Dysregulation, functional implications, and prognostic ability of the circadian clock across cancers

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Abstract
It has been proposed that the circadian rhythm generally plays important roles in tumor suppression, but there is also evidence that disruption of the canonical circadian pathway has anticancer effects. In this study, we systematically analyzed the aberrances of circadian clock genes across cancers based on data from The Cancer Genome Atlas (TCGA). These data showed that the frequencies of mutations and copy number alterations in core clock genes (PER1/2/3, CLOCK, CRY1/2, and ARNTL) were low, but that the expression levels of core clock genes were downregulated by the higher levels of DNA methylation in most tumors. The circadian clock index (CCI) was established through a principal component analysis, and this measure well represents the overall expression of the core clock genes. In fact, the CCI was significantly lower in hepatocellular carcinoma with HBV infection than in other cancers. Furthermore, pathways such as the MAPK, JAK-STAT, and immune-related signaling pathways were enriched in tumors with high CCI values. Interestingly, the CCI was generally positively related to the immunophenoscores and immunophenotypes of tumors. Additionally, the expression levels of core clock genes and the CCI were also generally positively related to survival across cancers. Taken together, the results of this study provide a comprehensive analysis of circadian clock aberrances in cancer, and the results should aid further investigations of the molecular mechanisms of cancer and the development of therapeutic strategies.

KEYWORDS
cancer biology, genomics, immunology, survival
1 | BACKGROUND

The 2017 Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for their leading discoveries of the molecular mechanisms controlling the circadian rhythm.1,2 The circadian clock is critical for the normal physiological functions of cells, and disruption of the circadian system has been proposed to pose an important cancer risk. Several recent studies have demonstrated that aberrations in the circadian rhythm are involved in various cancers, such as prostate cancer,3,4 breast cancer,5 endometrial cancer,6 colorectal cancer,7,8 liver cancer,9 lung cancer,10 and leukemia.11 For example, disruption of the circadian system could promote hepatocarcinogenesis through chronic jet lag-driven gene dysregulation and liver metabolic dysfunction,9 decrease lung cancer survival, and promote lung tumor growth and progression.10 Sulli et al have recently shown that REV-ERB agonists could serve as anticancer agents.12 Alternatively, Puram et al have found that disruption of the circadian pathway could exert antileukemic effects.11 Thus, an investigation of the circadian system could provide clues for improving the understanding of the molecular mechanisms underlying tumourigenesis and might provide helpful information for the development of cancer therapies.13-19

The circadian rhythm in humans is orchestrated by the autoregulatory transcription and translation feedback loops of core clock genes, which comprise the activator genes, including CLOCK and ARNTL (also known as BMAL1), and the repressor genes, including PER1, PER2, CRY1, and CRY2.20,21 Basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) transcription factors, CLOCK and its heterodimeric partner ARNTL, form a complex that binds to the regulatory elements of core clock genes, including repressors (PER1, PER2, and PER3) and the cryptochrome (CRY1 and CRY2),20,21 which are also regulated by the E3 ligase complex of SKP1-cullin-F-box protein-β-TrCP (SCF-β-TrCP)-based ubiquitination.20,21 In addition, the nuclear receptors NR1D1 (REV-ERBa) and NR1D2 (REV-ERBβ), which are regulated by CLOCK and ARNTL, could be driven by RORα and RORβ to repress the transcription of ARNTL and NFIL3, whereas NFIL3 could, in turn, repress D-box-binding protein (DBP) to regulate ROR nuclear receptors.20,21 Through comprehensive bioinformatics analyses, Lehmann et al recently constructed a regulatory network for the mammalian circadian clock.22 Thus, the orchestration of the circadian clock is complicated and involves many genes. In general, various genes, including CLOCK, ARNTL, PER1, PER2, and CRY1, form the core components of the mammalian circadian clock (CCMCCs), and these establish complicated molecular circuits that orchestrate the different phases of the circadian rhythm.20,21 Furthermore, the expression of a substantial fraction (~5%-20%) of genes is under the control of the circadian rhythm.20,21 According to the Circadian Gene Database (CGDB),21 nearly 2000 genes show rhythmic expression, and many of these have been implicated to play roles in cancer.

Krugluger et al have found that the downregulation of PER1 is correlated with high-grade colon tumors,24 and Huisman et al have revealed that the circadian rhythm is disrupted in colorectal liver metastases.7 Through a population-based case-control study, Zhu et al have demonstrated that SNPs in core clock genes are significantly associated with susceptibility to prostate cancer.25 In addition, melatonin can resynchronize the dysregulated circadian rhythm in prostate cancer cells and should thus be investigated as an agent for the treatment of prostate cancer.26 Altered expression of core clock genes was also observed in chronic myeloid leukemia,27 and both malignant and normal hematopoietic cells harbor an intact clock and undergo robust circadian oscillations.11 Methylome profiling in human hepatocellular carcinomas (HCCs) has revealed that PER3 is hypermethylated,28 and the overexpression of PER1 due to inhibition of miR-34a decreases the growth of cholangiocarcinomas.29 Hypermethylation-triggered epigenetic inactivation of ARNTL has been detected in hematologic malignancies,30 and the overexpression of ARNTL could increase oxaliplatin sensitivity in colorectal cancer.31 Relogio et al have found that RAS can deregulate the mammalian circadian clock32 and revealed a remarkable interplay between the circadian clock and pre-mRNA splicing in cancer.33 Recently, Shilts et al have performed comprehensive analyses that indicated widespread dysregulation of the circadian clock in cancer,34 and Ye et al have observed that most circadian clock genes are related to survival, oncogenic pathways, and anticancer drug sensitivity.35 Based on these findings, El-Azhem and Relogio have proposed that escaping circadian regulation might be an emerging hallmark of cancer.36 Taken together, the results strongly indicate that the rhythm orchestrated by the circadian clock is involved in tumourigenesis, cancer progression, and metastases, and detailed systematic analyses of circadian clock genes and rhythmic genes in cancer should be performed at different molecular levels.

In this study, we systematically analyzed the molecular dysregulation, functional implications, and clinical relevance of the circadian clock across 20 cancers based on data from The Cancer Genome Atlas (TCGA). The results revealed that the circadian clock genes were most often dysregulated through DNA hypermethylation-based disruption of expression rather than mutations or copy number alterations. Based on the principal component analysis, we established the circadian clock index to represent the overall expression of core circadian clock genes and discovered that it is closely related to viral infections, various signaling
pathways, such as the MAPK, JAK-STAT, immune-related, protein export, nucleotide excision repair, mismatch repair and cell cycle pathways, immunophenotypes, and survival. Our study provided a comprehensive analysis of the role of the circadian clock in cancer, and the results should be helpful for further investigations of circadian clock-related molecular mechanisms and the development of therapies for cancer.

2 | METHODS

2.1 | Gene set curation and data obtainment

The circadian rhythm-related genes were curated from a previous systematic review.21 The core clock genes include CLOCK, ARNTL, PER1, PER2, PER3, CRY1, and CRY2,20,21 whereas the CCMCCs are more extensive and contain 22 genes, including the core clock genes plus BTRC, CSNK1D, CSNK1E, CUL1, DBP, FBXL21, FBXL3, NFIL3, NR1D1, NR1D2, PRKAA1, PRKAA2, RORA, RORB, and SKP1.20,21 A gene list of 1350 rhythmic genes was downloaded from the CGDB, which claims that the transcript-level oscillations of these genes have been validated in previous publications through various methods, such as RT-PCR, northern blot, and in situ hybridization.23 In general, core clock genes and CCMCCs are the key orchestrators and important regulators of the circadian rhythm, respectively,21 and rhythmic genes are regulated by the circadian rhythm.23 All the data on mutations, copy number variations, DNA methylation, expression, and clinical outcomes were obtained from a level 3 dataset from TCGA at FireBrowse (http://gdac.broadinstitute.org, 2016 January). The analyses were performed using genomic and transcriptome data from both tumor and normal tissues for the following 20 types of cancer: bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), cervical and endocervical cancers (CESC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC). The somatic copy-number alterations (SCNAS) of the core clock genes were obtained from cBioPortal,37,38 and immunophenotype data were obtained from The Cancer Immunome Atlas (TCIA, https://tcia.at/) with substantial assistance from the authors.39

2.2 | Analysis of somatic mutations, driver genes, copy numbers, and DNA methylation

Mutation data were obtained from the downloaded MAF files, and only nonsilent mutations were reserved for the analysis. The frequency ratio was calculated as the mutation frequency divided by the mutation burden to eliminate the diversity of mutation burden among cancers. A frequently mutated gene (FMG) in one type of cancer was identified based on a mutation frequency greater than 5% in that type of cancer, whereas the enrichment of FMGs in the set of rhythmic genes was performed based on hypergeometric statistics. Driver gene analysis for each cancer type was conducted using MutSigCV v1.2 with the GenePattern online server (https://genepattern.broadinstitute.org)40 with a cutoff of P < 0.05. The correlation between mutations of 375 driver genes40 and the CCI was analyzed by regression method, while cancer types and tumor mutation burden were adjusted. The P values across 375 genes were corrected for multiple hypothesis testing using method of Benjamini & Hochberg, and only genes under 10% false discovery rate (FDR) were considered as significantly mutated. Genes and promoter regions were annotated with the Bioconductor (Release 3.7) R package IlluminaHumanMethylation450kanno.ilmn12.bg19 (version 0.6.0) for DNA methylation 450 K data. Only the methylation sites within the promoter regions of the core clock genes were considered in the differential methylation analysis, which was performed with Wilcoxon rank sum tests.

2.3 | Analysis of gene expression

Normalized RNA-Seq v2 data were downloaded for the gene expression analysis and are summarized in Table 1. The R package DESeq2 was employed to perform differential expression analysis between tumors and normal tissues,41 and the genes with adjusted P < 0.05 were defined as differentially expressed. The principal component analysis (PCA) was conducted with R packages.42 The principal component 1 (PC1) was employed as the circadian clock index (CCI) to represent the overall expression of the core clock genes, and the CCI values were performed with Wilcoxon rank sum tests. To test whether the mutational status of the driver genes40,43 was significantly associated with the CCI, rank-transformed CCI was modeled via linear regression as a function of the gene's mutational status, and the rank-transformed mutation burden was used to diminish confounding effects. The gene set enrichment analysis (GSEA) was carried out using GSEA software44 with default parameters to study differences in expression of the circadian clock genes between tumor and normal tissues and between high- and low-grade tumors and to investigate the pathway differences between tumors with high
The GSEA results were visualized with gseapy package (https://pypi.python.org/pypi/gseapy), and the other visualizations were generated with ggplot2 and other R packages such as pheatmap and ComplexHeatmap.

### 3 | RESULTS

#### 3.1 | Molecular alterations of the circadian rhythm across cancers

Since the circadian rhythm is critical for the physiological functioning of organs, we analyzed the molecular alterations of circadian clock genes. As shown in Figure 1A, the mutation frequencies of core clock genes varied notably among cancers. However, the mutation frequencies of almost all core clock genes were lower than 5%, with the following exceptions: PER2 in CHOL, STAD and UCEC, PER1 in CHOL, and PER3 in UCEC. Thus, since the mutation rates of core clock genes were found to be low, the circadian rhythm system might not be disrupted by mutation in cancers. In general, PER1/2/3 exhibited the highest mutation frequencies among the core clock genes. In fact, the mutation-based driver gene analysis showed that only PER3 might be a potential driver gene in CESC and ESCA. Furthermore, the mutation rates of other CCMCs in cancer were also low (Figure S1 and S8). To further dissect the genomic alterations of circadian rhythm, the copy number variations of core clock genes were also analyzed (Figure 1B), and the results showed that the copy number alterations of core clock genes were limited, although both amplifications and deletions of core clock genes were observed. The alteration frequencies of core clock genes were lower than 5% in most cancers, with the exception of PER3 in CHOL and CLOCK in LUSC. Taken together, the core clock genes appear to be relatively stable at the genomic level.

To investigate aberrances in rhythmic genes across cancers, the mutations in the 1350 experimentally identified rhythmic genes curated from the CGDB were systematically analyzed in various cancers. The genes with mutation frequencies greater than 5% were defined as FMGs, and enrichment analyses were then performed across cancers based on hypergeometric test. The results revealed that FMGs were enriched in rhythmic genes in cancers, including COAD (P = 0.0343), ESCA (P = 0.0339), KIRC (P = 0.0060), and UCEC (P = 0.0074), which indicated that rhythmic genes have relatively high mutation rates in these cancers. Furthermore, the results obtained using MutSigCV software showed that a number of rhythmic genes were driver genes in these cancers, although rhythmic genes were not significantly enriched with driver genes. For example, more than 10% of driver genes were also circadian genes in KIRP and CLOCK in LUSC. Thus, rhythmic genes might be increasingly involved in rhythmic dynamics and could be preferentially mutated in several cancers.

#### 3.2 | Differential expression of circadian clock genes across cancers

The circadian rhythm has been generally proposed as a tumor-suppressive mechanism that is disrupted in cancers, the low frequencies of genomic alterations in core clock genes indicate that the circadian clock might be dysregulated at the gene expression level. The differential expression analysis of the core clock genes across cancers was performed with DESeq2 software, and the significant results (adjusted P < 0.05) are shown in Figure 2A. It was clear that the expression levels of core clock genes were

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**TABLE 1** The number of samples and abbreviations for the 20 types of cancers investigated in this study

| Cancer type                              | Abbreviation | Number of samples |
|------------------------------------------|--------------|-------------------|
| Bladder urothelial carcinoma             | BLCA         | 412               |
| Breast invasive carcinoma                | BRCA         | 1098              |
| Cervical and endocervical cancers        | CESC         | 307               |
| Cholangiocarcinoma                       | CHOL         | 51                |
| Colon adenocarcinoma                     | COAD         | 460               |
| Esophageal carcinoma                     | ESCA         | 185               |
| Glioblastoma multiforme                  | GBM          | 613               |
| Head and Neck squamous cell carcinoma    | HNSC         | 528               |
| Kidney Chromophobe                       | KICH         | 113               |
| Kidney renal clear cell carcinoma        | KIRC         | 537               |
| Kidney renal papillary cell carcinoma    | KIRP         | 323               |
| Liver hepatocellular carcinoma           | LIHC         | 377               |
| Lung adenocarcinoma                      | LUAD         | 585               |
| Lung squamous cell carcinoma             | LUSC         | 504               |
| Pancreatic adenocarcinoma                | PAAD         | 185               |
| Prostate adenocarcinoma                  | PRAD         | 499               |
| Rectum adenocarcinoma                    | READ         | 171               |
| Stomach adenocarcinoma                   | STAD         | 443               |
| Thyroid carcinoma                        | THCA         | 503               |
| Uterine Corpus Endometrial Carcinoma     | UCEC         | 560               |
significantly downregulated in almost all cancers. A limited number of exceptions were observed, and these included the overexpression of \textit{PER1/2} in KIRC, \textit{ARNTL} in KRIC and HNSC, THCA and CRY2 in KICH, and \textit{CLOCK} in CESC and LUAD. Thus, the expression levels of core clock genes are generally downregulated in tumors. Since there is no timepoint information for the samples in TCGA, the observed differences in expression should be considered as the differences between tumor and normal tissues. Furthermore, we performed a GSEA with the core clock genes among cancers, and the results shown in Figure S2 reveal that the core clock genes were significantly downregulated in seven cancers, namely, UCEC, BRCA, HNSC, BLCA, CESC, STAD, and GBM.

To dissect the potential cause of the low expression of the core clock genes in cancers, we analyzed the DNA methylation status of the promoters of the core clock genes. DNA methylation data were available only for \textit{PER1/2/3} and \textit{ARNTL}, and the analysis showed that the promoter regions of \textit{PER1/2/3} and \textit{ARNTL} were generally highly methylated (Figure 2A) in most cancers and that the expression level and methylation status were highly correlated. Thus, this high degree of methylation might be the major cause of the low expression of \textit{PER1/2/3} and \textit{ARNTL} in tumors. In addition, the low expression of other CCMCCs was consistent with their hypermethylation in most cancers (Figure S3A). Furthermore, we established the circadian clock index (CCI) for core clock genes based on the PCA. According to the results shown in Figure S3B, all the proportions of PC1 in 27 cancers were greater than 66%, and the proportions in READ, GBM, COAD, UCEC, PRAD, BRCA, and CHOL were higher than 85%. Thus, the expression of core clock genes could be well represented by PC1, which indicated that PC1 could be employed as the CCI. The differences between tumor and normal tissues were then analyzed. As shown in Figure 2B, the CCI was generally significantly lower in almost all tumors, which was consistent with the differential expression analysis results. Because viral infection might contribute to aberrant gene expression, we analyzed the correlation between the CCI and viral infections. The results presented in Figure 2C show that the CCI was significantly lower in hepatitis B virus (HBV)-infected liver tumors than in normal tissues, which indicate that HBV infection might contribute to the dysregulation of the circadian rhythm in liver cancer. Furthermore, we analyzed the correlation between the CCI and driver gene mutations through regression analysis. As the results shown in Figure 2D, the CCI was negatively correlated with mutations of driver genes including \textit{TP53} in BRCA, LIHC, and LUAD, \textit{XIRP2} in STAD, \textit{MUC4} in KIRC and \textit{FAM46C} in HNSC, while negatively correlations were observed for \textit{RNF43} in CESC, \textit{PIK3CA} in BRCA, \textit{FRG1} in HNSC, and \textit{EGFR} in BLCA.

Furthermore, the expression and methylation of CCMCCs were analyzed, and the results presented in Figure S8 show that the expression of CCMCCs was disrupted in tumors and that hypermethylation contributed...
substantially to this disruption. In tumor tissues, dysregulated circadian rhythm would disrupt the oscillatory expression of circadian genes; therefore, it was necessary to investigate the differential expression of rhythmic genes. DESeq2 was employed to identify the differentially expressed genes (DEGs) in 20 cancers using an adjusted $P$ value less than 0.05 as the cutoff value. Enrichment analyses of DEGs in rhythmic genes across cancers were based on using a hypergeometric test. It was observed that DEGs were enriched in rhythmic genes in various cancers, including GBM ($P = 7.87E-07$), CHOL ($P = 0.0021$), PAAD ($P = 0.0024$), and CESC ($P = 0.0142$), and thus indicated that disruption of the circadian rhythm might strongly influence gene expression in these cancers. Although the regulation of gene expression is complicated, the disruption of oscillatory expression in cancer is likely to be related to the dysregulation of gene expression in tumors.

### 3.3 | Signaling pathways correlated with the disrupted circadian rhythm

Since circadian rhythm is critical for normal physiological functioning and is disrupted in tumors, we analyzed the signaling pathways correlated with a disrupted circadian rhythm for the results might be helpful for future studies aiming to understand underlying molecular mechanisms and functions of the circadian rhythm in cancer. GSEA of signaling pathways was performed between high- and low-CCI tumors (Figure 3A-B, Figure S4). As shown in Figure 3A, various pathways were up- or downregulated in high-CCI tumors compared...
with low-CGI tumors. The mammalian circadian rhythm pathway was significantly upregulated in almost all high-CGI tumors, with the exception of BRCA, and these findings provide further evidence showing that the CCI can accurately represent the circadian rhythm. Pathways such as the MAPK, JAK-STAT, VEGF, ErbB, and Notch signaling pathways were positively correlated with circadian rhythm, whereas protein export, mismatch repair, nucleotide excision repair, and cell cycle were negatively related to circadian rhythm. Furthermore, CCI was positively correlated with a large number of metabolic pathways in LIHC (Figure S4A), which was consistent with the findings of previous studies.46-50

The circadian rhythm recently emerged as an important regulator of the immune system.51,52 The GSEA results showed that a number of immune pathways were differentially activated in high- and low-CGI tumor tissues (Figure 3B). For example, leukocyte transendothelial migration, hematopoietic cell lineage, cytokine-cytokine receptor interaction, intestinal immune network for IgA production, and chemokine signaling pathway were upregulated in high-CGI tumor tissues, whereas protein export, mismatch repair, nucleotide excision repair, and cell cycle were negatively related to circadian rhythm. Furthermore, CCI was positively correlated with the immunophenoscore (IPS), which represents the immunogenicity of the tumor.39 The correlations between CCI and four categories, namely, suppressor cells (including Tregs and MDSCs, SC), checkpoint (CP), effector cells (including activated CD8+ T cells, CD4+ T cells, Tem CD8+, and Tem CD4+ cells, EC) and HLAs (MHC) were analyzed (Figure 3C). We also observed that CCI was positively correlated with the repression of signals, including those to SC and CP in THCA, KIRP, LIHC, LUAD, GBM, and KIRC, but negatively correlated with the repression of signaling in COAD, STAD, CESC, BLCA, and LUSC. In contrast, the CCI was negatively correlated with...
the activation of signaling, including signals to EC and MHC, in THCA, LIHC, LUAD, and KIRC, but positively associated with the activation of signaling in PRAD, HNSC, UCEC, COAD, STAD, CESC, BLCA, and LUSC. Unexpectedly, the CCI was positively correlated with both CP and MHC in BRCA. Thus, the relationship between the circadian clock and immune signaling might be close but complicated, and more studies should be conducted to elucidate this network.

Furthermore, based on estimations of the abundances of immune cell types by CIBERSORT, the correlations between the CCI and immune cell types were analyzed, and the results are shown in Figure S5. The correlations were generally miscellaneous, while there were also special patterns that could be detected. For example, the CCI was positively correlated with resting mast cell, naïve B cell, resting memory CD4 T cell, and CD8 cell in most cancers and negatively correlated with macrophage M1, resting dendritic cell, follicular helper cell, and regulatory T cell in most cancers. The CCI was extremely positively correlated with activated dendritic cell in GBM and LIHC, activated mast cell in PRAD, plasma cell in GBM, and resting memory CD4 T cell in LIHC, and these correlations were either weaker or negative in other cancers. Strongly negative correlations were observed between the CCI and macrophage M0 in LIHC, macrophage M1 in KIRP, and neutrophil in KICH. Taken together, these lines of evidence show that the circadian rhythm system is strongly correlated with the immune status, but the relationship between circadian rhythm and immunophenotypes is complicated, and further investigations are needed in these cancers.

### 3.4 | Clinical relevance of the circadian rhythm across cancers

A previous study found that the circadian rhythm is associated with survival in colorectal cancer patients. For circadian rhythm is crucial to cell physiology and the core clock genes were dysregulated at mutation, SCNA, DNA methylation and expression levels, the prognostic ability of the circadian system in cancers should be investigated. Survival analyses were performed based on the expression of the core clock genes with the best cutoff values across cancers. As summarized in Figure 4A, high expression levels of most core clock genes indicated low hazard ratio (HR) in most cancers except PRAD and STAD, whereas CLOCK was negatively correlated with survival in most cancers. Thus, it is obvious that high expression levels of the core clock genes generally predict better survival among cancers. The exceptions might be related to the multifunctional roles of core clock genes and need further investigation. Furthermore, the prognostic ability of the CCI was also studied. The survival analysis with the best cutoff value showed that a high CCI predicted better survival in BRCA, CESC, KIRP, and LIHC (Figure 4B-E). Besides, we carefully analyzed relationships between circadian and clinical characteristics including smoking, alcohol, BMI, age, and tumor stages. It was observed that cigarette smoking was positively correlated with CCI in ESCA (Pearson $r = 0.24$, $P = 0.02$) and LUSC ($r = 0.10$, $P = 0.04$), while BMI was negatively correlated with CCI in READ ($r = 0.25$, $P = 0.03$). Furthermore, positive correlation was observed between age and THCA ($r = -0.10$, $P = 0.03$). However, no significant association between the CCI and alcohol was observed across cancers. Furthermore, we analyzed the correlation among tumor stages and circadian rhythm through GSEA of the core clock genes between high- and low-stage tumors among cancers and found that the downregulated core clock genes were significantly enriched in high-stage tumors in KICH and KIRC (Figure S6).

To further reveal the prognostic ability of the circadian clock genes, the survival analysis was performed among the CCMCCs. As shown in Figure S6, most CCMCCs were positively related with survival among cancers, although miscellaneous results were also observed. For example, in KIRC, high expression level of FBXL3 predicted better survival, while RORB, CSNK1D, and CSNK1E significantly predicted poor survival (Figure S7). However, high expression of FBXL3 was related to poor survival in STAD (Figure S7). Thus, the functional roles of CCMCCs in cancer survival should be further investigated.

## 4 | DISCUSSION

Although it has long been known that the circadian rhythm plays crucial roles in physiology, this system had not attracted much attention until recently. Common sense, epidemiological evidence, and biomedical studies all indicate that circadian rhythm and its orchestrating circadian clock genes are associated with cancer. In this study, we systematically analyzed the dysregulation of circadian clock genes at different molecular levels across 20 types of cancer. Circadian clock genes were dysregulated at the expression level, which agreed with the findings of another recent comprehensive study. In general, the expression of circadian clock genes was downregulated in tumor tissues and correlated with their hypermethylation. Currently, the TCGA data have no timepoint information, which makes it unclear whether different timepoints could influence the expression of core clock genes in tumor tissues. The principal component analysis revealed that the CCI could well represent the expression of circadian clock genes, and the CCI was correlated with viral infection, various signaling pathways, immunophenotypes, and cancer patient survival.

Previously, Taniguchi et al. reported that in hematologic malignancies ARNTL was transcriptionally silenced by the hypermethylation of its promoter CpG island. TCGA data showed that the expression of ARNTL was significantly lower in most solid tumors than in nontumor tissues; in addition, hypermethylation of ARNTL was also observed in...
PRAD (Figure 2A) and STAD, while the methylation status of ARNTL in other cancers was not available. Furthermore, hypermethylation of PER1/PER2/PER3 was observed in most cancers, although the hypermethylation of PER3 was only statistically significant in HNSC, BRCA, and THCA. Thus, the main cause of the lower expression levels of circadian clock genes might be hypermethylation of ARNTL and PER1/PER2/PER3, while the mechanisms for CLOCK and CRY1/CRY2 require further investigation.

In general, the proportions of PC1 were sufficiently high to represent the expression of the core clock genes in PCA (Figure S3A). However, CRY1 in GBM, PER1/PER2 in KIRC, and CRY2 in KICH had significantly higher expression levels in tumors than in nontumor tissue, and the CCI was also higher in tumors (Figure 2B). Thus, aberrances of the circadian clock in these three cancers should be more carefully studied. Interestingly, PER1/PER2, and CRY2 are negative arms of the circadian feedback loop, while the role of CRY1 is unclear. The clock correlation distance model proposed by Shilts et al might be helpful for further analyses of aberrances of the circadian clock in these three cancers. Furthermore, this study clarified that the CCIs of the core clock genes were closely correlated with immunophenotypes and cancer patient survival. However, the relationships were not consistently positive or negative among cancer types. Thus, further detailed investigations should be performed to determine the mechanisms and functions of the dysregulation of the circadian clock in different cancer types.

5 | CONCLUSIONS

The circadian rhythm and its regulatory circadian clock genes play crucial roles in cancer, and further investigation...
could advance the understanding of the functional roles of the circadian clock and the circadian rhythm in cancer and aid the development of potential therapies.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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