of the CRY1 gene, while missense mutations were detected in two different exon regions of the CRY2 genes. CRY1 and CRY2 genes contain evolutionarily conserved DNA photolyase homology domain (close to the C-terminal region) and FAD binding domain (closer to the N-terminal region). These two domains are termed the photolyase homology region (PHR). CRY1 interacts directly with CLOCK and BMAL1 through two different sites in PHR, while the PHR domain of CRY1 interacts with the C-terminal regions of PER1 and PER2.[21–26] Two missense mutations were detected for the CRY1 gene, especially in the parts covering these regions. While we can say that the p.S44P missense mutation in exon-1 in the DNA photolyase domain has a tolerable effect on the function of the protein, we can state that the mutation in p.G377A in exon-7 in the FAD binding domain region may have a negative effect on the function of the protein. In the CRY2 gene, the missense mutation (p.R192L) in the DNA photolyase homology domain is likely to have a negative effect on the function of the protein, while the effect of the missense mutation in the FAD binding domain (p.Y274F) on the function of the protein is tolerable. Due to the fact that two regions function together, mutations in the domains are likely to have a negative effect on the PER1 and PER2 interaction in the HCC patient group. CRY1 and CRY2 gene mRNA expression levels were relatively low in the HCC patient group when compared to healthy samples, but this was not statistically meaningful. In addition, decreased CRY1 gene mRNA expression levels were not statistically correlated with OS, while decreased CRY2 gene mRNA expression levels were associated with shorter OS. A previous study has indicated that disruption in CRY2 gene expression levels is an independent predictor of OS in HCC patients.[7] The missense mutation (p.R192L) in the CRY2 gene is the mutation that has the most effect on the structure of the protein among all SNP/missense mutations examined in this study.

Therefore, considering these effects, we can say that CRY2 gene mutation may have a meaningful correlation with the mortality of patients with HCC.

NPAS2, on the other hand, contains bHLH domain and PAS domains, just like BMAL1 and CLOCK. NPAS2 has a critical role in HCC cell survival and tumor growth, mainly mediated by transcriptional upregulation of CDC25A. With this feature, increased NPAS2 expression levels in HCC patients accompany poor prognosis. Therefore, it is likely to serve as a potential therapeutic target.[27–29] In the study, one FS deletion (P121Rfs*10) in the PAS-A domain of the NPAS2 gene and one IF deletion in exon-8 were detected. In HCC patients, it is possible that the termination code and truncated protein are formed as a result of FS deletion and subsequent IF deletion, especially in the PAS-A region, which is 100% conserved in the evolutionary process. In addition, it is highly possible that the p.K380M missense mutation in the exon-12 region causes a disorder in the protein's function. As a result of our analysis, NPAS2 gene mRNA expression levels were relatively increased in HCC patients compared to healthy samples, but this increase in mRNA levels was not statistically significant. In addition, relatively increased NPAS2 gene mRNA levels were statistically associated with shorter OS.

Genetic and epigenetic changes in circadian clock genes have been shown to trigger carcinogenesis in different mutant and knock-out mouse models. Therefore, studies have suggested that PER2, CRY1, and CRY2 are critical in controlling hepatic carcinogenesis.[30,31]

Analysis of miRNA profiles in liver from Clock gene mutant mice reveals that Clock gene-regulated miRNAs may play a role in cancer initiation or progression by controlling genes involved in cell proliferation, invasion, and/or metabolism in mouse liver. Thus, liver cancer development may occur in Clock gene regulation disorder.[32] In addition, NPAS2, a core circadian molecule analog of CLOCK, is upregulated in HCC and facilitates cancer cell survival. Mechanistically, NPAS2 heterodimerizes with BMAL1 and induces the expression of cyclin-dependent kinases (CDK)2, CDK4, and CDK6, as well as the phosphatase CDC25A, which dephosphorylates Bcl-2, thereby stimulating cell proliferation and inhibiting mitochondria-dependent intrinsic apoptosis, respectively.[33]

Therefore, NPAS2 is an important contributor to the poor prognosis of HCC and may constitute a potential therapeutic target in HCC patients.[34]

Conclusion

We used several high-throughput bioinformatics databases to analyze and investigate CLOCK, BMAL1 PER1, PER2, PER3, CRY1, CRY2, and NPAS2 gene expressions and effects. Considering the mutation types and their effects, we can say that mutations in PER1, CRY2, and NPAS2 genes are negative prognostic factors because they decrease the mean OS time of HCC patients. On the contrary, we can state that the mutations detected in the PER2 gene in our study are an independent positive prognostic factor for HCC patients. In light of this information, the present study may help us to understand the molecular functions of circadian rhythm-related factors in HCC better. In addition, knowing these molecular changes well may provide possible molecular targets for HCC in chemotherapy and immunotherapy. However, we think that studies with driver gene mutations in HCC are needed to evaluate these genes as a treatment option.

Ethics Committee Approval: The data used in our study were obtained from public database TCGA, therefore, ethical approval was not required.

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