Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (1): 39-44
Published online May 11, 2014 (http://www.cellmolbiol.com)
Received on January 14, 2014, Accepted on May 1, 2014.
doi : 10.14715/cmb/2014.60.1.7

Lentivirus-Mediated Knockdown of Tumor Protein D52-like 2 Inhibits Glioma Cell Proliferation

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Abstract

TPD52L2 (tumor protein D52–like 2) is a member of TPD52 family which has been implicated in multiple human cancers. Recently, TPD52 protein was shown to be associated with several malignancies, but very little is known about the function of TPD52L2 in cancers, especially in glioma to date, and its roles in glioma occurrence and progression remain to be elucidated. In the present study, we employed lentivirus-mediated RNA interference (RNAi) to knock down TPD52L2 expression in human glioma cell line U251. We found that knockdown of TPD52L2 significantly not only inhibited cell proliferation and colony formation, but also induced G0/G1 cell cycle arrest in vitro. Taken together, these findings suggest that TPD52L2 might play an important role in glioma tumorigenesis.

Key words: TPD52L2, glioma, RNA interference, proliferation.

Introduction

Malignant glioma is the most devastating brain tumor in adults with the incidence from five to ten per 100000 general populations (1). It commonly produces profound and progressive disability and leads to death in most cases (1). Despite significant advances in neurosurgery, radiotherapy, and chemotherapy, the prognosis of malignant glioma remains dismal, with an estimated median survival rate of <1 year approaching zero (2). With the emergence of biologically target-based therapies, it is increasingly important to identify the pathogenic molecules and (or) signal transduction pathways that regulate the tumorigenesis and malignant development, and further to decipher new therapeutic targets and develop novel targeted treatments to battle this deadly disease (3).

Tumor protein D52-like proteins are small hydrophilic proteins including the coiled-coil motif of approximately 50 amino acids which is required for multimer formation and heterologous interaction with other proteins, and N- and C-terminally located PEST sequences, which have been linked with regulating protein stability (4). TPD52 is a founding member of the family found to be overexpressed in several human cancers, including prostate cancer (5), breast cancer (6), lung cancer (6), colon cancer (7), ovary cancer (8) and melanoma (9). Increased TPD52 expression has been found to increase anchorage-independent colony formation, cell proliferation and tumor migration/invasion (10, 11). TPD52L2 (tumor protein D52-like 2) is another member of human TPD52-like family consisting of four members, hD52 (TPD52, PrLZ), hD53 (TPD52L1), hD54 (TPD52L2), hD55 (TPD52L3/NYD-SP25) (4). TPD52L2 expression has been reported to have prognostic significance in both breast and pancreatic carcinoma (12), and to be increased in colon cancer (13). However, the expression and function of TPD52L2 in glioma tumorigenesis remain unclear. Recently, TPD52L2 was also shown to mediate the positive regulation of hABCF3 on cell proliferation of human liver cancer cell lines in vitro through the interaction with hABCF3 (14). As it shares functional characteristics with TPD52, TPD52L2 may have similar roles in enhancing cancer-associated phenotypes such as proliferation and anchorage-independent growth. Therefore, it is necessary to further explore the role of TPD52L2 in cell growth.

In this study, we have investigated the effect of TPD52L2 knockdown on cell proliferation, colony formation and cell cycle progression through lentivirus-mediated RNA interference (RNAi) technology in glioma cells. We found that the downregulation of TPD52L2 by short hairpin RNA (shRNA) significantly decreased the viability and proliferation of U251 glioma cells in vitro. Furthermore, flow cytometry analysis showed that TPD52L2 was involved in cell cycle control. In conclusion, our data firstly indicate that TPD52L2 may play an important role in glioma pathogenesis.

Materials and methods

Cell culture

Human glioma cell line U251 was obtained from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C.

Construction of recombinant lentivirus and gene silencing

The shRNA sequence(5'-GCGGAGGGTTTGAAA-

GAATATCTCGAGATATTCTTTCAAACCCTC-CGCTTTTTT-3') was selected for human TPD52L2 (NM_199360) after screening to validate potential shRNA and it was inserted into the lentiviral expression vector pFH-L (Shanghai Hollybio, China) which contains a green fluorescent protein (GFP) gene as a reporter gene. Non-silencing siRNA (5'- TTCTCC-GAACGTGTCACGT-3') was used as negative control. To rule out the possible off-target effects of shRNA, another shRNA sequence(5'-CTCTACAAGAAGACT-CAGGAACTCGAGTTCCTGAGTCTTCTTGTA-GAGTTTTTT-3') was used to get comparable results. Recombinant lentivirus was generated by triple transfection of 80% confluent U251 cells with modified pFH-L vector and packing plasmids pVSVG-I and pCMVΔR8.92 (Shanghai Hollybio, China) using Lipofactinmine 2000 (Invitrogen) according to the manufacturer's recommendation. U251 cells (50000 cells per well) were seeded into 6-well plates and transfected with TPD52L2 shRNA (Lv-shTPD52L2), TPD52L2 shRNA 2 (Lv-shTPD52L2-s2) or control shRNA (Lv-shCon) expressing lentivirus at a multiplicity of infection (MOI) of 10.

RNA extraction and real-time quantitative PCR

Total RNA was isolated from U251 cells after 5 days infection using Trizol reagent (Gibco, USA) according to the manufacturer's instruction. Five μg total isolated RNA was used to synthesize the first strand of cDNA using SuperScript II RT 200 U/ml (Invitrogen, USA). TPD52L2 mRNA expression was evaluated by RT-qPCR on the BioRad Connet Real-Time PCR platform with SYBR Green PCR core reagents. β-actin was used as the internal reference control. The PCR primers were as following: TPD52L2-F: TTCACAGGCAG-GACAGAAGA, TPD52L2-R: TTGAAGGTCGCA-GAGTTCCT; β-actin-F: GTGGACATCCGCAAA-GAC, β-actin-R: AAAGGGTGTAACGCAACTA. Data were analyzed using $2^{-\Delta\Delta Ct}$ method. Results were presented as C_T values, which were defined as the threshold PCR cycle number at which an amplified product is first detected. The average C_T was calculated for both TPD52L2 and β-actin, and ΔC_T was determined as the ratio of the mean of the triplicate C_T values for TPD52L2 to the mean of the triplicate C_T values for β-actin.

Western blot analysis

U251 cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in 2X SDS sample buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS, 10% Glycerol) for 1 h at 4°C. The lysates were clarified by centrifugation at 13,000 × g for 30 min at 4°C and the supernatants were employed for further analysis. The total protein concentration was estimated using BCA protein assay kit. Protein samples (30 μg) were loaded and electrophoresed in a 10% SDS-PAGE at 50 V for 3 hours, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) at 300 mA for 1.5 h. After being blocked with Tris buffered saline Tween-20 (TBST) (20 mM Tris (pH7.6), 150 mM NaCl, 0.01% Tween-20) containing 5% nonfat dry milk for 1 hour at room temperature, membranes were probed with primary antibodies, goat anti-TPD52L2 (1: 500 dilution, Sigma, St. Louis, MO, USA) and rabbit anti-GAPDH (1: 60000 dilution, Proteintech Group, Inc) overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The membranes were analyzed using super ECL detection reagent (Applygen, Beijing, China).

MTT cell viability assay

Briefly, U251 cells from different groups (Lv-shTPD52L2, Lv-shCon) were seeded in 96-well plates at a density of 2000 cells/well. At indicated time points, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added into each well at a final concentration of 5 mg/ml for 4 h. Acidic isopropanol (10% SDS, 5% isopropanol and 0.01mol/L HCl) was then added to stop the reaction and measured with an ELISA reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 595 nm. Viability of cells was calculated from theoretical absorbance.

Colony formation assay

In order to assay monolayer colony formation, stably transfected U251 cells from different groups (Lv-shTPD52L2, Lv-shCon) after 96 h infection were plated in 6-well plates at a density of 500 cells per well. After culture for 9 days, cells were fixed with methanol and stained with crystals purple. The number of colonies was counted.

Cell Cycle Analysis

Cell cycle analysis was determined by Propidium iodide (PI) staining method using flow cytometry (FACS Calibur, BD Biosciences). Briefly, after 3 days of infection, U251 cells were reseeded in 6-cm dishes (200000 cells per dish) and cultured for 40 h at 37°C, respectively. After trypsinization treatment, U521 cells in each well were harvested followed by washing with ice-cold PBS and fixing with 70% cold alcohol. After centrifugation, cells were resuspended in PI/RNase/PBS (100 μg/mL propidium iodide and 10 μg/mL RNase A) solution and incubated in dark for 30 min at room temperature. The suspension was filtered through a 50-mm nylon mesh, and the DNA content of stained nuclei was analyzed by PI staining. PI uptake was analyzed by fluorescence activated cell sorting on flow cytometer (FACS Calibur, BD Biosciences). Tests were performed in triplicate for each sample.

Statistical Analysis

All statistical analyses were performed using SPSS13.0 software. The differences between groups were compared using Student's *t*-test, and data was expressed as mean ± SD of three independent experiments. Statistically significant difference was accepted at $p<0.05$.

Results

Lentivirus-mediated RNAi inhibits TPD52L2 expression in glioma cells

As TPD52L2 protein is aberrant to express in glioblastoma (GBM) identified by the exon expression array (15), we sought to determine the functional role of TPD52L2 knockdown in glioma. Firstly, shRNA

targetting *TPD52L2* gene was inserted into pFH-L to generate the recombinant lentivirus. Efficiency of lentivirus infection was quite high as measured by GFP expression (Figure 1A). TPD52L2 mRNA expression was then measured with RT-qPCR. As shown in Figure 1B, endogenous TPD52L2 mRNA was significantly reduced in Lv-shTPD52L2 group with 87.3% of inhibition rate, compared to Lv-shCon group ($p<0.01$). Western blot analysis was used to further confirm the suppression rate of TPD52L2 shRNA. TPD52L2 protein level was significantly downregulated following TPD52L2 knockdown treatment (Figure 1C). Similarly, the expression of TPD52L2 in U251 cells infected with Lv-shTPD52L2-s2 was markedly decreased by 77.5% compared with control cells ($p<0.01$, Figure S1A). Therefore, TPD52L2 expression was successfully knocked down by lentivirus-mediated RNAi in glioma cells.

TPD52L2 knockdown inhibits glioma cell proliferation and colony formation

To elucidate the functional role of TPD52L2 in glioma tumorigenesis, we examined whether the downregulation of TPD52L2 influenced the malignant phenotype of U251 cells. Cell proliferation rate was first examined in the lentivirus infected U251 cells by MTT assay. It was shown that the proliferation rate of TPD52L2 knockdown cells after 4 and 5 days was significantly lower than that of control cells, respectively (0.436 ± 0.015 vs. 0.762 ± 0.030, $p<0.001$; 0.569 ± 0.012 vs. 0.891 ± 0.077, $p<0.001$), suggesting the efficient inhibition of cell proliferation in Lv-shTPD52L2 transfected U251 cells (Figure 2A). Similarly, the proliferation rate of U251 cells was reduced by 36.4% after Lv-shTPD52L2-s2 infection ($p<0.001$, Figure S1B).

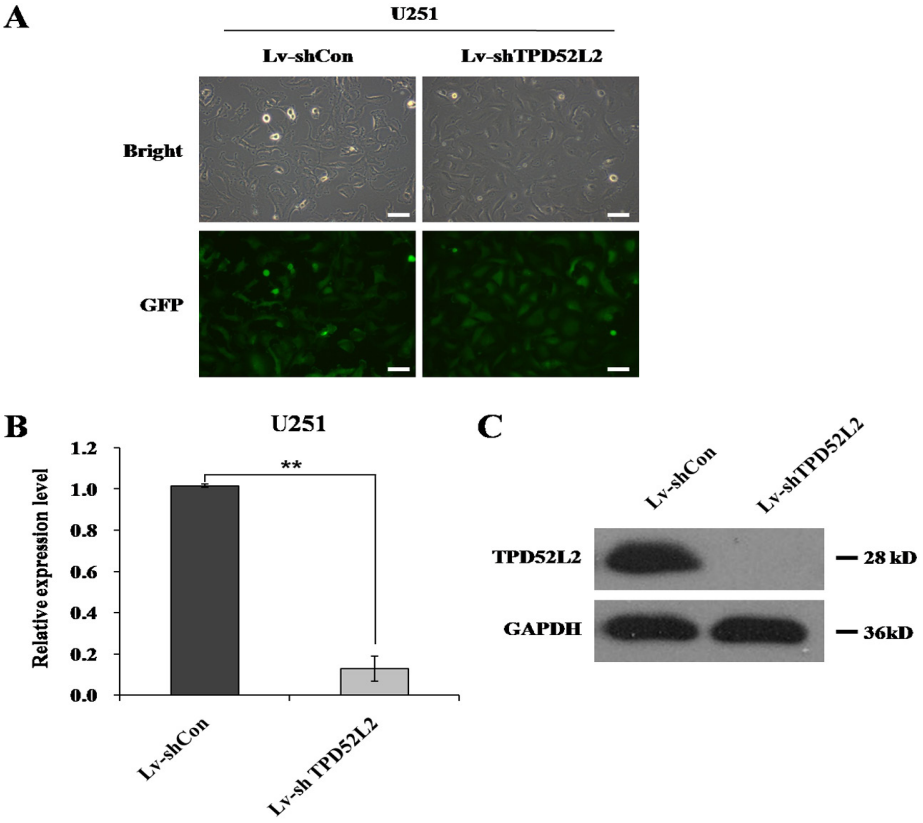

Figure 1. Knockdown of TPD52L2 expression through lentivirus-mediated RNAi. (A) Representative graphs of U251 cells infected with indicated lentivirus at MOI of 10 were shown. Efficiency of lentivirus infection was reflected from green fluorescence of GFP. Scale bar: 50 μm. (B) RT-qPCR analysis of TPD52L2 mRNA level in U251 cells after Lv-shTPD52L2 infection. (C) Western blot analysis of TPD52L2 protein level in U251 cells after Lv-shTPD52L2 infection. **$p<0.01$, compared to Lv-shCon.

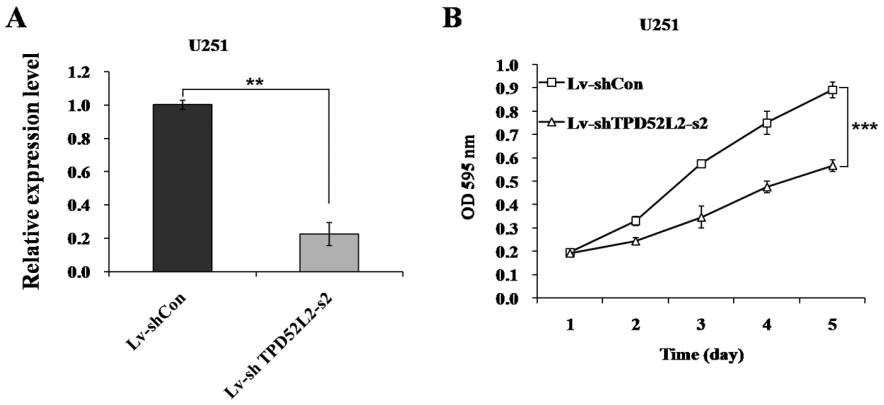

Figure S1. Off-target exclusion assay of TPD52L2 using Lv-shTPD52L2-s2 in U251 cells. (A) RT-qPCR analysis of TPD52L2 mRNA level in U251 cells after Lv-shTPD52L2-s2 infection. (B) Growth curves of U251 cells in Lv-shTPD52L2-s2 and Lv-shCon groups measured by MTT assay. **$p<0.01$, ***$p<0.001$, compared to Lv-shCon.

Furthermore, the effect of TPD52L2 knockdown on the growth of U251 cells was tested by colony formation assay. As shown in Figure 2B, Lv-shTPD52L2 could strongly suppress both the size of single colony and the number of colonies formed in U251 cells. The colonies numbers of TPD52L2 knockdown cells in monolayer culture were significantly less than those of control cells (16.0±1.0 vs. 56.7±3.2, p<0.001) (Figure 2C). These data strongly suggest that TPD52L2 expression is indispensable to the growth of U251 cells. Downregulation of TPD52L2 is detrimental to the cell proliferation and colony formation of glioma cells, indicating an exciting finding of a potential pathogenic target of glioma.

Downregulation of TPD52L2 induces the cell cycle arrest of U251 cells

Given that the dysregulation of cell cycle progression is strongly related with the abnormal cell proliferation, we investigated the cell cycle progression to further explore the mechanisms underlying the inhibition of cell growth by TPD52L2 knockdown. The cell cycle distribution of U251 cells after TPD52L2 knockdown was analyzed through FACS assay. As shown in Figure 3A and B, about 61.25% of TPD52L2 knockdown cells were in G0/G1 phase compared to 50.26% of control cells (p<0.01), whereas 16.65% of TPD52L2 knockdown cells were in S phase compared to 24.35% of control cells (p<0.01). Additionally, the percentage of Lv-shTPD52L2 infected cells distributed in G2/M phase was also lower than that of control cells (22.10% vs. 25.38%, p<0.01). The above evidence demonstrated that cell cycle was blocked in G0/G1 phase in U251 cells after TPD52L2 knockdown.

Discussion

TPD52L2 is a member of TPD52-like protein family. Although the function of TPD52L2 has not been reported previously, TPD52, the first member of this family, was much described and overexpression of such protein

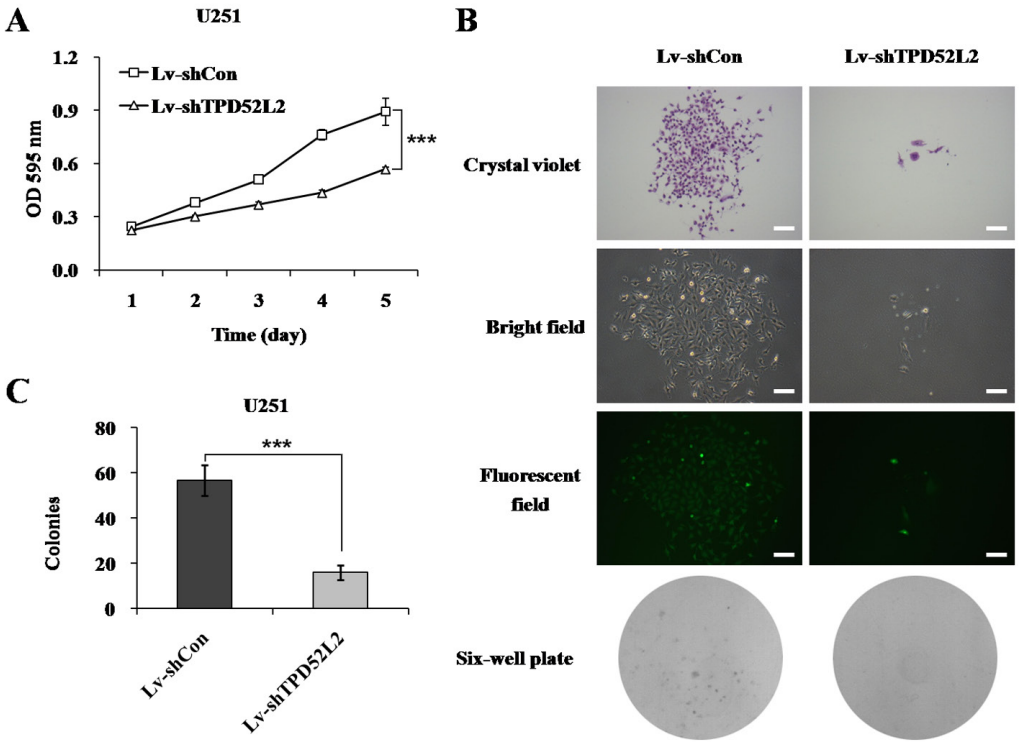

Figure 2. Effects of TPD52L2 knockdown on U251 cell proliferation and colony formation. (A) Growth curves of U251 cells in Lv-shTPD52L2 and Lv-shCon groups measured by MTT assay. (B) Representative photographs of single colony and total colonies in plates were shown. Scale bar: 125 μm. (C) Statistical analysis of the average number of colonies in each plate. ***P<0.001, compared to Lv-shCon.

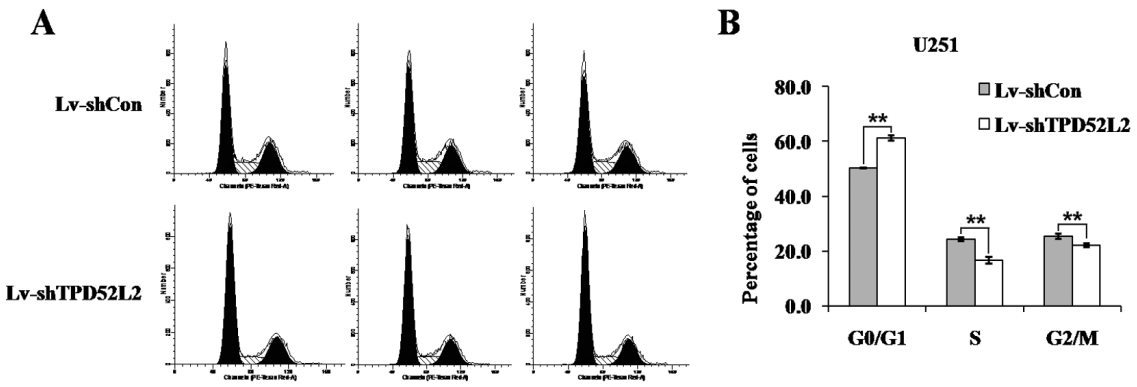

Figure 3. Downregulation of TPD52L2 induces cell cycle arrest. (A) Cell cycle distribution of U251 cells was analyzed by FACS. (B) The percentage of U251 cells in G0/G1 phase was significantly more in Lv-shTPD52L2 group that that in Lv-shCon group. **p<0.01, compared to Lv-shCon.

has been revealed in many kinds of human cancers via in situ hybridisation, northern blot, immunohistochemical analysis or expression microarray studies (6, 8, 16). Clinically, TPD52 overexpression has been shown to be associated with poor prognosis for patients with breast cancer or prostate cancer (17, 18). Moreover, exogenous expression of TPD52 isoforms in various cell lines has been shown to lead to increases in anchorage-independent colony formation, cell proliferation, metastatic ability post-inoculation, tumor volume post-inoculation and migration/invasion rate (5, 10, 19). These evidence strongly supported that TPD52 overexpression may contribute to the tumorigenesis through promoting the cell proliferation, growth and migration, and these malignant phenotype may be associated with the establishment of cell subcellular functions of TPD52 protein, such as activation of Akt/protein kinase B and androgen signaling, regulation of transforming growth factor (TGF)-βR1 (receptor) expression and TGF-β1 secretion, and plasma membrane-based exocytic and endocytic functions and protein trafficking (10, 11, 19). Similar to TPD52, TPD52L1 (D53), a TPD52-like protein, is also positively regulated by estradiol in breast carcinoma cells (20). Moreover, it is maximally expressed in early prophase breast carcinoma cells and rapidly reduced thereafter, in parallel with cyclin B1, suggesting that it acts as a cell cycle regulated protein whose deregulated expression can adversely affect the completion of mitosis (21). Additionally, TPD52L1 was shown to promote the ASK1-induced apoptosis by activating caspase signaling in mammalian cells (22). Taken together, these reports suggest that TPD52 family members may serve as novel markers of malignant tumors, and that these proteins may play crucial roles in human cancers. Future studies are necessary to further investigate the precise function of TPD52-like proteins beyond TPD52 in tumorgenesis.

TPD52-like sequences share about 50% nucleotide sequence homology (23), indicating that TPD52-like proteins shares functional characteristics with TPD52 (4, 24). Indeed, TPD52L2 has been shown to possess similar function in enhancing cancer-associated phenotypes such as proliferation and anchorage-independent growth. TPD52L2 interacted with ABCF3 to regulate the cell proliferation of human liver cancer cell lines in vitro (14). In addition, Yoshiki Mukudai recently revealed that TPD52L2 affects oral squamous cell carcinoma (OSCC) cell attachment to the extracellular-matrix (ECM), OSCC cell migration, and Akt/PKB activation by modulating integrin activation via a talin1-mediated inside-out signal of the ECM (25). Consistent with above the tumorigenesis potential of TPD52L2 at molecular levels, the protein has been reported to have prognostic significance in both breast and pancreatic carcinoma (26). However, the differential upregulation of TPD52-like genes expression in human cancer may also indicate critical functional differences between TPD52-like proteins (4). Here we hypothesized that there may be functional complementation in TPD52 family.

In spite of these findings, however, as yet little is known about the direct effects of the TPD52L2 on the mechanisms underlying cell growth, invasion and metastasis, particularly in glioma. In the present study, downregulation of TPD52L2 by lentivirus-mediated RNAi was found to significantly attenuate the transformed phenotype of glioma cells, such as cell proliferation and colony formation. Given that the decreased surviving colony of TPD52L2 knockdown cells may also result from elevated apoptosis, therefore, future work is necessary to perform apoptosis analysis on signaling molecular levels in U251 cells. On the other hand, TPD52L2 knockdown induced the cell cycle arrest in G0/G1 phase, suggested that TPD52L2 may act as a novel G0/G1 phase-related cyclin to regulate the cell cycle. Additional work should focus on the identification of the interacting partner of TPD52L2 in the G0/G1 phase of U251 cells.

In summary, our results firstly demonstrated that downregulation of TPD52L2 inhibited cell proliferation and colony formation, as well as cell cycle progression in human glioma cells. TPD52L2 may function as an oncogene in glioma and it may represent a novel therapeutic target for glioma treatment.

Acknowledgements

The authors are thankful for the financial support from the National Natural Science Foundation of China (No. 81101909, No. 81001016).

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