Int J Clin Exp Pathol 2020;13(9):2297-2304
www.ijcep.com /ISSN:1936-2625/IJCEP0116077

Original Article

The expression of the circadian gene TIMELESS in non-small-cell lung cancer and its clinical significance

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Received June 11, 2020; Accepted July 20, 2020; Epub September 1, 2020; Published September 15, 2020

Abstract: We aimed to explore the relationship between the circadian gene TIMELESS (TIM) in non-small-cell lung cancer (NSCLC) and prognosis, so as to provide a reference for subsequent targeted therapy and patient prognosis. A total of 72 patients with NSCLC were selected as the study cohort. Immunohistochemistry and Western blot were used to measure the expressions of TIM in NSCLC and para-carcinoma tissues (PCT). RT-qPCR was used to measure the mRNA expression of TIM. The Kaplan-Meier method was further utilized to generate survival curves for a log-rank test single-factor analysis and a Cox regression multi-factor analysis. The relationship between the TIM gene expression and the clinical features and survival were explored. The results indicated that the mRNA and protein expressions of TIM in the NSCLC tissues were higher than those in the PCT ($P < 0.01$). An immunohistochemistry assay showed that the expression rate of TIM in the NSCLC tissues of the 72 cases was 84.72%. The TIM expression was significantly different in terms of the NSCLC differentiation level, the tumor size, the lymph node metastasis, the TNM stage, and the survival rate ($P < 0.05$, $P < 0.01$). A Cox regression analysis indicated that the degree of TIM expression can be used as an independent observation index that indicates the prognosis of NSCLC. The TIM expression was significantly increased in the NSCLC tissues, and the higher the degree of positive expression, the worse the clinical prognosis. The expression of TIM is of certain auxiliary value in determining the degree of NSCLC malignancy, the lymph node metastasis, and the evaluation of the prognosis.

Keywords: Non-small-cell lung cancer, timeless, immunohistochemistry, Western blot, RT-qPCR, prognosis, survival rate

Introduction

Lung cancer is the malignant tumor with the highest morbidity and mortality in China [1]. About 80% of the pathological subtypes of lung cancer are non-small-cell lung cancer (NSCLC). Patients with NSCLC have few obvious symptoms in the early stages, and most are diagnosed at an advanced stage, so it is difficult to treat and has a high mortality rate. Circadian rhythm and its associated clock genes had been recognized as an important physiological endogenous adaptation mechanism. They regulate the metabolic processes of the cells in the body alternately day and night, and they are closely related to the occurrence and development of tumors [2-5]. TIMELESS (TIM) is one of the core circadian rhythm genes, and its effect on the proliferation, apoptosis, and DNA damage of tumor cells is a hot spot of current research [2, 6]. According to recent studies, TIM is abnormally expressed in various tumor diseases such as liver cancer, colorectal cancer, breast cancer, and lung cancer [6, 7], but so far there are still few studies that examine TIM expression and the clinical prognosis of NSCLC. This study attempts to further analyze the correlation between TIM and the clinical pathological features and prognosis of NSCLC by measuring the expression of TIM in NSCLC, in order to lay the groundwork for the subsequent exploration of the role of TIM in the occurrence and development of NSCLC, the determination of the clinical diagnosis and the evaluation of the prognosis.

Materials and methods

Case information

With the consent of the patients and their families, and with a review and the approval of the

ethics committee of The First Affiliated Hospital of The Yangtze University Health Science Center, the carcinoma tissues and para-carcinoma tissues (PCT) (> 5 cm from the tumor edge) of 72 patients who underwent surgical treatment for NSCLC in The First Affiliated Hospital of The Yangtze University Health Science Center between January, 2015 and December, 2017 were collected. All cases were included or excluded according to the following criteria: A. Inclusion criteria: a. Patients who had not received radiotherapy, chemotherapy, targeted therapy, or any other anti-tumor treatments before the surgery; b. Patients whose clinical information was complete and who voluntarily received follow-up for three months or more; c. Those who were diagnosed with NSCLC by 2 pathologists after the surgery. B. Exclusion criteria: a. Lung cancer patients at TNM stage IV; b. Patients with severe cardiovascular disease, cerebrovascular disease, pulmonary dysfunction, coagulopathy, kidney disease, or with other complex lesions; c. Patients who currently have or previously had other tumor diseases; d. Patients whose clinical information was incomplete, or who refused to receive follow-up. Among the included patients, 46 were male and 26 were female, and they ranged in age from 33 to 68 years old, with an average age of (52.69±7.92) years. There were 45 cases without a history of smoking and 27 cases with a history of smoking. The tumor sizes of 20 of the patients were less than or equal to 3 cm, and the tumor sizes of the remaining 52 cases were greater than 3 cm. In terms of histological type, the included patients had adenocarcinoma (60 cases), squamous cell carcinoma (10 cases), and adenosquamous carcinoma (2 cases). According to the 2011 WHO lung tumor classification standard pathological grade, there were 54 well and moderately differentiated cases and 18 poorly differentiated cases. Regarding the clinical TNM stages, 50 cases were at stages I-II, and 22 were at stage III. There were 48 cases with lymph node metastasis and 24 cases without. All the experimental samples were divided into 2 parts immediately after surgery. One part was fixed in 4% neutral-buffered formaldehyde to make a paraffin block, and the other part was immediately soaked in liquid nitrogen for preservation. Long-term outpatient or telephone follow-up was conducted for all the patients until the patient died of NSCLC recurrence or metastasis or until Dec 31, 2019. As of this writing, no follow-up was lost.

Immunohistochemical detection of the TIM expressions in the NSCLC tissues

Before the staining, the NSCLC and para-carcinoma tissues were fixed in 4% neutral-buffered formaldehyde at 4°C for 24 hours, and then dehydrated with gradient alcohol, made transparent with xylene and embedded in paraffin. Then the sections with a thickness of 4 μm were made. The sections were baked at 60°C for 4 hours after the anti-slice escaping treatment with polylysine, and then deparaffinized using the normal method. The antigen retrieval was performed with a microwave in a pH 6.0 citrate buffer, the immunohistochemistry adopted the EnVision two-step method (Gene Tech Shanghai Company Limited, China), and the TIM dilution ratio was 1:1000 (anti-rabbit TIM polyclonal antibody, Abcam, USA). For the specific steps, refer to the kit instructions. It was developed using DAB for 5-8 minutes (Maxim Technologies Co. Ltd., Fuzhou, China), counterstained, and mounted, and then observed under a microscope. The TIM protein expression was localized in the nuclei, with a brown or tan diffuse distribution. Five fields (10 × 20) were randomly selected in each slice, and 100 cells were counted respectively. The experimental results were determined according to the proportion of positive tumor cells and the intensity of the staining. The specific criteria were as follows: as regards the percentage of positive cells, no stained cells scored 0 points, 1 point for ≤ 10%, 2 points for 11%-50%, 3 points for 51%-75%, and 4 points for > 75%; as regards the staining intensity, unstained cells scored 0 points, 1 point for light yellow, 2 points for brown, and 3 points for tan. The two categories of scores were added to obtain a comprehensive score, where 0 points was defined as negative, 1-4 points as weak positive (+), 5-8 points as medium positive (++), and 9-12 points as strong positive (+++) **Figure 1A-C**.

Western blot determination of the TIM protein expression

Total proteins were extracted through the NSCLC and para-carcinoma tissues protein cleavage. The protein concentration was measured using the BCA method (Pierce® BCA Protein Assay Kit, Thermo, USA). SDS-PAGE gel electrophoresis was then performed. The proteins were transferred to nitrocellulose membranes, rinsed using PBST, and then blocked at

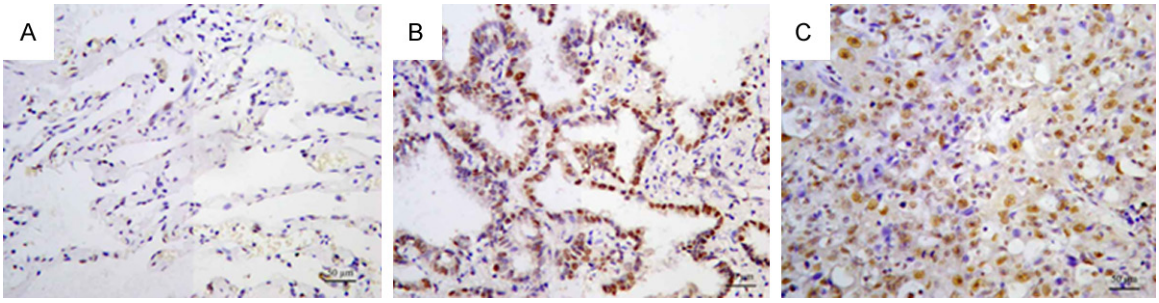

Figure 1. TIM expressions in human NSCLC tissues (EnVision × 200). The immunohistochemical detections showed that the TIM expressions were located in the nuclei with a brown or tan diffuse distribution. A. TIM showed sporadic epithelial cell expressions in the para-carcinoma tissues (0-+). B. The TIM expressions in the highly-moderately differentiated adenocarcinoma (++). C. TIM was diffusely strongly positive in poorly differentiated adenocarcinoma (+++).

room temperature for 2 hours. The NC membrane was cut, and the NC membrane with proteins was mixed with I antibody (TIM 1:100, Abcam, USA, β-actin 1:100, Sigma, USA), then incubated overnight in a shaker at 4°C. Then, we continued to incubated it at room temperature for 2 hours after adding II antibody (1:10,000; Sigma-Aldrich, USA). An efficient chemiluminescence kit Enhanced (Thermo, USA) was used for the protein signal detection and Image-pro Plus 6.0 was used for the quantitative analysis of the bands.

RT-qPCR detection of the TIM mRNA expression

Total RNA was extracted using the TRIzol method (Tripure isolation reagent 200 Trizol, Roche, Switzerland) after obtaining 100 mg homogenate of NSCLC and the para-carcinoma tissues. The RNA was reverse-transcribed into cDNA with reference to the instructions of the RT-PCR kit (Promega, USA), and a 10 μl RT-qPCR amplification system was constructed using SYBR® Green Supermix (Bio-Rad, USA), cDNA, and primers (synthesized by Sangon Biotech Shanghai Co., China), taking GAPDH as the internal reference. The relative expression of the target gene was calculated using the 2-ΔΔCt algorithm. The TIM upstream primer sequence was: 5'-CGACGAAGAGGAAGATGATGAGG-3', and the TIM downstream primer sequence was: 5'-TCCAACTCACATCGGTCAAACA-3'; the GAPDH upstream primer sequence was: 5'-GACTCATGACCACAGTCCATGC-3', and the GAPDH downstream primer sequence was: 5'-AGAGGCAGGGATGATGTTCTG-3'. The expression of the TIM mRNA was determined using a Fluorescent PCR instrument (Real time fluorescence quantitative PCR instrument, Bio-Rad, USA).

Statistical analyses

SPSS 20.0 Software was utilized for the data analysis. The measurement data were expressed as the mean ± SD. T tests were used for the comparisons between groups, and a rank-sum test was used for the ranked data. The prognoses were analyzed by using Kaplan-Meier method and the Cox regression model. A value of $P < 0.05$ was considered significant, and $P < 0.01$ was considered very significant.

Results

Immunohistochemical determination of the expression of TIM in the NSCLC and para-carcinoma tissues

The immunohistochemistry showed that the TIM expressions in the NSCLC tissues were localized in the nuclei (**Figure 1**). In the 72 NSCLC tissue samples, the positive expression rate of TIM was 86.11% (62/72), among which the weak positive expression rate was 29.17% (21/72), the medium positive rate was 27.78% (20/72), and the strong positive expression rate was 29.17% (21/72). In the study comparing the expression of TIM with the clinicopathological features, the results of the rank sum test analysis showed that the intensity of the TIM expression was closely related to the tumor size, degree of differentiation, TNM stage, and lymph node metastasis. The intensity of the TIM expression in the patients with a tumor size ≥ 3 cm was stronger than it was in the patients with a tumor size < 3 cm (mean rank 40.53 >

Table 1. A comparison of the TIM expressions with the clinicopathological features

Clinicopathological Features	N	TIM Negative	TIM Positive			Z values	*P* values
			+	++	+++		
Gender						-0.835	0.403
Male	46	8	8	14	16		
Female	26	3	5	6	12		
Age (years)						-0.273	0.785
≤ 50	32	5	5	9	13		
> 50	40	6	8	11	15		
Differentiation degree						-1.973	0.048
Moderately-Highly	54	9	11	18	16		
Poorly differentiated	18	2	2	2	12		
TNM stage						-2.197	0.028
I-II	50	9	9	16	16		
III	22	2	4	4	12		
The largest tumor diameter (cm)						-2.76	0.006
< 3	20	5	6	6	3		
≥ 3	52	6	7	14	25		
Smoking or not						-0.805	0.421
T	27	3	5	7	12		
N	45	8	8	13	16		
Lymph node status						-2.178	0.029
Metastasis	48	9	11	13	15		
No metastasis	24	2	2	7	13		

26.03, Z = -2.760, P = 0.006), and in the poorly differentiated group it was stronger than it was in the well differentiated group (mean rank 44.56 > 33.81, Z = -1.973, P = 0.048), and in patients at TNM stage III it was stronger than it was in patients at stages I-II (mean rank 44.27 > 33.08, Z = -2.197, P = 0.028), and in the patients with lymph node metastasis it was stronger than it was in the patients without lymph node metastasis (mean rank 43.75 > 32.88, Z = -2.178, P = 0.029). However, there were no statistically significant differences in the TIM expressions among the different ages, genders, or smoking statuses (**Table 1**).

Differential expression of the TIM protein and mRNA in the NSCLC and para-carcinoma tissues

The Western blot showed that the relative expression of the TIM protein in the NSCLC tissues was 0.98±0.02, and the level in the para-carcinoma tissues was 0.39±0.05. The expression of the TIM protein was significantly increased in the NSCLC tissues compared with the expression in the para-carcinoma tissues, and the difference was significant (P < 0.01) (**Figure 2A**, **2B**). According to the RT-qPCR detection of the TIM mRNA expression, the relative expression of TIM mRNA in the NSCLC tissues was 1.25±0.02, and the value was 0.35± 0.01 in the para-carcinoma tissues. Similarly, compared with the para-carcinoma tissues, the TIM mRNA expressions in the carcinoma tissues was significantly increased (P < 0.01) (**Figure 2C**).

Prognosis analysis

By the end of the follow-up, there were 11 NSCLC patients with a survival period of more than 60 months. The survival curves of the NSCLC patients are depicted by using the Kaplan-Meier method. As shown in **Figure 3A**, **3B**, the overall survival (OS) and the disease-free survival (DFS) in the TIM high expression group were significantly lower than they were in the TIM medium-low expression group. The results of the log-rank single-factor analysis were similar (X^2 = 14.554, P < 0.01). The factor with P < 0.05 in the single-factor ANOVA was further included in the Cox regression model for the multi-factor analysis. The results indicated that TIM expression intensity, tumor size,

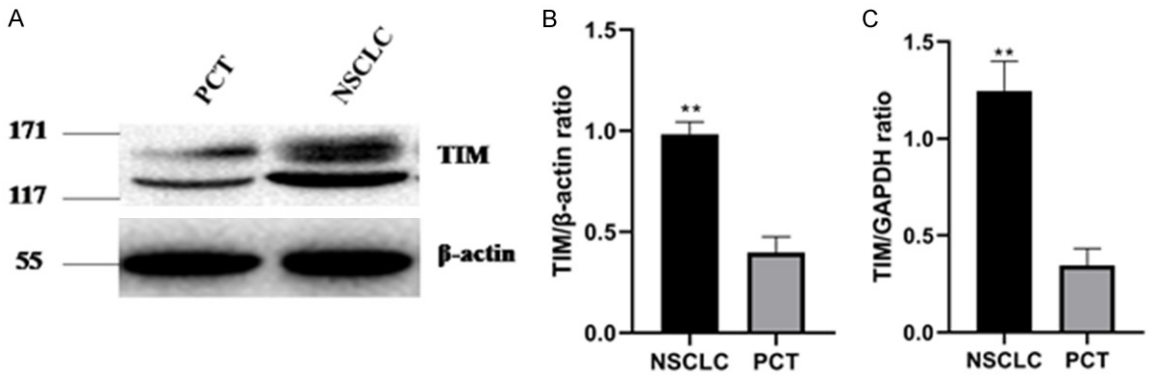

Figure 2. The expression differences of TIM in the NSCLC and paracancerous tissues (PCT) were compared using Western blot and RT-qPCR. A. Western blot showed that the relative expression of the TIM protein in the NSCLC tissues was significantly increased compared with the expression in the adjacent tissues. B. The relative expression of the TIM protein in the NSCLC tissues was 0.96±0.07, and the expression in the paracancerous tissues was 0.34±0.08. Compared with paracancerous tissues, the TIM protein expression in the NSCLC tissues was significantly increased, and the difference was significant ($P < 0.01$). C. The results of the TIM mRNA expression determined by RT-qRCR showed that the relative expression of the TIM mRNA in the NSCLC tissues was significantly higher than it was in the paracancerous tissues, with a significant difference ($P < 0.01$).

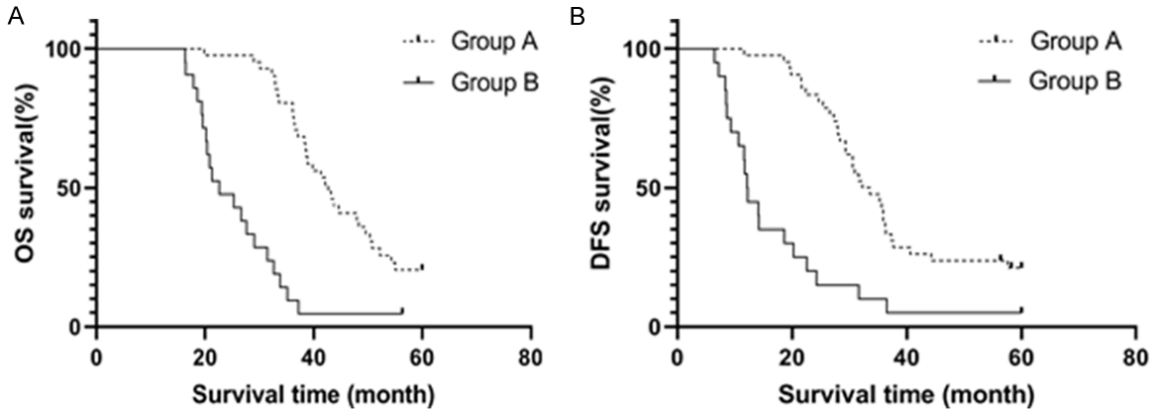

Figure 3. The relationship between the TIM expression levels and the OS and DFS of the NSCLC cases. A. The overall survival (OS) of the TIM strong positive group (group B) was significantly lower than it was in the TIM weak-medium positive expression group (group A). B. The disease-free survival (DFS) of the TIM strong positive group (group B) was significantly lower than it was in the TIM weak-medium positive expression group (group A).

degree of differentiation, TNM stage, and lymph node metastasis can be used as independent risk factors that affect the prognosis of NSCLC (**Table 2**).

Discussion

Most NSCLC patients are in the advanced stages at the time of their diagnoses. Although the treatment regimen has progressed from traditional chemoradiotherapy to molecular targeted therapy and immunotherapy in recent years, the advanced NSCLC survival rate has not been improved satisfactorily [8]. Therefore, it is urgent to find more effective targets for early diagnosis and treatment, so as to clarify the early diagnosis of NSCLC and improve patients' prognoses.

An important part of the circadian clock genes, TIM was first discovered in Drosophila and is widely distributed in organisms [9, 10]. In humans, Timeless is located on chromosome 12, with a full length of about 43 kb. The encoded product is the Timeless protein, referred to as TIM, which can form a heterodimer with another circadian clock gene, Per, to exercise the function of regulating biological rhythm

Table 2. Multivariate analyses of variance for the prognoses of the 72 NSCLC cases

Items	Log-rank univariate analysis		Cox model multivariate analysis	
	X^2	*P* values	HR (95% CI)	*P* values
The expression of TIM (Positive vs Negative)	6.56	0.011	1.917 (1.033-2.801)	0.014
Differentiation degree (Moderately-Highly vs Poorly differentiated)	14.554	< 0.001	2.431 (1.408-3.454)	< 0.001
tumor size (≥ 3 cm vs < 3 cm)	38.537	< 0.001	10.414 (4.452-24.360)	< 0.001
TNM stage (III vs I-II)	10.14	0.001	0.426 (0.248-0.732)	0.001
Lymph node status (Metastasis vs No metastasis)	21.246	< 0.001	0.219 (0.109-0.440)	< 0.001

[11], but it can also be used as a cofactor to participate in the process of gene transcriptional regulation [12]. Recent studies have found that TIM is abnormally expressed in various tumors, but its expression level varies in different tissues. In relevant studies on kidney cancer and pancreatic duct adenocarcinoma, it has been suggested that the entire family of circadian clock genes, including TIM, may be involved in the occurrence and development of tumors, as they show a significantly low expression and are closely related to the low survival of patients [13, 14]. In liver cancer, timeless expression was also significantly down-regulated, which may be related to promoter methylation, and the TIM expression level was related to tumor size [15]. However, the TIM expression was found to present at a high level in colorectal cancer, as opposed to the low expression of the other circadian clock genes in the family, and it was significantly related to lymph node metastasis and tumor TNM stage [16, 17]. Research on breast cancer also obtained results to those of colorectal cancer [18]. All the histological types of tissues in lung cancer showed a tendency to overexpress TIM, and a knockout of the TIM gene can significantly inhibit the growth of cancer cells and enhance the sensitivity of cells to cytotoxic drugs [6, 19, 20].

The results of Western blot and RT-qPCR in our research revealed that the expressions of the TIM protein and mRNA in the NSCLC tissues are higher than they are in the para-carcinoma tissues, which was consistent with the experimental results described above, indicating that TIM may be involved in the occurrence and development of NSCLC. The immunohistochemical test results from the postoperative samples of the NSCLC patients further showed that TIM expression is closely related to NSCLC tumor size, degree of differentiation, TNM stage, and lymph node metastasis, but the differences in age, gender, and smoking status were not statistically significant, suggesting that TIM expression is closely related to the invasion and metastasis of NSCLC. In terms of the correlation between TIM expression and the survival rate of NSCLC, the Kaplan-Meier method was adopted to depict the survival curves of the NSCLC patients, and log-rank tests were used for the single-factor analysis. The results revealed that the postoperative survival rate of the TIM positive expression group was significantly lower than the rate of the negative group, and the postoperative survival rate of the TIM medium-high positive expression group was lower than the rate of the TIM low positive expression group. The difference was statistically significant. The factor with $P < 0.05$ in single-factor ANOVA was further included in the Cox regression model for the multi-factor analysis. The results indicated that TIM expression intensity, tumor size, degree of differentiation, TNM stage, and lymph node metastasis can be used as independent risk factors that affect the prognosis of NSCLC. The above experimental results suggest that the determination of the expression level of TIM is of great importance in determining the degree of malignancy of NSCLC, the risk of metastasis, and the prognosis.

In summary, the TIM expression is significantly increased in NSCLC tissues, and the higher the degree of positive expression, the worse the clinical prognosis. The expression of TIM has a certain auxiliary value in determining the NSCLC's degree of malignancy, the lymph node metastasis status, and the prognosis, and it lays a foundation for the subsequent exploration of the molecular mechanism of TIM in the occurrence and development of NSCLC and targeted therapy for NSCLC.

Acknowledgements

This work was partly supported by the Natural Science Foundation of Hubei Province (No. 2019CFB817).

Disclosure of conflict of interest

None.

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