RESEARCH ARTICLE

Altered expression of circadian clock genes in head and neck squamous cell carcinoma

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Abstract Head and neck squamous cell carcinoma (HNSCC) means a group of cancers developed from the upper aerodigestive tract, and 90% of them are squamous cell carcinomas. HNSCC is the tenth most commonly diagnosed form of cancer in males worldwide, but it is the seventh most common cause of cancer-related death. The circadian clock regulates daily rhythmic variations in various physiologic processes including sleep and activity, appetite, hormone levels, metabolism, and gene expression. Many recent studies have demonstrated that the disruption of circadian rhythm is associated with cancer development and tumor progression, such as chronic myeloid leukemia, hepatocellular carcinoma, endometrial carcinoma, and breast cancer. However the direct links between aberrant circadian clock gene expression and human malignancies, including HNSCC, remain largely unknown. In this study, the expression profiles of nine circadian clock genes of cancer tissue and noncancerous part from 40 patients of HNSCC were investigated. The expression of *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CKIε*, and *BMAL1* showed significant downregulation in the cancer tissues ($p<0.005$). Downregulated *PER3*, *CRY2*, and *BMAL1* expression was correlated with more advanced cancer stages ($p<0.05$). Downregulated *PER3* and upregulated *TIM* expression correlated with larger tumor size ($p<0.05$), and lower expression of *PER3* correlated with deeper tumor invasion ($p<0.05$). Poor survival was related to lower expression of *PER1* ($p<0.05$) and *PER3* ($p<0.01$). These results indicate a possible association of circadian clock gene, especially *PER3*, expression with the pathogenesis of HNSCC.

Keywords Head and neck squamous cell carcinoma · Circadian clock genes · Circadian rhythm · Real-time quantitative RT-PCR

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Introduction

Head and neck squamous cell carcinoma (HNSCC) includes the malignancy of the oral cavity, pharynx (naso-, oro-, and hypopharynx), larynx, and paranasal sinuses. HNSCC is the tenth most commonly diagnosed form of cancer in males

worldwide, but it is the seventh most common cause of cancer-related death [1]. In Taiwan, HNSCC represented the fourth leading cause of cancer death in men and the sixth most prevalent cancer types in both sexes in 2006 [2]. HNSCC is highly curable if detected early, usually with some form of surgery although chemotherapy and radiation therapy may also play an important role. Even though a lot of research and the advances were made in the field of oncology and surgery, the mortality rates remain unchanged [3]. Epidemiological studies recognized the major risk factors for development of HNSCC include tobacco, betel nuts, viral infection, and genetic polymorphism or damage [4, 5]. Alteration of genes has also been implicated in HNSCC, such as *LKB1*, *hMLH1*, *TP53* [6].

Circadian rhythm is present in humans and almost all eukaryotes with a 24-h cycle. Various physiological processes, such as sleep and activity, appetite, hormone levels, metabolism, and gene expression, display daily rhythmic changes [7]. In mammals, the central pacemaker is located at the suprachiasmatic nuclei (SCN) of the anterior hypothalamus and the SCN clock, and the daily light–dark cycle is entrained to 24-h day through the retina-to-SCN neural pathways [8, 9]. Peripheral oscillators in body cells similar to those operating in the SCN have also been found [10].

The regulations of central and peripheral circadian oscillators use transcriptional–translational feedback loops which consist of at least nine core circadian clock genes including *PERIOD1* (*PER1*), *PERIOD2* (*PER2*), *PERIOD3* (*PER3*), *CLOCK*, *CRYPTOCHROME1* (*CRY1*), *CRYPTOCHROME2* (*CRY2*), *BMAL1*, *CASEIN KINASE 1ε* (*CK1ε*), and *TIM-LESS* (*TIM*) [7, 8, 11, 12]. The loops rely on positively regulating genes (such as *CLOCK* and *BMAL1*) and negatively regulating genes (such as *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *TIM*) in the oscillators. During daytime, *CLOCK* and *BMAL* form heterodimers and bind to E-boxes and drive the transcription of the three *PER* genes and the two *CRY* genes. In the cytoplasm, PER and CRY proteins are translated and form PER-CRY complexes and subsequently translocate into the nucleus to suppress the BMAL1- and CLOCK-mediated transcription. Therefore, the marked characteristic of circadian systems is the prominent daily cycling of circadian clock gene mRNA expression, and downstream circadian clock-controlled gene mRNA and protein expression [10]. It has been noticed that disruption of circadian rhythms is associated with cancer development and tumor progression [9, 13, 14]. Epidemiologic studies on night-shift workers have revealed that circadian disruption is a critical risk factor for the tumorigenesis of breast cancer [15, 16], colorectal cancer [17], and prostate cancer [18]. Many recent studies also demonstrated that expression of circadian clock genes was disturbed in various types of cancers [19]. Therefore, in this study, we have investigated the expression of circadian clock genes in human HNSCC tissue and to elucidate the association between circadian clock gene expression and cancer behavior. Real-time quantitative RT-PCR was used to analyze the expression of the nine circadian clock genes in the cancer and noncancerous tissue from 40 HNSCC patients.

Materials and methods

Patients and samples

Tumor samples and the adjacent noncancerous tissues were obtained from 40 patients (39 men and 1 woman), aged 30–82 years (mean ± SD, 53.31±10.83), diagnosed with HNSCC undergoing surgery at the Department of Otolaryngology, Kaohsiung Chang Gung Memorial Hospital from 2009 throughout 2011. Clinical pathologic characteristics, including patient's age, sex, TNM staging, tumor size, depth of invasion, and survival, are listed in Table 1. The HNSCC tissue specimens were obtained at the following time points: 30 cases were obtained between 1400 and 1600 hours, and 10 cases were obtained between 1000 and 1200 hours. Immediately after resection, the specimens were obtained and snap-frozen in liquid nitrogen and stored until use. Informed consent was obtained from all patients prior to tissue acquisition. This study was approved by the Institutional Review Board of the Kaohsiung Chang Gung Memorial Hospital.

Real-time quantitative RT-PCR analysis of circadian clock genes

Total RNA was extracted from cancer tissue and noncancerous tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The 2-μg RNA input for cDNA synthesis was determined by spectrophotometric OD_{260} measurement, and cDNA was generated with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacture's protocols. The cDNA sequence of the nine circadian clock genes was evaluated, and the specific forward and reverse primers and MGB TaqMan® probe were designed using Primer Express software version 1.5 (Applied Biosystems, Foster City, CA, USA). TaqMan® MGB probes were synthesized and labeled with FAM fluorescent dyes (Applied Biosystems). Sequences of the forward and reverse primers and TaqMan® MGB probes are listed in Table S1. Expression of human housekeeping genes, *ACTB* (β-actin), was used for normalizing circadian clock gene expression in real-time quantitative RT-PCR. All reactions were carried out in a 20-μL final volume containing 50 ng cDNA (as total input RNA), 400 nM each primer, 200 nM probe, and 10 μL 2× TaqMan® Universal PCR Master Mix (Applied Biosystems). Real-time quantitative PCR was performed in an ABI 7500

Table 1 Characteristics of HNSCC patients

Sex	Number	Median age, years (range)	Staging	Number	Tumor size (cm)	Number	Tumor invasion (cm)	Number	Survival	Number
Male	39	53.31±10.83	I	5	<3	17	<1	15	Expired	6
Female	1		II	6	>3	23	>1	25	Survived	34
			III	10						
			IV	9						

Fast Real-Time System (Applied Biosystems), and the PCR cycling parameters were set as follows: 95°C for 10 min followed by 40 cycles of PCR reactions at 95°C for 20 s and 60°C for 1°min. The expression levels of the circadian clock genes were normalized to the internal control *ACTB* to obtain the relative threshold cycle (ΔC_T), and the relative expression between cancer tissue and noncancerous part was calculated by the comparative C_T ($\Delta\Delta C_T$) method.

Statistical analysis

A paired *t* test was used to detect the differences between two groups in each circadian clock gene expression, and the values of ΔC_T were used for all the statistical analyses. The test was two sided with statistical significance set at 0.05, and all computations were made using SPSS for Windows Release 13.0 software (SPSS, Chicago, IL, USA).

Results

Analysis of circadian clock gene expression in HNSCC with real-time quantitative RT-PCR

Cancer parts and noncancerous parts of tissues from 40 HNSCC patients were examined for the expression of the nine circadian clock genes using real-time quantitative RT-PCR to elucidate if the expression levels of circadian clock genes were altered in cancer tissues. Our data demonstrated that the expression levels of *PER1*, *PER2*, *PER3*, *CRY2*, and *BMAL* were significantly downregulated in HNSCC ($p<0.01$) (Table 2 and Fig. 1a). Among the seven down-regulated genes, *CRY2* was the most downregulated gene; a ninefold decrease than in noncancerous tissue was detected (Fig. 1b).

Disease severity and circadian clock gene expression in HNSCC patients

Disease severity depends on cancer staging. The TNM staging system was established by the American Joint Committee on Cancer which includes tumor (T), neck lymph node (N), and metastasis (M). The stages from I to IV represent the general cancer status from mild to severe

and is more close to prognosis and therapy response. We divided the patients into stage I and II (stage I/II) and stage III and IV (stages III/IV) groups for correlation analysis with circadian clock gene expression. We found down-regulated *PER3*, *CRY2*, and *BMAL1* expression was correlated with more advanced cancer stages ($p<0.05$) (Fig. 1b).

Tumor conditions and circadian clock gene expression in HNSCC patients

To investigate whether the circadian clock gene expression shows a tumor-dependent variation in HNSCC, we ana-lyzed the pathologic report of the 40 HNSCC patients and correlated with their circadian clock gene expression levels. Patients with tumor size <3 or >3 cm and tumor invasion depth <1 or >1 cm were divided into two different groups for analysis, respectively. The paired *t* test indicated that among the nine circadian clock genes analyzed, transcripts of *PER3* and *TIM* displayed a tumor size-dependent variation pattern, and *PER3* displayed an invasion depth-

Table 2 The expression levels of the nine circadian genes in paired cancer and noncancerous parts of 40 HNSCC patients were determined by real-time quantitative RT-PCR

Gene	$\Delta\Delta C_T$ [ΔC_T (cancer part–*ACTB*) − ΔC_T (noncancerous part–*ACTB*)]	Expression decreased in cancerous part (folds)	*p* value (*t* test)
PER1	2.65±0.41	6.28	0.0000
PER2	2.45±0.35	5.49	0.0000
PER3	2.33±0.58	5.02	0.0000
CRY1	1.79±0.40	3.39	0.0049
CRY2	3.19±0.49	9.13	0.0000
CK1ε	0.69±0.25	1.61	0.0078
CLOCK	0.71±0.41	1.64	0.1998
BMAL1	2.34±0.41	5.06	0.0000
TIM	0.16±0.26	1.12	0.6059

The amount of circadian gene was normalized to the endogenous reference *ACTB* to obtain the ΔC_T value for each sample. The normalized circadian gene expression (ΔC_T) of the cancer part was first related to the ΔC_T of the normal part to obtain the relative threshold cycle ($\Delta\Delta C_T$), and then the relative expression levels ($2^{-\Delta\Delta C_T}$) were calculated. Results are mean ± SE
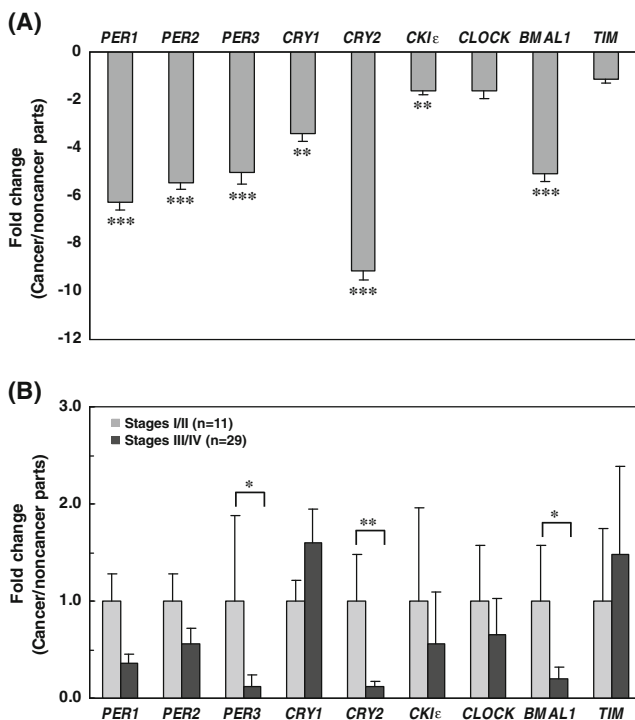
Fig. 1 Expression of circadian clock genes in HNSCC determined by real-time quantitative RT-PCR. **a** Expression of the nine circadian clock genes in paired cancerous and noncancerous tissues from 40 HNSCC patients. The *y*-axis represents the relative mRNA expression level. The value of mRNA expression in noncancerous part is designated 1, and the level of mRNA expression in cancerous tissues is calibrated to obtain the folds changed in cancerous tissues. Statistical significance: ***$p<0.001$ and **$p<0.01$ **b** Disease severity and circadian clock gene expression in HNSCC patients. The 40 HNSCC patients were divided into two groups by their disease stages, stages I/II and stages III/IV, for correlation analysis with circadian clock gene expression. The *y*-axis represents the relative mRNA expression level. The relative expression in cancerous tissues is calculated by $\Delta\Delta C_T$. The expression in stages I/II is designated 1, and the relative expression in stages III/IV is calibrated to obtain the folds changed. Statistical significance: *$p<0.05$ evaluated with *t* test

dependent variation pattern (Fig. 2a and b). Decreased *PER3* and increased *TIM* expression correlated with larger tumor size ($p<0.05$) (Fig. 2a), and decreased *PER3* expression correlated with deeper tumor invasion ($p<0.05$) (Fig. 2b).

Survival and circadian clock gene expression in HNSCC patients

The patients' survival status was followed up for 2 years after surgery. Among the 40 HNSCC patients, 6 patients died from the disease, and 34 patients survived. We also analyzed the correlation between patients' survival status and circadian clock gene expression. Downregulated *PER1* ($p<0.05$) and *PER3* ($p<0.01$) were correlated with poor survivals (Fig. 2c).

Age and circadian clock gene expression in HNSCC patients

To rule out the possibility that the altered circadian clock genes expression was due to age differences, we divided the patients into young (30–45 years old), middle age (45–60 years old), and old age (60–80 years old) groups for correlation with analysis with circadian clock gene expression. We found the expression of *CKIε* was significantly downregulated in middle-aged patients than in young-aged patients ($p<0.05$) (Fig. 2d). In addition, the expression of *TIM* was also significantly impaired in middle-aged patients than in young-aged ($p<0.05$) and old-aged patients ($p<0.05$) (Fig. 2d). Nevertheless, expression of genes that correlated with disease severity (*PER3*, *CRY1*, and *BMAL1*), tumor invasion (*PER3*), and survival (*PER1* and *PER3*) was not different among different age groups of patients.

Discussion

Circadian rhythms regulate various functions of the human body [14, 20]. Since many genes involved in proliferation are under the control of circadian clock, maintaining the circadian clock rhythms can be a critical control point for cancer development and tumor progression [21]. The aberrant expression of circadian clock genes has recently been associated with many different types of human cancers [17–20, 22, 23]. In our previous studies, we have demonstrated deregulated expression of different circadian clock genes in different cancers, including CML [22], breast cancer [23, 24], endometrial cancer [25], and hepatocellular carcinoma [19]. We have also demonstrated an abolished daily expression oscillation of circadian clock genes in CML [22]. However, there is no report that links the aberrant expression of circadian clock genes to the HNSCC. Therefore, in this study we aimed to make this link and hope to further understand the underlying molecular mechanism of HNSCC. In the present study, we analyzed and compared the expression levels of the nine circadian clock genes in 40-paired cancerous and noncancerous tissues of HNSCCs. We found that seven genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CKIε*, and *BMAL1*) were significantly downregulated in HNSCC cells compared to their noncancerous counterpart cells (Table 1). Among these seven downregulated genes, *CRY2* was the most downregulated gene in cancer tissue, which was decreased about ninefold than in non-cancerous tissue. A recent study indicated that *CRY* mutation activates p53-independent apoptosis pathways (NF-κB signaling pathway), which eliminate premalignant and malignant cells [26]. Therefore, circadian clock genes, like *CRY*, were
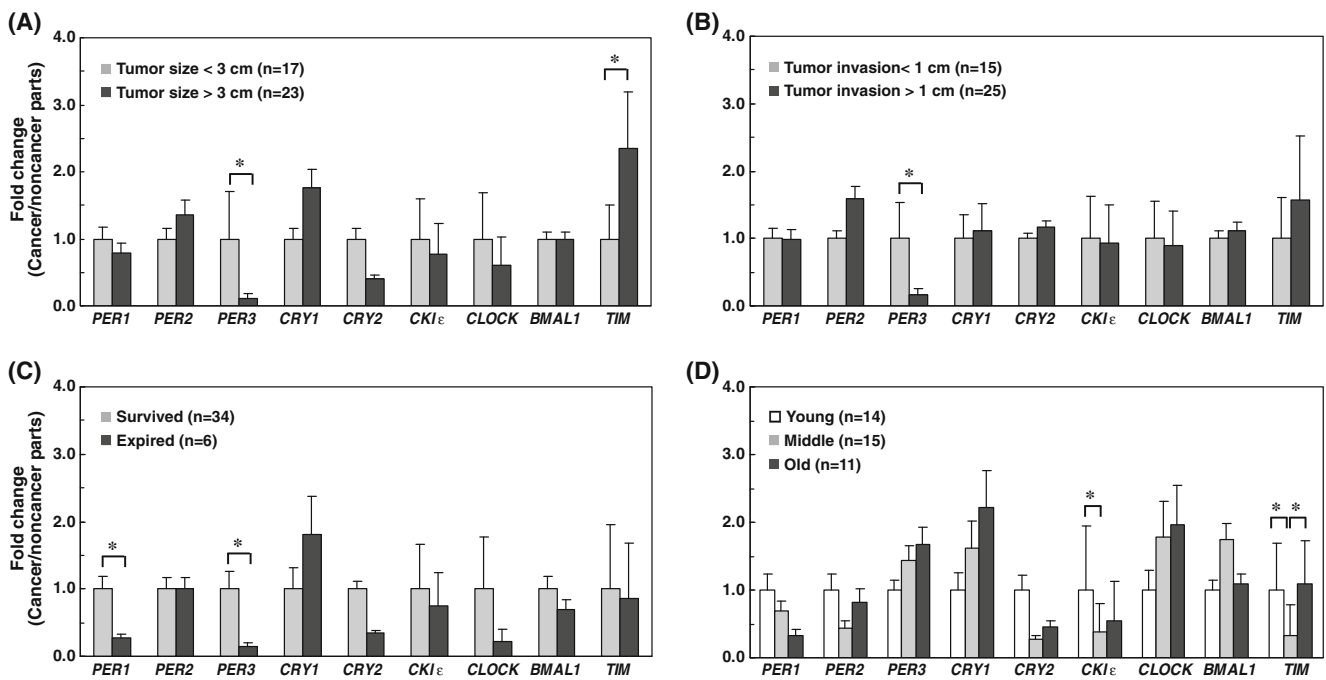
Fig. 2 Tumor conditions and circadian clock gene expression in HNSCC patients. Tumor size (a), tumor invasion (b), survival (c), and age (d) of the 40 HNSCC patients were correlated to the expression of the nine circadian clock genes. The *y*-axis represents the relative mRNA expression level. The relative expression in cancerous tissues is calculated by $\Delta\Delta C_T$. The expression in tumor size <3 cm (a), tumor invasion <1 cm (b), and survival (c) is designated 1, and the relative expression in tumor size >3 cm (a), tumor invasion >1 cm (b), and expired (c) is calibrated to obtain the folds changed, respectively. Statistical significance: *$p<0.05$ evaluated with *t* test

suggested to play a role as tumor suppressor genes that enhance apoptosis. In HNSCC, the downregulated and disrupted circadian clock genes possibly lost their function of eliminating premalignant and malignant cells, hence leading to the accumulation of malignant cells and evolution of malignancies. In a recent study, loss of *BMAL1* has been shown to reduce the expression of *PER1*, *PER2*, *PER3*, and *p53* [27]. The downregulated circadian clock gene *BMAL1* also accelerates the development of tumors and may influence the response to anti-cancer drugs [27]. Therefore, it is reasonable to hypothesize that circadian clock genes can regulate or suppress other circadian gene in the certain mechanism and this may also explain why most circadian genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, and *BMAL1*) are downregulated at the same time in HNSCC.

Results from our correlation analysis of circadian clock gene expression with patients' disease severity, tumor size, tumor invasion, survival, and age revealed that different altered genes correlated with different clinical parameters. Interestingly, only *PER3* was correlated with all the parameters analyzed, which is also in consistent with our previous finding in CML [22] and hepatocellular carcinoma [19]. What is the role of *PER3* in human cancers? The *PER1*, *PER2*, and *PER3* genes belong to the same Period gene family. *PER1* and *PER2* are important in regulating the circadian clock, but the exact role of *PER3* has not been

well described. In a recent study by Sato et al., *PER1* was found to be more strongly expressed in human gingival cancer cells than in adjacent non-tumor tissues, and the expression of *PER3* was less in tumor cells than in adjacent non-tumor cells [28]. *PER1* and *PER3* were shown to have anti-apoptotic and pro-apoptotic effects in human gingival cancer CA9-22 cells, respectively. Therefore, it was suggested that the balance of *PER1* and *PER3* may control apoptotic reactions in gingival cancer cells [28]. However, our results showed that *PER1* and *PER3* are both downregulated in cancer ($p<0.00001$). Although an insignificant role was displayed in studies of *Per3*-knockout mice [29], it seems that altered *PER3* has a critical role in human cancers. Since the role of *PER3* in circadian clock is insignificant, it may suggest that *PER3* affects tumorigenesis in a circadian-independent way. Structural variation of *PER3* exon 18 has been shown to be associated with increased risk of breast cancer among premenopausal women [30], suggesting genetic alteration may be a possible reason for the altered *PER3* function. However, hypermethylation of gene promoter leading to abnormal silencing of *PER3* transcription has also been demonstrated in CML [22], hepatocellular carcinoma [19], and breast cancers [23]. Further studies on genetic and epigenetic analysis of *PER3* will elucidate the reasons for the downregulated *PER3* expression in HNSCC.

Although it is still controversial whether the circadian rhythm disturbance is a carcinogenic effect itself, an aberrant circadian rhythm between normal and malignant tissues is commonly observed [31, 32]. Cell proliferation without circadian rhythmicity is commonly seen in patients with advanced cancers, and the circadian rhythm disturbance may be both a cause and an effect of the disease [33]. It is possible that alteration of the circadian clock gene expression rhythms breaks the balance that promotes cell division, resulting in proliferation of tumor cells as previous studies have shown that the circadian clock regulates several critical cell cycle and check-point-related protein and controls cell proliferation and physiological conditions like DNA repair and oxidative stress response [34]. Therefore, it is reasonable to suggest that difference in the circadian rhythm in cancerous cells of HNSCC may result in disrupting regulation of the central pacemaker and cell cycle-related genes leading to a favorable status for proliferation of the cancerous cells of HNSCC. On the other hand, tumor cells might accelerate their own growth by disrupting circadian rhythms, and therefore recovery of circadian rhythms in cancer patients may be helpful in improving their prognoses. A recent study of mouse embryonic stem cells has shown that the mammalian circadian molecular oscillator develops during cellular differentiation, and this process can be reversed by expression of the reprogramming genes (*Sox2*, *Klfr*, *Oct3/4*, and *c-Myc*) [35]. The authors suggested that there are some functional cross-talk between cellular differentiation and circadian clock formation in mammals. Since reprogramming of somatic cells can result in cells with properties of cancer stem cells [36], the alteration of cell state may also contribute to the altered circadian clock genes expression in HNSCC. The controversy of the cause or effect between circadian rhythm disturbance and tumorigenesis in HNSCC remains elusive, but our study provides clues for the existence of a link between the development of HNSCC and disturbance of circadian clock genes expression.

Conflicts of interest   None.

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