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Original Article

Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) positively regulates the progression of NSCLC via promoting cell proliferation

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Abstract: Toll-Interleukin 1 Receptor Domain Containing Adaptor Protein (TIRAP) is an adaptor protein for Toll-like receptors-2 and-4 (TLR2/4) which are engaged in transducing the signal to downstream molecules. Toll-like receptors (TLR) are overexpressed in many types of cancer cells. However, the detailed role of TIRAP in non-small cell lung cancer (NSCLC) has not been elucidated. In this study, Western blot and immunohistochemistry (IHC) staining showed that TIRAP was up-regulated in NSCLC tissues compared with adjacent non-tumor tissues. IHC analysis indicated that TIRAP was significantly associated with clinical pathologic variables. Kaplan-Meier analysis showed that high expression of TIRAP was related to poor prognosis of lung cancer patients. And TIRAP along with other clinicopathological variables was an independent prognostic indicator for patients' overall survival by multivariate analysis. In vitro studies using serum starvation-refeeding experiment, TIRAP-siRNA transfection assay, and flow cytometry analysis demonstrated that TIRAP expression promoted proliferation of NSCLC cells, while TIRAP knockdown led to inhibition of cell growth. Our results indicated that TIRAP was involved in the tumorigenesis of NSCLC and might be a potential therapeutic target of NSCLC.

Keywords: Non-small cell lung cancer, TIRAP, prognosis, proliferation

Introduction

Lung cancer is one of the most common causes of cancer death in the world [1]. Non-small cell lung cancer (NSCLC) accounts for about 80% among all lung cancer cases, which including adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, and large cell carcinoma [2]. Despite advances achieved in lung cancer treatment and tumor molecular biology, these patients often have low fiow-year survival rate which is often less than 15% [3, 4]. Therefore, it is urgent to identify new molecular targets of lung cancer which will benefit both diagnosis and treatment of NSCLC [5].

Toll-like receptors (TLRs) play an important role in the recognition of microbes during host defense [6]. All TLRs have a Toll-interleukin 1 receptor (TIR) domain, which is responsible for signal transduction. In humans there are 10 different TLRs, and three being cytosolic proteins termed MyD88, TIR domain-containing adapter protein (TIRAP), also termed MyD88 adapter-like (Mal) and TIR-domain-containing adapter inducing interferon-β (TRIF), which act as adapters [7-10]. It has been demonstrated that both TIRAP is necessary for the activation of the NF-κB pathway in response to signaling through TLR2 and TLR4 [11]. Upregulation of TIRAP has been observed in several cancers, including colorectal cancer, gastric cancer [12, 13]. However, the detailed role of TIRAP in NSCLC has not been elucidated.

In this study, we aim to investigate the role of TIRAP in NSCLC's development. We determined the expression level of TIRAP in NSCLC tissues and evaluated its correlation with clinicopathological features, as well as its implication for

Table 1. Expression of TIRAP in 140 human lung adenocarcinoma carcinoma tissues

Clinicopathological parameters	Total	TIRAP		*P*-value
		Low (N=78)	High (N=62)	
Gender				
Male	69	9	60	0.148
Female	71	15	56	
Age				
<60	61	12	49	0.317
≥60	79	12	67	
Tumor size (cm)				
<3	62	19	43	0.000*
≥3	78	5	73	
Smoking status				
Yes	20	1	19	0.101
No	120	23	97	
Lymph node metastasis				
No	83	17	66	0.15
Yes	57	7	50	
Distal metastasis status				
M0	137	78	59	0.084
M1	3	0	3	
Pathology grade				
Well	29	18	11	0.000*
Mod	94	6	88	
Poor	17	0	17	
Clinical stage				
I+II	122	24	98	0.002*
III+IV	18	0	18	
Ki67				
Low	25	22	3	0.000*
High	115	2	113	

*Statistical analyses were performed by the Pearson χ^2 test. P<0.05 was considered significant.

clinical prognosis. Furthermore, we also investigated the role of TIRAP expression in cancer cell proliferation. This study provides the first demonstration that TIRAP expression promotes NSCLC proliferation.

Materials and methods

Tissue samples

One hundred and twenty lung cancer sections, eight lung cancer tissue samples and eight normal tissue samples from patients underwent surgery between 2008 and 2013 at Department of Pathology, Affiliated Hospital of Nantong University. Affiliated Hospital of Nantong University Hospital were good at formalin-figoo and paraffin-embedded for histopathologic diagnosis and immunohistochemical study. The main clinical and pathologic variables are shown in **Table 1**. Eight couples of fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at -80°C until used for Western blot analysis.

Western blot analysis

Tissue and cell protein were collected with 2 lysis buffer which containing 50 mM Tirs-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 60 mM b glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, and complete protease inhibitor cocktail (Roche Diagnostics), and then incubated for 20 min at 4°C while rocking. Lysates were collected after centrifugation (15 min 12,000 rpm, 4°C). Protein concentrations were measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Then the same total protein was separated by SDS-PAGE and transferred to a polyvinylidenefluoride (PVDF) membrane (Immbilon; Millipore). The membranes were first blocked with 5% no-fat milk in TBST (150 mM NaCl, 20 mM Tris, 0.05% Tween-20) and the membranes were washed with TBST for three times after 2 h at room temperature, and then incubated overnight with the primary antibodies. Then LumiGLO Regent and Peroxide (Cell Signaling) is used as the secondary antibodies. The band was then detected by the ECL (enhanced chemiluminescence) detection systems (Pierce, Rockford, IL, USA). The band intensity was measured by ImageJ analysis system (Wayne Rasband, National Institutes of Health, USA) [14].

Immunohistochemical staining

Surgically excised tissues were fixed with 10% formalin and embedded in paraffin, and 4-μm-thick specimen sections were prepared on glass slides. Then the sections were depara-

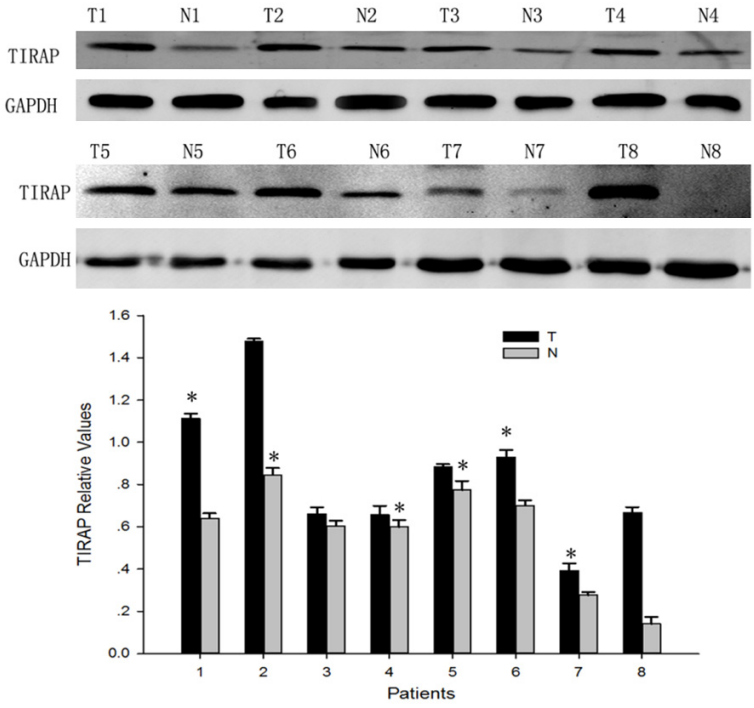

Figure 1. Expression profiles of TIRAP in NSCLC and non-tumorous adjacent tissues. Western blot was performed to detect expression of TIRAP and PCNA in eight representative paired NSCLC tumor tissues (T) and adjacent non-tumor tissues (N). GAPDH was used as a loading control. The bar chart demonstrates the relative levels of TIRAP protein to GAPDH by densitometry. The data are mean ± SEM (*P<0.05, compared with adjacent tumor tissues). The same experiment was repeated at least three times.

ffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was performed by heating to 121°C for 3 min in 10 mmol/l citrate buffer (pH 6.0) with an autoclave. Endogenous peroxidase activity was blocked by hydrogen peroxide (0.3%) for 20 min after cooling. After that, the sections were incubated with rabbit antihuman TIRAP antibody (diluted 1:50) for 2 h at room temperature. Washed with phosphate-buffered saline (PBS), tissues were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig polymer as a second antibody (Dako, Hamburg, Germany) for 20 min at room temperature, according to the manufacturer's instructions. Finally, the peroxidase reaction was visualized by incubating the sections with DAB (0.1% phosphate buffer solution, 0.02% diaminobenzidine tetrahydrochloride, and 3% H_2O_2). After being rinsed in water, the sections were counterstained with hematoxylin, dehydrated, and mounted in resin mount [15].

Evaluation of the results of immunohistochemical staining

We use a microscope to observe the stained sections. All of the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the patients by three pathologists. For assessment of TIRAP more than 1000 cells from fise high-power fihigh in each specimen were selected randomly, and the staining were examined to determine the mean percent. IHC staining was scored according to the following provision: The intensity of TIRAP staining was scored as 1 (negatively or poorly stained), 2 (moderately stained), 3 (strongly stained). Percentage scores were assigned as 1 (1-25%), 2 (26-50%) and 3 (51-100%) for univariate and multivariate analysis. Then, multiplied the two scores, and divided patients into two groups according to the scores (average = 4.5): high expression group and low expression group [16].

Cell cultures

The human NSCLC cell lines A549 were obtained from the institute of Cell Biology, Academic Sinica and all cells were cultured in the 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37°C.

Plasmids and shRNA for transient transfection

The human NSCLC cell line A549 was grown in dishes until they reached 80% confluence. The medium was replaced 24 h later with fresh medium for transfection. The TIRAP-shRNA and control-shRNA were purchased from Genechem (Shanghai). The target sequences for TIRAP gene: TIRAP-shRNA#1 was 5'AGACTAGATTCACTGCCTA3'. TIRAP-shRNA#2 was 5'CCACCCAAT-

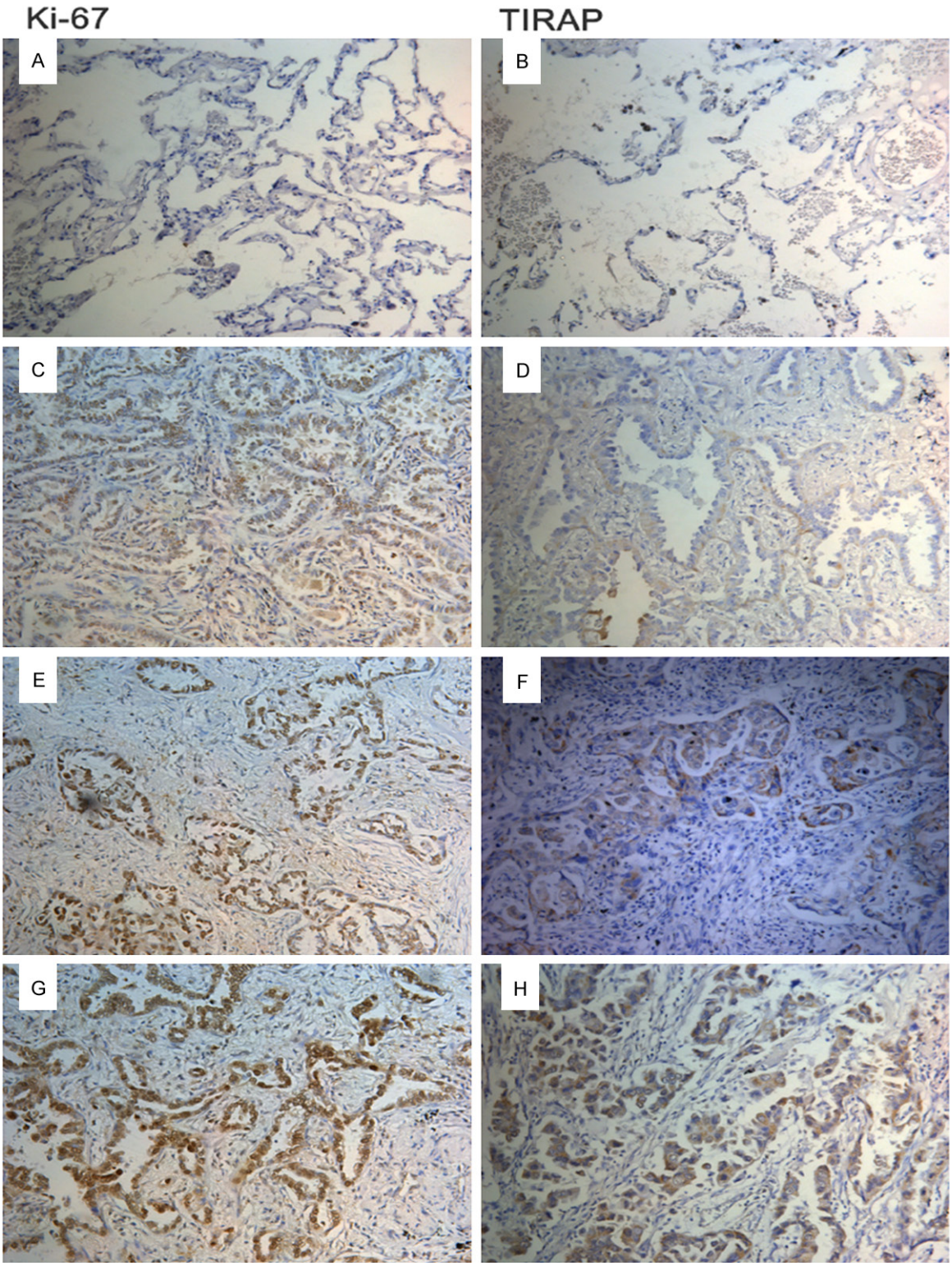

Figure 2. Immunohistochemical stain of TIRAP and Ki-67 in NSCLC tissues. Paraffin-embedded tissue sections were stained with antibodies against TIRAP and Ki-67 and counterstained with hemotoxylin. TIRAP and Ki-67 expression in adjacent non-tumor tissues (A, B), well differentiated (C, D), moderate differentiated (E, F), and poor differentiated (G, H) NSCLC tissues (×200).

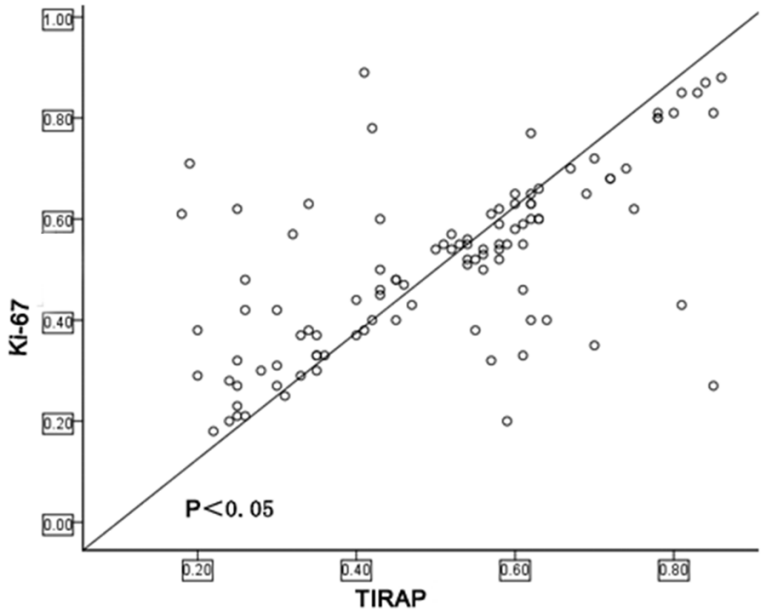

Figure 3. Correlation between TIRAP and Ki-67 expression in NSCLC patients. The correlation between TIRAP and Ki-67 expression in NSCLC was further evaluated by Spearman rank correlation test (P<0.05).

GGTAAATCAT3'. TIRAP-shRNA#3 was 5'ACAGAAGATAAGCGCTTAA3'. TIRAP-shRNA#4 was 5'TCACGAAATCGGACATTTA3'. Cells were collected for Western blot, wound healing assays and transwell assays after transfection for 36 h [17].

Cell viability assay

After treatment according to the protocols, cells were seeded at 2×10^4 per well in 100 μL medium in 96-well plates and incubated overnight. Then Counting Kit-8 reagents (Dojindo, Japan) was added to each well, and incubated at 37°C for 2 h. The absorbance was recorded at 450 nm [18].

Flow cytometric analysis

For cell cycle analysis, cells were collected and fixed with 70% cold ethanol at -20°C overnight. Then cells were incubated with 1 mg/mL RNase A for at 37°C 30 min. Subsequently, cells were stained with propidium iodide (50 μg/mL PI) in PBS, 0.5% Tween-20, and analyzed using a Becton-Dickinson flow cytometer BD FACScan (San Jose, CA, USA) [19].

Statistical analysis

To analyses the association between TIRAP expression and clinicopathological features χ^2 test was used. Survival curves were calculated by the Kaplan-Meier method, and the log-rank test was used. Multivariate analysis was performed using Cox's proportional hazards model. Relationship between TIRAP and Ki-67 expression in NSCLC was measured using the Spearman rank correlation test. Other data were analyzed with Student 'st test. P<0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS 13.0 software.

Result

TIRAP was overexpressed in NSCLC tumor tissues

Western blot analysis was performed to detect different expression level of TIRAP in eight paired tumor and adjacent non-tumor tissues of NSCLC. As shown in **Figure 1**, in most cases, TIRAP expression was significantly higher in tumor tissues than in adjancent non-tumorous tissues. To further evaluate TIRAP expression and its clinical significance in NSCLC, IHC assay was performed to determine the expression of TIRAP and Ki-67, which is also a cell proliferation index, in 120 NSCLC samples. Immunoreactivity of TIRAP and Ki-67 was seen predominantly in the nucleus (**Figure 2**). Moreover, the expression of TIRAP and Ki-67 also showed the same stain tendency. Both of their expression was up-regulated in NSCLC tissues compared with non-tumor tissues, which showed rare or almost none expression. In addition, less differentiated tumor tissues showed much significant higher expression of TIRAP (**Figure 2**).

Correlation of TIRAP expression with clinicopathologic variables in NSCLC

To further explore the role of TIRAP in NSCLC, Pearson χ^2 test was performed to analyze the association of TIRAP expression with clinicopathologic variables. Clinicopathologic data of the patients were summarized in **Table 1**. Consistent with its expression results showed in **Figure 2**, the expression level of TIRAP was

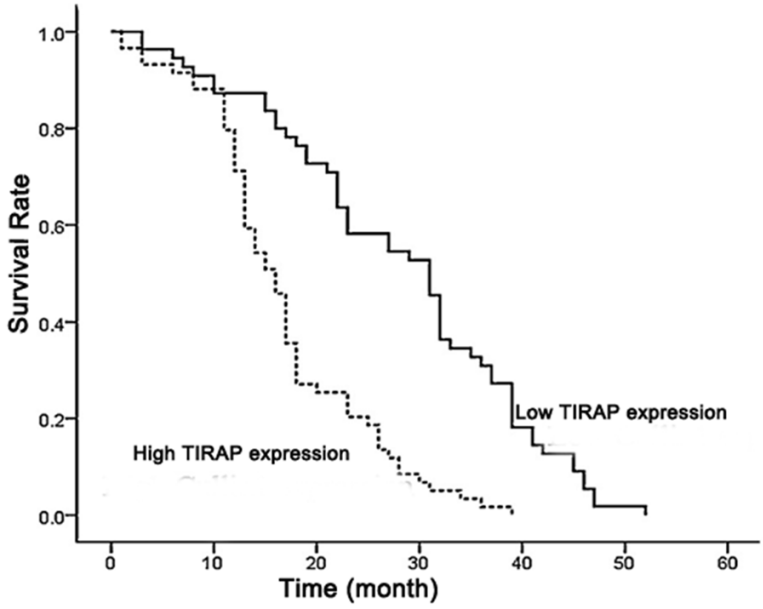

Figure 4. Correlation between TIRAP expression and patients' survival. Kaplan-Meier survival curves for high versus low TIRAP expression on 120 patients with NSCLC showed a highly significant separation between curves (P<0.05).

associated with histological differentiation (P<0.000) and clinical stage (P=0.002, **Table 1**). Moreover, there was significant positive correlation between the expression level of TIRAP and that of Ki67 (P=0.000, **Table 1**). In addition, the correlation between TIRAP and Ki67 expression in NSCLC was further evaluated by Spearman rank correlation test (P<0.05, **Figure 3**). However there was no significant relation between TIRAP expression and other clinical factors (**Table 1**).

High expression of TIRAP predicted poor prognosis of NSCLC patients

To evaluate the prognostic significance of TIRAP expression, Kaplan-Meier analysis was performed. The result showed that high expression of TIRAP was significantly associated with poor overall survival rate of NSCLC patients (P= 0.034, **Figure 4**).

TIRAP expression promoted proliferation of A549 cells

Given that TIRAP was reported to promote gastric tumourigenesis [20], and that TIRAP expression was positively correlated with the expression of Ki-67 which is a cell proliferation marker, we suppose that TIRAP expression might contribute to cell cycle progress of NSCLC. Therefore, we further evaluated the expression of TIRAP during cell cycle progression in A549 cells. A549 cells were cultured in serum-deprived condition for 72 h and then recovered serum refeeding. Flow cytometry analysis was performed to analyze the cell cycle progression of A549 cells. It showed that after serum starvation, cells were arrested in the G0/G1 phase. The percentage of cells in the G0/G1 phase was more than 70%. Then upon serum refeeding, cells in the G0/G1 phase decreased, with concomitant increase of cells in the S phase (**Figure 5A**). Next, Western blot assay were performed to analyze the expression of TIRAP, PCNA and Cyclin A. As expected, TIRAP expression was increased as early as 4 h and reached the highest level 12 h after serum re-addition, which was consistent with PCNA (a marker of proliferation) and Cyclin A (a marker of cell cycle) (**Figure 5B**, **5C**). Thus, these results indicated that TIRAP might have an impact on the proliferation of NSCLC cells in a cell cycle-dependent pathway.

Knockdown of TIRAP inhibited proliferation of A549 cells

To further investigate the effects of TIRAP expression on NSCLC cell proliferation, A549 cells were transiently transfected with TIRAP-shRNA and control-shRNA. The efficiency of TIRAP-shRNA was confirmed by Western blot assay 48 h after transfecting. As shown in **Figure 6A**, TIRAP protein level decreased maximally in TIRAP-shRNA#3 transfected A549 cells. So TIRAP-shRNA#3 got the best interference efficiency. Therefore we used TIRAP-shRNA#3 for subsequent experiments. In addition, Western assay also revealed that with TIRAP down-regulation following siRNA transfection, the expression of cell proliferation marker PCNA was decreased and cell apoptosis marker cleaved caspase 3 increased (**Figure 6B**). In addition, knockdown of TIRAP also lead

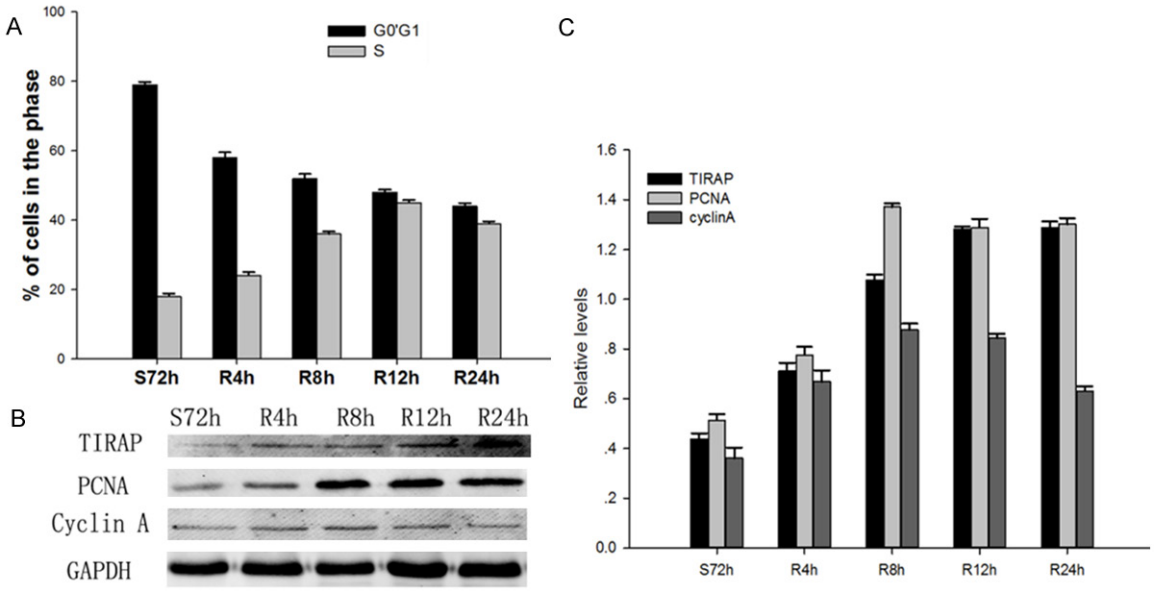

Figure 5. TIRAP expression promoted proliferation of A549 cells. A. The S72h A549 cells were released by refeeding with serum, and cell lysates were prepared and analyzed by Western blot using antibodies against TIRAP, cyclin A, PCNA, and GAPDH. B. The bar graph indicates density of TIRAP/cyclin A/PCNA versus GAPDH at each time point. C. Cells synchronized at G1 progressed into the cell cycle when serum was added for 0, 4, 8, 12, 24 h. Data are presented as mean ± SEM of three independent measurements. *, #, ^*P* <0.05, compared with control cells serum starved for 72 h (S72h). S, serum starvation, R, serum release, SEM, standard error of the mean.

to reduced NSCLC cell growth rate demonstrated by CCK-8 assay (**Figure 6C**). To explore the mechanism of the decreased cell growth rate affected by TIRAP-shRNA transfection, cell cycle distribution was determined by fluorescence-activated cell sorting (FACS). The percentage of cells in the S phase of TIRAP knocked down cells was obviously decreased (15.52%) as compared with that of control siRNA cells (28.43%), suggesting that TIRAP may be able to promote the G0/G1-S transition and thus cell growth (**Figure 6D**). Taken together, these data suggested that the expression of TIRAP might promote G0/G1-S transition, and therefore be responsible for NSCLC cell proliferation.

Discussion

The initiation and progression of NSCLC is a comprehensive pathologic process involving complex alterations in oncogenes and tumor suppressor genes that play roles in cell proliferation and cell apoptosis. In spite of the development in therapy methods such as sourgical resection, chemotherapy and radiation therapy, this disease is rarely curable and prognosis is poor [21]. A deeper understanding of the genes associated with NSCLC development is of great necessity. In this study, we aimed to investigate the role of TIRAP in the development of NSCLC.

In this study, we demonstrated that TIRAP might be an important regulator in NSCLC. Firstly, Western blot analysis using samples from eight paired fresh NSCLC tumor and adjacent non-tumor tissues revealed that TIRAP was overexpressed in NSCLC tumor tissues (**Figure 1**). Secondly, immunohistochemistry stain of 140 NSCLC sample showed that immunoreactivity of TIRAP was seen predominantly in the nucleus, and the positive ratio of its expression was increased with clinical stage (**Figure 2**). In addition, immunohistochemistry analysis revealed that TIRAP expression was correlated with Ki-67 and tumor grade. Multivariate analysis with the Cox's proportional hazards model indicated that TIRAP could be an independent prognostic factor for the survival of NSCLC patients. Accordingly, Kaplan–Meier analysis revealed that TIRAP overexpression predicted poor survival. Furthermore, serum starvation and release experiments showed that TIRAP expression was positively related with cell proliferation, and knock down of TIRAP would result in concomitant reduced

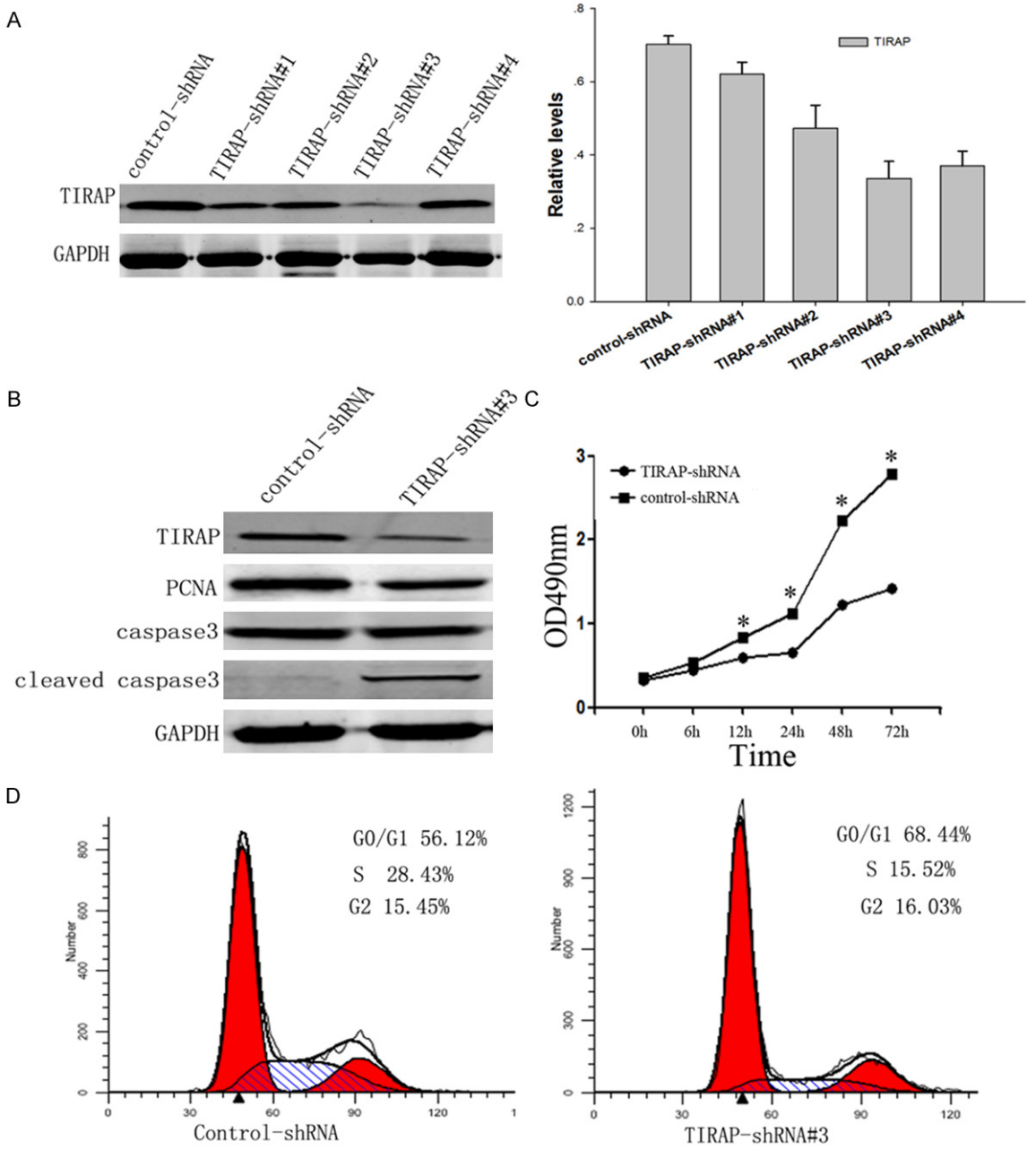

Figure 6. Knock down of TIRAP expression inhibited A549 cell proliferation. A. TIRAP expression was determined by Western blot following TIRAP-shRNA transfecting in A549cells. The bar chart below demonstrated the ratio of TIRAP to GAPDH by densitometry. The data are mean ± SEM (*P<0.05 compared with the control). B. The expression of TIRAP, PCNA, caspase3 and cleaved-caspase3 was determined following control-shRNA and LYAR-shRNA#3 transfection. C. CCK-8 assay was performed to determine cell vitality of A549 cells transfected with TIRAP-shRNA#3 exhibited significantly weakened proliferation. D. Flow cytometric analysis of cell cycle distribution following control-shRNA and TIRAP-shRNA#3 transfection. All these data are representative of at least three independent experiment.

expression of PCNA, and inhibited proliferation of A549 cells. Taken together, these findings supported our previous hypothesis that TIRAP might play an important role in promoting the progression of NSCLC.

TLRs are overexpressed on many types of cancer cells, including colorectal cancer cells, grastic cancer and breast cancer [12, 13, 22]. TLRs are defined by the presence of a Toll/interleukin-1 (IL-1) receptor (TIR) domain in their cyto-

solic regions, which is involved in signal transduction. It is now known that individual TLRs use different combinations of adaptor proteins to activate distinct signaling pathways. Previous study have shown that TIRAP controls activation of MyD88-independent signaling pathways downstream of TLR4 [23]. It has been demonstrated that both TIRAP and MyD88 are necessary for the activation of the NF-κB pathway in response to signaling through TLR2 and TLR4 [24]. However, further studies are necessary to elucidate the molecular mechanisms of TIRAP in NSCLC pathogenesis.

In summary, our studies showed that TIRAP was upregulated in NSCLC and associated with poor prognosis. Downregulation of TIRAP inhibited proliferation and induced apoptosis of A549 cells. Therefore, TIRAP might serve as a novel molecular target for the diagnosis and treatment of NSCLC.

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Disclosure of conflict of interest

None.

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