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The Expression and Prognostic Significance of Circadian Gene *NR1D1/NR1D2* in Patients with Gastric Cancer

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ABSTRACT

Objective: The dysregulation of circadian rhythms is proved to be associated with the development of and may affect the treatment of tumors. However, the biological role of (Nuclear Receptor Subfamily 1, Group D, Member 1) *NR1D1/NR1D2* (Nuclear Receptor Subfamily 1, Group D, Member 2) remains elusive in gastric cancer. We analyzed the effects of *NR1D1/NR1D2* expression with occurrence and prognostic value for gastric cancer.

Methods: By using the online databases including UALCAN (The University of Alabama at Birmingham Cancer Data Analysis Portal), KM plotter (Kaplan-Meier plotter), STRING database and FunRich software, the effects of *NR1D1/NR1D2* on the occurrence, clinical features and prognosis of gastric cancer were evaluated in gastric cancer.

Results: Based on the analyses of data obtained from online database, both the mRNA expression of *NR1D1 and NR1D2* were higher in stomach carcinoma than in normal tissue ($p<0.001$). The level of *NR1D1* expression was significantly increased in female patients, T (Tumor), N (Nodes), M (Metastasis) and Lauren classification in gastric cancer patients ($p=0.017$, 0.000, 0.014, 0.005 and 0.004) and the *NR1D2* was significantly higher in male patients, *HER2* status, tumor stage, T, Lauren classification, and differentiation in gastric cancer patients ($p=0.000$, 0.000, 0.000, 0.001, 0.000 and 0.000). The elevation of *NR1D1/NR1D2* was significantly correlated with worse OS (Overall Survival), PPS (Post-Progression Survival) and FP (First Progression) in gastric cancer patients ($p<0.001$). Furthermore, we constructed protein-protein interaction networks of genes coexpressed with *NR1D1 and NR1D2*. FunRich analyses suggested that the *NR1D1 and NR1D2* was possibly involved in (Brain and Muscle ARNT-Like 1) *BMAL1*: *CLOCK* (Circadian Locomotor Output Cycles Kaput)/*NPAS2* (Neuronal PAS Domain Protein 2) activates gene expression and circadian clock pathway.

Conclusion: Our study suggests that *NR1D1 and NR1D2* may be potential biomarkers for the diagnosis and prognosis of gastric cancer.

Keywords: Gastric cancer; *NR1D1; NR1D2*; mRNA expression; Prognosis

INTRODUCTION

Gastric cancer has become to be a very common cause of mortality and ranks the fifth cancer incidence rate of the WHO statistics in 2020 [1,2]. The carcinoma patients have already received many kinds of traditional therapies, including surgery, chemotherapy, and targeted therapy, but it remains poor outcomes for metastatic gastric cancer patients [3,4]. In recent years, the number of gastric cancer patients has increased rapidly, but the treatment of advanced gastric cancer is still limited.

Therefore, we have been trying to find more appropriate diagnostic and therapeutic targets related to the prognosis of patients. The pathogenesis of gastric cancer is a complex process with multiple factors, multi gene regulation and multi-step participation [5]. The unlimited proliferation of cancer cells is the basis of tumorigenesis and development. Understanding the molecular mechanism of gastric cancer cell proliferation is of great significance for the prevention and treatment of gastric cancer.

Circadian rhythm factors form a Transcription Translation Feedback Loop (TTFL) to produce circadian rhythm and the body regulates various physiological functions through this way [6]. The circadian clock generates 24 h rhythms in gene expression and play an important role in physiology and behavior. Dysregulation of circadian rhythms may be associated with the development of cancer and may affect the treatment of tumors [7,8].

The uncontrolled growth of tumor cells is the most basic feature of malignant tumors, and biological behaviors such as invasion and metastasis are also closely related to it. A large number of gene expression microarray studies have shown that circadian rhythm genes are related to cell cycle progression, proliferation and apoptosis, but the specific regulatory mechanisms are still not fully understood [9].

The circadian rhythm is regulated by a series of biological rhythm factors. *CLOCK* and *BMAL1* will form a heterodimer to activate the transcription of clock control genes *PER* (Period), *CRY* (Cryptochrome) and other genes. *NR1D1* (known as REV-ERBα) is one of the core components of circadian rhythm, which is regulated by *CLOCK*. The gene encoded on chromosome 17q21.1, activated for transcription by *BMAL1*. *NR1D2* (known as REV-ERBβ) is a variant of *NR1D1* and characterized as a repressor [10,11].

The heterodimer complex *CLOCK/BMAL1* binds to E-box elements in the promotor region of the genes including *NR1D1 and NR1D2*, which interconnect to form positive and negative transcriptional/translational feedback loops [12-14]. *NR1D1* is mainly expressed in adipose tissue, liver tissue, skeletal muscle system and brain tissue [15].

It is documented that *NR1D1 and NR1D2* are closely related to a variety of cellular physiological processes, including cell differentiation, lipid metabolism, inflammatory response and rhythm regulation, making it a potential therapeutic target for cancer and inflammatory diseases [16-18]. Studies have shown that *NR1D1* is also highly expressed in melanoma and non-small cell lung cancer than normal tissues [19]. The expression of *NR1D1* between liver cancer tissues with metastasis is significantly higher than that without metastasis [20]. It is reported that *NR1D2* was abundant in human glioblastoma GBM (Glioblastoma Multiforme) tissue and cell lines but not primary human astrocyte [21].

However, there is no report on the relationship between the expression of *NR1D1 or NR1D2* and the prognosis of gastric cancer patients. We use several bioinformatics web-based resources to analyze the expression of the genes in gastric cancer patients and its effect on prognosis.

MATERIALS AND METHODS

The expression analysis of *NR1D1* and *NR1D2* across stomach adenocarcinoma and normal tissues were examined using a web-based tool UALCAN. UALCAN is a comprehensive, user-friendly, and interactive web resource for analyzing cancer OMICS data based on TCGA (The Cancer Genome Atlas), MET500 (Metastatic cancer), CPTAC (Clinical Proteomic Tumor Analysis Consortium) and CBTTC (Children's Brain Tumor Tissue Consortium) [22]. The relationship between clinicopathological parameters and *NR1D1 or NR1D2* was expression was analyzed by ULCAN and KM plotter.

We used an online database to analyze the correlation of *NR1D1* expression to the OS, PPS and FP in gastric cancer patients by KM plotter. The KM plotter is capable to assess the correlation between the expression of 30K genes and survival from 21 tumors including gastric cancer, breast, ovarian and lung with the databases include GEO (Gene Expression Omnibus), EGA (European Genome-phenome Archive), and TCGA [23].

The protein-protein association data of the *NR1D1* and *NR1D2* interaction partners was collected and integrated from the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database [24], and the functional enrichment analysis was conducted to identify genes coexpressed with the two genes by using the FunRich tool [25].

The *NR1D1 or NR1D2* expression between tumor and normal tissue was analyzed by student's t-test. The chi-square tests were performed to analyze the associations between *NR1D1 or NR1D2* expression and clinicopathologic characteristics by SPSS 22.0 software (IBM Analytics, USA). The Kaplan-Meier analysis was used to assess the survival of gastric cancer patients. The Pearson correlation coefficient was used for analyzing the correlations between genes. In general, p-values<0.05 were considered statistically significant.

RESULTS

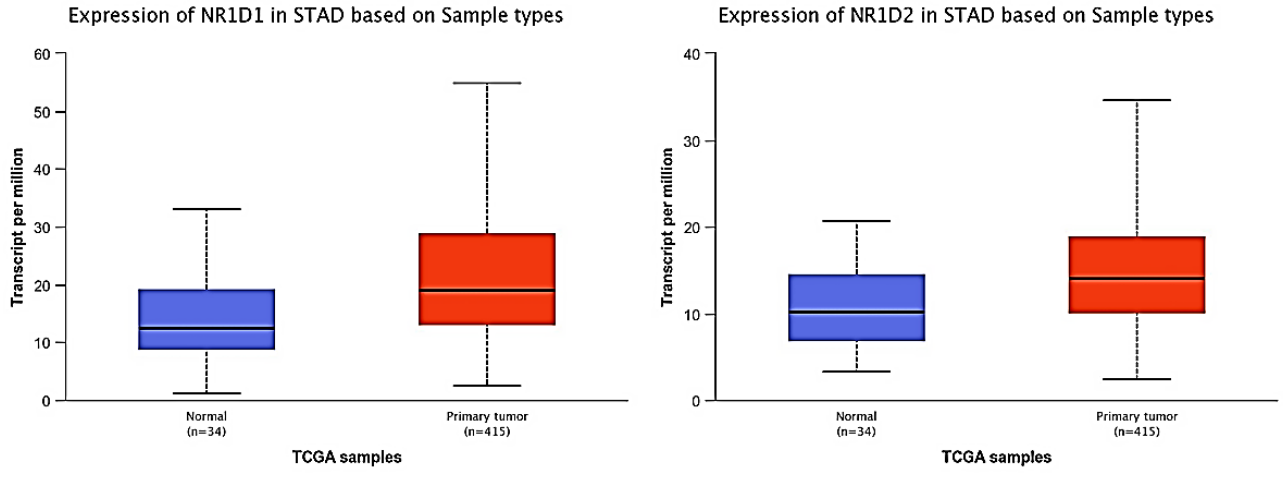
The expression of *NR1D1 and NR1D2* in stomach adenocarcinoma and normal tissues

To estimate the different expression levels of *NR1D1* and *NR1D2* between gastric cancer and normal tissues, we used the UALCAN database to identify the expression profiles. In the database, the Stomach Adenocarcinoma (STAD) was analyzed instead of gastric cancer. The *NR1D1 and NR1D2* mRNA expression data in 415 stomach adenocarcinoma and 34 normal tissues from TCGA were analyzed.

The expression of *NR1D1* was significantly higher in primary stomach adenocarcinoma tissue than in normal tissue (p=3.97e-09). The similar result was found with *NR1D2* expression in stomach adenocarcinoma and normal tissues (p=9.68e-03) (Figure 1).

Figure 1. The analysis of the *NR1D1/NR1D2* expression levels in STAD and normal tissues.

Note: ■ Normal; ■ Primary tumor.



The association of *NR1D1* and *NR1D2* expression with clinicopathological parameters in gastric cancer patients

We downloaded the dataset from KM plotter database to investigate the relationship between the expression of *NR1D1 or NR1D2* and clinical characteristics. The level of *NR1D1* expression was significantly increased in female patients, T, N, M and Lauren classification in gastric cancer patients (p=0.017, 0.000, 0.014, 0.005, and 0.004), whereas the *NR1D1* expression did not significant associations with other features (p>0.05, Table 1).

Table 1. The expression of *NR1D1* with different clinicopathological features in gastric cancers.

Gastric cancer	*NR1D1* (204760_s_at)		p
	Low expression	High expression	
Gender			
Male	388 (71.3%)	156 (28.7%)	0.017
Female	148 (62.7%)	88 (37.3%)	
***HER2* status**			
Negative	365 (68.6%)	167 (31.6%)	0.232
Positive	222 (64.7%)	121 (35.3%)	
TNM stage			
I	49 (73.1%)	18 (26.9%)	0.038
II	97 (69.3%)	43 (30.7%)	
III	188 (61.6%)	117 (38.4%)	
IV	109 (73.6%)	39 (26.4%)	
T stage			
T2	172 (71.4%)	69 (28.6%)	0
T3	118 (57.8%)	86 (42.2%)	
T4	16 (42.1%)	22 (57.9%)	
N			
N0	55 (74.3%)	19 (25.7%)	0.014
N1-3	250 (59.2%)	172 (40.8%)	
M			
M0	294 (66.2%)	150 (33.8%)	0.581
M1	35 (62.5%)	21 (37.5%)	
Lauren classification			
Intestinal	237 (74.1%)	83 (25.9%)	0.005
Diffuse	148 (61.4%)	93 (38.6%)	
Mixed	20 (62.5%)	12 (37.5%)	
Differentiation			
Poorly	85 (51.5%)	80 (48.5%)	0.004
Moderately	47 (70.1%)	20 (29.9%)	
Well	12 (37.5%)	20 (62.5%)	
Poorly	85 (51.5%)	80 (48.5%)	0.202
Moderately-well	59 (59.6%)	20 (40.4%)	

The expression of *NR1D2* was significantly higher in male patients, *HER2* status, tumor stage, T, Lauren classification, and differentiation in gastric cancer patients (p=0.000, 0.000, 0.000, 0.001, 0.000 and 0.000). The other features didn't show significantly correlations with *NR1D2* (p>0.05, Table 2).

Table 2. The expression of *NR1D2* with different clinicopathological features in gastric cancers.

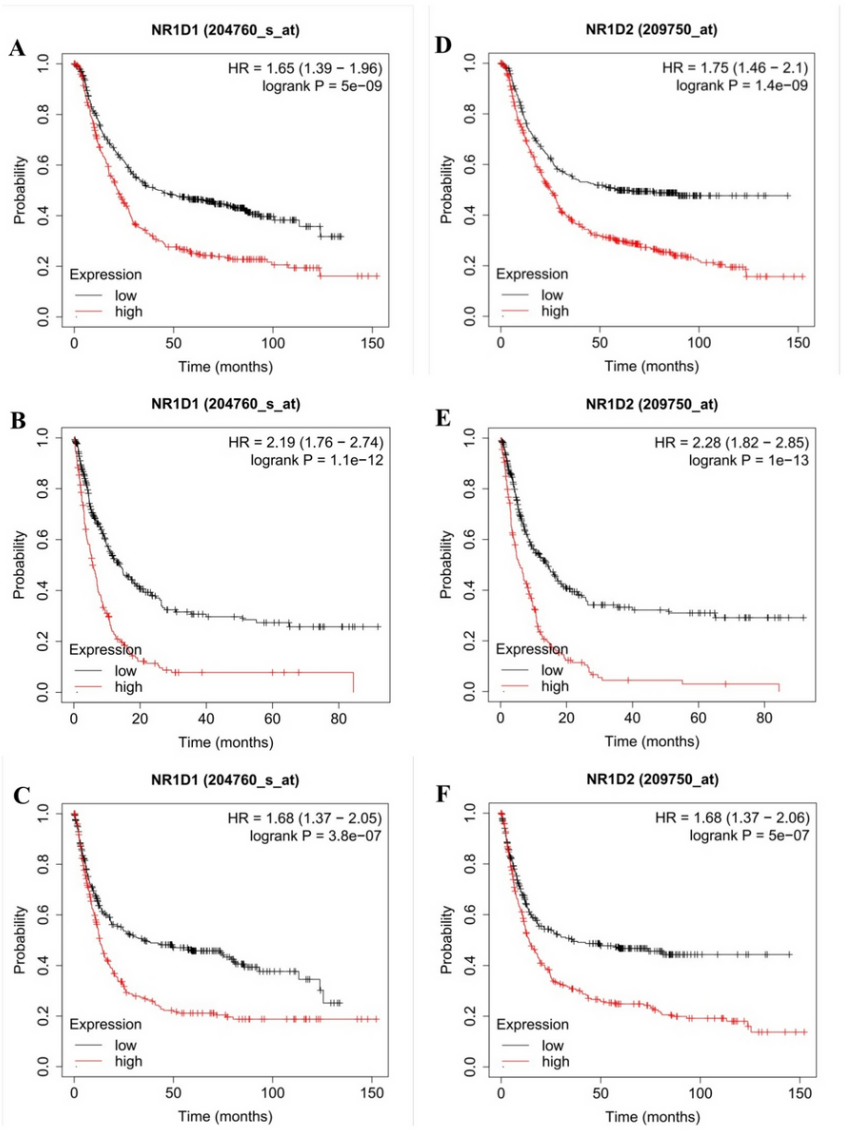
Gastric cancer	*NR1D2* (209750_at)		p
	Low expression	High expression	
Gender			
Male	152 (27.9%)	392 (72.1%)	0
Female	104 (44.1%)	132 (55.9%)	
***HER2* status**			
Negative	229 (43.0%)	303 (57.0%)	0
Positive	99 (28.9%)	244 (71.1%)	
TNM stage			
I	37 (55.2%)	30 (44.8%)	0
II	68 (48.6%)	72 (51.4%)	
III	100 (32.8%)	205 (67.2%)	
IV	70 (47.3%)	78 (52.7%)	
I-II	105 (50.7%)	102 (49.3%)	0.001
III-IV	170 (37.5%)	283 (62.5%)	
T stage			
T2	156 (64.7%)	85 (35.3%)	0.001
T3	116 (56.9%)	88 (43.1%)	
T4	13 (34.2%)	25 (65.8%)	
N			
N0	43 (58.1%)	31 (41.9%)	0.424
N1-3	224 (53.1%)	198 (46.9%)	
M			
M0	241 (54.3%)	203 (45.7%)	0.173
M1	25 (44.6%)	31 (55.4%)	
Lauren classification			
Intestinal	151 (47.2%)	169 (52.8%)	0
Diffuse	174 (72.2%)	67 (27.8%)	
Mixed	15 (46.9%)	17 (53.1%)	
Intestinal	151 (47.2%)	169 (52.8%)	0
Diffuse-mixed	189 (69.2%)	84 (30.8%)	
Differentiation			
Poorly	99 (60.0%)	66 (40.0%)	0
Moderately	16 (23.9%)	51 (76.1%)	
Well	18 (56.2%)	14 (43.8%)	
Poorly	99 (60.0%)	66 (40.0%)	0
Moderately-well	34 (34.3%)	65 (65.7%)	

The prognostic values of *NR1D1* and *NR1D2* expression in gastric cancer patients

We analyzed the survival data of gastric cancer patients with the expression of *NR1D1* and *NR1D2* by Kaplan-Meier at the website www.kmplot.com. The Affymetrix ID for *NR1D1* is: 204760_s_at, and the *NR1D2* ID is 209750_s_at. The survival curves of gastric cancer patients based on *NR1D1* mRNA expression level were plotted in Figure 1. The high expression of *NR1D1* was significantly correlated with worse OS in gastric cancer patients (p=5e-09). The PPS and FP were also with worse results for high expression of *NR1D2* (p=1.1e-12, 3.8e-07). The survival rate of gastric cancer patients with high expression level of *NR1D2* mRNA was significantly lower (p=1.4e-09). The same results were found in PPS and PF of gastric cancer patient analyses (p=1e-13, 5e-07) (Figure 2).

Figure 2. A-C). The Kaplan-Meier analysis of OS, PPS and FP according to *NR1D1* expression; D-F) The Kaplan-Meier analysis of OS, PPS and FP according to *NR1D2* expression.

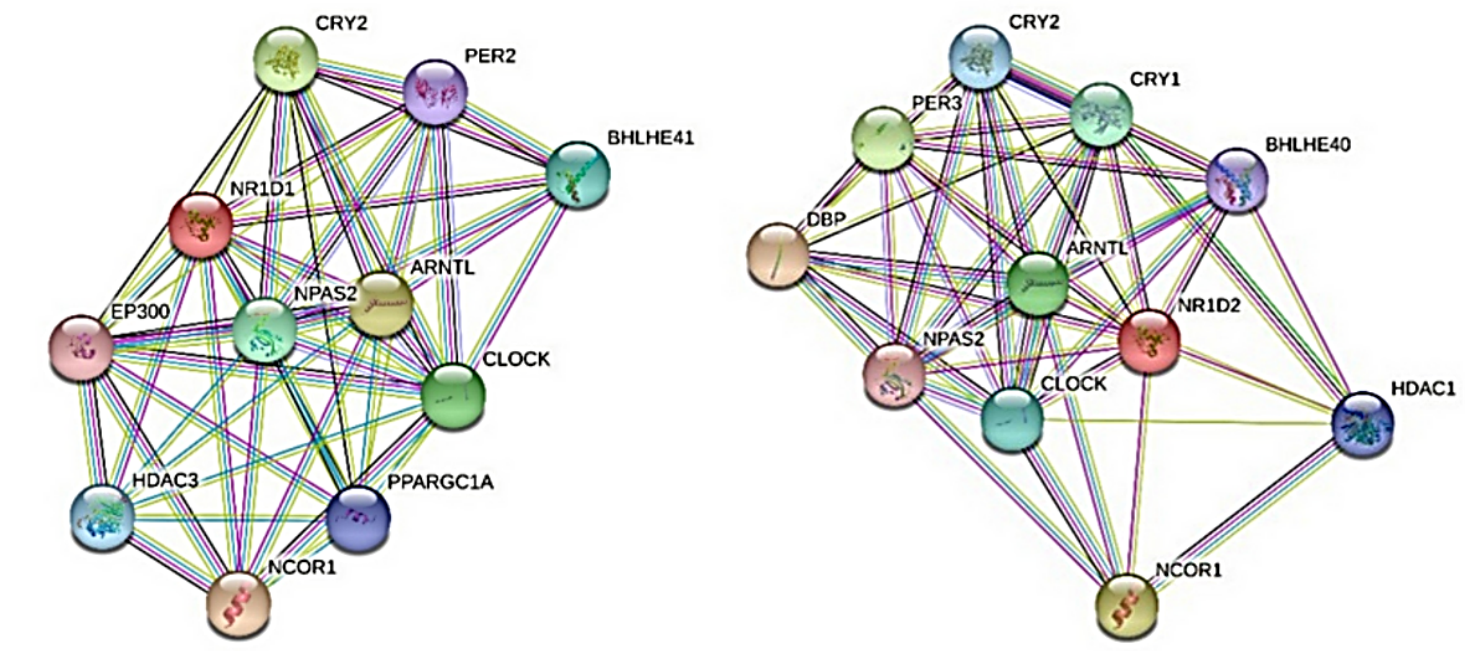
Note: — Low; — High.



The analysis of genes coexpressed with *NR1D1/NR1D2* in gastric cancer

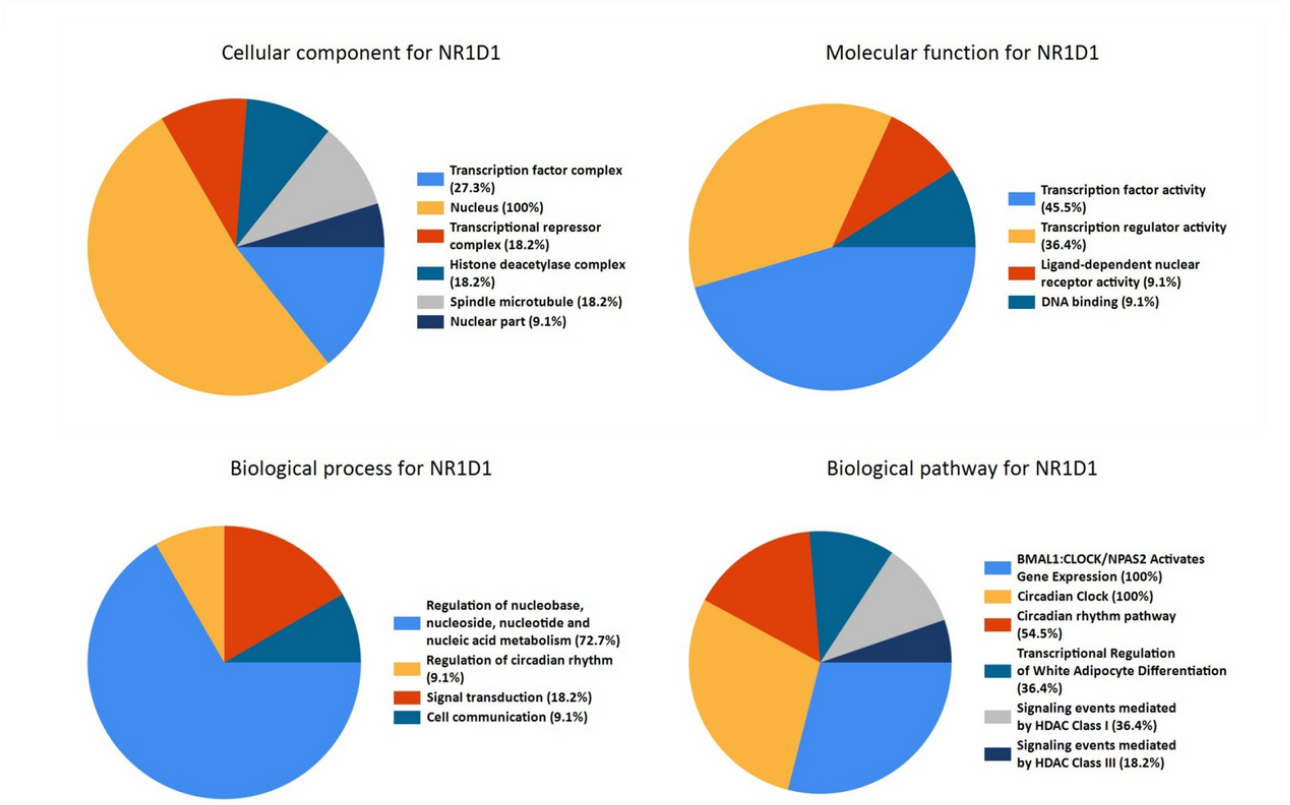
Then we analyzed the coexpressed genes with *NR1D1* to construct a Progein-Protein Interaction (PPI) network using the STRING database. The similar analyzed the coexpressed genes with *NR1D2* also using the STRING database (Figure 3).

Figure 3. The PPI network of the *NR1D1/NR1D2* interaction partners according to the STRING database.



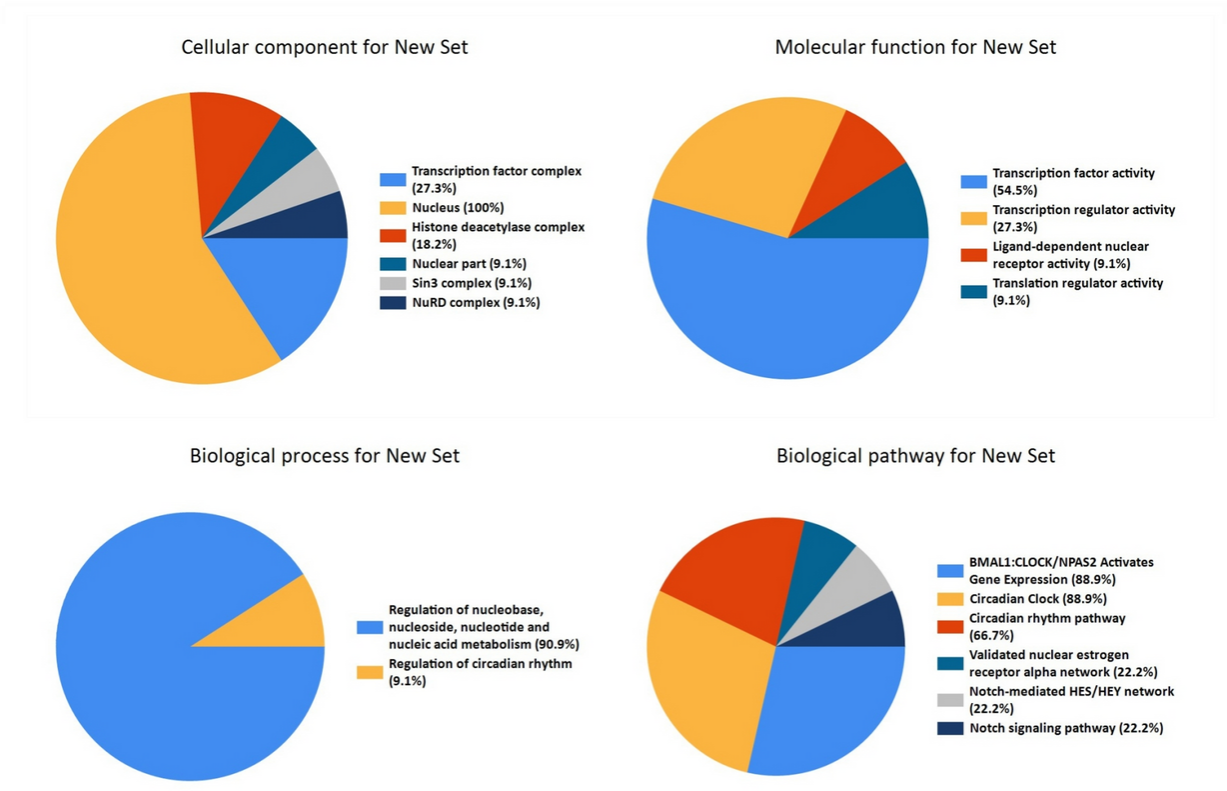
To further investigate the biological role of the coexpressed genes with *NR1D1*, we performed functional enrichment analysis using FunRich. Our results suggest that the top enriched biological processes were regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (72.7%), signal transduction (18.2%), regulation of circadian rhythm (9.1%), and cell communication (9.1%). We also found that the expression protein was mostly distributed in the nucleus (100%), followed by transcription factor complex (27.3%), transcriptional repressor complex (18.2%), histone deacetylase complex (18.2%) and spindle microtubule (18.2%). Transcription factor activity (45.5%), transcription regulator activity (36.4%), ligand-dependent nuclear receptor activity (9.1%), and DNA binding (9.1%) were the main functions among these coexpressed genes. The biological pathway analysis suggested that the genes that coexpressed with *NR1D1* were functionally enriched in several pathways, mostly those involving *BMAL1*: *CLOCK/NPAS2* activates gene expression (100%) and circadian clock (100%) (Figure 4).

Figure 4. FunRich software identified the biological roles of the coexpressed genes with *NR1D1* in gastric cancer.



For *NR1D2*, we also investigated the functional enrichment analysis for the biological role of the coexpressed genes with it by FunRich. The top enriched biological processes were regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (90.9%), and another process is the regulation of circadian rhythm (9.1%). The results showed that the expression protein was mostly distributed in the nucleus (100%), followed by transcription factor complex (27.3%), histone deacetylase complex (18.2%), nuclear part (9.1%), Sin3 complex (9.1%) and NuRD complex (9.1%). Transcription factor activity (54.5%), transcription regulator activity (27.3%), ligand-dependent nuclear receptor activity (9.1%) and translation regulator activity (9.1%) were the main functions among these coexpressed genes. In the biological pathway analysis, the genes that coexpressed with *NR1D2* were functionally enriched in several pathways, mostly those involving *BMAL1*: *CLOCK/NPAS2* activates gene expression (88.9%), circadian clock (88.9%) and cicadian rhythm pathway (66.7%) (Figure 5).

Figure 5. FunRich software identified the biological roles of the coexpressed genes with *NR1D2* in gastric cancer.



DISCUSSION

Gastrointestinal carcinoma was rapidly increasing in incidence recently, and the advanced carcinoma are limited with available and effective treatments. Gastric cancer is a highly heterogeneous tumor with high malignant potential. Radical surgery is the most important treatment for gastric cancer, but at present, the effect of simple surgery, traditional chemotherapy and radiotherapy is not ideal. Based on the continuous progress in the field of immunity in recent years, it plays an increasingly important role in the field of gastric cancer treatment. Refining and optimizing the beneficiaries of immunotherapy is still the key direction of precision treatment and individualized treatment of gastric cancer [26-29].

More and more studies have shown that circadian clock genes play an important role in the occurrence and development of a variety of tumors [30,31]. Overexpression of *PER2* can inhibit the progression of esophageal cancer by activating autophagy pathway, and *BMAL1* can promote the invasion and metastasis of breast cancer by up regulating the expression of *MMP9* (Matrix Metalloproteinase 9) [32,33]. The biological clock system is composed of a series of biological clock genes, including the above-mentioned biological clock core gene *BMAL1*, negative feedback gene *PER1-3*, as well as target genes and clock control genes *NR1D1, NR1D2* [34]. Previous studies have shown that the expression of *NR1D1* in tumor tissues is higher than that in normal tissues, while the expression in patients with metastasis is even higher [19,20]. However, there are only a few studies on the expression and prognosis of *NR1D1* in carcinomas, while even fewer studies on *NR1D2*.

To date, there are no reports on the function of *NR1D1 or NR1D2* in association with gastric cancer. In our study, we first demonstrate that *NR1D1 and NR1D2* are significantly upregulated in gastric cancer. Both the two genes were higher in gastric cancer with advanced stage. Furthermore, we used KM-plotter database to confirm the prognostic value of the genes, and the highly expressed *NR1D1 and NR1D2* were significantly associated with a worse prognosis. In order to construction of the *NR1D1/NR1D2* related genes expression network and the results of functional enrichment analysis facilitated the identification of possible and important biological processes. For biological pathway analysis, circadian clock pathway was found to be the most likely pathway associated with *NR1D1/NR1D2* in gastric cancer. The circadian clock genes have been confirmed to promote the occurrence and development of tumors by interfering with the cell cycle, damaging the DNA structure, participating in cell metabolism, stem cell maintenance, and promoting inflammatory reaction in many ways [35-37].

Malfunctions of the circadian clock may directly or indirectly trigger abnormal cellular processes, and eventually lead to the onset and progression of cancer. Several studies have proved the interaction between circadian regulation and cancer with implications on the treatment response [38,39]. The *NR1D1* gene locate in the *ERBB2* (Erythroblastic Leukemia Viral Oncogene Homolog 2) amplicon, which is a predictor of aggressive tumor phenotype, and its expression is correlated with poor clinical outcomes [40,41]. Previous research has suggested that high expression of *NR1D1* is not only closely related to tumor progression, but also plays an important role in inflammation. The high expression of *NR1D1* in arthritis can promote the release of inflammatory factors, especially the release of matrix-degrading enzymes, which directly aggravates the destruction of the matrix around the joint and aggravates the disease [42]. In immune regulation, elevated *NR1D1* expression can upregulate the secretion of CCL-2 (C-C Motif Chemokine Ligand 2), IL-6 (Interleukin 6) and IL-1b (Interleukin 1 Beta), regulating the development of inflammation [43,44].

In a chronic inflammatory response, inflammatory cells release a large number of inflammatory factors, thereby activating cell signal transduction pathways, changing the cell survival microenvironment, regulating cellular metabolic processes, and further promoting the growth, proliferation and metastasis of tumor cells [3]. The occurrence and development of gastric cancer is a gradual evolution process, which is the result of the joint action of many factors. Chronic inflammation, as a common predisposing factor of gastric cancer, plays an important role in the progression of gastric cancer [45-47]. *NLRP3* (NOD-like Receptor Family, Pyrin Domain containing 3) is an important downstream of the rhythmic core molecule *NR1D1*, a multifunctional protein complex composed of ASC (Apoptosis-associated Speck-like protein containing a CARD) and casepase-1, which plays a central role in the innate immune response activated by pathogen-associated pattern recognition molecules and is also mediated by macrophages. It is a key part of the innate immune and inflammatory regulators, and plays an important role in the occurrence and development of ulcerative colitis [48].

The previous researches have shown that macrophages are the most abundant immune cells in the tumor microenvironment and play a key role in the occurrence and development of tumors. In tumor tissues, macrophages are the most typical tumor-infiltrating immune cells, and play a significant role in promoting the early tumor-to-tumor metastasis [49,50]. Another study pointed that *NR1D1* was playing as an enhancer of cancer invasiveness, and acting as a role of circadian disruption in proliferation, apoptosis, and migration in CRC cells both *in vitro* and *in vivo* [51].

On the contrary, high *NR1D1* expression is associated with better clinical outcomes in breast cancer patients, and the circadian clock system is closely linked to the sensitivity of chemotherapy. It suggested that *NR1D1* could be a therapeutic target for breast cancer treatment, especially in those patients treated with ROS-inducing chemotherapeutic agents [52]. Our study indicates that the suppression role of *NR1D1 and NR1D2 via* circadian clock pathway is warranted to exploration regarding gastric cancer. However, the mechanism of action of *NR1D1/NR1D2* in the occurrence and development of gastric cancer is not clear. With the continuous updating the database of gastric cancer, we believe that there will be a large sample size to confirm our observations in the future.

CONCLUSION

In conclusion, by analyzing the KM plotter databases, this is the first report that *NR1D1/NR1D2* is upregulated in gastric cancer, which is correlated with advantage stage and poor outcome through the circadian clock pathway. Our findings suggest that *NR1D1/NR1D2* serve as a promising prognostic factor for gastric cancer and promote the tumorigenesis, and provide new potential targets for targeted drug development.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the ethics committee of the Beijing Jishuitan Hospital, and informed consent was obtained from all individuals. All the methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

All authors agreed to publish this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets generated and/or analyzed during the current study are available in the web-based database of ULCAN, KM plotter, and STRING.

COMPETING INTEREST

The authors declare that there is no conflict of interest.

FUNDING STATEMENT

None.

AUTHORS CONTRIBUTIONS

ZhiXue Zheng conceived and designed the experiments, analyzed and interpreted the data, and wrote the paper, Xuan Cai and Yaqi Liu, Jingtao Bi revised the manuscript. All authors reviewed the manuscript.

DATA ACCESS STATEMENT

Data supporting this study are openly available from ULCAN and KM plotter database.

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