Identification of Circadian-Related Gene Mutation And Expression Patterns in Skin Cancer

Cilt Kanserinde Sirkadiyen İlişkili Gen Mutasyonu ve İfade Paternlerinin Belirlenmesi

Dilara Fatma Akın[1], Didem Özkan[2]

ABSTRACT

Aim: Many peripheral organs have endogenous rhythms that are modulated by circadian rhythm; the skin is one of these peripheral organs. Irregularities in rhythm can reprogram cellular pathways and lead to cell proliferation, resistance to apoptosis, metastasis, elimination of immune system cells, and increased angiogenesis. The aim of this study was to comprehensively investigate the mutational and mRNA profile of core circadian rhythm genes in SKCM samples.

Methods: The genome sequences and gene expression profiles of SKCM (n:442) patients were downloaded from the TCGA database. Mutation frequency and mutation patterns of target genes were determined in detail. PolyPhen-2 and SNAP tools were used to estimate the oncogenic properties of the mutations we detected for SKCM. Simultaneously, mRNA expression profiles were determined to asses the functional effects of mutations in circadian rhythm genes. Finally, STRING network analysis was performed to better understand the functional relationships of mutated proteins in cellular processes.

Results: There were 152 missense mutations, 13 nonsense mutations, 6 splice region mutations and, 2 fusion gene mutations among the 173 mutations found in 10 genes, and 62 of them had pathogenic properties. The mRNA expression levels of BMAL1, CRY2, PER1, PER2, and PER3 were down-regulated in SKCM tissue samples, while TIMELESS was up-regulated compared to the the healthy group ($p<0.01$). The effect of mRNA expression, considered a prognostic marker, on overall survival was found to be significant for decreased ARNTL ($p=0.00038$) level and increased TIMELESS ($p=0.00033$) level.

Conclusion: Determining this molecular perspective may be useful in developing targeted drug therapies and personalized medicine.

Keywords: Circadian; cryptochrome; period; skin cancer; clock genes; mutation

ÖZ

Amaç: Birçok periferal organın sirkadiyen ritim tarafından modüle edilen endojen ritimleri vardır; deri de bu periferik organlardan biridir. Ritmdeki düzensizlikler hücresel yolları yeniden programlayabilir ve hücre çoğalmasına, apoptoza direnç, metastaz, bağışıklık sistemi hücrelerinin yok edilmesi ve anjiyogenezin artmasına neden olabilir. Bu çalışmanın amacı, SKCM örneklerinde çekirdek sirkadiyen ritim genlerinin mutasyonel ve mRNA profilini kapsamlı bir şekilde araştırmak olarak belirlenmiştir.

Method: SKCM (n:442) hastalarının genom dizileri ve gen ekspresyon profilleri TCGA veri tabanından alındıktan sonra hedef genlerin mutasyon sıklığı ve mutasyon paternleri detaylı olarak belirlendi. SKCM için tespit ettiğimiz mutasyonların onkojenik özelliklerini tahmin etmek için PolyPhen-2 ve SNAP biyoinformatik araçları kullanılırken eş zamanlı olarak, sirkadiyen ritim genlerindeki mutasyonların fonksiyonel etkilerini değerlendirmek için mRNA ekspresyon profilleri belirlendi. Son olarak, mutasyona uğramış proteinlerin hücresel süreçlerdeki fonksiyonel ilişkilerini daha iyi anlamak için STRING ağ analizi yapıldı.

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[1]Nigde Ömer Halisdemir University, Faculty of Medicine, Medical Biology, Nigde, Turkey, Mail: dilarafatmaakin@gmail.com, ORCID: 0000-0002-0903-0017

[2]Istanbul Okan University, Vocational School of Health Services, Istanbul, Turkey. Mail: didemtorun@gmail.com, ORCID: 0000-0003-4210-5073

Sorumlu yazar/Correspondence: Didem Özkan, didemtorun@gmail.com

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Bulgular: 10 gende bulunan 173 mutasyondan; 152 misssense mutasyon, 13 nonsense mutasyon, 6 splice mutasyonu ve 2 füzyon gen mutasyonu olarak belirlendi ve bu mutasyonların 62'sinin patojenik özelliklere sahip olduğu görüldü. BMAL1, CRY2, PER1, PER2 ve PER3'ün mRNA ekspresyon seviyelerine bakıldığında, SKCM doku örneklerinde downregüle olurken, TIMELESS sağlıklı gruba kıyasla upregüle olduğu tespit edildi($p<0.01$). Prognostik bir belirteç olarak kabul edilen mRNA ekspresyonunun genel sağkalıma etkisi, azalan ARNTL (p=0,00038) seviyesinde ve TIMELESS (p=0,00033) seviyesinde artışta anlamlı bulundu.
Sonuç: Bu moleküler bakış açısının belirlenmesi, hedefe yönelik ilaç tedavileri ve kişiselleştirilmiş tıp gelişiminde faydalı olabilir.

Anahtar Kelimeler: Sirkadiyen; kriptokrom; ritm; cilt kanseri; saat genleri; mutasyon

Introduction

The circadian rhythm is the cell-autonomous timekeeping system that generates a periodic rhythm of about 24 hours, that is preserved in almost all forms of life, from single-celled organisms to humans (1-4). This rhythm in the cell consists of a system of positive and negative feedback loops. There is a close interaction between the circadian clock mechanism at the molecular and cellular levels and many processes such as the cell cycle, DNA repair mechanism, apoptosis, senescence, autophagy, and other oncogenic and immune pathways (3-5). Recent studies have also shown that changes in circadian rhythm modulate cell cycle timing, alter DNA replication fork progression, impair genome stability by affecting DNA damage response (DDR) and DNA repair efficiency (5-7). Negative changes in circadian rhythm can reprogram these processes and ultimately lead to uncontrolled cell proliferation, resistance to apoptosis, metastasis, elimination of immune system cells, and enhanced angiogenesis. All of these features are the same as the characteristic features of cancer. The circadian rhythm is built into every cell as molecular machine capable of adapting to external changes through its extraordinary plasticity (6-8).

The incidence of skin cancer is increasing worldwide due to changes in our lifestyle and environment. The main cause of skin cancer is exposure to the UV component of sunlight. Ultraviolet radiation (UVR) produces two major lesions in DNA, cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct [(6-4) PP]; both are known to be major causes of skin cancer and have mutagenic and carcinogenic effects in animal models (4,5,9). In mice and humans, nucleotide excision repair (NER) is the only repair system available to remove CPDs and (6-4) PPs from DNA. NER activity in the skin is under the control of circadian rhythm, reaching a maximum at 4-6 during the day and decreasing to a minimum at 4-6 am at night (5,9,10). However, genetic abnormalities in genes encoding key proteins (CLOCK, NPAS2, ARNTL, PER1, PER2, PER3, Bmal1/ARNTL, CRY1, CRY2, TIMELESS, and CSNK1E) responsible for this rhythm ensure that this clock functions properly and thus the cellular processes it regulates, and can cause rhythm disruption. To this end, in this study we aimed to uncover the comprehensive genetic profile of the genes encoding the molecules that ensure the proper functioning of the chronobiological function of the skin in the group of skin cancer. We believe that identifying the influence of circadian rhythm on the pathogenesis of skin cancer is a crucial step that will allow physicians to improve therapeutic decisions and maximize the effectiveness of prescribed treatments.

Material Methods

Data Collection

The data set of the Skin Cutaneous melanoma (SKCM) was obtained from the cbioPortal database. Raw data is accessed through cBioPortal. 442 SKCM patients' genome sequence and gene expression profiles were downloaded from the TCGA (TCGA PanCancer Atlas n:448) database. Six patients were not included in the study because there

was no detailed information in the database. An overview of research design is presented in **Figure-1**.

Mutation Profile Analysis

cBio Cancer Genomics Portal (http://cbioportal.org); the mutation data, the number of copies changes, microarray and RNA sequencing-based mRNA expressions are an open-access bioinformatics tool that provides DNA methylation values with data from The Cancer Genome Atlas (TCGA) (11). In this regard, comprehensive mutation profile analyzes of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E genes were performed using OncoPrint, Cancer Types Summary, and Mutation tabs provided by cBioPortal. The types and frequency of the determined genetic changes were analyzed for each gene.

Analysis of the functional/pathogenic analysis of detected mutations

The probable pathogenicity of the mutations found in the to determine the possible pathogens and clinical characteristics of the mutations detected in ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E was determined using scores from the PolyPhen-2, SIFT, and the COSMIC databases. PolyPhen-2 predicts that a combination of these features could cause damage to the mutation on the protein, and provides the user with a score (probably damaging, possibly damaging, benign or unknown) (12). The SIFT algorithm (https://sift.bii.a-star.edu.sg/) is a bioinformatics tool that predicts whether the location of an amino acid affects protein function based on array homology and the physical properties of amino acids. The program classifies an amino acid exchange as tolerable or harmful according to its protein function (13). Furthermore, the Catalogue of Somatic Mutations In Cancer (COSMIC) of the detected mutations has been scanned in its database to determine pathogenic scores (14).

Gene Expression Profile Analysis

GEPIA (http://gepia.cancer-pku.cn/) is an on-line data database that contains the 9736 tumor sample and the 8587 healthy sample expression profiles (15). It is an interactive bioinformatics tool developed to provide customizable analyzes such as differential expression analysis in tumors or normal tissues, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis, and dimensionality reduction analysis. The gene expression profile ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E were analyzed as box plot graphs generated by the GEPIA database using SKCM (n:442) sample from TCGA and GTEx data, and data from 558 healthy tissue samples. Moreover, the cbio portal mRNA expression module was used to compare the expression levels of altered and unaltered individuals for each gene to determine the effect of the detected mutations on mRNA expression levels. Box graphs was plotted using the RNASeq V2 RSEM normalized expression values and statistical testing on the Portal was automatically calculated. The p-values were automatically calculated by the tool in both analyses, and p-values below 0.05 were considered statistically significant.

Protein-protein interaction analysis

The STRING database (https://string-db.org) is used to evaluate protein-protein interaction information (16). The predicted interactions of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E proteins were performed by this database which defines direct (physical) and indirect (functional) relationships between proteins.

Statistical Analytics

All statistical analyzes used in the evaluation of study data were performed on the GEPIA database. The one-way ANOVA test was used to measure differential expression. Overall survival analysis was carried out using the Kaplan-Meier curves. GEPIA uses Log-rank test, the Mantel–Cox test, for hypothesis test. To compare low and high expression groups, the log-rank test was used. In all tests, the statistically significant value was accepted as $p < 0.05$.

Results

Characteristics of the Study Group

The demographic, clinical, and genetic data for patient group are summarized in **Figure-2**. In the patient group consisting of 169 women and 273 men; While 367 patient samples are in the

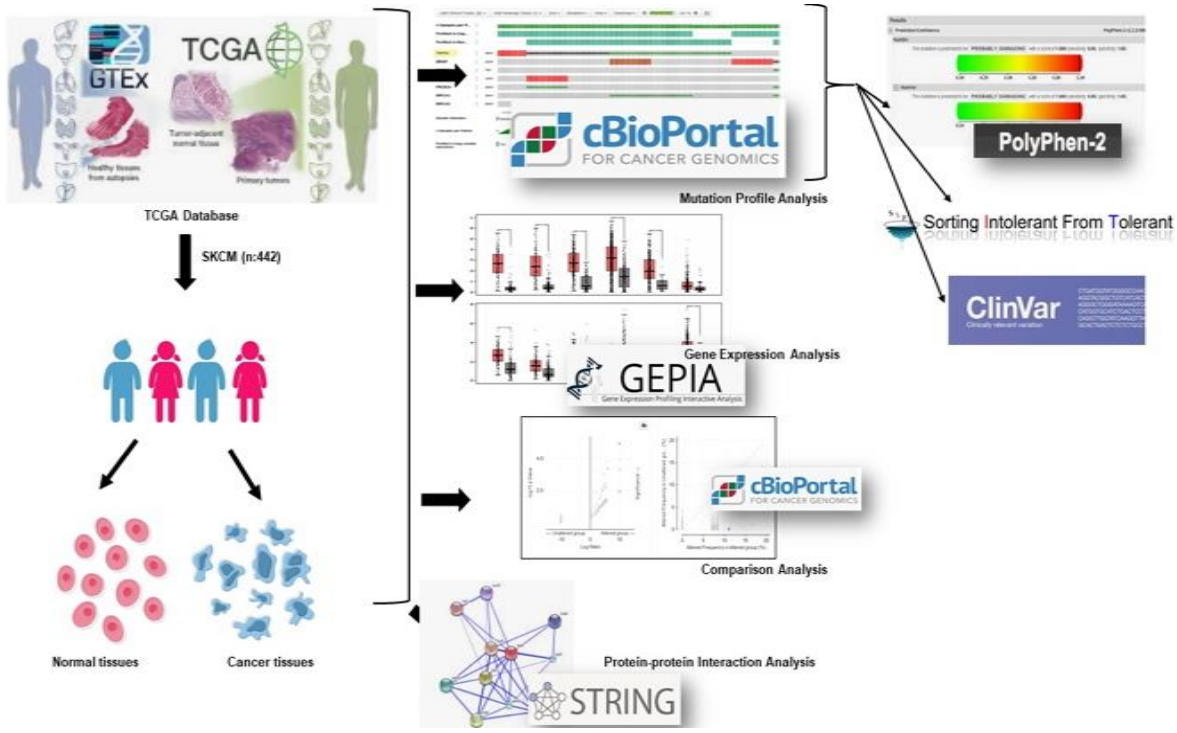

Figure-1. Flowchart of the implemented workflow illustrates the methods that have been followed. TCGA, The Cancer Genome Atlas.

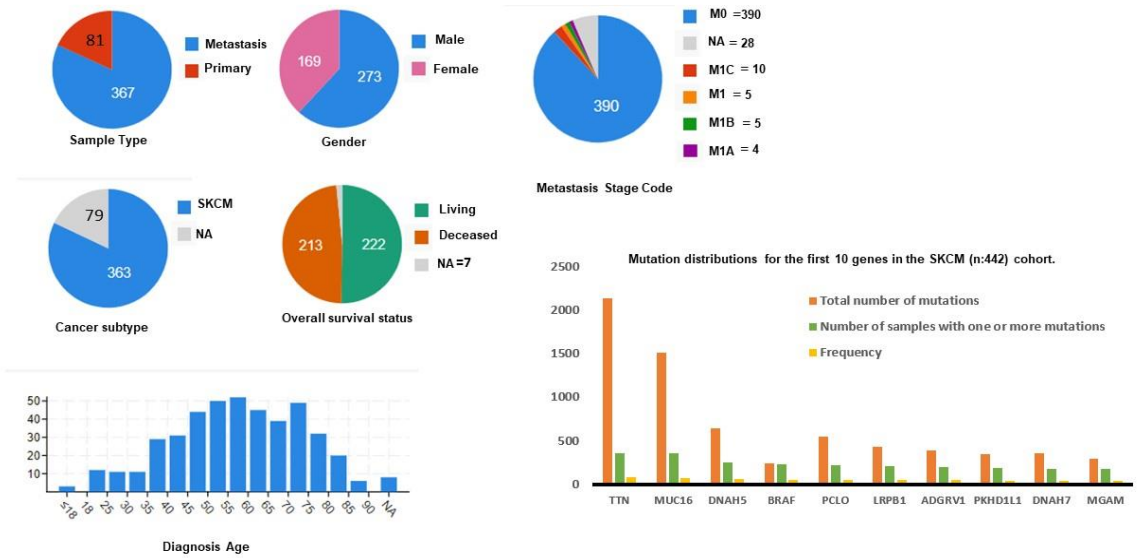

Figure-2. Clinico-pathological and epidemiological data of 442 patients with SKCM

metastatic state, 81 are in the primary state. When the patients were evaluated according to their metastasis stage code, it was seen that MO=390 M1C=28, M1=5, M1B=5, M1C=10 and M1A=4 stage. In the study group, 363 patients were found to have SKCM. When compared according to the mutation distribution, the TTN gene was found to have the highest mutation frequency, while the MGAM gene was determined to be the least gene.

Results of Mutation Profile Analysis

The cBioPortal interface was used to analyse the genome sequencing data of 442 patients to identify genetic alterations in the ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E in SKCM patient group. At least one genetic alteration (missense mutation, non-sense mutation, frame shift mutation, deletion, and gene amplification) was detected in 36% of SKCM patients. As a result of the detailed examination of the mutations, a total of 173 different mutations (152 missense mutations, 13 nonsense mutations, 6 splice regions, 2 fusion gene mutations) in ten genes were determined and the features of these mutations are thoroughly listed in **Table-1** (Supplemental material). Distribution of mutations in ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E genes in SKCM patients from cBioPortal is shown in **Figure-3** and **Figure-4.** The localization of mutations detected in the domains of proteins belonging to the study genes in SKCM patient is shown in **Figure-4.**

PER1, PER2, and PER3 analysis

The PER family, the most important gene of the circadian rhythm, is responsible for the formation of the rhythm and is known to coordinate the basic activities of life. These 3 genes, which belong to the Period gene family, have phostolyase homology domain (PAS) (17). A total of 19 different missense mutations were detected in *PER1*. p.L388F, p.401N, p.H409Y, and p.W441L missense mutations were detected on the PAS domain.

The frequency of somatic mutation detected in this gene in SKCM patient group is 6%. In PER2, 32 different mutations (26 missense mutations, 5 nonsense mutations, 1 splice sregion mutation) were determined. Since p.X386_splice is located in the splice region, which is 100% conserved in the evolutionary process between species. It is possible that this mutation causes anomaly in the PER2 gene expression. The frequency of somatic mutations detected in PER2 gene in SKCM cohort was 7%. In addition, the nonsense mutations p.E179* and p.E305 resulted in a termination codon at amino acid in PER2, leading to the formation of a truncated protein.

In PER3, 23 different mutations (20 missense, 2 humans and 1 splice regions) were detected. The frequency of somatic mutation detected in this gene in SKCM patient group was 9%. Because the p.X290_splice region, which was detected between the 7th exon and the 8th intron and encodes the PAS domain is located in the splice region that is 100% protected in the interspecies evolutionary process, this mutation was likely to cause an abnormality in PER3 gene expression. p.Q460* and p.G1029* might lead to the formation of a truncated protein because of the early termination of the PER3 polypeptide. Deep deletion was detected in all 3 period genes, resulting in homozygous allelic loss and gene amplification in PER3.

CRY1 and CRY2 analysis

Cryptochromes are the basic components of the molecular clock that generates the circadian rhythm. CRY1 and CRY2 are transcriptional regulators associated with DNA repair enzymes. Two major homologous domains (DNA photolyase domain and FAD binding) are present (18) A total of 12 mutations (11 missense mutations, 1 splice region mutation) were detected in the CRY1 gene. The p.K22N missense mutation on DNA photolyase domain was detected. The p.X429_splice region mutation was detected at the 9th intron boundary of 8 exons encoding the FAD _binding domain. The mutation in question may lead to an alternative branching site, resulting in intron retention, exon skipping, and intronic extension of the exon, leading to the formation of dysfunctional transcripts. Missense mutations were detected in the FAD _binding domain (p.R220C, p.P282S, p.M309I, p.W330L, p.A458V, and p.M470L). CRY1 and PER1 mutations were mutually exclusive in the SKCM patient group ($p < 0.05$).

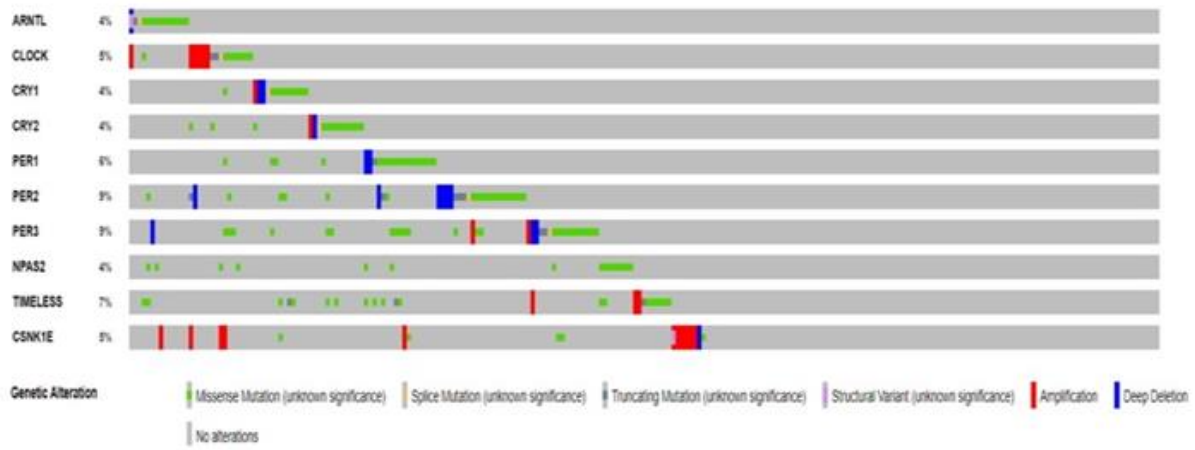

Figure-3. Genetic alterations of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E genes in SKCM patients. Percentages of overall mutations for each gene are given on the left**.**

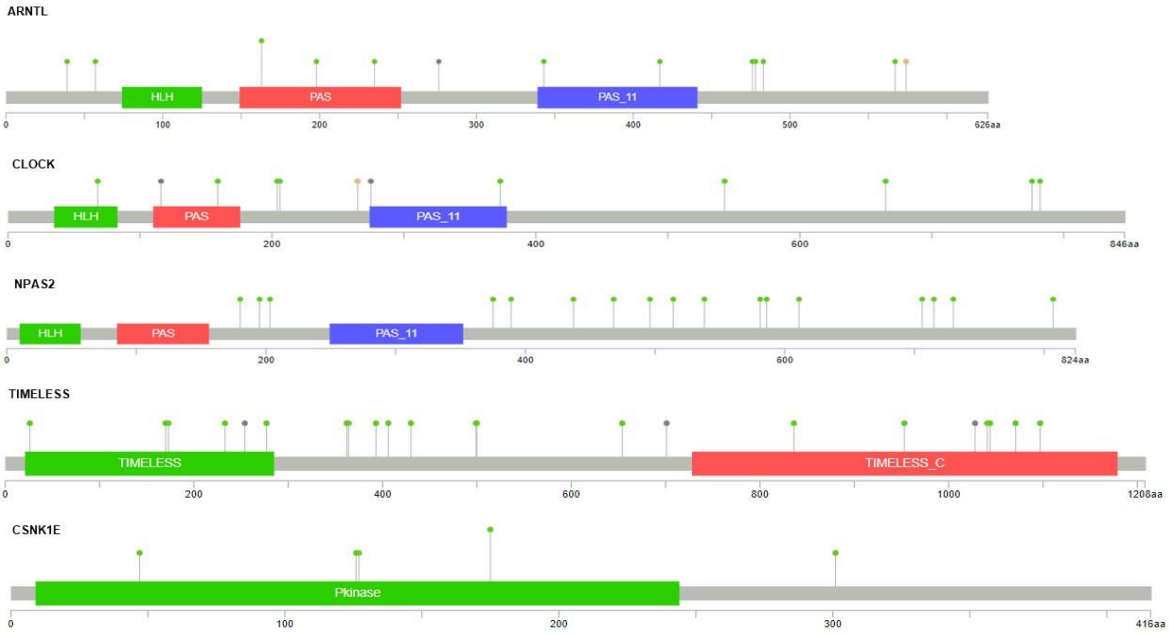

Figure-4. The localization of mutations detected in the domains of proteins belonging to the study genes in SKCM patient.

Thirteen mutations (12 missense, 1 splice region) were detected in *CRY2*.The p.X130_splice was detected at the 4th intron boundary of 3 exons encoding the DNA photolyase domain. On the other hand, 7 different amino acid codon modifying missense mutations were detected in the FAD _binding domain (p.V401G/M, p.S441F, p.R461C, p.P480C, p.S493L and p.M524. The frequency of CRY1 and CRY2 somatic mutations was determined as 4%. In addition, deep deletions were detected in both cryptochrome genes, resulting in gene amplification and loss of homozygote allele.

BMAL1, NPAS2, CLOCK, TIMELESS, and CSNK1E analysis

The BMAL1, NPAS2, and CLOCK genes belong to the transcription factor family, which includes the basic helix-loop-helix (bHLH) and PAS A and B domains. BMAL1 is a fundamental factor in the regulation of circadian rhythms (19). TEAD1-ARNTL fusion gene abnormality was detected, and 14 different mutations (12 missense, 1 splice region and 1 nonsense) were detected. The frequency of somatic BMAL mutation was 4%.

Twelve different mutations (9 missense mutations, 2 nonsense mutations, and 1 splice region mutation) were detected in the CLOCK gene. The p.E116* and p.E275* mutations detected in the PAS domains are of insufficient function and may result in truncated protein production. The p.X265_splice mutation, located at the 11-exon intron boundary, is able to cause the formation of non-functional transcripts, which is 100% conserved between species in the evolutionary process, an by creating an alternative exon splice region. Furthermore, CLOCK gene amplification abnormalities were detected. The frequency of somatic mutation frequency was 5%.

The largest circadian rhythm gene is *NPAS2* encoding neuronal PAS domain protein 2 (NPAS2), which is located at chromosome 2p11.2-2q13 (20). In the NPAS2, localized to 2q11.2, 17 different missense mutations were detected. The frequency of somatic mutation was 4%.

TIMELESS, as a circadian clock gene, was originally identified in *Drosophila melanogaster* as a crucial part of the circadian rhythm (21). Twenty-two different (19 missense, 3 nonsense) mutations were determined in TIMELESS gene. The p.E254* nonsense mutation detected on the TIMELESS domain probably causes loss of function of the protein to be formed with premature stop codon formation. Eleven missense mutations were located on sequences encoding TIMELESS domains. The frequency of somatic mutation was 6%.

Casein kinase 1 epsilon (CSNK1E), located in chromosome 22q13.1, is a member of the clock gene family (22). Six different missense mutations and fusion gene product were detected in CSNK1E gene. Five of the missense mutations were located on sequences encoding the protein kinase domain (Pkinase). The frequency of somatic mutation was 5%.

Results of Functional Impact Analysis of Detected Mutations

According to the analysis results of Poly-Phen2 and SIFT tools, the 152 missense mutations out of 64 mutations may be pathogenic since their pathogenic scores are close to 1 and they have "affected" properties and they may have disease-causing properties. **Table-1** shows the mutations that have pathogenic properties.

Results of Gene Expression Profile Analysis

The gene expression profiles of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E were determined as a result of comparing SKCM patients (n:461) with the healthy group. According to our results, the expression levels of ARNTL, CRY2, PER1, PER2, and PER3 were significantly down-regulated in SKCM patient group compared with the healthy group (**Figure-5**). However, the expression level of TIMELESS was up-regulated in the patient group in constrast to the other genes ($p<0.01$). In analysis to determine the effect of genetic mutations on mRNA expression levels, patients were divided into altered and unaltered groups for the study genes. When comparing the mRNA expression levels of these groups, the mRNA expreesion levels of PER3 and CSNK1E mRNA were higher in the unaltered patient group than in the altered group ($p <0.05$) (**Figure-6**).

Results of Protein-Protein Interaction Partners

STRING network analysis was performed to determine the functional interactions of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS and CSNK1E proteins in cellular processes. According to this analysis, target genes; have been shown to interact with SIRT, the NAD+ dependent class III deacetylase enzyme responsible for chromatin remodulation, histone acetyltransferase P300, the MCM gene family, which is a DNA replication licensing factor, and WDHD, which acts as adapter/regulatory modules in pre-m RNA processing and cytoskeleton formation (23,24). Target genes also interact with NONO, which is involved in the regulation of gene expression and DNA repair mechanism, and FBXL3 and BTRC, which are involved in the ubiquitin-protein ligase complex. Finally, target genes have been shown to interact with CDC45, the factor that initiates DNA replication, and with proteins involved in many important cellular processes that regulate mitosis (25,26) (**Figure-7**).

Discussion

Studies conducted in recent years show that there is a close link between circadian rhythm, cell cycle regulation, DNA damage response, and repair. Any disruption of this balance triggers replication stress and alters DNA repair capacity, leading to genome instability and eventually cancer (1,2,5,7,8,17-22, 27). In this study, the genetic profiles of ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E, which are key molecules of circadian rhythm, were examined in detail using the genome and transcription results of SKCM patients. As a result of genotyping analyzes in the SKCM cohort consisting of 442 patients; a total of 173 different mutations were determined in 10 genes (152 missense, 13 Nonsense, 6 splice regions, 2 fusion gene mutations). A review of the literature shows that as a result of genome profiling studies on circadian rhythm genes, variants of CLOCK1, CRY1, CRY2, NPAS2, PER1, RORA, and TIMELESS are associated with breast cancer risk, whereas BMAL1, CLOCK1, CRY1, CRY2, CSNK1E, NPAS2, PER1, PER2, and PER3. SNPs have been reported to be associated with prostate cancer risk (3-8,17-22,27-30). We investigated the mRNA expression profiles of circadian rhythm genes and proteins in the group of SKCM patients not only from a genome-wide perspective but also from a functional perspective.

BMAL1, one of the biological clock genes, is a transcription factor that plays an important role in regulating circadian rhythms. The C-terminal transactivation domain (TAD) of BMAL1 is required for the circadian cycle, and mutations in TAD affect the circadian cycle. BMAL1-TAD interacts with CRY and prometes transcriptional repression of CLOCK: BMAL1 [19,31,32]. The pathogenic mutations p.S567P and p.X574_splice, which we detected in the SKCM patients group, on the TAD domain may cause uncontrolled gene activation. BMAL1 gene expression was found to be downregulated. BMAL1 could directly bind to the promoter region of the p53, thereby transcriptionally activating the p53-dependent tumor suppressor pathway to induce apoptosis and cell cycle arrest (33). We found that decreased BMAL1 expression was statistically significantly associated with shortened survival in the group of SKCM patients, and we think that decreased BMAL1 mRNA expression is a poor prognostic marker in the development of SKCM because it cannot sufficiently activate the tumor suppressor pathway.

CLOCK p.M68I and BMAL1 p.R39L are missense mutations at the E-box binding site of the p.P57H target genes. The E-box facilitates transcription of several genes involved in cell proliferation, muscle and neural differentiation, immunoglobulin production, and circadian rhythms (19,31-33). NPAS2 is a paralog of the CLOCK protein and can functionally substitute CLOCK to regulate circadian rhythms. NPAS2 has a bHLH domain similar to CLOCK and BMAL1 in its N-terminal region and 2 PAS domains (PAS-A and PAS-B) in the amino acid region of 260-310. Studies have reported an association between SNPs in PAS domain and cancer risk (20,28,31-33). However, we did not find mutations/SNPs in the PAS domains in the SKCM patients.

Cryptochromes are a component of the negative feedback loop of the circadian cycle and are essential for the maintenance of the

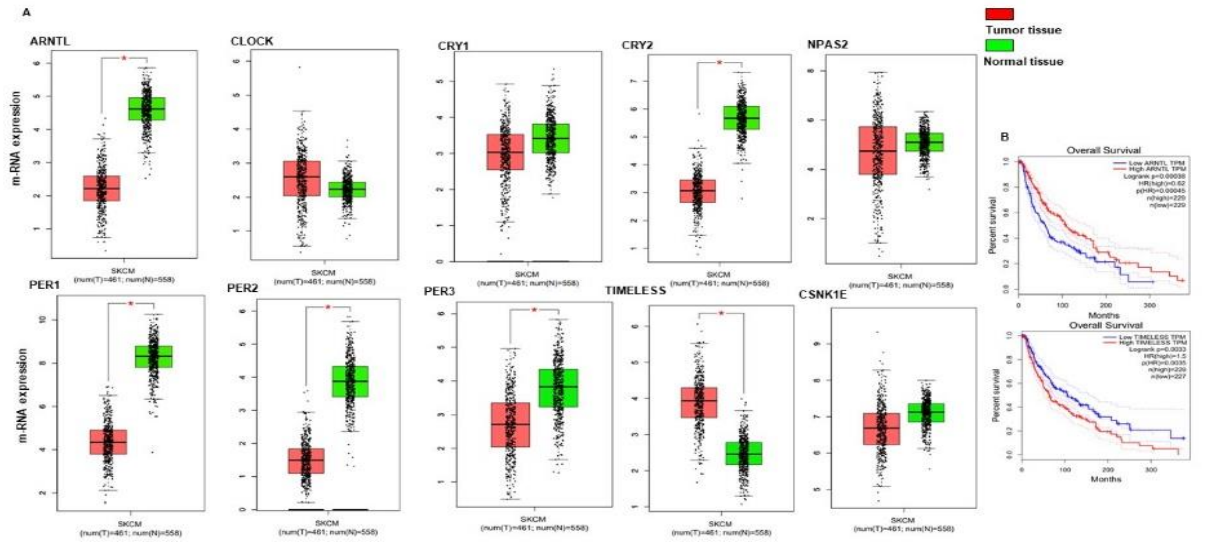

Figure-5.
Gene Expression Profiling Interactive Analysis (GEPIA) was performed to validate higher expressi on of seven hub genes (ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E) in SKCM samples compared with normal samples. The red and green boxes represent SKCM and normal skin tissues respectively. *represented p<0.01.

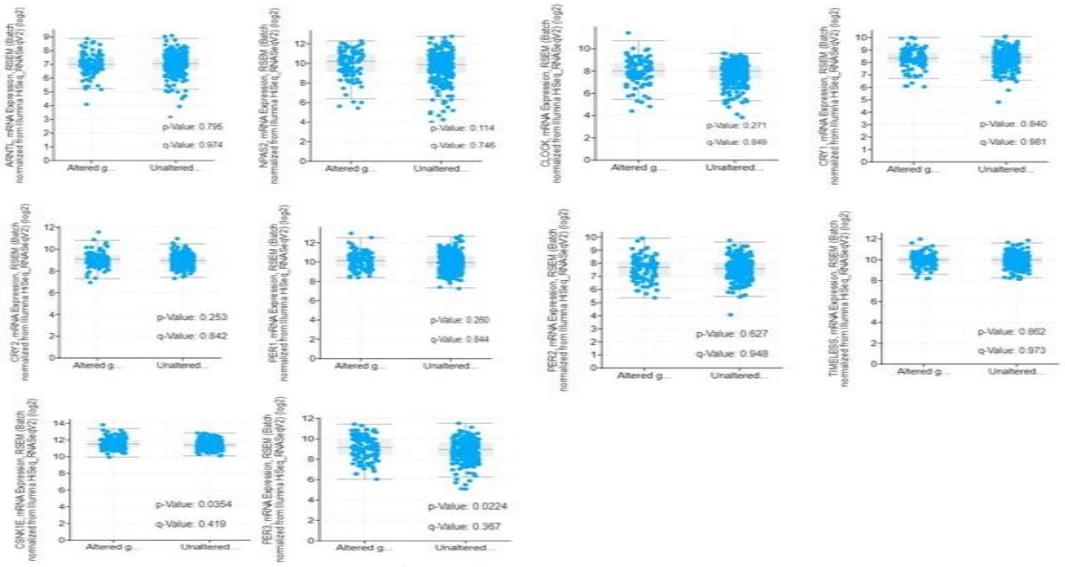

Figure-6. Mutational status for each gene is shown and the fold change indicates expression levels in the altered groups normalized to the expression levels in the unaltered groups.

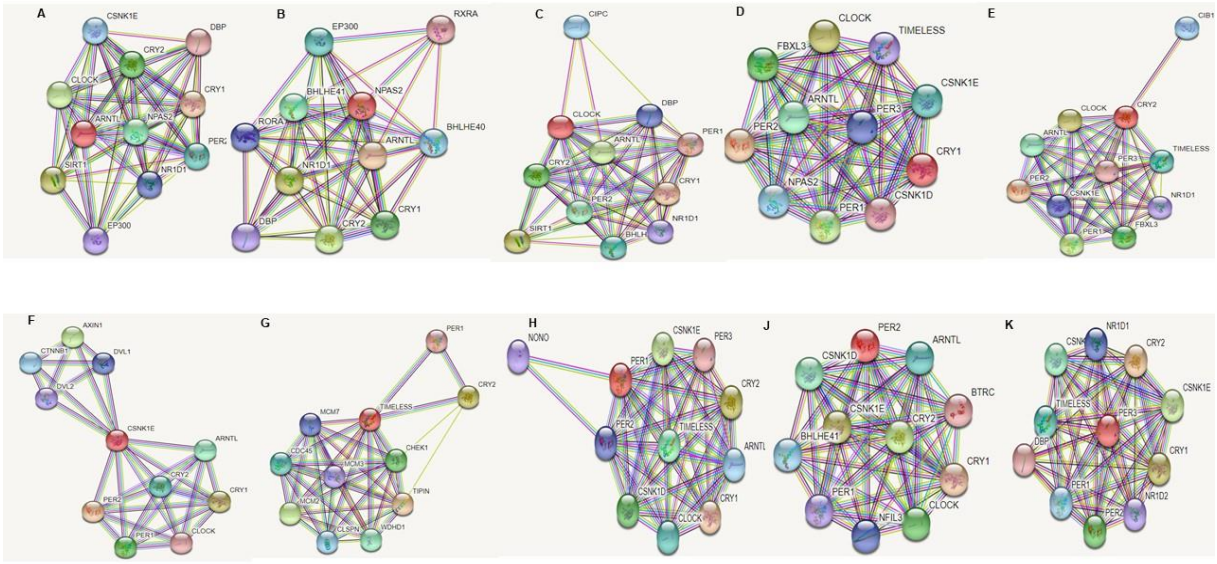

Figure-7. Schematic representation of known and predicted protein-protein interactions with the ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, PER3, NPAS2, TIMELESS, and CSNK1E genes. Each line has features. [Red line-indicates the presence of fusion evidence; Green line- neighborhood evidence; Blue line- cooccurrence evidence; Purple lineexperimental evidence; Yellow line- textmining evidence; Light blue line—database evidence; Black line—coexpression evidence.].

circadian rhythm. CRY1 and CRY2 contain the primary FAD binding domain and the DNA photolyase homology domain near the C-terminal region. CRY1 interacts directly with CLOCK and BMAL1 through two distinct regions in the PHR, photolyase homology region (PHR) domain. The PHR domain of CRY1 interacts with the C-terminal regions of PER1 and PER2 (18,34,35). Mutations in p.K22N, p.R135S, p.P144L, and p.X130_splice in this domain may affect the interaction of PER1 and PER2 in the SKCM patients group. In addition, p.M470L, p.A458V, and p.X429_splice mutations at amino acid residues 371-470, which are critical for the transcriptional inhibitory activity of CRY1, were detected in SKCM patiens. Cryptochromes are involved in the regulation of the cell cycle and the cellular response to DNA damage by controlling the expression of specific cell cycle genes [16,9,18,34-36]. In this study, we observed a significant upregulation of CRY2 in the group of SKCM patients. We think that this might lead to an impairment of cell cycle protein expression. Considering the results of STRING protein-protein analysis, FBXL3 is known to control cryptochrome transcription via ubiquitin-dependent degradation, which is one of the posttranslational modifications. Abnormalities in mRNA expression of CRY2 and mutations that alter the ubiquitination point could affect FBXL3-dependent degradation.

Many studies have shown that deregulation of PER1, PER2, and PER3, other circadian rhythm genes, plays an important role in the development of cancer (17,36,37). Casein kinase 1 (CK1) plays a central role in regulating the period of the circadian clock. PER2 protein expression is regulated by CK1-mediated phosphorylation and proteasomal degradation via the p.Ser478 residue of PER2 protein (38). The p.E179*, p.E305* and p.X386_splice mutations that we detected in current study, which can lead to the formation of stop codons and truncated proteins, have the features that can lead to the loss of the Ser478 phosphorylation site by altering the reading frame. In the SKCM patient group, we identified several amino acid codon altering missense mutations in the domains forming the PER1 and PER3 proteins, nonsense mutations that might result in truncated PER proteins, and

splicing mutations that might cause intron-exon boundary disruption. In SKCM patients, PER1, PER2, and PER3 were found to be statistically significantly downregulated. In particular, PER2 acts as an important tumor suppressor in tumor suppression and in vivo DNA damage response. Neoplastic growth of cancer cells can be restricted and their apoptotic rate increased by overexpression of PER proteins (17,37). We see that downregulation of PER2 mRNA expression in the SKCM cohort has lost its tumour suppressive property, as in cancers such as HCC, CML, and HNSCC, pancreas and breast. Moreover, it has been reported that downregulation of PER2 expression is often associated with poor prognosis (17,37-41). In addition, STRING protein-protein interaction analysis reveals that SIRT1 regulates circadian clock gene expression by deacetylating PER2.

The CSNK1E gene encodes the CK1ε protein whose main function is to regulate the circadian rhythm by phosphorylating other clock gene products. In addition, CSNK1E is known to phosphorylate key proteins in cancer signaling pathways such as p53 and β-catenin (42). In the SKCM patients group, 5 different missense mutations (p.P47S, p.R127W, p.G175S, p.H126Y, p.G175D) were identified in the protein kinase domain that phosphorylates CSNK1E. The p.P301S mutation is located in the autoinhibitory domain responsible for controlling kinase activation. However, due to its benign nature, we can say that there is no possible pathogenic effect. TIM is the best characterized gene in DNA replication and damage repair because it controls DNA replication and maintains the stability of the replication fork and genome. TIM contains TIMELESS, TIMELESS C, and regions defined as DNA-binding domain (DDT) that allow itto function as a transcription factor (21,43).

In the SKCM patients group, we identified p.H364Y, p.K362N, p.A836V, p.S1097F missense mutations on DDT subdomains (342-365 a.a, 814-866 a.a, 1084-1144 a.a). Because these mutations are located at DNA binding sites, they may reduce and/or inhibit the activation of MCM7 and CHEK1, the target genes of TIMELESS. Recent studies have shown that TIMELESS plays a role in cancer progression as an oncogene (21,43). In present study, it was found that the TIMELESS mRNA level was significantly increased in the SKCM patient group compared with the healthy group, confirming the oncogenic character of TIMELESS for this type of cancer. In the SKCM patients group, upregulated TIMELESS mRNA expression is a poor prognostic marker associated with shortened overall survival (p=0.0033). In addition, STRING protein-protein interaction analysis shows that TIMELESS, one of hub proteins, appears to be a transcription factor that interacts with key proteins responsible for DNA replication. For example: MCM proteins (MCM2-7) are a family of DNA-binding proteins found in the nucleus and are the licensing factor of the replication initiation complex that initiates DNA synthesis in all eukaryotes (44). Impaired expression of TIMELESS may stimulate uncontrolled DNA replication. Finally, according to the results of protein-protein interaction analysis: it is shown that NR1D2 has a one-to-one interaction with the key molecules of circadian rhythm. NR1D2 is a ligand-dependent transcriptional repressor of metabolism and inflammatory response and has been shown in recent studies to have an impaired expression profile in various cancers. This could be one of the triggering factors in the pathogenesis of SKCM.

Conclusion

In summary, 152 missense mutations, 13 nonsense mutation, 6 splice region mutations and, 2 fusion gene mutations among the 173 mutations found in 10 genes, and 62 of them had pathogenic properties. We argue that ARNTL and TIMELESS levels might be an independent risk factors for SKCM due to its pathogenicity. Circadian rhythm plays an important role in skin physiology, and changes in molecular clockwork have a major impact on the development of skin cancer. These results indicate that the function-altering pathogenic mutations and abnormal expression levels we detected in key circadian rhythm genes in SKCM tissues may be the factors underlying the tumor development process and could serve as molecular targets for the diagnosis and especially the treatment of SKCM

Supplemental material: https://saglikbilimleri-my.sharepoint.com/:w:/g/personal/esra_pehlivan_sbu_edu_tr/Efd4qxO0sT9FvgppY5e13KMBzp2fs-Esxj1KS_qTKiRnGw?e=uWd3s

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