Prognostic Significance of Circadian Gene Expression in Lung Cancer

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Abstract

Objective To explore the expression and prognostic significance of circadian genes (PER1, PER2, PER3, CRY1, CRY2, CLOCK and BMAL1) in lung cancer.

Methods mRNA expressions of circadian rhythm genes in lung cancer and normal tissues were analyzed using the OMINE database. The functions of circadian genes in lung cancer were analyzed using the cBioPortal platform and OncoPrint. The association between the expression level of circadian genes and overall survival (OS) of lung cancer patients was analyzed using the Kaplan-Meier Plotter database.

Results Analysis of the ONCOMINE database showed that the mRNA expression levels of PER1-3, CRY-2, CLOCK and BMAL1 were lower in lung cancer tissues than those in normal tissues. The cBioPortal platform and OncoPrint database showed that each circadian gene underwent a change ranging from 2.5% to 19.1% with the type of change including gene mutation, amplification, deletion, fusion and multiple alterations, of which gene mutation was the most prevalent. Kaplan-Meier analysis revealed that a decreased circadian gene expression level was significantly correlated with a shorter OS ($P < 0.05$). In addition, the expression level of circadian genes also varied with the lung cancer subtype.

Conclusions Circadian genes showed low expression levels in lung cancer, which was significantly correlated with a shorter survival. In addition, the aberrant expression of circadian genes in lung cancer is mainly caused by gene mutation.

Introduction

Lung cancer is one of the most malignant tumors in the world in terms of the incidence and mortality. In 2020, 2.2 million new cases of lung cancer were reported worldwide, accounting for 11.4% of all new tumor cases, and 1.8 million deaths occurred, accounting for 18.1% of all tumor-related deaths¹. Situation of lung cancer in China is not optimistic either; in 2020, over 820,000 new cases of lung cancer were reported, and 710,000 lung cancer deaths occurred, accounting for 23.8% of all cancer-related deaths². Primary lung cancer consists of non-small cell lung cancer (NSCLC) and small cell lung cancer, with the former accounting for 80%-85% of lung cancer. In recent years, more epidemiological and genetic data have shown that disruption of circadian rhythms is closely related to the development of lung cancer and directly or indirectly drives the course of tumors³.

Circadian rhythm is an intrinsic regulatory mechanism gradually formed by the earth's organisms during the long evolutionary process with the change of day and night. It is regulated by circadian genes. Under normal circumstances, cell proliferation, division and other physiological activities can be coordinated orderly under the regulation of circadian genes to complete the coordination work. However, disturbances of the circadian rhythm in the human body can lead to the occurrence of hormone secretion disorders, cancer, diabetes, and autoimmune diseases⁴. Circadian genes can influence people's susceptibility to cancer by affecting the regulation of DNA damage and repair, the metabolic processes of substances, the

biological pathways of the cell cycle, and cell apoptosis^{5,6}. Several circadian genes have been identified in the human body, such as PER1-3, CRY1-2, CLOCK, BMAL1, Timeless, NPAS2 and NR1D1⁷. The number of studies exploring the regulation of circadian genes on the organism is increasing year by year, but these studies have not reached uniform results. The specific expression regulation mechanisms of circadian genes in lung cancer remain unclear and need to be further investigated. This study aimed to further explore the relationship of the expression level of circadian genes with survival and prognosis of lung cancer patients by using the Cancer Genome Atlas database, and search for the functions and pathways of related rhythm proteins.

Material And Methods

1.1 ONCOMINE database

The ONCOMINE database⁸ (<http://www.oncomine.Org>) is a cancer microarray database and web-based data-mining platform designed to develop and compare transcriptomic data from genome-wide expression analysis of various types of cancer with their respective normal tissues. Up to now, the database has collected 715 gene expression datasets and 86,733 cancer and normal tissue sample data. In this study, we used Analysis Type: Cancer versus Normal Analysis; Data Type: mRNA; Gene: PER1-3, CRY1-2, CLOCK, BMAL1 as the main filters and selected "Lung Cancer " as the cancer type by setting the selection threshold to fold change >2, Gene rank = ALL, and p-value <0.05 as a statistically significant difference.

1.2 Kaplan-Meier online survival analysis

The Kaplan-Meier mapper⁹ (<http://kmplot.com/analysis>) is capable to assess the effect of 54,000 genes on survival in 21 cancer types by relying on the GEO, EGA, and TCGA databases. We used this method to analyze the correlation between the expression of each circadian gene and overall survival (OS) in lung cancer. The screening conditions in this study were Cancer: Lung Cancer; Gene: PER1-3, CRY1-2, CLOCK, BMAL1; Split Patients by: Auto select best cut off; Survival: OS. p<0.05 was considered a statistically significant difference.

1.3 PrognoScan database

The PrognoScan database¹⁰ (<http://www.prognoscan.org/>) is a publicly available cancer microarray dataset that can be used to analyze the correlation of gene expression and survival. At present, the database had covered datasets of more than 40 different cancer types, including bladder cancer, prostate cancer and lung cancer¹¹. This study aimed to investigate the relationship between the expression of each circadian gene and prognosis in lung cancer, with the threshold set at COX p-value < 0.05, and the results of the analysis are summarized in Figure 4.

1.4 STRING database

The STRING database¹² (<http://string-db.org/>) aims to collect and integrate the relationships between proteins through protein-protein association data from large numbers of organisms, thus providing a systematic understanding of cellular functions. These protein-protein associations included direct and indirect associations, and the data information was mainly derived from high-throughput experimental data, predictions based on genomic context analysis, and interactions between other primary databases. In this study, protein function was analyzed by using the STRING database, and accessed under the protein name: PER2 protein, selected organism: human.

1.5 The cBioPortal platform

The CBioPortal¹³ (<http://cbioportal.org/>) is an open portal that allows interactions between multiple genetic platforms, providing visualization and analysis of samples and data types. In total, this database integrates more than 200 cancer genomics studies, including gene mutations, DNA copy data (gene amplification and gene deletions), mRNA and miRNA expression, protein levels, and DNA methylation clinical data. We examined the expression changes of seven circadian genes (PER1-3, CRY1-2, CLOCK, and BMAL1) in lung cancer tumors with the help of the cBio Portal platform and OncoPrint.

1.6 Statistical analysis

SPSS 22.0 software was used for statistical analysis. Continuous variables were compared using t-test. Kaplan-Meier and Log-rank tests were used for survival analysis. Statistical significance was set as $p < 0.05$.

Results

2.1 Screening of target circadian rhythm genes PER1-3, CRY1-2, CLOCK and BMAL1

Selection of target genes: Among the many genes obtained from the ONCOMINE database according to the screening conditions that we set, we selected the genes according to the median rank order from front to back. After excluding genes that have already been reported in detail, we finally selected the genes PER1, PER2, PER3, CRY1, CRY2, CLOCK, and BMAL1.

2.2 Results of circadian gene expression in common tumor types

A total of 2,946 datasets were included in the ONCOMINE database regarding the expression levels of PER1 (423), PER2 (392), PER3 (385), CRY1 (452), CRY2 (415), CLOCK (433), and BMAL1 (446) in different tumor tissues (Figure 1). Among them, 46 studies showed statistically significant results regarding PER1 expression, 40 of which showed lower expressions of PER1 in tumor tissues, and we recorded it as PER1 (40/46). For the other genes, the result was PER2 (45/48), PER3 (33/37), CRY1 (17/35), CRY2 (37/39), CLOCK (11/16), and BMAL1 (18/36).

2.3 Results of circadian gene expression in lung cancer

Using the ONCOMINE database, we investigated the transcript levels of PER1-3, CRY1-2, CLOCK, and BMAL1 in lung cancer and normal samples. The results showed that the mRNA levels of the above circadian genes were lower in lung cancer tissues (Table 1). Specifically, genes PER1-3 and CRY2 showed significantly lower expression in lung squamous carcinoma and adenocarcinoma. Expression of CRY1 was significantly lower in lung adenocarcinoma and small cell lung cancer. BMAL1 showed significantly lower expression mainly in lung squamous carcinoma, and CLOCK showed significantly lower expression in lung carcinoid tumors (Figure 2).

Table 1
Expression levels of circadian genes in lung cancer

Gene Type	Cancer Subtype	P-value	Fold Change	Rank(10%)	Sample
PER1	Small Cell Lung Carcinoma	7.49E-6	-8.101	3%	203
	Lung Adenocarcinoma	3.35E-5	-5.555	5%	203
	Lung Carcinoma Tumor	3.68E-4	-3.922	28%	203
	Lung Adenocarcinoma	8.29E-19	-2.125	3%	107
PER2	Squamous Cell Lung Carcinoma	4.35E-14	-2.430	6%	156
	Large Cell Lung Carcinoma	6.44E-7	-2.691	11%	156
	Lung Adenocarcinoma	0.005	-2.213	24%	203
	Small Cell Lung Carcinoma	0.001	-3.007	27%	203
PER3	Large Cell Lung Carcinoma	0.009	-2.006	8%	73
	Lung Adenocarcinoma	1.05E-9	-2.024	9%	156
	Squamous Cell Lung Carcinoma	2.74E-6	-2.204	19%	156
	Large Cell Lung Carcinoma	3.60E-4	-2.061	20%	156
CRY1	Lung Adenocarcinoma	1.39E-6	-2.058	6%	66
	Lung Carcinoma Tumor	0.001	-2.984	34%	203
	Small Cell Lung Carcinoma	0.007	-2.047	39%	203
CRY2	Squamous Cell Lung Carcinoma	3.80E-18	-2.335	3%	156
	Lung Adenocarcinoma	2.47E-5	-3.450	9%	66
CLOCK	Lung Carcinoma Tumor	0.004	- 2.382	43%	203
BMAL1	Squamous Cell Lung Carcinoma	5.67E-5	-2.526	4%	73

2.4 Relationship between circadian gene expression and prognosis in lung cancer

We analyzed the correlation between the expression level of each circadian gene and OS in lung cancer patients using the Kaplan-Meier-Plotter online database. The results showed that OS was shortened in lung cancer patients in the low-expression group of each circadian gene (PER1-3, CRY1-2, CLOCK, and BMAL1) compared with the high-expression group ($p<0.05$) (Figure 3). The PrognScan database was further used to explore the relationship between the transcript level of each circadian gene and cancer prognosis. The result showed that patients with low-expressions of PER1-3, CRY2 and CLOCK had worse OS than high-expression patients($p<0.05$) (Figure 4). The above data predicted a poor prognosis for lung cancer patients with low expression of circadian rhythm genes and a better prognosis for lung cancer patients with high expression.

2.5 Co-expression analysis of circadian gene-related proteins

SRTING is a database that can be used to predict protein-protein interactions. Co-expression analysis showed that PER2 gene was co-expressed with BMAL1 (ARNTL), BHLHE41, CLOCK, CRY1, CRY2, CSNK1D, CSNK1E, NFIL3, NPAS2, and PER1 genes (Figure 5), indicating that there was a coordinated interaction between circadian genes in the body. These circadian genes together regulated the biological clock changes in the body. In addition, the KEGG pathway analysis revealed that PER2 protein was involved in the regulation of multiple signaling pathways, especially the circadian signaling pathway (Table 2).

Table 2
Analysis of the KEGG pathway of the PER2 protein

KEGG Pathways			
Pathway	Description	count in gene set	false discovery rate
hsa04710	Circadian rhythm	10 of 30	4.91e-26
hsa05168	Herpes simplex infection	4 of 181	1.45e-05
hsa04340	Hedgehog signaling pathway	2 of 46	0.0013
hsa04713	Circadian entertainment	2 of 93	0.0037
hsa04728	Dopamine synapse	2 of 128	0.0056
hsa04390	Hippo signaling pathway	2 of 152	0.0065

2.6 Circadian gene signature in human lung cancer

The expression levels of circadian rhythm genes have been demonstrated to affect OS and the prognostic status of lung cancer patients through Kaplan-Meier and PrognScan databases. Further, using the cBioPortal database, a total of 6509 samples were investigated in 22 datasets to explore the genetic alterations of circadian genes such as PER1-3, CRY1-2, CLOCK, and BMAL1 (ARNTL) in various lung cancer subtypes. The results showed that the types of genetic alterations in these 22 datasets included

mutations, amplifications, deletions, fusions, and multiplex alterations (Figure 6). The OncoPrint interface was used to provide statistics on the percentage of alterations of each gene, which ranged from 2.5–19.1% from each dataset. Among them, mutation was found to be the most common alteration.

Discussion

This study used bioinformatics data to explore the role of each circadian gene in lung cancer and its association with OS. The results showed that the expression levels of circadian genes PER1-3, CRY1-2, CLOCK, and BMAL1 were generally lower in lung cancer tissues than those in normal tissues. The expression levels of the circadian genes were positively correlated with patient OS, and the lower the levels, the worse their prognosis. Through the STRING database, we found that BHLHE41, CRY1 and CRY2 proteins had the strongest association with PER2 protein. The analysis of the KEGG pathway revealed that PER2 protein played an important role in the molecular regulation of circadian rhythmogenesis.

It was found in our study that the expression of circadian rhythm genes in tumor tissues was significantly different from that in normal tissues, demonstrating that they played important roles in cancer development. Some scholars used a genetically engineered mouse model (GEMM) of lung adenocarcinoma to study the effect of circadian rhythm disorders on lung tumorigenesis. They disrupted the physiological rhythm of mice through jet lag, and found that physiological rhythm disorders could accelerate the progression of lung tumors. Meanwhile, they used a germline mutant allele of Per2 and BMAL1, and found that deficiency of PER2 and BMAL1 genes significantly reduced the survival rate of mice ($P < 0.05$) and increased the number of lung tumor cells¹⁴. Loss of circadian genes including CLOCK, PER1-3, CRY2, NPAS2 and RORC was associated with a poorer prognosis in breast cancer¹⁵. Hsu et al¹⁶ investigated the expression profile of circadian genes in patients with head and neck squamous carcinoma and found that the expression of PER1-3, CRY1-2, CK1 ϵ and BMAL1 in cancer tissues showed a significant downregulation. Some researchers also analyzed the expressions of circadian genes in abdominal tumors (liver, gastric, pancreatic, colorectal, and renal cancers) and their impact on patient prognosis using the TCGA database. The results showed that downregulation of most circadian genes was associated with a poorer prognosis in patients with abdominal malignancies, and different rhythm genes were associated with different prognoses for different cancer types¹⁷. Tokunaga et al¹⁸ found that the expression levels of PER1-3 and CLOCK were significantly lower in ovarian cancer than those in normal ovaries by RT-qPCR assay, whereas the expression of CRY1 was higher in cancer tissues than that in normal ovarian tissues. Similarly, a study by Zeyu Wang¹⁹ demonstrated that knockdown or disruption of circadian rhythm genes promoted the development, invasion and metastasis of lung tumors.

Circadian genes play an important role in cell cycle regulation, proliferation and apoptosis, and DNA repair. Per1 is a tumor suppressor gene and can promote cell apoptosis or cell cycle arrest in colorectal cancer cells by interacting with DNA damage response proteins ATM and CHK2, and cell cycle regulators Wee1, Cyclin B1 and Cdc2²⁰. BMAL1 is also a tumor suppressor gene, and its overexpression can inhibit

the proliferation of cancer cells. It has been reported in the literature that BMAL1 induces apoptosis mainly by activating the ATM pathway and then blocking the G2/M phase of the cell cycle. During anticancer therapy, this gene can help increase the sensitivity of colorectal cancer cells to chemotherapeutic agents and enhance the therapeutic effect²¹. It is reported in the literature that overexpression of CLOCK significantly inhibited apoptosis in intestinal cancer cells^{22,23}. To be more specific, the expression levels of the pro-apoptotic genes Bax and Bid were significantly reduced and the expression of the anti-apoptotic gene p-AKT was significantly increased in intestinal cancer cells overexpressing CLOCK. In vitro experiments showed that silencing PER2 gene significantly decreased the apoptotic capacity of human oral squamous carcinoma cells and the number of G1/G0 phase cells. Meanwhile the expression of Ki-67, MDM2, c-Myc, Bcl-2, MMP2 and VEGF mRNA was increased, and the expression of oncogenes p53, Bax and TIMP-2 mRNA was decreased²⁴.

All these data suggest that circadian genes contribute to the body homeostasis through different regulatory pathways. Disturbance of circadian rhythms promote the development of a variety of cancer and affect the disease progression and prognosis. Numerous studies have reported that circadian genes BMAL1^{25,26}, PER2²¹, or PER1^{27,28} are tumor suppressor genes, and downregulation of their expressions promoted tumor development and vice versa when they were overexpressed. These results are consistent with the results in our findings. The merit of this study is the use of a database of large sample analysis, thus effectively avoiding the problem of bias error and small sample size in a single-center. The results obtained in our study may provide useful experimental clues for the clinical treatment of lung cancer and other cancer types. In addition, this study provide provides our experience about intensive and effective control and management of databases and how to establish an effective data model for data analysis. However, the specific molecular mechanism of how circadian genes precisely affect lung cancer development is not well defined in this study, and further clinical experiments are needed to gain deeper insights into it. In conclusion, this study has fully demonstrated the association between circadian rhythms and lung cancer, thus providing a new direction for the treatment and early prevention of lung cancer.

Declarations

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Author Contributions

Conception and design: L.L., B.B.W., Hongxia Duan and S.S.X. Acquisition, statistical analysis or interpretation of the data: all authors. Drafting of the manuscript: L.L., B.B.W., Hongxia Duan and S.S.X. All authors reviewed and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available through the ONCOMINE database (<http://www.oncomine.Org>).

Ethics approval and consent

All procedures performed in this study involving human participants are in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. For this type of study, formal consent is waived.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no potential conflicts of interest related to this study.

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Figures

Figure 1

Transcription levels of various circadian genes in common tumor types (Oncomine Database). The left red column represents the number of datasets with overexpression of circadian genes (the tumor VS normal), and the right blue column represents the number of datasets with low expression of circadian genes (the tumor VS normal).

Figure 2

Analysis of the ONCOMINE database revealed that the mRNA expressions of circadian genes such as PER1, PER2, PER3, CRY1, CRY2, CLOCK and BMAL1 in lung cancer tissues were lower than those in normal lung tissues.

Figure 3

A correlation exists between the expression levels of circadian genes and the overall survival of lung cancer patients (Kaplan Meier-Plotter). (A)PER1, (B)PER2, (C)PER3, (D)CRY1, (E)CRY2, (F)CLOCK, (G)BMAL1. The survival curves for patients with high (red) and low (black) expression of rhythmic genes were compared from the Kaplan-Meier database ($P < 0.05$).

Figure 4

Relationship between the transcription levels of circadian genes and the prognosis of lung cancer (Prognoscan Database). (A) PER1, (B)PER2, (C)PER3, (D)CRY2, (E)CLOCK. Survival curves for patients with high (red) and low (blue) expression of circadian genes in lung cancer were plotted from the Prognoscan database ($P < 0.05$).

Figure 5

Identification of known and predicted structural proteins (STRING) based on the function of PER2 protein. Nodes in each color represent the associated protein molecules.