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Original Article

TPD52L2 silencing inhibits lung cancer cell proliferation by cell cycle G2/M phase arrest

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Abstract: Tumor protein D52-like 2 (TPD52L2), as a most important member of TPD52 family, has been reported to be involved in multiple human cancers. However, its expression and roles in lung cancer remains unclear. Therefore, the aim of this study was to investigate the biological functions and molecular mechanisms of TPD52L2 in lung cancer. TPD52L2 expression was firstly observed to be upregulated in human lung cancer tissues compared with normal tissue by Western blot analysis. Then the expression of TPD52L2 was specific knocked down via lentivirus-mediated short hairpin RNA (shRNA) in two lung cancer cell lines, A549 and 95D. Knockdown of TPD52L2 significantly suppressed cell proliferation and colony formation, as determined by MTT and colony formation assays. Furthermore, TPD52L2 silencing induced cell cycle arrest at G2/M phase through regulating the protein levels of CDK1, Cyclin B1 and p21, as confirmed by flow cytometry and Western blot assay. These results suggest that TPD52L2 plays an important role in lung cancer cell proliferation and its silencing might serve as a promising gene therapy for lung cancer treatment.

Keywords: Lung cancer, TPD52L2, cell proliferation, cell cycle

Introduction

Lung cancer is the most leading cause of death from cancer worldwide, almost 85% of which are from non-small cell lung cancer (NSCLC) [1, 2]. It was estimated that NSCLC may remain to be the highest mortality of cancer death in the next 50 years, because of most NSCLC tumors are generally diagnosed in the late stages which have malignant proliferation and distant metastasis [3]. NSCLC patients often die of cancer invasion or metastasis after surgery, radiotherapy, and chemotherapy. Thus, there is a need to identify new and early biomarkers for NSCLC to establish prognostic sets needing in different therapeutic intensity.

TPD52 family is constitute of tumor protein D52 (D52, HD52, Protein N8, prostate and colon associated protein 3, prostate leucine zipper PC-1, PrLZ, N8L), tumor protein D52-like 1 (D53, HD53), tumor protein D52-like 2 (D54, HD54, TPD54, HCCR-binding protein 2) and tumor protein D52-like 3 (HD55, testis development protein NYD-SP25, protein kinase NYD-SP25, NYDSP25) [4]. TPD52L2 is a most important member of TPD52 family. These protein typically include a coiled-coil motif which is required for homo and heteromeric interactions with other D52-like proteins (D52 or D53), and other heterologous partners (hABCF3 or 14-3-3 proteins) [5-8]. In addition, both the N and C termini encompass PEST sequences, which regulate protein stability [9]. As TPD52L2 shares functional characteristics with D52 [5, 10], TPD52L2 and its family members form homomeric and heteromeric interactions to play a proto-oncogene role in the dependent of numerous solid tumors such as breast, colon, liver cancer and pancreatic carcinoma [4, 11, 12]. However, it is a negative regulator of extra cellular matrix-dependent migration and attachment in oral squamous cell carcinoma-derived cell lines (SAS, HSC2, HSC3 and HSC4) [13]. TPD52L2 were located in the genomic regions (20q13) which are frequently gained in many cancer types and harbor multiple ampli-fication targets. Overexpression of TPD52L2 may therefore be largely produced by genetic mechanisms targeting a shared D52-like function, such as promoting proliferation. However,

the function of the TPD52L gene in the dependent of NSCLC is unclear. Lentiviral vectors have become the most widely used vectors in biological and functional genomics research, and they have shown great promising for clinical applications [14, 15].

In the present study, we introduced an effective lentivirus mediated-shRNA that repression of TPD52L2 long term in A549 and 95D NSCLC cells lines to provide additional evidence for the function and preliminary mechanism of TPD52L2 in the context of proliferation.

Materials and methods

Patient specimens

Total 4 pairs of fresh tumor tissue samples with paired non-cancerous lung tissue samples were obtained in operation from the Yishui Central Hospital of Linyi (Shandong, China) between September 2015 and December 2015. None of these patients had received radiotherapy or chemotherapy prior to surgical treatment. All participants involved in the study have signed written informed consent. Western blot analysis was used to determine the protein levels of TPD52L2 in fresh lung cancer tissues with paired non-cancerous lung tissues.

Cell lines and culture

Human lung cancer cell lines, A549 and 95D, as well as HEK293T cells were from Cell Bank of Chinese Academy of Science (Shanghai, China) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. A549 and 95D cells were cultured in RPMI-1640 (Hyclone, Biowest) supplemented with 10% fetal bovine serum (FBS). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) plus 10% FBS.

Lentiviral vector construction and transfection

To knock down the expression of TPD52L2, two short hairpin (shRNA) sequences were specific designed for targeting human TPD52L2: shTPD52L2-1 (5'-CCGGGACCATAAAGTCTAAGGTTGTCTCGAGACAACCTTAGACTTTATGGTCTTTTTG-3') and shTPD52L2-2 (5'-GCGGAGGGTTTGAAAGAATATCTCGAGATATTCTTTCAAACCCTCCGCTTTTTT-3'). A non-silencing sequence (5'-TTCTCCGAACGTGTCACGT-3') was used as a negative control (NC). The corresponding recombinant lentiviruses (shTPD52L2-1, shTPD52L2-2 or NC) were transfected into HEK293T cells together with packaging plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two days after transfection, the supernatant was collected, centrifuged for 10 min (4000 g, 4°C), and filtered through a 0.45 μm cellulose acetate filter. The recombinant lentivirus was stored at -80°C.

For lentivirus infection, A549 and 95D cells were plated in a six-well plate at a density of 8×10^4 cells per well and then infected with the recombinant lentivirus. After infection for 96 h, cells were collected to confirm the knockdown efficiency of TPD52L2 using Western blot analysis.

Western blot analysis

All proteins were extracted from tissues samples and cells using ice-cold RIPA butter (Beyotime, Shanghai, China). After centrifuging at 12,000 g for 15 min at 4°C, the protein concentration was determined by enhanced BCA protein assay kit (Beyotime, Shanghai, China). Approximately 30 μg protein was loaded each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and then blocked with 5% milk for 2 h at room temperature. The membranes were probed with specific primary antibodies including anti-TPD52L2, Cyclin B1, CDK1, P21 and GAPDH overnight at 4°C, and next incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The expression of targeted proteins was visualized by enhanced chemiluminescence detection reagent (Millipore, Billerica, MA). GAPDH was used as the loading control.

MTT assay for cell viability

To determine whether TPD52L2 plays an important role in lung cancer cell viability, MTT assay was performed in A549 and 95D cells after lentivirus infection. Briefly, cells were seeded in 96-well plates at a density of 2500 cells per well in triplicates and added 20 μl MTT solution following incubation for 1, 2, 3, 4

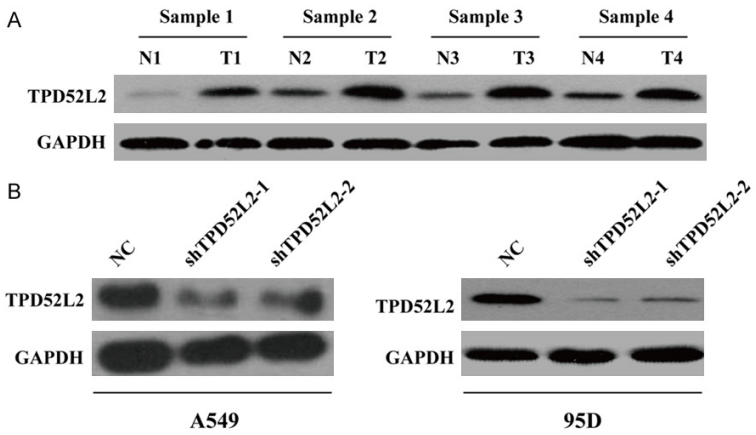

Figure 1. The expression of TPD52L2 was effectively knocked down in lung cancer cells. A: The protein level of TPD52L2 was determined in four paired lung cancer tissues and nomal tissues using Western blot analysis. B: The protein level of TPD52L2 was measured in A549 and 95D cells after three treatments (NC, shTPD52L2-1 and shTPD52L2-2) using Western blot analysis. GAPDH was used as internal control.

and 5 days, respectively. After incubation for another 4 h, each well was removed the medium and MTT and added 100 μl acidic isopropanol to stop the reaction. The optical density (OD) value in each well was measured with an ELISA reader (Bio-Rad, USA) at a wavelength of 595 nm.

Colony formation assay

To detect whether TPD52L2 has proliferative effect on lung cancer cells, a colony formation assay was performed in A549 and 95D cells after lentivirus infection. Cells (200 cells/well) were reseeded in six-well plates in triplicate and cultured for 8 days. Subsequently, cell were washed and fixed by with 4% paraformaldehyde. Fixed cells were washed twice and stained with crystals purple for 10 min. Then cells were washed three times with ddH_2O_2 and photographed with a digital camera. The number of colonies containing more than 50 cells was counted under the light/fluorescence microscope.

Flow cytometry assay for cell cycle

The cell cycle distribution of lung cancer cells after lentivirus infection was analyzed using flow cytometry assay. Briefly, A549 and 95D cells were seeded into 6-cm dishes at a density of 80000 cells per dish and then cultured for 4 days to reach 80% confluence. Cells were harvested, fixed in 70% pre-cold ethanol for 12 h and then washed with PBS. The cells were then stained with PBS containing propidium iodide (PI) and RNase A. Then samples were detected using a flow cytometer (FACS Calibur, BD Biosciences).

Statistical analysis

All the data was presented as mean ± standard deviation (SD) of three independent experiments using SPSS software version 10.0 (SPSS Inc, United States). The paired Student's *t*-test was used to evaluate differences between groups. Statistically significant differences were accepted at $P<0.05$.

Results

The expression of TPD52L2 was effectively knocked down in lung cancer cells

To investigate the role of TPD52L2 in lung cancer, the protein levels of TPD52L2 were firstly determined in four paired lung cancer tissue and normal tissue using Western blot analysis. As shown in **Figure 1A**, the protein levels of TPD52L2 were clearly increased in all the tumors tissues in comparison to the paired normal tissues, indicating there might be a closely relation between TPD52L2 expression and lung cancer development. To verify this speculation, the expression of TPD52L2 was then specific knocked down using lentivirus-mediated shRNAs in lung cancer cell lines, A549 and 95D cells. Knockdown efficiency was further confirmed by Western blot analysis. As shown in **Figure 1B**, the protein level of TPD52-L2 was significantly down-regulated in shTPD52L2-1 and shTPD52L2-2-treated lung cancer cells (A549 and 95D) compared with negative control. These data suggest that both shTPD52L2-1 and shTPD52L2-2 could specifically and efficiently reduce TPD52L2 expression in lung cancer cells.

Knockdown of TPD52L2 impaired cell viability of lung cancer cells

The cell viability was determined by MTT assay and observed for consecutive 5 days in A549

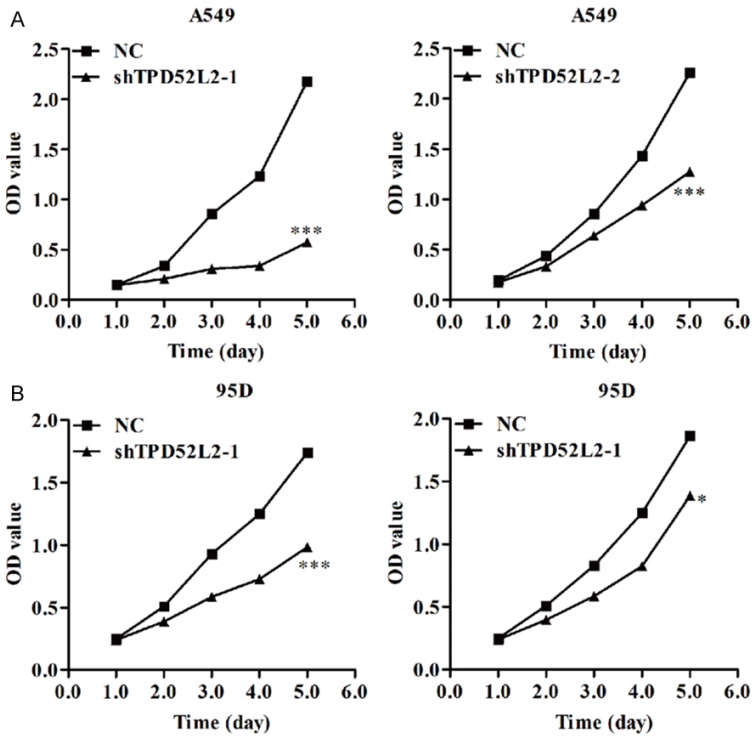

Figure 2. Knockdown of TPD52L2 significantly suppressed cell viability of A549 and 95D cells. Cell viability in NC, shTPD52L2-1 and shTPD52L2-2-treated A549 (A) and 95D (B) was measured using MTT assay. Data were expressed as mean ± standard deviation (SD) of three independent experiments performed in triplicate. ***P<0.001 versus NC.

and 95D cells from different treatments (NC, shTPD52L2-1 and shTPD52L2-2). As shown in **Figure 2A**, the growth rate of shTPD52L2-1 and shTPD52L2-2-treated A549 cells was significantly suppressed from the second day, and reduced by 71.9% and 41.2%, respectively, compared with NC-treated cells on the fifth day (P<0.001). Similarly, cell viability was also obviously decreased by shTPD52L2-1 and shTPD52L2-2, respectively (**Figure 2B**, P<0.001 and P<0.05). These findings suggest that TPD52L2 might play a positive role in lung cancer cell viability.

Knockdown of TPD52L2 inhibited the colony formation of lung cancer cells

The effect of TPD52L2 on colony formation ability was determined on two lung cancer cell lines. For A549 cells, representative images of colonies from different treatments (NC, shTPD52L2-1 and shTPD52L2-2) indicated knockdown of TPD52L2 significantly reduced the number and size of colonies (**Figure 3A** and **3B**, P<0.001). Similarly, the ability of colony formation was remarkably impaired by shTPD52L2-1 and shTPD52L2-2, respectively (**Figure 3C** and **3D**, P<0.001 and P<0.01). Notably, shTPD52L2-1 exhibited a significant inhibition in lung cancer cell proliferation, which was used for the subsequent mechanism study.

Knockdown of TPD52L2 induced cell cycle G2/M phase arrest in lung cancer cells

To further investigate the underlying mechanisms of inhibited cell proliferation and colony formation induced by TPD52L2 knockdown, flow cytometry with PI staining was used to analyze cell cycle distribution profiles of lung cancer cells infected with shTPD52L2-1. As shown in **Figure 4A**, there were obvious differences in the cell cycle distribution profiles between shTPD52L2-1-treated cells and NC-treated cells in both A549 and 95D cells. Further analysis (**Figure 4B**) showed that knockdown of TPD52L2 significantly decreased the percentage of cells in G0/G1 phase from 56.74% to 28.95% in A549 cells (P<0.001), and from 65.23% to 51.26% in 95D cells (P<0.001), while increased the percentage of cells in G2/M phase from 19.23% to 38.86% in A549 cells (P<0.001), and from 16.53% to 28.74% in 95D cells (P<0.001). Collectively, we suggest that TPD52L2 knockdown inhibited lung cancer cell proliferation through inducing cell cycle G2/M phase arrest.

To further illuminate these findings, we detected the expression alterations of several cell cycle regulators using Western blot analysis. As shown in **Figure 5**, the protein levels of CDK1 and Cyclin B1, associated with G2-M transition, were down-regulated in the shTPD52L2-1-treated A549 and 95D cells. In addition, p21, as cell cycle regulation inhibitor, was obviously upregulated in shTPD52L2-1-treated A549 and 95D cells. These data indicate TPD52L2 could pro-

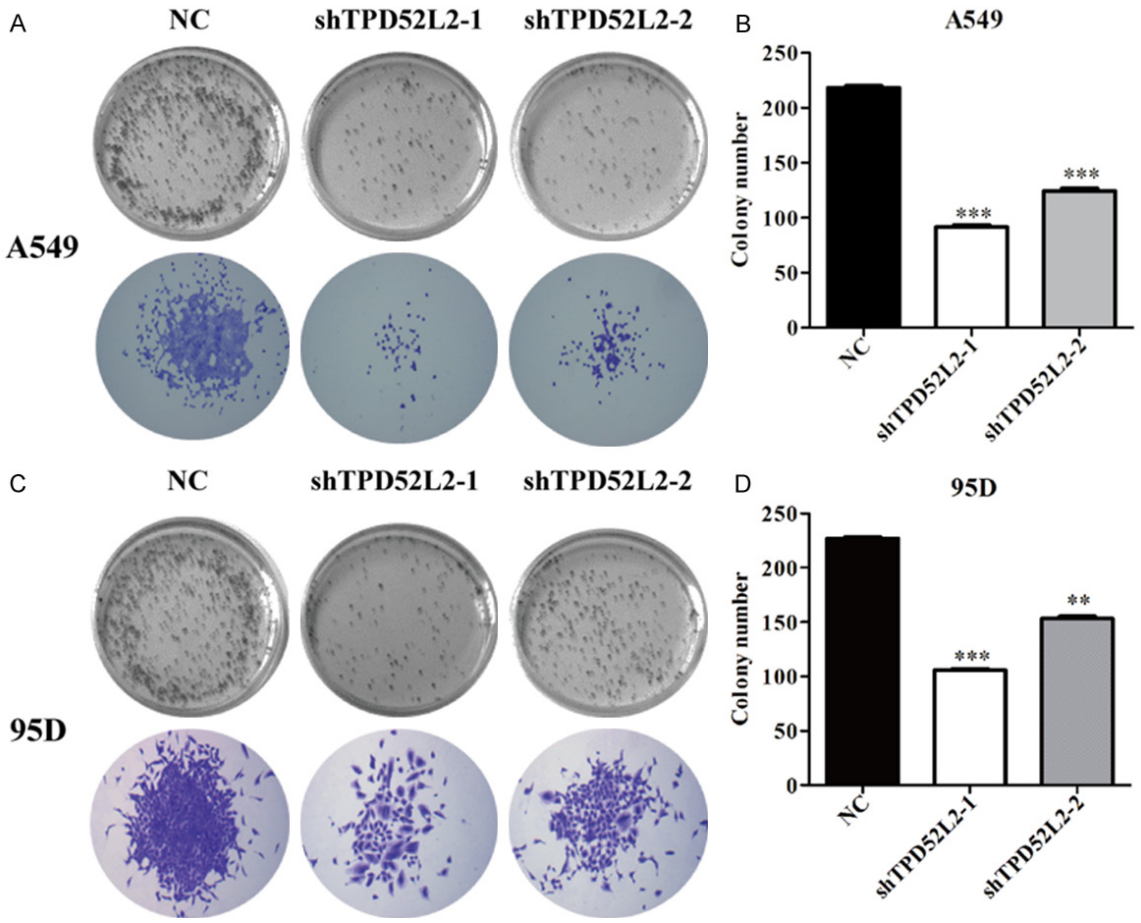

Figure 3. Knockdown of TPD52L2 impaired colony formation ability of A549 and 95D cells. The size and number of colonies in A549 (A) and 95D (C) were photographed under a light/fluorescence microscope. Statistical analysis of colonies numbers in NC, shTPD52L2-1 and shTPD52L2-2-treated A549 (B) and 95D (D). Data were expressed as mean ± standard deviation (SD) of three independent experiments performed in triplicate. $^{**}P<0.01$; $^{***}P<0.001$ versus NC.

mote tumor cell proliferation via cell cycle regulation.

Discussion

Lung cancer is the leading cause of cancer death worldwide due to its high incidence and mortality. As the most common lung cancer, NSCLC is a terrible threat to human health [16]. Therefore, discovery of new therapeutic target and development of novel therapeutic regimens that can more effectively inhibit cancer cell proliferation or metastasis appear to be of central importance. Recently studies showed that TPD52L2 overexpression in numerous tumors [4, 11, 12, 17-19]. Interestingly, our result also showed that TPD52L2 was overexpressed in lung cancer tissue. However, TPD52L2 as a potential target in NSCLC has still not been reported. This study was carried out to investigate whether TPD52L2 overexpression in NSCLC cells A549 and 95D. A noteworthy observation is that TPD52L2 were strong positive expression in highly metastatic breast cancer using 2-DE and LC-IT-MS [11]. The most of TPD52 family members act as oncogene in various tumors by (de) regulating cell cycle progression, activation of the Stat3/Bcl-2 pathway and other mechanisms [9, 20-29].

We hypothesized that TPD52L2 may be play a key role in the proliferation of NSCLC. Thus, in order to investigate the role of TPD52L2 in NSCLC, A549 and 95D cells were employed and infected with TPD52L2 lentivirus and control lentivirus to knock down TPD52L2. The selected shRNA-containing vector efficiently suppressed at both mRNA and protein expression

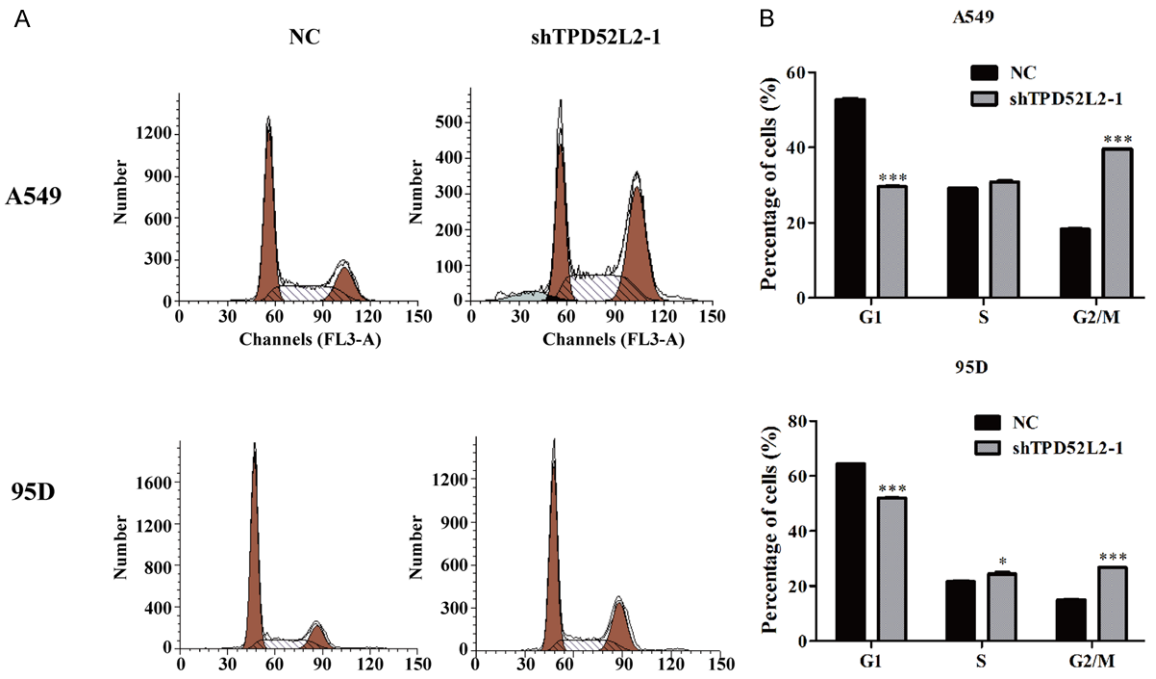

Figure 4. Knockdown of TPD52L2 induced cell cycle G2/M phase arrest. A: The representative images showed different cell cycle distribution profiles in A549 and 95D cell following TPD2L2 knockdown. B: Statistical analysis of the percentage of cells in G0/G1, S and G2/M phase in NC, shTPD52L2-1 and shTPD52L2-2-treated A549 and 95D cells. Data were expressed as mean ± standard deviation (SD) of three independent experiments performed in triplicate. **P*<0.05; ****P*<0.001 versus NC.

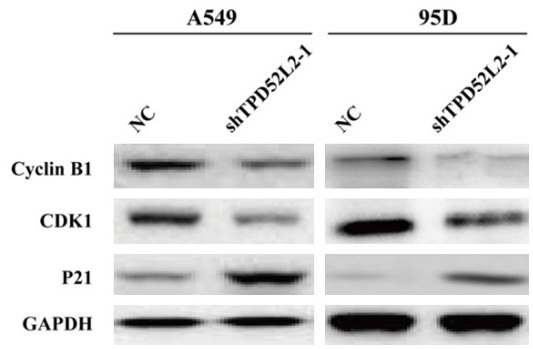

Figure 5. Knockdown of TPD52L2 altered protein levels of cell cycle regulators. Western blot analysis of CDK1, Cyclin B1 and p21 expression in A549 and 95D cells from different treatments (NC, shTPD52L2-1 and shTPD52L2-2). GAPDH was used as internal control.

level of TPD52L2. Then, the effect of TPD52L2 knockdown on cellular functions of A549 and 95D cells was explored. MTT assay results showed that A549 and 95D cells were observed to have dampened cell proliferation and impaired colony formation infected with TPD52-L2-targeted shRNA. As we known, the accurate cell division cycle is essential for successful cell proliferation. One of the control cell division cycle mechanism was range from changes in protein activity (for example, the modifications of CDK1 and Cyclin B1 phosphorylation status) to changes in protein stability or localization [30]. We detected that the effect of TPD52L2 knockdown on cell cyclein A549 and 95D cells using flow cytometry with PI staining. Results showed that suppressed TPD52L2 expression in A549 and 95D led to G1 phase cell cycle arrest and decreased percentage of cells in S phase and G2/M phase. We also detected that the protein levels of CDK1 and Cyclin B1, associated with G2-M transition, were downregulated in the shTPD52L2-1-treated A549 and 95D cells. What more, p21, as cell cycle regulation inhibitor, was obviously upregulated in shTPD52L2-1-treated A549 and 95D cells. The same as TPD52L1 [21], TPD52L2 promoted tumor cell proliferation via cell cycle regulation. Importantly though, our results in vitro are consistent with previous clinical data as mentioned above.

Conclusions

This study demonstrated that the Lv-shTPD-52L2 was successfully established to knockdown TPD52L2 expression in A549 and 95D

cells and this exerted anti-proliferative effect and caused cell cycle arrest in G0/G1 phase. Although further studies are needed, results from this study already suggest that TPD52L2 knockdown may a potential therapeutic approach in NSCLC particularly in highly metastatic NSCLC treatment and may help to better understand NSCLC progression or be used with other conventional chemotherapeutics to increase therapeutic efficacy.

Disclosure of conflict of interest

None.

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