Modulatory Effects of Circadian Rhythms in the Lung Adenocarcinoma Tumor Microenvironment

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Abstract

Background: Dysregulated circadian dynamic balance is strongly associated with cancer development. However, biological functions of circadian rhythms in lung adenocarcinoma (LUAD) have not been elucidated. This study aimed at valuating the modulatory effects of circadian rhythms in the LUAD tumor microenvironment.

Methods: Multiple open-source bioinformatics research platforms are used to comprehensively elucidate the expression level, prognosis, potential biological function, drug sensitivity, and immune microenvironment of circadian clock genes in LUAD.

Results: Most circadian clock genes in LUAD are dysregulated and are strongly correlated with patient prognosis, and missense mutations at splicing sites of these genes. Besides, these genes are closely associated with some well-known cancer-related marker pathways, which are mainly involved in the inhibition of the Apoptosis, Cell cycle, and DNA Damage Response Pathway. Furthermore, functional enrichment analysis revealed that circadian clock genes regulate transcription factor activities and circadian rhythms in LUAD tissues. As for drug sensitivity, high expression of CLOCK, CRY1, and NR1D2 as well as suppressed PER2 and CRY2 expression levels are associated with drug resistance. The expression levels of circadian clock genes in LUAD correlate with immune infiltration and are involved in the regulation of immunosuppression.

Conclusions: Our multi-omics analysis provides a more comprehensive understanding of the molecular mechanisms of the circadian clock genes in LUAD and provides new insights for a more precise screening of prognostic biomarkers and immunotherapy.

Introduction

Globally, lung cancer is associated with high morbidity and mortality, and has a five-year survival rate of less than 20% (Bray et al., 2018). Lung adenocarcinoma (LUAD) is the most common pathological type, accounting for about 40% of lung cancer cases (Xu et al., 2020). Due to its high morbidity and mortality rates, LUAD is a major public health concern. Studies have proposed two therapeutic approaches have been advanced, targeted therapy and immunotherapy. However, these strategies have not significantly improved the overall survival outcomes for LUAD patients (Pao and Hutchinson, 2012). There is a need to identify potential prognostic biomarkers and possible therapeutic targets for LUAD in order to guide the treatment and prediction of prognosis of LUAD.

Circadian rhythms are based on circadian clock genes and related proteins. In oncology, circadian clock genes and their downstream genes regulate various cancer-related biological functions, such as tumor metabolism, immunity, DNA damage repair, and cell cycle (Gu et al., 2017). Dysregulated circadian rhythms promote abnormal cell proliferation, migration, and invasion, which affects tumorigenesis and progression (Gu et al., 2018; Mocellin et al., 2018). In cancer treatment, dysregulated circadian rhythms also regulate tumor drugs sensitivities (Xiang et al., 2015). It has been reported that BMAL1 increases the sensitivity of paclitaxel in tongue squamous cell carcinoma and inhibits its progression (Tang et al., 2017). The chemotherapeutic potential of Paclitaxel (PTX) nanoparticles (PTX-NPs) against lung cancer cells at different times of the day has also been evaluated using the chronological administration principle. There were diurnal differences in anti-cancer effects, with PTX-NPs exhibiting the most potent anti-tumour activity at 15 Hours After Light Onset (15 HALO) by reducing liver damage and inducing apoptosis through the upregulation of Per2 expression (Hu et al., 2017; Zhao et al., 2019). Immune responses are also associated with the regulation of circadian clock genes. Under normal physiological conditions, the proliferation and activity of immune cells follow a robust circadian rhythm, while disruption of the circadian rhythm causes immunosuppression (Fu and Kettner, 2013; Allen et al., 2019). For example, mice deficient in both Cry1 and Cry2 showed elevated pro-inflammatory cytokines levels, which are more likely to cause chronic inflammation, a common underlying mechanism for cancer development (Narasimamurthy et al., 2012). Chloé C Nobis et al. reported that CD8 + T-cell response intensities varied after vaccination, while this circadian rhythm phenomenon was not shown in mice lacking the circadian clock genes (Nobis et al., 2019). In glioblastoma (GBM), CLOCK and BMAL1 trigger pro-tumor immunity by transcriptionally upregulating OLFML3, which recruits immunosuppressed microglia into the tumor microenvironment, suggesting that CLOCK may be a new therapeutic target for GBM (Chen et al., 2020). Based on the above findings, we evaluated the interactions between the circadian clock and LUAD immune checkpoints.

Due to the rapid advances in sequencing technology and the enhanced application of bioinformatics tools, we performed a multi-omics analysis of the core circadian clock genes ARNTL (also known as BMAL1), CLOCK, CRY1, CRY2, NPAS2, NR1D1, NR1D2, PER1, PER2, PER3, RORA, and TIMELESS through public databases. The data for these genes were obtained from public databases. Moreover, a comprehensive analysis of circadian gene expression, prognosis, immune regulation and drug sensitivity was performed to establish the potential for crosstalk between the LUAD tumor microenvironment and the circadian clock.

Methods

Data sets and data availability

LUAD samples and normal lung samples were extracted from The Cancer Genome Atlas¹, the Gene Expression Omnibus² and the Genotype-Tissue Expression³ databases. Multiple circadian clock aspects such as gene expression levels, potential prognostic biomarkers, potential function, and immune infiltration in LUAD were analyzed using open-source bioinformatics research platforms.

Gene expression

Using the UALCAN database⁴, the student's t test was used to generate a p value for significance of during the expression analysis (Chandrashekar et al., 2017). $p \leq 0.05$ was set as the threshold for significance. Expression levels of the circadian clock genes at the protein level were validated using The Human Protein Atlas database⁵ (Uhlen et al., 2017).

Prognostic analysis

The Kaplan-Meier Plotter database⁶ and Prognoscan database⁷ were used to establish survival curves to show the differences associated with high and low expression levels of individual genes. (Mizuno et al., 2009; Nagy et al., 2018). The significance of each circadian clock gene in overall survival and first progression was performed by Kaplan–Meier survival curves. Hazards ratio, 95% confidence interval and log-rank P were analyzed with Cox PH Model.

Single Nucleotide Variation (SNV), drug sensitivity, and methylation analysis

SNV summary and oncoplot waterfall plot was generated by maftools. The Spearman correlation was used to analyze the gene expression correlates with the drug sensitivity through GSCALite database⁸ (Liu et al., 2018). The Spearman's rank correlation coefficients of transcript levels and AUCs were normalized using Fisher's Z transformation. A Bonferroni-corrected, two-tailed distribution with family-wise error rates less than 0.025 in each tail for z-scored. The Spearman rank correlation coefficients for annotated drug-target pairs were compared to the same number of correlation pairs generated by random sampling correlations. The UALCAN database was used for comparisons of the differences in gene methylation levels between normal and LUAD tissues (Chandrashekar et al., 2017).

Enrichment analysis

Circadian clock genes were submitted to DAVID 6.8⁹ for enrichment analysis (Huang da et al., 2009), including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. String¹⁰, an open-source database for known protein-protein interactions (PPI) and predicting PPI, was used to analyze PPI networks of the circadian clock genes (Szkarczyk et al., 2019).

Immune infiltrate analysis

Through the TIMER database¹¹ (Li et al., 2017), the correlation between core circadian genes and infiltration by immune cells was analyzed using Spearman's rank correlation analysis. A somatic copy-number alterations (SCNA) module compares tumor infiltration levels among LUAD with different somatic copy number alterations in core circadian genes. The infiltration level of each SCNA category was compared to the normal using a two-sided Wilcoxon's rank-sum test.

¹TCGA: <https://cancergenome.nih.gov>.

²GEO: <https://www.ncbi.nlm.nih.gov/geo/>.

³GTEX: <https://gtexportal.org>.

⁴<http://ualcan.path.uab.edu/>.

⁵<https://www.proteinatlas.org>.

⁶<http://kmplot.com/analysis/>.

⁷<http://dna00.bio.kyutech.ac.jp/PrognScan/index.html>.

⁸<http://bioinfo.life.hust.edu.cn/web/GSCALite/>.

⁹<https://david.ncifcrf.gov/>.

¹⁰<https://string-db.org/>.

¹¹<https://cistrome.shinyapps.io/timer/>.

Results

Circadian rhythms of core circadian genes in LUAD

Twelve circadian clock genes (ARNTL, CLOCK, CRY1, CRY2, NR1D1, NR1D2, NPAS2, PER1, PER2, PER3, RORA, and TIMELESS) were used in this study. Through the Circadb database, we used RNA sequences to analyze the expression levels of 11 biological clock genes (except TIMELESS) at different times in lung tissue through Circadb database¹² (Pizarro et al., 2013). The results were shown that the expression levels of these genes significantly fluctuated with time (Supplementary Figure 1).

Defining circadian clock genes in LUAD and normal tissue

In the UALCAN database, compared to normal tissues, the expression levels of CLOCK, NPAS2, NR1D1, and TIMELESS were found to be up-regulated in LUAD patients, while the expression levels of CRY1, CRY2, NR1D2, PER1, PER2, PER3, and RORA were down-regulated, with statistically significant differences ($P < 0.05$) (Figure 1). The expression levels of circadian clock genes in LUAD tissues and normal tissues were detected by immunohistochemistry (IHC). Expression levels of CLOCK, NPAS2, NR1D1, and TIMELESS in LUAD tissues were found to be higher than those in normal tissues while the expression levels of CRY1, CRY2, and NR1D2 in LUAD tissues were lower than those in normal tissues (Figure 2). Regarding the correlation between differentially expressed core circadian clock genes in LUAD and normal tissues, TIMELESS, NR1D1, NPAS2, PER1, and PER2 were partially negatively expressed in tumor tissues while NPAS2 and PER2 were partially negatively expressed in normal tissues (Figure 3).

Prognostic values of circadian clock genes in LUAD patients

Circadian genes have a definite prognostic value. Table 1 shows the models for predicting prognosis using circadian genes. Up-regulated genes, including CRY1 (HR = 0.58, $P = 0.001$), CRY2 (HR = 0.51, $P = 0.001$), NR1D2 (HR = 0.49, $P = 0.001$), PER2 (HR = 0.44, $P = 0.001$), PER3 (HR = 0.5, $P = 0.001$), RORA (HR = 0.45, $P = 0.001$) were correlated with improved overall survival; while up-regulated CLOCK (HR = 1.4, $P = 0.001$), NPAS2 (HR = 2.19, $P = 0.001$), and TIMELESS (HR = 1.88, $P = 0.001$) were correlated with poor survival outcomes (Supplementary Figure 2). The time to first progression of the following up-regulated genes was also found to be increased: CRY1 (HR = 0.61, $P = 0.002$), CRY2 (HR = 0.5, $P = 0.001$), NR1D2 (HR = 0.63, $P = 0.001$), PER1 (HR = 0.58, $P = 0.001$), PER2 (HR = 0.51, $P = 0.001$), PER3 (HR = 0.47, $P = 0.001$), and RORA (HR = 0.57, $P = 0.001$); while the time to first progression of the following up-regulated genes was decreased: NPAS2 (HR = 1.74, $P = 0.001$), NR1D1 (HR = 1.54, $P = 0.007$), and TIMELESS (HR = 1.8, $P = 0.001$) (Supplementary Figure 3). We also validated the Overall Survival (OS) of the genes by the PrognScan database (Table 2). There was a significant correlation between the expression levels of CLOCK, CRY2, NPAS2, NR1D1, NR1D2, PER1, PER3, RORA, and TIMELESS and OS. Moreover, the OS prognostic values of CRY2, NPAS2, NR1D2, PER3, RORA, and TIMELESS genes were comparable to those of the Kaplan-Meier Plotter database. LUAD patients with elevated expressions of CRY2, NR1D2, PER3, and RORA and suppressed expressions of NPAS2 and TIMELESS exhibited better survival outcomes ($P < 0.05$).

Circadian clock genes associated with cancer hallmarks in LUAD

To reveal the potential impact of circadian dysregulation in LUAD, we evaluated the role of the circadian clock in LUAD. SNV analysis revealed that missense mutations and splicing sites were the most common types of circadian clock gene variations in LUAD (Figure 4). In addition, the methylation levels of CRY2 and NR1D1 DNA in LUAD tissues were lower than those in normal

tissues, while the methylation levels of NPAS2, PER1, PER3, and RORA in LUAD tissues were higher than those in normal tissues, with statistically significant differences ($P < 0.05$)(Figure 5).

Then, we determined the expression levels of circadian clock genes in the typical cancer-related signaling pathways (Apoptosis, Cell Cycle, DNA Damage Response, Epithelial-mesenchymal transition (EMT), Hormone androgen receptor (AR), Estrogen receptor (ER), Phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT), RAS/ Mitogen-activated protein kinase (MAPK), Receptor tyrosine kinase (RTK), Tuberous sclerosis complex (TSC)/ Mammalian target of rapamycin (Mtor)). The circadian clock genes were closely associated with most of the typical cancer-related marker pathways. Among them, CRY2 activates PI3K/AKT. RORA, PER2, CRY2, and NPAS2 activate RAS/MAPK, while most of the Circadian clock genes are involved in inhibiting Apoptosis, Cell cycle, and DNA Damage Response Pathway(Figure 6A). We also established the correlation between circadian clock gene expression and drug sensitivity. Elevated expression levels of CLOCK, CRY1, and NR1D2 as well as suppressed expression levels of PER2 and CRY2 were associated with drug resistance (Figure 6B).

Enrichment analysis of circadian clock in LUAD

We used GO and KEGG enrichment analysis to evaluate the potential roles of the circadian clock genes. These genes exert a major impact on the circadian rhythm and biological process, such as on the transcription factor complex of cellular component, and on transcription corepressor binding, transcription cofactor binding, and E-box binding of molecular function (Figure 7A). KEGG enrichment analysis showed that circadian clock genes are involved in circadian regulation of gene expression, circadian rhythms, and rhythmic process (Figure 7B). PPI network results showed that they are associated with circadian rhythm, rhythmic Process, circadian regulation of gene expression, and regulation of circadian rhythm and regulatory region DNA binding (Figure 7C). These findings imply that circadian rhythm genes can regulate the transcription factor activity and circadian rhythm in LUAD.

Immune infiltrates analysis of circadian clock in LUAD patients

Few studies have evaluated the role of the biological clock in the LUAD immune microenvironment. We analyzed the correlations between clock genes and the abundance of six types of immune cells(Table 3). The expression levels of ARNTL and CLOCK were positively correlated with the abundance of infiltration of B cells ($r = 0.158$, $P = 0.001$ / $r = 0.287$, $P = 0.001$), CD8+T cells ($r = 0.221$, $P = 0.001$ / $r = 0.144$, $P = 2.45e-03$), CD4+T cells ($r = 0.232$, $P = 0.001$ / $r = 0.272$, $P = 0.001$), Macrophages ($r = 0.207$, $P = 0.001$ / $r = 0.478$, $P = 0.001$), Neutrophils ($r = 0.321$, $P = 0.001$ / $r = 0.446$, $P = 0.001$), and Dendritic cells ($r = 0.315$, $P = 0.001$ / $r = 0.361$, $P = 0.001$) (Supplementary Figure 4A/B). However, CRY1 expression was only positively correlated with the infiltration abundance of Macrophage ($r = 0.134$, $P = 3.03e-03$) and Neutrophils ($r = 0.155$, $P = 0.001$) (Supplementary Figure 4C). And CRY2 expression also was not correlated with CD8 + T cells and neutrophil, but positively correlated with B cells ($r = 0.281$, $P = 0.001$), CD4 + T cells ($r = 0.297$, $P = 0.001$), Macrophages ($r = 0.171$, $P = 0.001$) and Dendritic cells ($r = 0.187$, $P = 0.001$) (Supplementary Figure 4D). In addition, the expression levels of NPAS2 and NR1D1 were positively correlated with CD4 + T cells ($r = 0.187$, $P = 0.001$ / $r = 0.251$, $P = 0.001$) and Dendritic cells ($r = 0.118$, $P = 9.33e-03$ / $r = 0.24$, $P = 0.001$) (Supplementary Figure 4E/F). As found in ARNTL and CLOCK, NR1D2 expression was positively correlated with B cells ($r = 0.204$, $P = 0.001$ / $r = 0.339$, $P = 0.001$ / $r = 0.368$, $P = 0.001$), CD8 + T cells ($r = 0.269$, $P = 0.001$ / $r = 0.134$, $P = 0.001$ / $r = 0.258$, $P = 0.001$), CD4 + T cells ($r = 0.239$, $P = 0.001$ / $r = 0.4$, $P = 0.001$ / $r = 0.394$, $P = 0.001$), Macrophages ($r = 0.259$, $P = 0.001$ / $r = 0.23$, $P = 0.001$ / $r = 0.23$, $P = 0.001$), Neutrophils ($r = 0.298$, $P = 0.001$ / $r = 0.232$, $P = 0.001$ / $r = 0.325$, $P = 0.001$) and Dendritic cells ($r = 0.348$, $P = 0.001$ / $r = 0.419$, $P = 0.001$ / $r = 0.419$, $P = 0.001$) (Supplementary Figure 4G/J/K). On the contrary, PER1 expression was not correlated with the infiltration abundance of immune cells (Supplementary Figure 4H). PER2 expression was only positively correlated with CD4 + T cells ($r = 0.163$, $P = 0.001$), but was not correlated with the other immune cells (Supplementary Figure 4I). As for TIMELESS, its expression positively correlates the abundance of Neutrophils ($r = 0.114$, $P = 0.001$) (Supplementary Figure 4L).

Besides, changes in somatic copy number (SCNA) of circadian rhythm genes inhibited immune infiltration, including CD8+T cells, neutrophils, dendritic cells, macrophages, CD4+T cells, and B cells. These results suggest a possible association between the circadian clock and immune infiltration in LUAD (Figure 8). We also assessed the association between clock genes with a different expression and immune cell infiltration. Using the Cox proportional risk model, we adjusted for confounding factors: B cells, CD4 + T cells, macrophages, neutrophils, dendritic cells, ARNTL, CLOCK, CRY1, CRY2, NR1D1, NR1D2, PER1, PER2, RORA, NPAS2, and TIMELESS. LUAD patients were correlated with elevated expression levels of B cells ($P < 0.001$), CD4+ T cells ($P = 0.047$), CRY2 ($P = 0.02$), PER3 ($P = 0.014$), RORA ($P = 0.013$), NPAS2 ($P < 0.001$), and TIMELESS ($P = 0.025$) (Table 4).

Discussion

The circadian clock regulates all human life aspects, including sleep, exercise, hormone secretion, and immune regulation (Asher and Sassone-Corsi, 2015). Due to different night and day work shifts, the frequent dysregulation of circadian rhythms leads to a significant increase in the risk of some cancers, including breast cancer, lung cancer, pancreatic cancer, and so on (Fu and Kettner, 2013). Studies have shown that another feature of circadian control is cell metabolism in the tumor micro-environment and organisms (Verlande and Masri, 2019). Despite significant advances in targeted therapy and immunotherapy for LUAD (Xu et al., 2020), the molecular mechanism between circadian rhythm and LUAD development has not been fully elucidated. Using comprehensive bioinformatics analysis, the roles of circadian clock in lung adenocarcinoma can be established and used to inform new therapeutic targets.

In all tissues, circadian rhythm regulation depends on the transcription and translation of circadian clock genes. Studies have shown that tumor cell growth exhibits a distinct rhythm, which is different from that of normal cells. We first evaluated the expression levels of circadian clock genes in LUAD, and found that NPAS2, NR1D1, TIMELESS were highly expressed in cancer tissues, while CRY1, CRY2, PER1, PER3, RORA, NR1D2 and PER2 were suppressed. Similar findings were found for differences in circadian clock gene protein expression in the HPA database. In addition, there were significant differences in the methylation levels of circadian clock genes in LUAD patients. CRY2 and NR1D1 in cancer tissues exhibited low DNA methylation levels than normal tissues, while NPAS2, PER1, PER3 and RORA exhibited higher levels of methylation than normal tissues. Abnormal expression of core circadian genes is associated with methylation deactivation in human cancers (Yang et al., 2006; Salavaty, 2015). Moreover, DNA hypermethylation is a potential mechanism for suppressing PER1 silencing in non-small cell lung cancer (Gery et al., 2007). The above findings imply that circadian rhythm dysregulation plays an important role in lung tumor development.

There is a strong correlation between circadian gene expression and cancer prognosis. TIMELESS was significantly correlated with the differentiation level, tumor size, lymph node metastasis and TNM stage of non-small cell lung cancer. The higher the expression level, the worse the clinical prognosis (Zhang et al., 2020). In non-small cell lung cancer, PER1, PER2 and PER3 downregulation is associated with tumor progression and metastasis, resulting in poorer prognosis (Liu et al., 2014; Xiang et al., 2018). Core circadian genes, PER2 and BMAL1, exert cell-autonomous tumor suppressive roles in lung tumor progression (Papagiannakopoulos et al., 2016). We constructed survival curves using the TCGA data and found that elevated expression levels of NPAS2, NR1D1, and TIMELESS, and suppressed expression levels of CRY1, CRY2, NR1D2, PER2, PER3, and RORA were negatively correlated with LUAD prognosis, consistent with previous studies. Therefore, clock genes are strongly associated with tumor development, progression, and prognosis (Fu and Kettner, 2013). Given that clock genes can influence each other's expression through feedback loops, these core clock genes can associate with each other to influence LUAD development. Therefore, these genes may be novel molecular targets for LUAD treatment.

Missense mutations and splice sites were the most common types of circadian clock gene variants in LUAD. Most circadian clock genes are involved in the inhibition of apoptosis, cell cycle, and DNA Damage Response pathways. CRY2 activates PI3K/AKT, whereas RORA, PER2, CRY2, NPAS2 activate RAS/MAPK. Functional enrichment analysis revealed that circadian genes are involved in transcription factor activity and regulation of circadian rhythms. The loss of circadian rhythm may mediate cancer susceptibility through a variety of cellular functions, including cell proliferation, apoptosis, and DNA damage responses (Angelousi et al., 2019). Moreover, evidence suggests a molecular connection between the two oscillators, which are the cell cycle and the biological clock, and in normal cells, coupling of the two manifests themselves as timed mitosis and rhythmic DNA replication. In cancer cells, they are often misregulated, leading to dysregulated cell proliferation and aberrant circadian gene expression levels (Gaucher et al., 2018; Farshadi et al., 2020).

We constructed a drug sensitivity model and found that a high expression level of CLOCK, CRY1 NR1D2 and a low expression level of PER2 and CRY2 were associated with drug resistance. This finding was also reported by Victoria Y Gorbacheva et al. They found that CLOCK, BMAL1 gene deficient mice were highly sensitive to treatment with the anticancer drug, cyclophosphamide (Gorbacheva et al., 2005). In addition, with reference to the resting activity cycle, chronotherapy has been shown to reduce the incidences of chemotherapy-related side effects by applying chemotherapeutic agents according to the expected time of minimum drug toxicity (Lévi, 2006). These studies suggest that the clock gene may be a potential biomarker for the development of anti-cancer

drugs. Under normal physiological conditions, the redistribution of inflammatory factors and immune cells (including T-lymphocytes, B-lymphocytes, NK cells, Dendritic cells, and Macrophages) in the body follows a robust circadian rhythm (Labrecque and Cermakian, 2015). Dysregulation of circadian rhythms is closely linked to immunosuppression (Sephton and Spiegel, 2003). Dysregulation of circadian rhythms can cause an overall overexpression of immunosuppressive checkpoints (such as PD-L1 and CTLA-4), which may lead to T-cell failure (Wu et al., 2019). However, immune functions of the biological clock in the LUAD tumor microenvironment have not been fully elucidated. We found a strong correlation between circadian clock genes and immune cell infiltration, and most of them were positively correlated with the infiltration abundance of B cells, CD8 + T cells, CD4 + T cells, Macrophages, Neutrophils, and Dendritic cells, respectively. Thus, the circadian clock plays an important role in immune responses, which informs advances in immunotherapy.

This study has some limitations. First, circadian rhythms can be interfered with by external environmental factors, which may bias the results. Second, this study lacks in vitro or in vivo experimental validation. Studies should be performed to validate our results.

In conclusion, most circadian clock genes are dysregulated and strongly correlated with LUAD prognosis. Circadian rhythms are also associated with a variety of cell functions, and cell cycles and are involved in immune responses in the tumor microenvironment. This study elucidates the molecular mechanisms of clock genes in LUAD which informs therapeutic development.

Abbreviations

LUAD : Lung adenocarcinoma, GO, Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes, BP: Biological processes, CC: Cellular components, MF: Molecular functions, PPI: Protein-protein interaction, SNV: Single Nucleotide Variation, OS: Overall Survival, SCNA, Somatic copy-number alterations, GBM: Glioblastoma, IHC: Immunohistochemistry, NSCLC: Non-small cell lung cancer, MFS: Metastasis-free survival, EMT: Epithelial-mesenchymal transition, AR: Androgen receptor, ER: Estrogen receptor, PI3K: Phosphatidylinositol 3-kinase, AKT: Protein kinase B, MAPK: Mitogen-activated protein kinase, RTK: Receptor tyrosine kinase, TSC: Tuberous sclerosis complex, Mtor: Mammalian target of rapamycin.

Declarations

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Author's contributions

Qianzhun Huang, Jian Huang and Xiaoyang Su performed data analysis work and aided in writing the manuscript. Qianzhun Huang designed the study, assisted in writing the manuscript. Ning Fang edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Maoming People's Hospital.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Tables

Table 1 The prognostic value of circadian clock genes in lung adenocarcinoma (KM plotter)

Gene	Overall survival (N=719)		First progression (N=461)	
	Hazard ratio	P-value	Hazard ratio	P-value
ARNTL	0.82(0.65-1.03)	0.086	1.06(0.78-1.45)	0.7
CLOCK	1.4(1.1-1.77)	0.0054	0.97(0.71-1.33)	0.85
CRY1	0.58(0.46-0.74)	6.1E-06	0.61(0.45-0.84)	0.002
CRY2	0.51(0.4-0.65)	2.5E-08	0.5(0.36-0.69)	1.9E-05
NPAS2	2.19(1.73-2.79)	4.7E-11	1.74(1.27-2.39)	0.00051
NR1D1	1.26(0.99-1.59)	0.058	1.54(1.12-2.11)	0.007
NR1D2	0.49(0.39-0.63)	3.3E-09	0.63(0.46-0.87)	0.0043
PER1	1.05(0.83-1.32)	0.68	0.58(0.42-0.8)	0.00081
PER2	0.44(0.35-0.57)	2.4E-11	0.51(0.37-0.7)	2.2E-05
PER3	0.5(0.4-0.64)	1.4E-08	0.47(0.34-0.64)	1.9E-06
RORA	0.45(0.35-0.58)	3.2E-10	0.57(0.42-0.79)	0.00046
TIMELESS	1.88(1.48-2.38)	1.4E-07	1.8(1.31-2.48)	0.00022

Bold values indicate P≤0.05

Table 2 The prognostic value of circadian clock genes in lung adenocarcinoma (PrognScan)

Gene	Endpoint	Hazard ratio	P-value	N	Reference
CLOCK	OS	0.10(0.02 - 0.60)	0.00088202	204	GSE31210
CRY2	OS	0.29(0.14 - 0.63)	0.000535485	204	GSE31210
NPAS2	OS	2.37(0.60 - 9.30)	0.0092596	82	Jacob-00182-CANDF
NR1D1	OS	0.21(0.05 - 0.83)	0.000658546	86	MICHIGAN-LC
NR1D2	OS	0.34(0.14 - 0.79)	0.000172833	204	GSE31210
PER1	OS	0.29(0.13 - 0.62)	0.00119373	204	GSE31210
PER3	OS	0.30(0.18 - 0.50)	8.57E-05	204	GSE31210
RORA	OS	0.60(0.44 - 0.81)	0.00404378	117	GSE13213
TIMELESS	OS	2.65(1.30 - 5.40)	0.0455948	204	GSE31210

OS, Overall Survival;

Table 3 The correlation between circadian clock genes and the abundance of six types of immune cells in lung adenocarcinoma (TIMER)

Gene	B cells		CD8+T cells		CD4+T cells		Macrophages		Neutrophils		Dendritic cells	
	Cor	P-value	Cor	P-value	Cor	P-value	Cor	P-value	Cor	P-value	Cor	P-value
ARNTL	0.158	0.001	0.221	0.001	0.232	0.001	0.207	0.001	0.321	0.001	0.315	0.001
CLOCK	0.287	0.001	0.144	0.002	0.272	0.001	0.478	0.001	0.446	0.001	0.361	0.001
CRY1	0.01	0.827	0.062	0.173	0.064	0.160	0.134	0.003	0.155	0.001	0.02	0.667
CRY2	0.281	0.001	0.046	0.313	0.297	0.001	0.171	0.001	0.078	0.087	0.187	0.001
NPAS2	-0.104	0.022	0.015	0.745	0.187	0.001	0.09	0.047	0.096	0.035	0.118	0.009
NR1D1	0.072	0.111	-0.019	0.671	0.251	0.001	0.082	0.071	0.171	0.001	0.24	0.001
NR1D2	0.204	0.001	0.269	0.001	0.239	0.001	0.259	0.001	0.298	0.001	0.348	0.001
PER1	0.049	0.285	-0.059	0.194	0.101	0.027	0.023	0.606	-0.056	0.216	-0.075	0.100
PER2	0.109	0.017	-0.032	0.479	0.163	0.001	-0.016	0.731	0.053	0.244	-0.054	0.235
PER3	0.339	0.001	0.134	0.003	0.4	0.001	0.23	0.001	0.232	0.001	0.419	0.001
RORA	0.368	0.001	0.258	0.001	0.384	0.001	0.23	0.001	0.325	0.001	0.371	0.001
TIMELESS	-0.05	0.273	0.009	0.836	0.04	0.378	-0.088	0.052	0.114	0.012	-0.006	0.889

Table 4 The cox proportional hazard model of six tumor-infiltrating immune cells and circadian clock genes in lung adenocarcinoma (TIMER)

	coef	HR	95%CI_l	95%CI_u	p.value	sig
B_cell	-5.337	0.005	0	0.062	0	***
CD8_Tcell	0.383	1.467	0.245	8.792	0.675	
CD4_Tcell	2.663	14.335	1.03	199.498	0.047	*
Macrophage	-0.161	0.851	0.066	10.901	0.901	
Neutrophil	-0.455	0.635	0.015	27.645	0.813	
Dendritic	-0.081	0.922	0.25	3.405	0.903	
ARNTL	-0.208	0.812	0.651	1.012	0.064	
CLOCK	0.079	1.082	0.883	1.326	0.449	
CRY1	0.009	1.009	0.801	1.272	0.938	
CRY2	-0.208	0.813	0.682	0.968	0.02	*
NR1D1	0.01	1.01	0.863	1.183	0.897	
NR1D2	-0.0111	0.895	0.739	1.084	0.256	
PER1	0.03	1.031	0.904	1.175	0.652	
PER2	0.062	1.064	0.879	1.287	0.526	
PER3	-0.173	0.841	0.733	0.966	0.014	*
RORA	-0.276	0.758	0.609	0.944	0.013	*
NPAS2	0.268	1.307	1.14	1.498	0	***
TIMELESS	0.178	1.194	1.023	1.395	0.025	*

*P < 0.05, ***P < 0.001

Figures

Figure 1

The expression of core circadian clock genes in normal tissue and LUAD tissue (UALCAN). $***p < 0.001$, $**p < 0.05$. LUAD, Lung adenocarcinoma.

Figure 2

The Expression of core circadian clock genes in LUAD in protein level (The Human Protein Atlas). CLOCK, NPAS2, NR1D1 and TIMELESS were significant upregulated in LUAD tissues compared with normal tissues. CRY1, CRY2, and NR1D2 were significant downregulated in LUAD tissues compared with normal tissues. LUAD, Lung adenocarcinoma.

Figure 3

The expression of circadian clock genes in LUAD tissue and normal tissue. (A) The expression of circadian clock genes in LUAD tissue. (B) The expression of circadian clock genes in normal tissues. (C) The co-expression of circadian clock genes in LUAD tissue. (D) The co-expression of circadian clock genes in normal tissues. LUAD, Lung adenocarcinoma.

Figure 4

The SNV analysis and methylation analysis of circadian clock genes in LUAD (GSCALite). (A) Summary plot displays the type of genetic variation and frequency of circadian clock genes in LUAD. (B) SNV waterfall plot shows the mutation distribution of circadian clock genes in LUAD and a SNV classification of SNV types. (C) Differences in methylation between tumor samples and normal samples. (D) The correlation between methylation and the core circadian gene expressions. KIRC, Clear cell renal cell carcinoma. LUAD, Lung adenocarcinoma; SNV, single nucleotide variation.

Figure 5

The degree of methylation of clock genes in normal tissue and LUAD tissue (UALCAN). The methylation levels of CRY2 and NR1D1 in LUAD tissues were lower than normal tissues, while NPAS2, PER1, PER3 and RORA were higher than normal tissues, and the differences were all statistically significant ($P < 0.05$). LUAD, Lung adenocarcinoma.

Figure 6

Cancer pathway activity and drug sensitivity analysis of circadian clock genes in LUAD (GSCALite). (A) The combined percentage of pathway activity in LUAD. (B) Spearman correlation represents and drug-related gene expression. A positive correlation means that gene expression is highly resistant to drugs. LUAD, Lung adenocarcinoma.

Figure 7

The enrichment analysis of circadian clock genes in LUAD. (A) Enrichment analysis of GO. (B) Enrichment analysis of KEGG. (C) PPI network of circadian clock genes (GeneMANIA). LUAD, Lung adenocarcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, Protein-protein interaction.

Figure 8

Associations of circadian clock genes with immune cells infiltration in LUAD (TIMER). LUAD, Lung adenocarcinoma.

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