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ORIGINAL ARTICLE

Role of Circadian Rhythm-Related Genes in the Pathogenesis of Breast Cancer

Meme Kanseri Patogenezinde Sirkadiyen Ritimle İlişkili Genlerin Rolü

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Abstract

Introduction: Circadian rhythm plays a crucial role in many physiological processes, such as genomic stability, DNA repair mechanisms, and apoptosis, and is often disrupted in breast cancer (BRCA). Therefore, this study aimed to elucidate the relationship between BRCA pathogenesis and circadian rhythm by comprehensively determining gene mutations, expression, and methylation profiles of circadian rhythms using bioinformatics tools.

Methods: The genome and expression profiles of the BRCA cohort (n: 1085) were obtained using bioinformatics tools providing data from The Cancer Genome Atlas. PolyPhen-2, SIFT, and Mutation Assessor tools were used to estimate the oncogenic–pathogenic effects of the detected mutations in BRCA pathogenesis. STRING analysis was performed to better understand the functional relationships of mutant proteins in cellular processes. In addition to genome profiling, gene expression and methylation profiles were generated.

Results: In total, 64 mutations were identified in 9 genes. Of these 64 mutations, 20 were classified as pathogenic or oncogenic. CRY1 and PER1 gene expression was downregulated in patients with BRCA whereas TIMELESS was upregulated compared with the healthy group ($p<0.01$). The effect of m-RNA expression, which is considered a prognostic marker, on overall survival was found to be significant for decreased CLOCK ($p=0.026$) and CRY1 ($p=0.025$) levels. STRING analysis revealed that hub proteins interact with the acetyltransferases P300 and NONO, which are involved in sister chromatid cohesion.

Discussion and Conclusion: The results indicate that CRY2 and PER1 gene expression is downregulated in BRCA and that circadian rhythm disruption may be associated with BRCA development.

Keywords: Breast cancer; Circadian rhythm; Cryptochrome; Gene expression; Mutation

Circadian rhythm is defined as the repetition of behavioral, mental, physiological, and biochemical rhythms in living organisms within a 24-h period. This rhythm function is like a molecular-based machine that determines the temporal regulation of physiology to maintain homeostasis.[1–3] It is critical for maintaining

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the normal physiology of an organism, including cell proliferation, cell cycle progression, DNA repair, and cellular metabolism.[1–4] It is controlled by circadian pathway genes. When the circadian clock is studied at the molecular level, it becomes clear that it is a product of clock genes in the transcription–translation regulatory system. Molecular rhythms play crucial roles in the expression and inhibition of proteins.[4,5] However, because these mechanisms are tightly regulated, the occurrence of circadian clock mutation alters the expression of proteins involved in the cell cycle. Therefore, disruption of circadian rhythm may be closely related to the development of several tumors, and oncogenic processes directly weaken the circadian rhythms.[3–6] Breast cancer (BRCA) is the most common type of cancer in women.[7] Some epidemiological studies have demonstrated a significant association between mutations in circadian genes (BMAL1/ARNTL, CLOCK, CRY1, CRY2, RORA, RORB, RORC, and PER1) and BRCA pathogenesis.[2–6,8]

Changes in circadian rhythm accelerate the proliferation of mammary epithelial stem cells, induce the development of mammary glands, and increase the incidence of spontaneous mammary tumors in mammals.[8,9] After BRCA is diagnosed by radiological imaging and pathological examination, various treatment options are available. Treatments depend on the characteristics and location of the tumor. Chemotherapy can be combined with other local treatments, such as radiotherapy and surgery, if necessary.[10] Studies conducted recently have shown that it is more effective to align cancer treatment protocols with the circadian clock. Therefore, the concept of chronochemotherapy has emerged, where treatment is administered at specific times of the day and according to the circadian period.[9,10] To achieve optimal efficacy with chronotherapy, the goal is to align the timing of drug administration with the corresponding phase of the circadian rhythm.

Chronotherapy administers chemotherapeutic agents at appropriate times of the circadian clock to achieve the best therapeutic index.[6,8–10] A study investigating the response to radiotherapy in BRCA with circadian disruption showed that circadian genetic variations and treatment duration influence the response to radiation and toxicity in women with BRCA.[6,8–10] Because various functions, including cell cycle and division, are at least partially controlled by components of the molecular clock (CLOCK, BMAL1/ARNTL, CRYs, and PERs), it is expected that the efficacy of chemotherapeutic agents will increase and their side effects will decrease if chemotherapy is administered at the right time. However, mutations in the genes encoding the hub proteins (CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS) that constitute this rhythm can lead to rhythm disruption and prevent the cellular processes regulated by this rhythm from functioning properly. With this in mind, we used bioinformatics tools to study the comprehensive genomic profile of the genes encoding molecules that ensure correct functioning of the body's chronobiological function in patients with BRCA.

Table 1. Demographic, clinical and genetic data of patients with BRCA

Characteristic	**Patient data n=1084 (%)**
Gender	
Male	12 (1.1)
Female	1072 (98.9)
Diagnosis age, years	
Cancer type	
Breast invasive ductal carcinoma	780 (72.0)
Breast invasive lobular carcinoma	201 (18.5)
Breast invasive carcinoma (NOS)	77 (7.1)
Breast invasive mixed mucinous Ca,	17 (1.6)
Metaplastic breast cancer	8 (0.7)
Invasive breast carcinoma	1 (<0.1)
Diagnosis type	
BRCA_LumA	499 (46)
BRCA_LumB	197 (18.2)
BRCA_Basal	171 (15.8)
BRCA_Her2	78 (7.2)
BRCA_Normal	36 (3.3)
NA	103 (9.5)
Overall survival status	
Living	933 (86.1)
Deceased	151 (13.9)
Total mutation frequency in BRCA	**Case (frequency %)**
Clock genetic alteration	1.3
ARNTL/BMAL1 genetic alteration	1.2
PER1 genetic alteration	1.2
PER2 genetic alteration	1.8
CRY1 genetic alteration	0.5
CRY2 genetic alteration	1.2
NPAS2 genetic alteration	1.3
SIRT1 genetic alteration	1.2
TIMELESS genetic alteration	1.1

BRCA: Breast cancer.

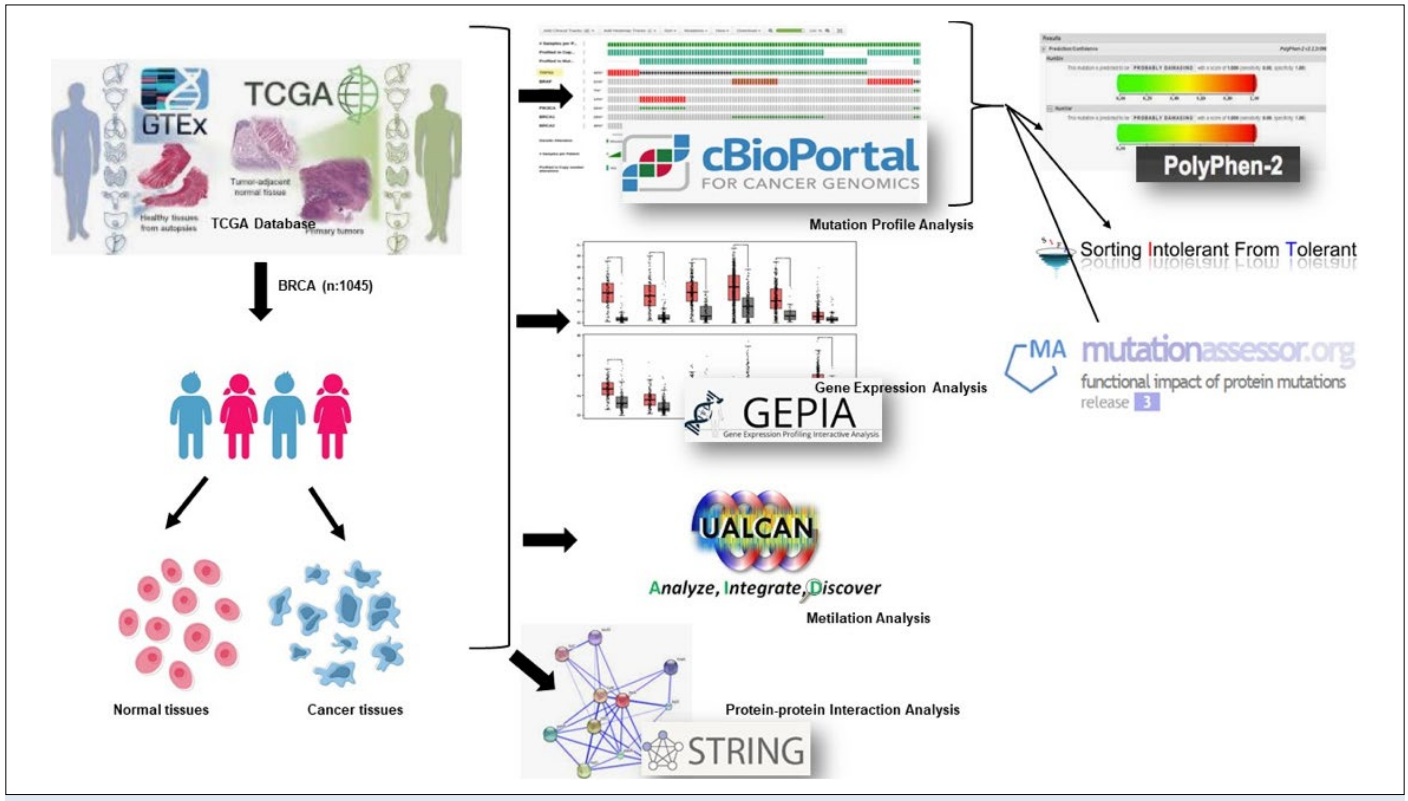

Figure 1. A flowchart of the implemented workflow illustrates the methods followed.

Materials and Methods

Study Group

The BRCA cohort (n=1085) was obtained from the cBioPortal (http://cbioportal.org) database. Figure 1 provides an overview of the research design and procedures. Data were downloaded on September 29, 2022. Detailed demographic and clinical characteristics of the dataset consisting of 1085 BRCA patients are presented in Table 1.

Genotyping Analysis

The cBio Cancer Genomics Portal is a bioinformatics tool that ensures that data are collected from The Cancer Genome Atlas (TCGA).[11] To comprehensively investigate the mutations in CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes in BRCA samples (n: 1085), BRCA was selected as the cancer type of interest in the web interface. Comprehensive mutation profile analyses were then performed using the OncoPrint, Cancer Type Summary, and Mutation tools provided by cBioPortal, with the functions of relevant genes provided by the interface.

Mutation Impact Analysis

The oncogenicity–pathogenicity of mutations identified in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS databases was determined using the PolyPhen-2, SIFT, and Mutation Assessor databases. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) is an online bioinformatics tool that supports the prediction of potential effects of mutations on protein stability and function through structural and comparative evolutionary analyses of the amino acid positions of possible mutations and SNPs.[12] SIFT (https://sift.bii.a-star.edu.sg/) is a bioinformatics tool that uses sequence homology and physical properties of amino acids to estimate whether the position of an amino acid affects protein function.[13] The Mutation Assessor (http://mutationassessor.org/r3/) estimates the functional effects of amino acid substitutions in proteins, such as mutations in cancer or polymorphisms. The functional effect was assessed on the basis of the evolutionary conservation of the affected amino acid in protein homologs.[14]

Gene Expression and Survival Analysis

GEPIA (http://gepia.cancer-pku.cn/) is a web server that allows users to perform differential expression analysis at the subtype level.[15] GEPIA is used to analyze the expression of genes and isoforms by comparing it with TCGA and Genotype Tissue Expression data. Therefore, we used this data provider to determine the differential expression of key genes in the BRCA cohort and healthy tissue samples.

Analysis of Promoter Methylation Levels

UALCAN is an interactive, freely accessible website for analyzing OMICS data. (http://ualcan.Path.uab.edu/index.html).[16] This website is based on PERL-CGI and can be used for approximately 6000 gene methylation levels. In this study, the promoter methylation levels of circadian rhythm genes in the BRCA dataset were analyzed.

Analysis of Protein-Protein Interactions

The STRING database (https://string-db.org) was used to determine protein–protein interactions.[17] The aim of this database is to create a comprehensive and objective network that includes physical and functional properties. The estimated interactions of the proteins CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS were performed using STRING, which revealed physical and functional relationships between proteins.

Statistical Analysis

All statistical analyses used to evaluate the study data were performed using the GEPIA database. The GEPIA database uses differential analysis to compare gene expression between tumor and healthy groups. One-way ANOVA was used to evaluate the study data. Overall survival was analyzed using Kaplan–Meier curves; p values <0.05 were considered statistically significant.

Results

Genotyping Results

In patients with BRCA, mutations in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes were analyzed using cBioPortal. It was found that 10.8% of patients with BRCA had at least one genetic mutation (missense, nonsense, frameshift mutation, deletion, and gene amplification) in their target genes. A total of 64 mutations were identified in detail in 9 genes (51 missense mutations, 7 frameshift insertions/deletions, 2 nonsense, 2 splice regions, and 2 fusion gene mutations) and are summarized in Appendix 1. The distribution of mutations in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes in patients with BRCA from the cBioPortal is shown in Figure 2a. Because the CLOCK p.X377_splice and BMAL1/ARNTL p.X47_splice region mutations are located in the splice site, which has been conserved between species during evolution, these mutations likely cause abnormalities in the expression of these genes. Additionally, the p.E162* and p.E819* nonsense changes detected in the coding sequences of the CLOCK gene may cause the polypeptide to terminate prematurely and form a truncated protein. Frameshift mutations that disrupt the reading frame were identified in the CLOCK, PER1, PER2, CRY1, NPAS2, and TIMELESS genes and were located in sequences that code for important domains. The somatic mutation frequencies of the target genes were found to be CLOCK (0.8%), BMAL1/ARNTL (0.4%), PER1 (0.8%), PER2 (1%), CRY1 (0.1%), CRY2 (0.3%), NPAS2 (0.7%), SIRT (0.4%), and TIMELESS (0.9%). CLOCK; TIMELESS, BMAL1/ARNTL; CRY2, PER2; TIMELESS and CLOCK; The coexistence of PER2 mutations was statistically significant (p values of 0.008, 0.008, 0.015, and 0.021). Abnormalities were observed in the ILKAP-PER2 fusion and the POLA1-TIMELESS fusion.

Mutation Impact Analysis Results

According to the results of the pathogenicity assessment analysis using the Poly-Phen2, SIFT, and Mutation Assessor tools, 51 of the 20 missense mutations determined in this study were classified as pathogenic in all three tools. Because the pathogenic scores of these mutations are close to 1 and "affected," they were classified to be potentially pathogenic and assessed as disease-causing. The pathogenic and oncogenic mutations are summarized in Appendix 1.

Gene Expression and Survival Analysis Results

The m-RNA expression patterns of BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS were analyzed in BRCA tumor samples (n=1085) and healthy tissues (n=291) matched to this cancer type using the GEPIA tool. Figure 2b shows that PER1 and CRY2 expression was statistically significantly downregulated in the patient group than in the healthy group. However, the expression of TIMELESS was upregulated in the patient group compared with that of the other genes ($p<0.01$). Survival analysis performed according to low and high gene expression profiles showed that individuals with low CLOCK and CRY1 expression had a longer life expectancy, and the differences were significant ($p<0.01$) (Fig. 2c).

Degree of Promoter Methylation Analysis Results

DNA methylation is an important prerequisite for epigenetic modification of the genome and is linked to the carcinogenesis process. The PER1 expression profile in BRCA tumor samples showed a low expression level. The results of the analysis performed using the UALCAN database to determine DNA methylation levels revealed

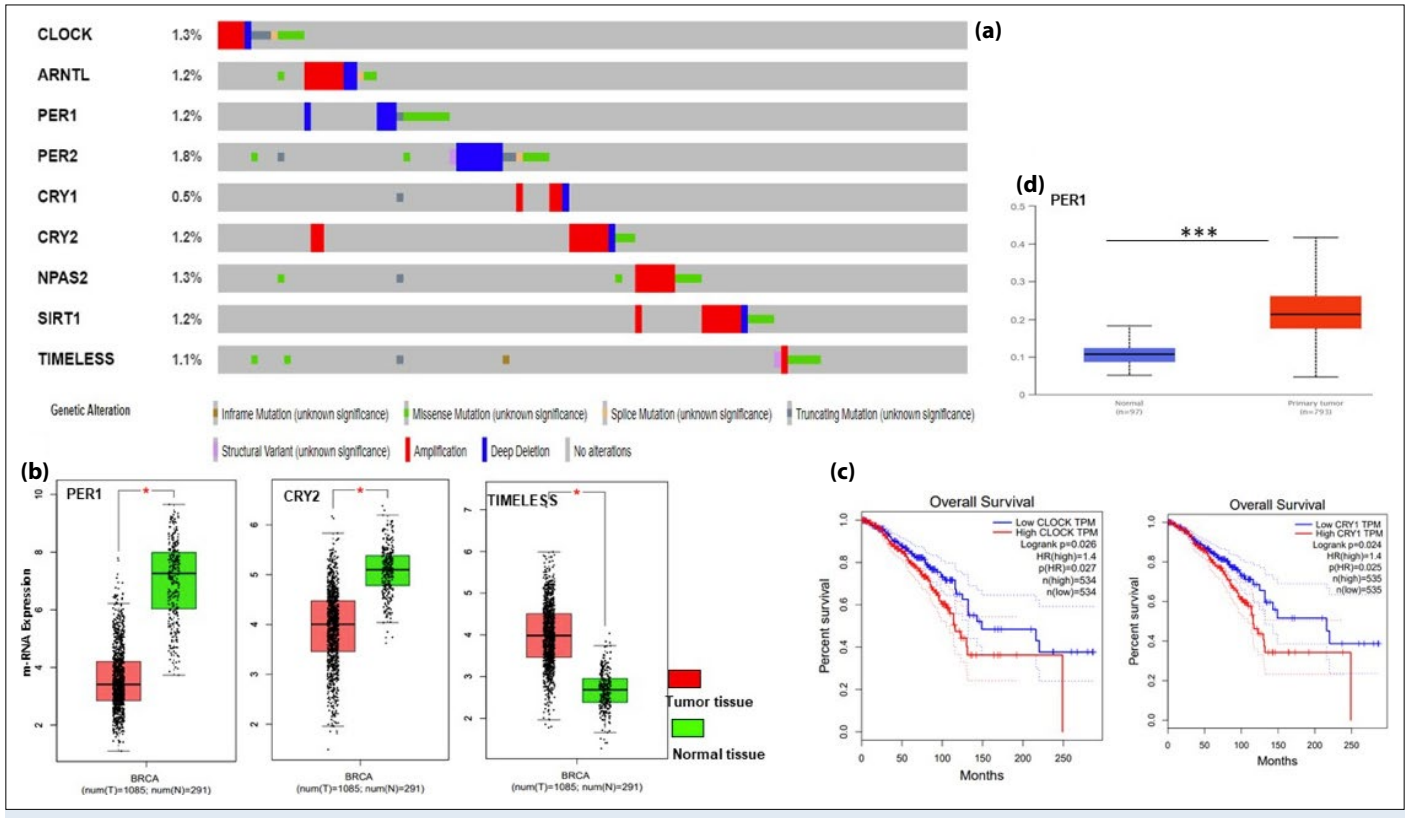

Figure 2. (a) Distribution of mutations in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes in TCGA breast cancer (BRCA) cohort from cBioPortal. The percentages of overall mutations for each gene are presented on the left. **(b)** Comparative analysis of m-RNA expression values of mutant and wild-type individuals in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes ($p<0.05$). **(c)** Comparison of Kaplan–Meier survival curves of high and low expressions of the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes in The Cancer Genome Atlas BRCA cohort ($p<0.05$). Red line, high expressions of m-RNA; green line, low expression of m-RNA. **(d)** Promoter methylation level of PER1 in the BRCA cohort ($p<0.05$).

that the promoter methylation level of PER1 was statistically significantly higher (hypermethylation) than that of healthy tissues (Fig. 2d).

Protein–Protein Interaction Analysis Results

STRING analysis was performed to determine the functional interactions of CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS in cellular processes. The structural domains of the proteins belonging to the genes investigated in the BRCA patient group and the localizations of the detected mutations were shown in Figure 3. According to this analysis, the target genes, as shown in Figure 4, include BHLH and DBP transcription factors; histone acetyltransferase P300; NONO, which is involved in the regulation of gene expression; P53, which is a tumor suppressor protein with transcriptional activation; and FBXL3, which is involved in the ubiquitin–protein ligase complex and is important for embryonic development, cellular differentiation, circadian lipid metabolism, circadian rhythm, and anabolic steroid rhythm regulation, such as RORA.

Discussion

Numerous studies have investigated various aspects of the circadian clock in relation to BRCA. The results of these studies indicate that genetic mutations and alterations in melatonin and circadian rhythm gene expression play an very important role in BRCA development.[4,8–10,18–21] Furthermore, epigenetic mechanisms accelerate the process of BRCA development by influencing these signaling pathways independently or together with genetic mutations.[19–23] In this study, the genomic profiles of CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS, which play crucial roles in the execution of the circadian rhythm, were examined in detail using genomic and transcriptional data in patients with BRCA. As a result of the genotyping analyses in the BRCA cohort, which consists of 1085 patients, 64 mutations were detected in 9 genes (7 frameshift insertions/deletions, 2 nonsense, 2 splice regions, and 2 fusion gene mutations). In the BRCA cohort of 1085 patients, 10.8% had somatic mutations in genes responsible for circadian rhythm.

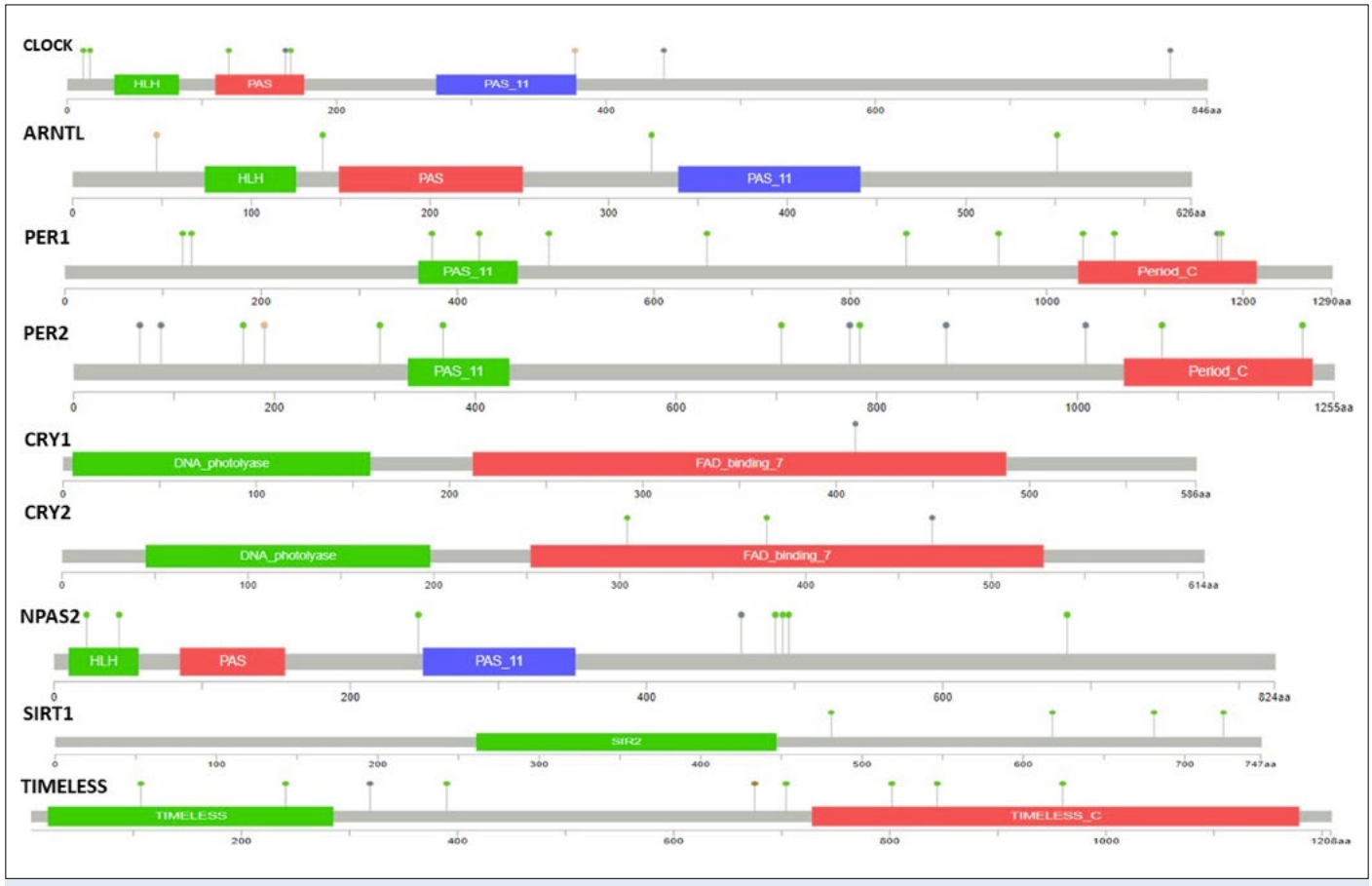

Figure 3. Schematic representation of the domain architectures of the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS proteins and mutations detected in The Cancer Genome Atlas breast cancer cohort.

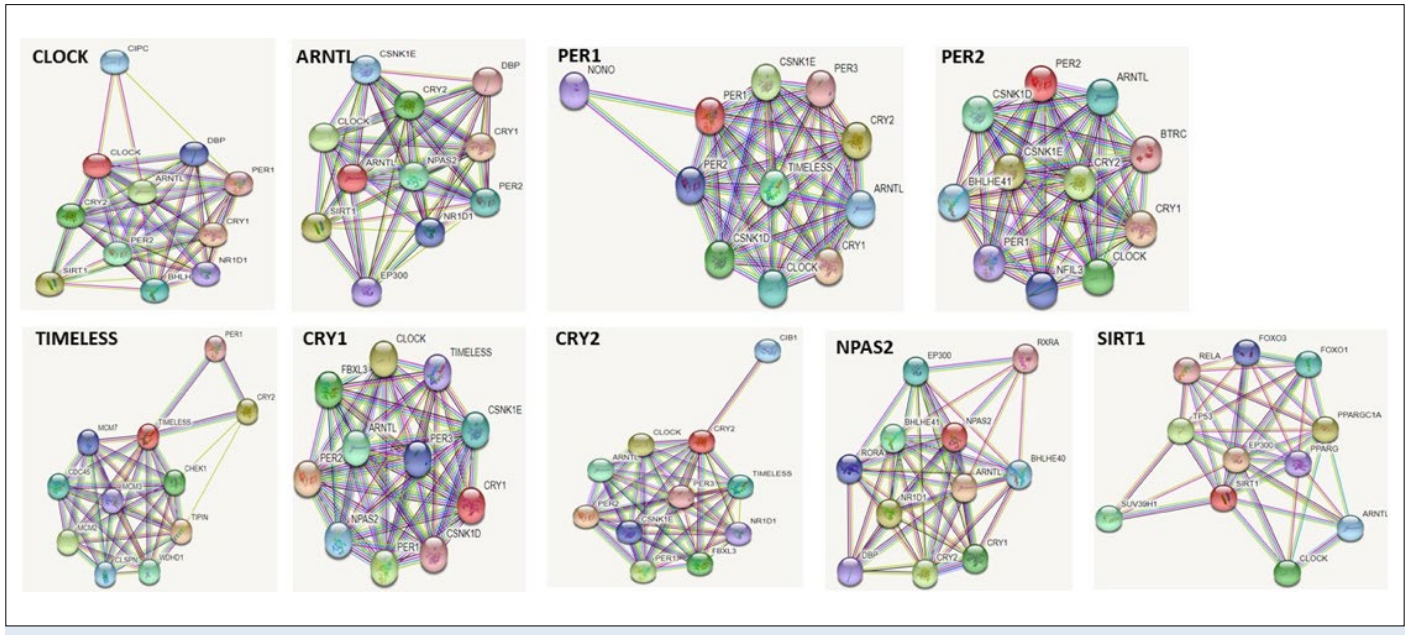

Figure 4. Schematic representation of known and predicted protein–protein interactions with the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes. Each line has the following characteristics: red line, the presence of fusion evidence; green line, neighbor-joining evidence; blue line, co-excursion evidence; purple line, experimental evidence; yellow line, text mining evidence; light blue line, database evidence; black line, co-expression evidence.

CLOCK is a histone acetyl transferase that is activated by heterodimerization with BMAL1/ARNTL. CLOCK and BMAL1/ARNTL proteins have bHLH and PAS domains that are homologous to each other, and they regulate the transcription of genes by binding to the transcriptional control regions of various genes via these domains.[24] The p.G120V, p.V166I, and p.E162* mutations in CLOCK are located in the PAS-A domain and can lead to the loss of sequences of the binding site. BMAL1/ARNTL is a member of nuclear receptor subfamily 1 group D that, like CLOCK, functions as a transcription factor. The C-terminal transactivation domain (TAD) of BMAL1/ARNTL is required for circadian rhythmicity.[25] The BMAL1/ARNTL-TAD pathway facilitates the transcriptional repression of CLOCK: BMAL1/ARNTL by interacting with CRY.[26,27] In the BRCA cohort, the 5 exon/6th intron border region p.X47_splice mutation may create an alternative branch point in the splice complex. This can ultimately lead to intron retention, exon skipping, and intronic exon expansion, resulting in dysfunctional transcripts with TAD deficiency. The CRY1, CRY2, and PER genes form the negative arm of the circadian feedback loop and are required for the maintenance of circadian timing.[26] CRY is involved in the control of DNA damage and cell cycle progression. CRY1 and CRY2 contain the primary FAD-binding and DNA photolyase homology domains near the C-terminal site. This domain regulates circadian rhythm by controlling the FAD levels.[3,5,6,26,27] The p.H411Tfs*26 frameshift mutation that we discovered in CRY1 is located on the FAD-binding domain and can cause a shift in the reading frame, leading to the formation of dysfunctional transcripts. Studies have shown that CRY2 is downregulated in BRCA tissue compared with that in surrounding cancer tissue and healthy controls. Therefore, low CRY2 expression in patients with BRCA has been associated with estrogen receptor negativity, higher tumor grade, and shorter overall survival.[27,28] CRY2 expression was found to be statistically significantly lower in the BRCA cohort, which is consistent with the literature. Therefore, it can be considered a prognostic candidate for poor prognosis.

TIMs interact with CRY and PER proteins and negatively influence the circadian rhythm. Additionally, TIM is a highly effective gene for DNA replication and repair in mammals by controlling DNA replication and maintaining the replication fork. The TIM protein contains regions defined as TIMELESS, TIMELESS C, the DDT domain, which functions as a transcription factor, and DNA-binding domains.[29,30] Mutations p.R107W, p.R241C, p.E820Q, and p.D844Y with oncogenic character are located in these domains, and MCM7, which is a target gene of TIMELESS, can decrease and/or inhibit CHEK1 activation. Although there are insufficient studies showing an association between the m-RNA expression level of TIM and BRCA, it has been shown that TIM is overexpressed differently in breast tumor samples compared with healthy tissues.[29,30] It has been reported that cell proliferation is significantly reduced in MCF-7 cell lines in which the TIM gene has been switched off. TIM also improves the invasion and migration abilities of BRCA cells, albeit in part by regulating the expression of MYC. This is because MYC can modulate the circadian system by binding to E-box elements. High MYC expression disrupts the circadian rhythm and increases proliferation.[29,31] Additionally, TIM can regulate sphingolipid metabolism in BRCA and control tumor cell growth via the Sp1/ACER2/S1P axis.[29] In agreement with the literature, the expression of TIM m-RNA was statistically upregulated in the BRCA cohort compared with that in healthy tissue. This finding could be one of the factors triggering BRCA pathogenesis.

SIRT1, a III (NAD+)-type dependent histone/protein deacetylase, modulates cell survival by inhibiting apoptosis or cellular senescence, including DNA damage and oxidative stress.[30] SIRT1 is required for circadian transcription of several important clock genes, including BMAL1/ARNTL, PER2, and CRY1.[32,33] The p.D481N missense mutation was discovered in the sirtuin homology/catalytic domain of SIRT, which is responsible for protein deacetylation. This mutation is oncogenic and can disrupt the activation of genes responsible for cell proliferation and survival.

The PER family is mainly responsible for the formation of rhythm and is known to organize the basic activities of life.[2,3,6,34,35] PER2 protein expression is modulated by CK1-regulated phosphorylation and proteasomal degradation via p.Ser478 of the PER2 protein. In this study, PER2 was the most frequently somatically mutated gene. p.M67Cfs*17, p.K87Nfs*25, and p.X190_splice mutations that can cause the formation of stop codons and truncated proteins, as we found in the BRCA cohort, can cause loss of the Ser478 phosphorylation site by altering the reading frame. Frameshift mutations, particularly in PER2, were identified in patients with metastasis. In the BRCA cohort, PER1 was statistically downregulated. Studies have shown that low PER1 expression levels are closely associated with estrogen receptor-negative, progesterone receptor-negative, human epidermal growth factor receptor 2-positive status, basal-like, and triple-negative status in BRCA samples.[35,36] Additionally, low PER1 expression levels were found to be associated with low survival in patients with BRCA, whereas high gene expression levels were associated with high and recurrence-free survival. PER1 was found to be hypermethylated in the BRCA cohort. This may be a reason for poor prognosis. The ILKAP-PER2 fusion

detected in the BRCA cohort was identified. ILKAP is a protein whose favorable phosphorylation states are crucial for cell proliferation. Therefore, it plays a crucial role in regulating cell cycle progression through substrate dephosphorylation. This protein selectively associates with integrin-associated kinase (ILK) to regulate cell adhesion and growth factor signaling.[37] Simultaneously, ILKAP inhibits the signaling axis of ILK-GSK3B and may play a crucial role in inhibiting oncogenic transformation.[35] It is possible that this leads to uncontrolled PER2 activation of the genetic abnormality.

NPAS2 is a paralog of the CLOCK protein that can functionally replace CLOCK to modulate circadian rhythms. NPAS2 is a transcriptional activator that can bind to DNA via its E-box sequence and form heterodimers with another circadian protein, BMAL1/ARNTL. NPAS2 has a bHLH domain and two 260–310 amino acid long PAS domains (PAS-A and PASB) in its N-terminal region, similar to CLOCK and BMAL1/ARNTL. PAS domains bind specifically to E-box sequences (CACGTG) in target DNA, and the bHLH domain stabilizes the DNA-binding complex.[38,39] In the BRCA cohort, the oncogenic missense mutations p.R22W and p.D44E are located on the bHLH domain and can interfere with the transcriptional activator of NPAS2.

Conclusion

On the basis of the findings using bioinformatics tools in a BRCA cohort of 1084 individuals, genetic abnormalities in the circadian signaling pathway may have an effect on BRCA pathogenesis and susceptibility. Mutations and polymorphisms in proteins encoding the circadian rhythm may be strong candidates for increased risk of BRCA because they are associated with circadian rhythm disruption and altered gene expression. This study may help us better understand the molecular functions of circadian rhythm-related factors in BRCA. A thorough understanding of these molecular changes may therefore allow us to evaluate potential molecular targets for BRCA chemotherapy and immunotherapy in the context of chronotherapy.

Ethics Committee Approval: Not applicaple. A bioinformatics study.

Authorship Contributions: Concept: DA, DFA; Design: DA, DFA; Supervision: DA, DFA; Data Collection or Processing: DA, EO, DFA; Analysis or Interpretation: DFA; Literature Search: DA, EO, DFA; Writing: DA, EO, DFA; Critical Review: DA, DFA.

Conflict of Interest: None declared.

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Appendix 1. Characteristics of the mutations detected in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes of the TCGA BRCA cohort

No	Gene	Nt alteration	Rs number	AT	Loc.	AA position	MS	Type of ca.	Clinical significance		
									Poly-Phen2 (score)	SIFT (score)	Mutation assesor (score)
M-1	CLOCK	c.49G>T	COS_3825996	Missense mutation	Exon-4	D17Y	M0	BRCA_Basal	Possibly Damaging (0.53)	Deleterious (0.00)	Low (1.79)
M-2	CLOCK	c.1327_1328del	COS_1486016	Frame Shift Deletion	Exon-15	T443Gfs*28	M0	BRCA_Her2	NA	NA	NA
M-3	CLOCK	c.359G>T	rs767225278	Missense mutation	Exon-7	G120V	M0	BRCA_LumA	Probably Damaging (1.00)	Deleterious (0.00)	High (3.75)
M-4	CLOCK	c.496G>A	NA	Missense mutation	Exon-8	V166I	M0	BRCA_LumB	Benign (0.00)	Tolerated (0.13)	Low (1.05)
M-5	CLOCK	c.35C>T	rs753109195	Missense mutation	Exon-3	S12L	MX	BRCA_LumA	Benign (0.00)	Deleterious (0.00)	Low (1.25)
M-6	CLOCK	c.1130+3A>G	NA	Splice_ Region Mutation	–	X377_splice	M0	BRCA_LumB	NA	NA	NA
M-7	CLOCK	c.2455C>T	NA	Nonsense mutation	Exon-22	Q819*	M0	BRCA_LumB	NA	NA	NA
M-8	CLOCK	c.484G>T	NA	Nonsense mutation	Exon-8	E162*	M0	BRCA_LumA	NA	NA	NA
M-9	ARNTL/BMAL1	c.418A>T	rs760404876	Missense mutation	Exon-9	T140S	M0	BRCA_Her2	Benign (0.00)	Tolerated (1.00)	Neutral (-1.80)
M-10	ARNTL/BMAL1	c.139_140+3del	NA	Splice_ Region Mutation	Exon-5	X47_splice	M0	BRCA_ LumB	NA	NA	NA
M-11	ARNTL/BMAL1	c.971T>C	NA	Missense mutation	Exon-13	V324A	M0	BRCA_LumA	NA	NA	Neutral (-1.63)
M-12	ARNTL/BMAL1	c.1651A>T	NA	Missense mutation	Exon-19	S551C	M0	BRCA_LumA	NA	NA	Low (1.87)
M-13	PER1	c.2570C>T	rs1353923775	Missense mutation	Exon-19	S857L	M0	BRCA_Her2	Benign (0.00)	Tolerated (0.23)	
M-14	PER1	c.1264C>T	rs535351039	Missense mutation	Exon-11	H422Y	M0	BRCA_Her2	Probably Damaging (0.87)	Tolerated (0.62)	Neutral (0.41)
M-15	PER1	c.3110C>T	COS_3821064	Missense mutation	Exon-20	S1032F	M0	BRCA_Her2	Possibly Damaging (0.84)	Deleterious (0.00)	Medium (2.06)
M-16	PER1	c.386C>T	COS_3821064	Missense mutation	Exon-4	S129L	M0	BRCA_Her2	Probably Damaging (0.87)	Deleterious (0.00)	Medium (2.71)

Appendix 1 (cont). Characteristics of the mutations detected in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes of the TCGA BRCA cohort

No	Gene	Nt alteration	Rs number	AT	Loc.	AA position	MS	Type of ca.	Clinical significance		
									Poly-Phen2 (score)	SIFT (score)	Mutation assesor (score)
M-17	PER1	c.3206C>G	COS_3821063	Missense mutation	Exon-20	S1069C	M0	BRCA_Her2	Possibly Damaging (0.68)	Tolerated (0.06)	Neutral (0.77)
M-18	PER1	c.1120G>C	COS_3821069	Missense mutation	Exon-9	D374H	M0	BRCA_Her2	Probably Damaging (1.00)	Deleterious (0.00)	High (3.76)
M-19	PER1	c.359C>T	COS_437841	Missense mutation	Exon-3	S120F	M0	BRCA_Her2	Probably Damaging (0.98)	Deleterious (0.00)	Medium (2.72)
M-20	PER1	c.3533G>A	rs199584851	Missense mutation	Exon-22	R1178Q	M0	BRCA_Basal	Benign (0.01)	Tolerated (0.07)	Low (1.70)
M-21	PER1	c.2852C>T	rs899874404	Missense mutation	Exon-19	S951L	M0	BRCA_Her2	Benign (0.00)	Tolerated (0.16)	Low (1.50)
M-22	PER1	c.1478T>C	NA	Missense mutation	Exon-12	I493T	M0	BRCA_ LumB	Probably Damaging (0.92)	Deleterious (0.00)	Medium (3.75)
M-23	PER1	c.1960T>C	rs1982444909	Missense mutation	Exon-16	S654P	MX	BRCA_Basal	Probably Damaging (0.96)	Tolerated (0.19)	Low (1.46)
M-24	PER1	c.3520del	NA	Frame shift_ deletion	Exon-22	S1174Lfs*26	M0	BRCA_LumA	NA	NA	NA
M-25	PER2	–	NA	Fusion	NA	ILKAP-PER2 Fusion	M0	BRCA_Basal	NA	NA	NA
M-26	PER2	c.3670G>A	COS_3839015	Missense mutation	Exon-23	E1224K	M0	BRCA_ LumB	Possibly Damaging (0.69)	Deleterious (0.01)	Medium (2.87)
M-27	PER2	c.2606del	NA	Frame shift_ deletion	Exon-19	P869Rfs*150	M0	BRCA_Basal	NA	NA	NA
M-28	PER2	c.1102G>A	COSV54524977	Missense mutation	Exon-10	V368M	M0	BRCA_Basal	Benign (0.23)	Tolerated (0.09)	Neutral (-0.20)
M-29	PER2	c.505G>C	NA	Missense mutation	Exon-5	D169H	M0	BRCA_Normal	Benign (0.08)	Deleterious (0.05)	Low (1.72)
M-30	PER2	c.2347G>A	COS_442591	Missense mutation	Exon-19	G783R	M0	BRCA_LumA	Benign (0.29)	Tolerated (0.13)	Medium (3.13)
M-31	PER2	c.2317C>T	COS_2908937	Missense mutation	Exon-18	R773*	M0	BRCA_LumA	NA	NA	NA
M-32	PER2	c.2114C>T	COS_442589	Missense mutation	Exon-18	A705V	M0	BRCA_LumA	Benign (0.00)	Tolerated (0.34)	Low (1.04)
M-33	PER2	c.913G>A	NA	Missense mutation	Exon-8	E305K	M0	BRCA_ LumB	Benign (0.01)	Deleterious (0.04)	Medium (2.73)

Appendix 1 (cont). Characteristics of the mutations detected in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes of the TCGA BRCA cohort

No	Gene	Nt alteration	Rs number	AT	Loc.	AA position	MS	Type of ca.	Clinical significance		
									Poly-Phen2 (score)	SIFT (score)	Mutation assesor (score)
M-34	PER2	c.570+1G>A	NA	Splice_ Region Mutation	NA	X190_splice	M0	BRCA_ LumB	NA	NA	NA
M-35	PER2	c.3250T>C	NA	Missense mutation	Exon-20	C1084R	MX	BRCA_Basal	Possibly Damaging (0.48)	Deleterious (0.00)	Medium (2.98)
M-36	PER2	c.3024de	NA	Frame shift_ deletion	Exon-19	T1009Pfs*10	MX	BRCA_LumA	NA	NA	NA
M-37	PER2	c.261del	NA	Frame shift_ deletion	Exon-3	K87Nfs*25	MX	BRCA_LumA	NA	NA	NA
M-38	PER2	c.198del	NA	Frame shift_ deletion	Exon-2	M67Cfs*17	MX	BRCA_LumA	NA	NA	NA
M-39	CRY1	c.1230del	NA	Frame shift_ deletion	Exon-8	H411Tfs*26	M0	BRCA_LumA	NA	NA	NA
M-40	CRY2	c.1137G>C	NA	Missense mutation	Exon-7	W379C	M0	BRCA_Basal	Probably Damaging (1.00)	Deleterious (0.00)	Medium (2.34)
M-41	CRY2	c.267C>G	NA	Missense mutation	Exon-1	I89M	M0	BRCA_Basal	NA	NA	Medium (2.31)
M-42	CRY2	c.1232G>T	NA	Missense mutation	Exon-7	W411L	M0	BRCA_Basal	Probably Damaging (0.96)	Deleterious (0.00)	Low (0.98)
M-43	NPAS2	c.64C>T	rs1683479934	Missense mutation	Exon-3	R22W	M0	BRCA_Basal	Probably Damaging (1.00)	Deleterious (0.00)	Medium (2.97)
M-44	NPAS2	c.1476G>C	NA	Missense mutation	Exon-15	M492I	M0	BRCA_ LumB	Benign (0.02)	Tolerated (0.14)	Medium (2.98)
M-45	NPAS2	c.1460G>A	NA	Missense mutation	Exon-15	S487N	M0	BRCA_Basal	Benign (0.00)	Tolerated (0.41)	Medium (2.97)
M-46	NPAS2	c.736G>A	rs751210882	Missense mutation	Exon-9	E246K	M0	BRCA_LumA	Possibly Damaging (0.88)	Deleterious (0.01)	Low (1.87)
M-47	NPAS2	c.1487C>T	rs530175072	Missense mutation	Exon-16	S496L	M0	BRCA_LumA	Possibly Damaging (0.79)	Deleterious (0.02)	Medium (2.95)
M-48	NPAS2	c.2052G>A	NA	Missense mutation	Exon-19	M684I	MX	BRCA_LumA	Benign (0.08)	Deleterious (0.02)	Low (1.76)

Appendix 1 (cont). Characteristics of the mutations detected in the CLOCK, BMAL1/ARNTL, PER1, PER2, CRY1, CRY2, NPAS2, SIRT1, and TIMELESS genes of the TCGA BRCA cohort

No	Gene	Nt alteration	Rs number	AT	Loc.	AA position	MS	Type of ca.	Clinical significance		
									Poly-Phen2 (score)	SIFT (score)	Mutation assesor (score)
M-49	NPAS2	c.132C>A	rs1288285417	Missense mutation	Exon-3	D44E	M0	BRCA_LumA	Possibly Damaging (0.89)	Deleterious (0.00)	Medium (2.10)
M-50	NPAS2	c.1392+1del	NA	Frame Shift Deletion	Exon-14	P464fs	M0	BRCA_LumA	NA	NA	NA
M-51	SIRT	c.1852G>A	COS_427772	Missense mutation	Exon-8	G618R	M0	BRCA_ LumB	Benign (0.04)	Tolerated (0.17)	Neutral (0.00)
M-52	SIRT	c.1441G>A	COS_3807493	Missense mutation	Exon-8	D481N	MX	BRCA_Her2	Possibly Damaging (0.57)	Tolerated (0.13)	Low (1.25)
M-53	SIRT	c.2170C>G	rs201854199	Missense mutation	Exon-9	Q724E	M0	BRCA_ LumA	Benign (0.00)	Deleterious (0.04)	Low (1.59)
M-54	SIRT	c.2043G>C	COS_1474709	Missense mutation	Exon-9	Q681H	M0	BRCA_Basal	Benign (0.00)	Tolerated (0.63)	Low (0.92)
M-55	TIMELESS	–	NA	Fusion	–	POLA1-TIMELESS	M0	BRCA_ LumB	NA	NA	NA
M-56	TIMELESS	c.2404G>C	COS_3812589	Missense mutation	Exon-19	E802Q	M0	BRCA_ LumB	Probably Damaging (0.99)	Deleterious (0.02)	Medium (3.16)
M-57	TIMELESS	c.2110G>A	rs780706303	Missense mutation	Exon-18	V704I	M0	BRCA_ LumB	Benign (0.01)	Deleterious (0.00)	Medium (2.77)
M-58	TIMELESS	c.2530G>T	NA	Missense mutation	Exon-21	D844Y	M0	BRCA_ LumA	Probably Damaging (1.00)	Deleterious (0.00)	Medium (3.16)
M-59	TIMELESS	c.1169G>A	rs780869332	Missense mutation	Exon-11	R390Q	M0	BRCA_ LumA	Probably Damaging (1.00)	Deleterious (0.00)	Medium (2.94)
M-60	TIMELESS	c.319C>T	rs751726906	Missense mutation	Exon-4	R107W	M0	BRCA_Her2	Probably Damaging (0.97)	Deleterious (0.01)	Medium (2.33)
M-61	TIMELESS	c.2022_2024dup	NA	Frame shift_ insertion	Exon-17	E678dup	M0	BRCA_Basal	NA	NA	NA
M-62	TIMELESS	c.721C>T	rs199809988	Missense mutation	Exon-8	R241C	MX	BRCA_ LumA	Possibly Damaging (0.74)	Deleterious (0.01)	Medium (2.89)
M-63	TIMELESS	c.2879C>T	rs374201861	Missense mutation	Exon-24	A960V	MX	BRCA_ LumA	Benign (0.01)	Tolerated (0.26)	Low (1.94)
M-64	TIMELESS	c.956del	NA	Frame shift_ deletion	Exon-10	P319Lfs*20	M0	BRCA_ LumA	NA	NA	NA

AT: Alteration type; Loc.: Localization; MS: Metastasis status; ca.: Cancer; M: Mutation; NA: Not applicable; MX: Metastasis cannot be measured; M0: Cancer has not spread to other parts of the body; M1: Cancer has spread to other parts of the body.