Int J Clin Exp Pathol 2016;9(12):12228-12239
www.ijcep.com /ISSN:1936-2625/IJCEP0037998

Original Article

PFTK1 promotes the progression of non-small cell lung cancer (NSCLC) through the Wnt/β-catenin signaling pathway

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Received July 17, 2016; Accepted October 10, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: PFTK1, as a Cdc-related serine/threonine protein kinase, is a crucial regulator of cyclins and cell cycle. However, the role of PFTK1 in non-small cell lung cancer (NSCLC) remains elusive. In the present study, Western blot and immunohistochemistry (IHC) assays demonstrated that PFTK1 was up-regulated in lung cancer tissues and cell lines. In addition, IHC analysis revealed that PFTK1 expression was associated with histological differentiation, lymph node status, TNM stage and predicted poor prognosis of NSCLC. In vitro studies using serum starvation-refeeding experiment and PFTK1 overexpression assay demonstrated that PFTK1 expression promoted proliferation of NSCLC cells, while PFTK1 knockdown assay showed that PFTK1 knockdown led to decreased cell growth rate. Besides, the transcription activity of Wnt/β-catenin pathway was enhanced when PFTK1 was overexpressed. Our finding supported that up-regulated PFTK1 might promote the progression of NSCLC via activating the Wnt/β-catenin signaling pathway.

Keywords: PFTK1, NSCLC, proliferation, Wnt

Introduction

Lung cancer is the leading cause of cancer-related death in developed countries [7] and is becoming a common malignancy in developing countries because of air pollution and a high cigarette smoking ratio, such as in China. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of all lung cancer occurrences [2]. Despite advances in early detection and standard treatment, the 5-year survival rate of patients suffering from NSCLC is merely 15%, which largely due to the fact that most of them are diagnosed at advanced stage [3]. Complete understanding of the molecular mechanism of NSCLC will contribute to the development of diagnostic technologies and new treatment methods.

PFTK1 is a member of Cdc2-related serine/threonine protein kinases, also named as PFTAIRE1, which share a highly conserved motif PSTAIRE and are crucial regulators of cyclins and cell cycle [33]. The gene of PFTAIRE1 is located at human chromosome 7q21.13, which is highly expressed in the brain, pancreas, kidney, heart, testis, and ovary and minimally expressed in the spleen and thymus. The function of PFTK1 was recently reported as a cyclin-dependent kinase (CDK), regulating cell cycle progression and cell proliferation by specifically interacting with members of cyclin proteins such as cyclin D3 (CCND3) and cyclin Y (CCNY) [14]. CCNY played an important role in *Drosophila* embryogenesis [15] and was associated with human NSCLC proliferation and tumorigenesis [34]. The interaction between PFTK1 and CCNY was reported to corporately mediate

Table 1. Relationship between PFTK1 expression and clinicopathological factors in 140 lung adenocarcinoma patients

Parameters	Total	PFTK1 expression		P
		Low	High	
Age (year)				0.197
< 60	61	32	29	
≥ 60	79	50	29	
Gender				0.13
Male	69	36	33	
Female	71	46	25	
Tumor size (cm)				0.106
< 3	62	41	21	
≥ 3	78	41	37	
Smoking status				0.069
Yes	20	8	12	
No	120	74	46	
Histological differentiation				0.025*
Poor	17	6	11	
Mod	94	54	40	
Well	29	22	7	
Lymph node status				0.001*
0	86	63	23	
> 0	54	19	35	
TNM stage				0.004*
I	92	64	28	
II	30	12	18	
III	15	5	10	
IV	3	1	2	
Ki67 expression				0.001*
Low	81	65	16	
High	59	17	42	

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

phosphorylation of low density lipoprotein receptor-related protein 6 (LRP6) in *Drosophila* [6]. It was well known that phosphorylation of transmembrane receptor LRP6 represented an initial step of the canonical Wnt signaling cascade [4, 31, 35]. It was reported that PFTK1-CCNY complex activated non-canonical Wnt signaling in hepatocellular carcinoma [26] and PFTK1 played an oncogenic role in promoting the cellular invasion and motility of HCC cells [20]. In addition, PFTK1 promoted tumor cell proliferation, migration and invasion in breast cancer [9]. However, whether PFTK1 affecting the progression of NSCLC remained unclear.

The Wnt pathway functions in multiple cellular biological processes and is involved in various human diseases including cancer [4]. The Wnt signaling is divided into two branches: the canonical β-catenin-dependent pathway and the non-canonical planar cell polarity (PCP) and Wnt/Ca^{2+} pathway [16]. The Wnt/β-catenin pathway is activated when a Wnt ligand binds to Frizzled (Fz) receptor and its co-receptor LRP6/5. The formation of Wnt-Fz-LRP6 complex, together with the recruitment of the scaffolding protein dishevelled (Dvl), results in LRP6 phosphorylation and activation and the recruitment of the Axin complex to the receptors [18]. Accumulated β-catenin in the cytoplasm travels to the nucleus and forms complexes with T cell factor/lymphoid enhancer factor (TCF/LEF), and activates the Wnt target genes including cyclin D1 and c-myc, which results in tumorigenesis [10, 28].

In this study, we found that the expression of PFTK1 was significantly increased in NSCLC cells and surgical tissues by assays of western blotting. We also investigated the association of PFTK1 with clinical and pathological parameters. In addition, PFTK1 expression was changed by small interference RNA to explore its role in the progression of NSCLC cells. Meanwhile, we also detected the role of PFTK1 in the activation of Wnt/β-catenin signaling pathway and its related protein expression, such as β-catenin, c-myc and cyclin D1. Therefore, we hypothesized that PFTK1 could promote the progression of NSCLC cell and would be a potential treatment target of NSCLC.

Materials and methods

Lung cancer tissue specimens and cell lines

Eight pairs of lung cancers and adjacent normal fresh tissues stored at -80°C until were analyzed by Western blot. Then 140 lung adenocarcinoma tissue specimens were obtained from the Department of Pathology, Affiliated Hospital of Nantong University from 2003 to 2008, and clinicopathologic data of these specimens was shown in **Table 1**. Every patient was asked to participate in the study, and the removed tissues for research purposes were obtained from the Ethics Committee of Nantong University Cancer Hospital. No patient was treated with chemotherapy or radiotherapy. The lung cancer cell lines A549, H1299 and SPCA1 were

obtained from the Institute of Cell Biology, Academic of China (Shanghai, China).

Western blot analysis and antibodies

Western blot assay was performed as previously described [17]. Tissue and cell protein were homogenized in a homogenization buffer containing 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, o.1 mM NaF, and complete protease inhibitor cocktail (Roche Diagnostic), and then placed on the ice for 0.5 h after being fully homogenized. Then the sample was centrifuged at 13,000 r for 15 min to collect the supernatant. Protein concentrations were measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The supernatant diluted in 2 × SDS loading buffer and boiled for 15 min. Proteins were separated with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and then transferred to polyvinylidenedifluoride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% non-fat milk for 2 h and then were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) for three times. Then the membranes were incubated with the primary antibodies overnight at 4°C. The next day, the membrane was washed with PBST and then incubated with the secondary antibodies. Immunoreactive bands were visualized by Odyssey Infrared Image. The band density was measured with a computer imaging system (Imaging Technology, Ontario, Canada). And the densities of bands were compared using ImageJ (NIH).

The antibodies used for Western blot analysis were listed below: anti-PFTK1 (1:500, Santa Cruz Biotechnology), anti-PCNA (1:500, Santa Cruz Biotechnology), anti-cyclinA (1:500, Santa Cruz Biotechnology), anti-CDK2 (1:500, Santa Cruz Biotechnology), anti-β-catenin (1:500, Santa Cruz Biotechnology), anti-cyclin D1 (1:500, Santa Cruz Biotechnology), anti-c-myc (1:500, Santa Cruz Biotechnology), and anti-GAPDH (1:3000, Santa Cruz Biotechnology).

Immunohistochemistry (IHC) and statistical analysis

The procedures were carried out similarly to previously described methods [9]. The antibodies used for immunohistochemical staining were as follows: anti-PFTK1 (1:100, Santa Cruz Biotechnology) and anti-Ki-67 (1:100, Santa Cruz Biotechnology). For assessment of PFTK1 and Ki-67, five high-power fields in each specimen were selected randomly, and then cytoplasm and nuclear staining were examined. The degree of immunostaining was viewed and scored separately by 2 independent investigators without knowledge of clinicopathologic data in a blind manner. Tumor cell proportion was scored as follows: 0 (no positive tumor cells); 1 (< 10% positive tumor cells); 2 (10-35% positive tumor cells); 3 (35-70% positive tumor cells) and 4 (> 70% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown) and 3 (strong staining = brown). Staining index (SI) was calculated as the product of staining intensity score and the proportion of positive tumor cells.SI score ≥ 6 was used to define tumors with the high expression of PFTK1, and an SI score ≤ 4 was used to indicate the low expression of PFTK1.

The SPSS18.0 statistical program was used for statistical analysis. The Chi-square test was performed to evaluate the relationship between PFTK1 expression and clinicopathological parameters in lung adenocarcinoma samples. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed by Cox's proportional hazards model, and the risk ratio and its 95%. All results represented three replications at least, and a P value < 0.05 was considered statistically significant. All data were represented as mean ± SEM [13].

Cell culture

The NSCLC cell lines (A549, H1299, SPCA1) were cultured in were cultured in Dulbecco's modified Eagle's medium (DMEM) (GibCo BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) supplemented with 2 mM L-glutamine and 100 U/mL penicillin-streptomycin mixture (GibCo BRL) at 37°C and 5% CO2. The medium was replaced 24 h later with fresh medium for transfection.

Plasmids and shRNA

Expression vectors for PFTK1 contain a full-length PFTK1 cDNA cloned into FLAG-CMV plasmid (Gene Chem Technology). The shRNA-

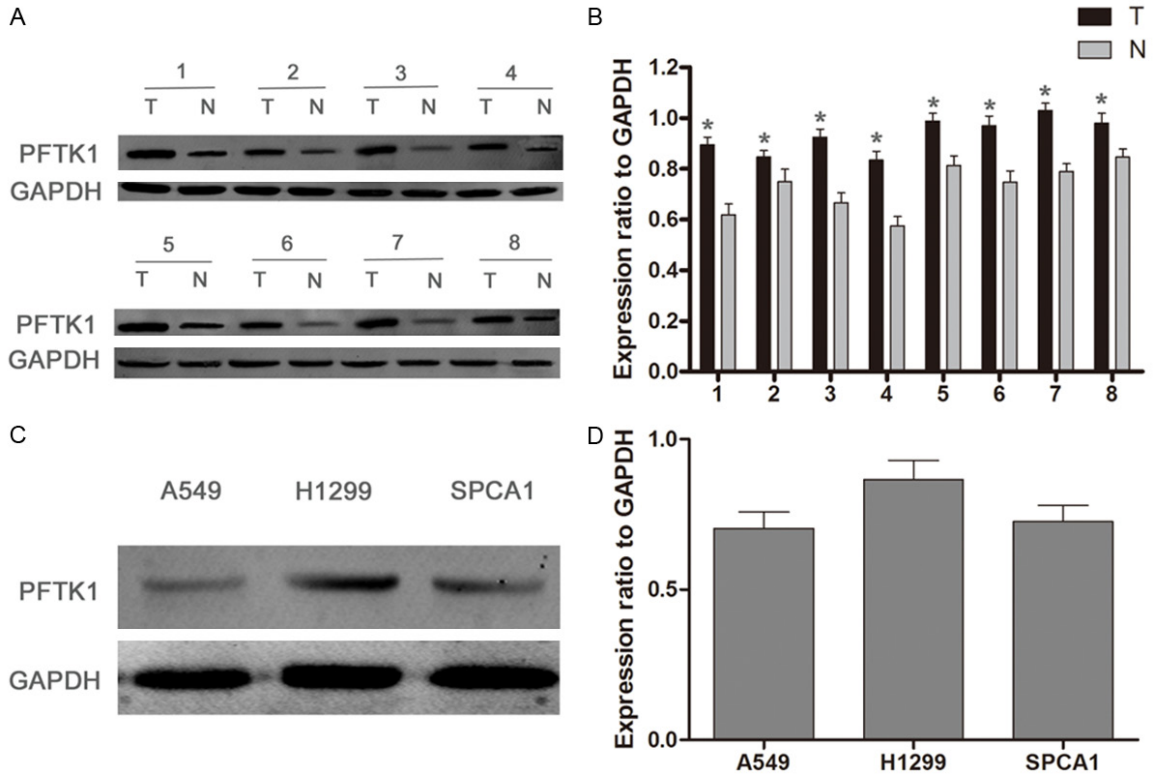

Figure 1. The expression of PFTK1 was upregulated in NSCLC tissues and cell lines. A. The protein level of PFTK1 in eight paired lung tumors (T) and their matched normal tissues (N) were detected by Western blot analysis. B. The bar chart demonstrated the ratio of PFTK1 to GAPDH by densitometry. The data were mean ± SEM (*$P < 0.05$, compared with adjacent normal tissues) of three independent experiments. C. PFTK1 protein in the different NSCLC cell lines was analyzed by Western blot analysis. GAPDH was used as a loading control. D. The bar chart demonstrated the ratio of PFTK1 to GAPDH by densitometry. The data were mean ± SEM of three independent experiments.

targeting PFTK1 sequence was: 5'-ACCCATAC-AGGAAATCC AA-3'. PFTK1 plasmids or shRNA were transfected into H1299 cells mediated by Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacture's protocol. Cells were harvested for Western blot, CCK8 and flow cytometry assays in 48 h after transfection.

Cell proliferation assay

Cell Counting Kit (CCK)-8 was used to detect cell proliferation. Briefly, cells were seeded into 96-well cell culture cluster plates (Corning, Inc., Corning, NY) at a concentration of 2×10^4 cells/well transfected with plasmids or shRNA for 48 h and then grown overnight. CCK-8 reagents (Dojindo, Kumamoto, Japan) were added to each well under different treatments, incubated for an additional 2 h at 37°C, and the absorbency was measured at a wavelength of 490 nm by an automated plate reader. All the data were obtained from three independent experiments.

Flow cytometric analysis

Cell cycle distribution was determined by flow cytometric analysis. Cells were prepared into a single-cell suspension. Cells were fixed in 70% ethanol over night at 4°C, and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Subsequently, cells were stained with 50 mg/ml propidium iodide (PI) in PBS and were analyzed by a FAC Scan flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA). The results were obtained from three independent experiments.

Real-time PCR analysis

Real-time PCR was performed using the Light Cycler 480 system with a 96-well plate. The primers used for real-time PCR were as follow: DKK1, 5'-CCGAGGAGAAATTGAGGAAAC-3'; 5'-TCTTGGACCAGAGTGTCTAGC-3'; Axin2, 5'-GGC-CAGTGAGTTGGTTGTCAC-3' and 5'-CCTTCATAC-ATCGGGAGCACC-3'; β-actin, 5'-AGGCCAACCGC-GAGAAGAT-3' and 5'-TCACCGGAGTCCATCACGA-3'.

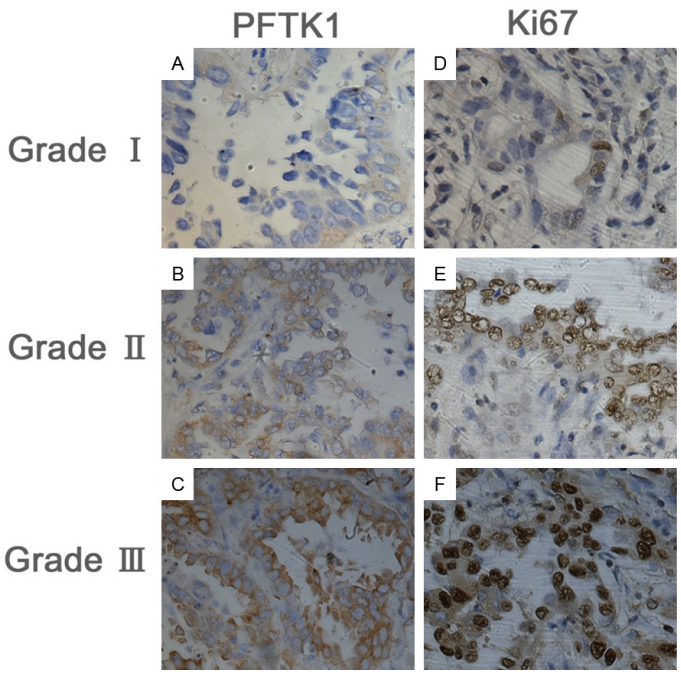

Figure 2. Immunohistochemical examined the expression of PFTK1 and Ki-67 in paraffin-embedded lung adenocarcinoma tissues. A, D. The immuoreactivity of PFTK1 and Ki-67 was low in grade I tissues. B, E. The immuoreactivity of PFTK1 and Ki-67 was moderate in grade II tissues. C, F. The immuoreactivity of PFTK1 and Ki-67 was high in grade III tissues (×400).

Table 2. Contribution of various potential prognostic factors to survival by Cox regression analysis in 140 specimens

	Hazard ratio	95.0% Confidence interval	P
Age	1496.	0.896-2.499	0.123
Gender	0.538	0.307-0.944	0.31
Smoking status	2.499	1.257-4.968	0.19
Tumor size	1.138	0.645-2.006	0.656
Histological differentiation	0.773	0.469-1.272	0.311
Lymph node status	1.040	0.594-1.818	0.892
TNM stage	1.451	1.071-1.967	0.016*
PFTK1 expression	2.707	1.478-4.957	0.001*
Ki-67 expression	55.383	16.732-183.316	< 0.001*

Note: Statistical analyses were performed by the Cox regression analysis. *$P < 0.05$ was considered significant.

Results

Expression of PFTK1 was up-regulated in NSCLC tissues and cell lines

To explore the relationship between PFTK1 and NSCLC, we first examined the expression of PFTK1 in eight paired human lung cancer tissues and non-tumor adjacent normal tissues. Western blot revealed that the protein expression of PFTK1 in lung cancer tissues was higher than the adjacent normal tissues (**Figure 1A**). The bar chart demonstrated the ratio of PFTK1 protein expression to GAPDH for the above by densitometry (**Figure 1B**). Moreover, we also investigated the protein expression of PFTK1 in NSCLC cell lines including A549, H1299 and SPCA1 by Western blot analysis (**Figure 1C**, **1D**). We found that the protein level of PFTK1 in H1299 cell line was higher than the other NSCLC cell lines. These findings showed that the expression of PFTK1 was up-regulated in NSCLC tissues and cells.

Immunohistochemical analysis of PFTK1 expression in NSCLC clinical samples and its relationship to clinicopathological parameters

To confirm the expression of PFTK1 in NSCLC, we investigated the expression of PFTK1 on 140 samples from patients with lung adenocarcinoma by using IHC. Representative examples of reactivity for PFTK1 and Ki-67 were shown in **Figure 2**. PFTK1 was expressed mainly in cytoplasm, whereas Ki-67 was located in the nucleus (**Figure 2**).

The clinicopathological data of patients were summarized in **Table 1**. We evaluated the relationship between PFTK1 and Ki-67 expression with clinicopathological variables. For statistical analysis, the expression of PFTK1 in the carcinoma specimens were divided into 2 groups, namely, high and low expressions, according to the percentage of PFTK1-positive cells and the intensity of PFTK1 cytoplasm staining. The results showed that PFTK1 expression was significantly associated with histological differentiation (P=0.025), lymph node status (P=0.001), TNM stage (P=0.004) and Ki-67 (P=0.001), but there was no correlation between PFTK1 expression and other prognostic factors such as age, gender, tumor size and smoking status.

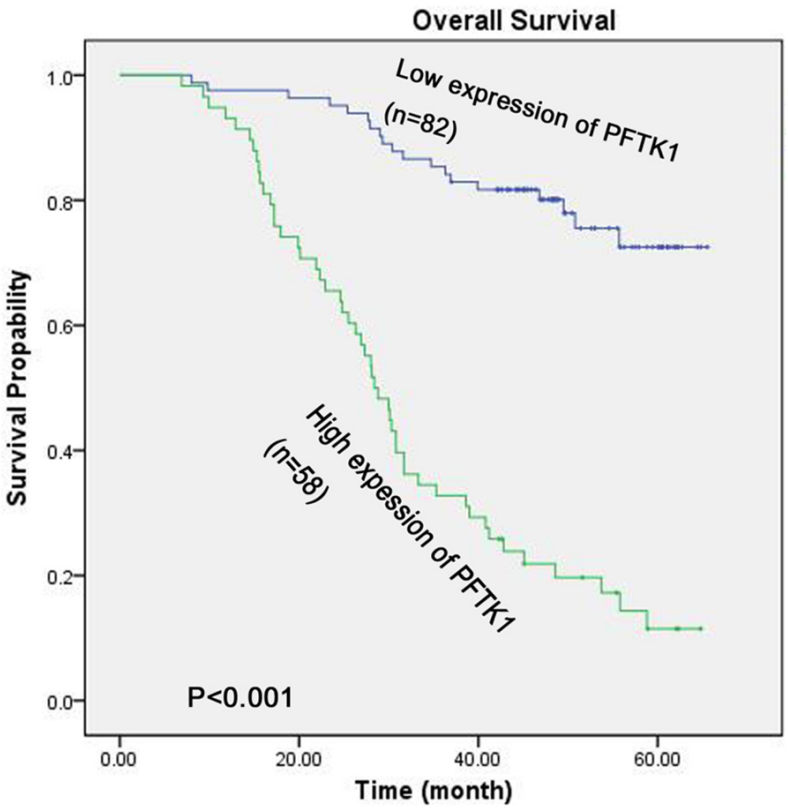

Figure 3. Kaplan-Meier survival analysis indicated that patients with the group of high expression of PFTK1 hand significantly shorter overall survival than the group of low expression of PFTK1.

PFTK1 was significantly associated with the survival of NSCLC patients

Survival analysis was restricted to 140 patients with follow-up data until death. After all variables were compared separately with survival status, we found that TNM stage (P=0.016), PFTK1 expression (P=0.001) and Ki-67 expression (P < 0.001) significantly influenced survival (**Table 2**). Kaplan-Meier analysis was performed to further investigate the prognostic significance of PFTK1 in NSCLC. The result revealed that high expression of PFTK1 was significantly associated with poor overall survival rate in NSCLC (P < 0.001; **Figure 3**). These suggested that PFTK1 might be an independent prognostic factor for overall survival of patients with NSCLC.

PFTK1 expression promoted proliferation of NSCLC cells

Previous study suggested that PFTK1 acted as a cyclin-dependent kinase (CDK) that regulated cell cycle progression and cell proliferation [25], we hypothesized that PFTK1 might promote cell proliferation and cell cycle progression in NSCLC cells. To verify that PFTK1 was involved in cell cycle, we constructed the serum starvation and releasing experiment (**Figure 4**). Flow cytometry showed that H1299 cells were mainly blocked at G0/G1 phase after serum starvation for 72 h. Then we added serum to medium founding cell released from G1 phase and entered the S phase (**Figure 4A**). We detected the expression of PFTK1 of H1299 cells in serum starvation and releasing experiment by using Western blot analysis. We found that the PFTK1 expression was upregulatedupon serum addition (**Figure 4B**, **4C**). Meanwhile, the expression of cyclin A and CDK2 were also up-regulated, which had been used as general markers of dividing cells (**Figure 4B**, **4C**). These results indicated that PFTK1 might have an impact on the proliferation of NSCLC cells.

PFTK1 knockdown inhibited proliferation of NSCLC cells

To further study the potential effects of PFTK1 on NSCLC cell proliferation, we used a small interference RNA (siRNA) to knockdown the expression of PFTK1 in H1299 cells. PFTK1-siRNA and control-siRNA vectors were stably transfected into the H1299 cells. The efficiency of PFTK1 silencing was detected by Western blot analysis (**Figure 5A**, **5B**). As well, the common proliferation maker PCNA [8] and the key cell cycle regulator cyclinA [27] were detected by Western blot analysis. The result indicated that the expression of PCNA and cyclin A were decreased in PFTK1-siRNA, compared with control-siRNA (**Figure 5A**, **5B**). Furthermore, flow cytometry analyses of cell cycle distribution revealed that the number of cells at G0/G1 in PFTK1-siRNA increased from 53.43% to 60.91%, with a concomitant decrease at S

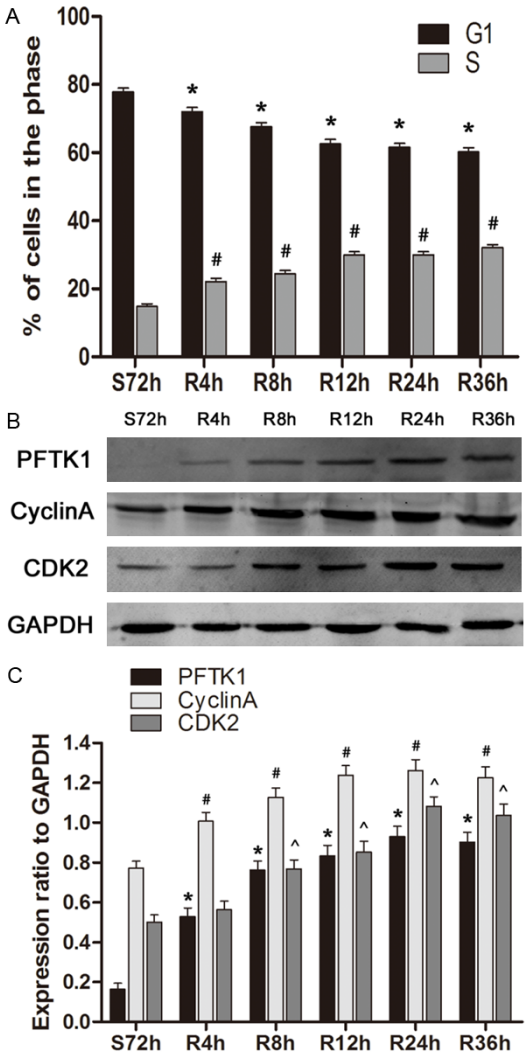

Figure 4. Expression of PFTK1 and cell-cycle related molecules in proliferating H1299 cells. A. The H1299 cells synchronized at G1 progressed into cell cycle when serum was added for 0, 4. 8, 12, 24, 36 h. Data were mean ± SEM of three independent experiments. *, #$P < 0.05$, compared with control cells serum staved for 72 h (72 h). B. Cell lysates were prepared and analyzed by Western blot using antibodies against PFTK1, cyclin A, CDK2 and GAPDH (loading control). C. The bar chart demonstrated the ratio of PFTK1/cyclin A/CDK2 to GAPDH by densitometry. The data were represented as means ± SEM of three independent experiments. *, #, ^$P < 0.05$, compared with control cells serum staved for 72 h (72 h). S, serum starvation; R, serum release; SEM, standard error of the mean.

phase from 32.67% to 19.20%, compared with control-siRNA (**Figure 5C**). We used CCK-8 to test the proliferation of NSCLC cells. The proliferation was dramatically inhibited in PFTK1-siRNA, compared with control-siRNA (**Figure 5D**).

Overexpression of PFTK1 promoted cell proliferation in NSCLC cell line

Based on the above data, we hypothesized that whether overexpression of PFTK1 could promote NSCLC cell proliferation. The H1299 cell line was transfected with Flag-PFTK1 (**Figure 6A**). Consistent with the increased expression of PFTK1, the expression of PCNA and cyclin was increased in Flag-PFTK1 transfected group compared with control one (**Figure 6A**, **6B**). Flow cytometry analysis revealed that overexpression of PFTK1 promoted the transition of cell cycle phase from G0/G1 to S (**Figure 6C**). Furthermore, cell vitality was determined by CCK-8 assay and the data showed that cell proliferation was enhanced due to over-expression of PFTK1 (**Figure 6D**). Taken together, these results indicated that PFTK1 might function as a regulator of cell proliferation in H1299 cells.

PFTK1 enhanced the activity of Wnt/β-catenin signaling in H1299 cells

It has been reported that PFTK1 could activate Wnt signaling in HCC cells [26]. Wnt/β-catenin signaling is well known to promote cell cycle progression and proliferation through transcriptional upregulation of target genes such as those encoding c-myc and cyclin D [5]. To determine whether PFTK1 had a similar effect on Wnt/β-catenin signaling pathway in NSCLC cells, we detected the protein expression of β-catenin, c-myc and cyclinD1 in PFTK1 upregulated H1299 cells by Western blot analysis. Overexpression of PFTK1 also led to increased expression of β-catenin, c-my and cyclinD1 (**Figure 7A**, **7B**). Moreover, Real-time PCR analysis was performed to imply that two inhibitors of Wnt signaling, such as DKK1 and Axin2, were upregulated when PFTK1 was overexpressed (**Figure 7C**).

Discussion

In this study, we found that PFTK1 promoted cell proliferation and progression of cell cycle in H1299 cells via activating the Wnt/β-catenin signaling pathway. We proved that PFTK1 expression was mostly up-regulated in human NSCLC tissues compared with the adjacent normal tissues by using Western blot and IHC analysis. High expression of PFTK1 was significantly correlated with histological differentiation, lymph node status, TNM stage and Ki-

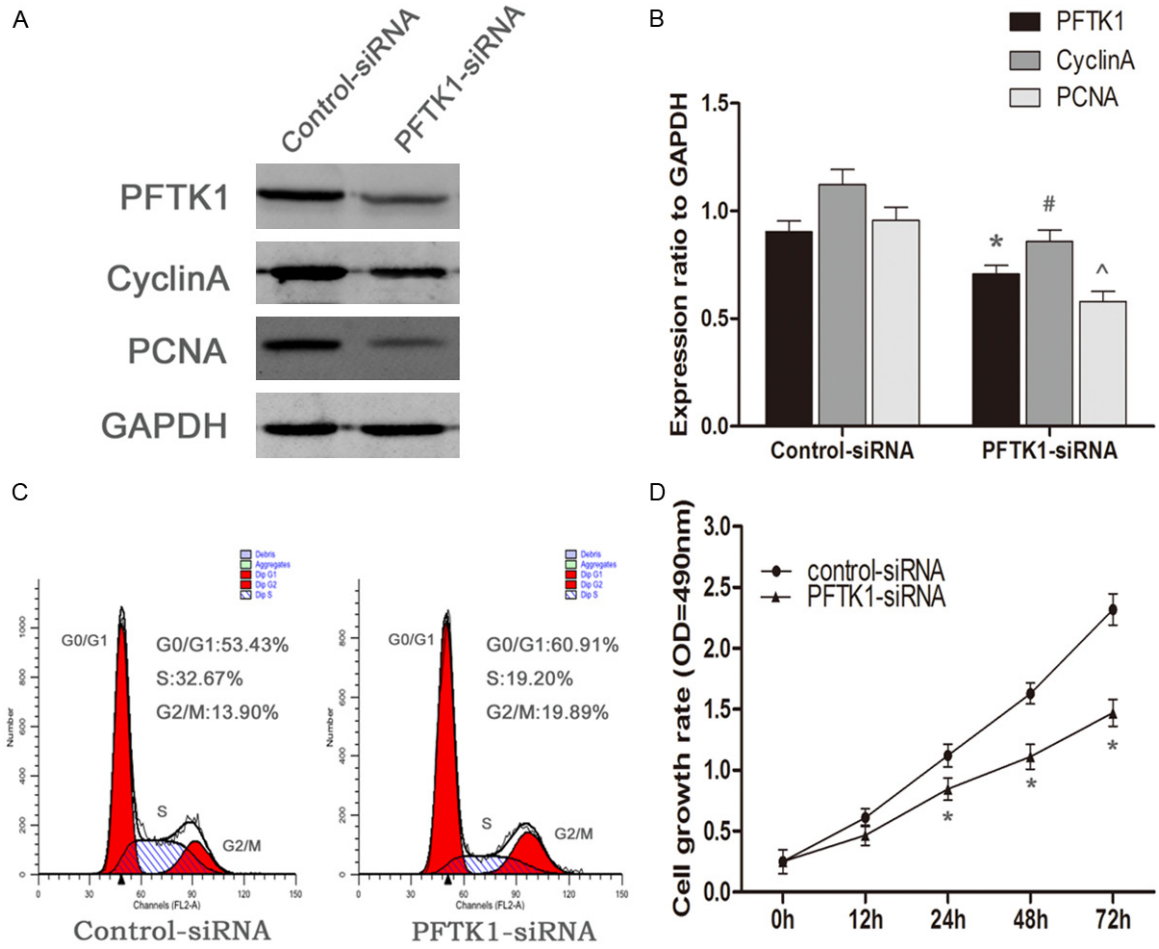

Figure 5. Knockdown of PFTK1 decreased the ability of cell proliferation in H1299 cells. A. H1299 cells were transiently transfected with PFTK1-siRNA or control-siRNA as described above for 48 h. The expression of PFTK1, cyclin A and PCNA were detected by western blot analysis. GAPDH was used as a loading control. B. The bar chart demonstrated the ratio of PFTK1/cyclin A/PCNA to GAPDH by densitometry. The data were mean ± SEM of three independent experiments. (*, #, ^$P < 0.05$, compared with control cells). C. Knockdown of PFTK1 restrained G1/S transition in H1299 cells. The cell cycle distribution of H1299 cells were detected by flow cytometric analysis. D. Effect of the knockdown of PFTK1 on proliferation of H1299 cells was analyzed by CCK-8 assay. The viability of cells transfected with PFTK1-siRNA was inhibited compared with the control cells. The data were mean ± SEM (n=3, *$P < 0.05$, compared with the control cells).

67, a proliferating cell maker [30]. Moreover, the high expression of PFTK1 was obviously correlated with poor prognosis in lung adenocarcinoma patients. These results demonstrated that PFTK1 was an independent prognostic factor for overall survival in patients with NSCLC and might participate in the progression of lung cancer.

PFTK1 was a Cdc 2-related serine/threonine protein kinase [33], while Cdc 2 was identified as the first CDK that was essential for G1/S and G2/M transitions in *Schizosaccharomycespombe* [24]. It was reported functional characterization of human PFTK1 as a CDK. The serum starvation and releasing experiment confirmed that PFTK1 promoted H1299 cells proliferation by regulating the cell cycle (**Figure 4**). Moreover, the knockdown of PFTK1 decreased the ability of H1299 cells proliferation and overexpression of PFTK1 increased the proliferative capability of H1299 cells by controlling the transition of G0/G1 to S phase in cell cycle (**Figures 5**, **6**). G1/S phase transition is a major checkpoint for cell cycle progression [29]. These results suggested that PFTK1 promoted cell proliferation and was involved in cell cycle in NSCLC cells.

Wnt signaling played an important role in tumor development and progression [1, 21]. β-Catenin is a key mediator in the Wnt signaling pathway

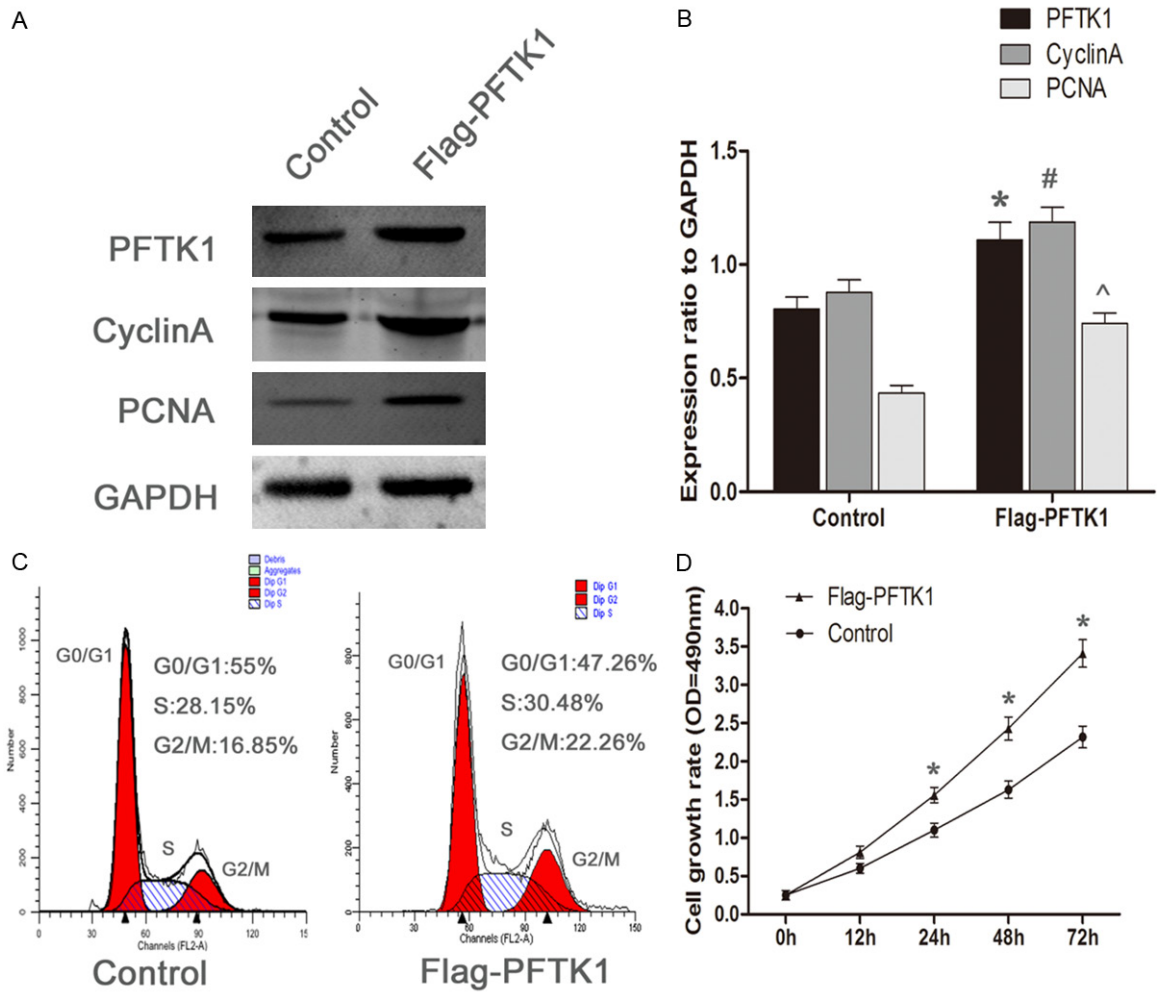

Figure 6. Overexpression of PFTK1 increased the ability of cell proliferation in H1299 cells. A. H1299 cell were transfected with Flag-PFTK1 plasmid or a control plasmid as described above for 48 h. Western blot analysis was performed to detect the expression of PFTK1, cyclin A and PCNA, GAPDH was used as a loading control. B. The bar chart demonstrated the ratio of PFTK1/cyclin A/PCNA to GAPDH by densitometry. The data were mean ± SEM (n=3, *, #, ^$P < 0.05$, compared with control cells). C. The upregulated expression of PFTK1 enhanced G1/S transition in H1200 cells. The cell cycle distribution of H1299 cells were detected by flow cytometric analysis. D. The cell viability was detected by using CCK-8. Up-regulated PFTK1 enhanced the H1299 cells proliferation compared with the control cells. The data were mean ± SEM (n=3, *$P < 0.05$, compared with the control cells).

[32]. When accumulating in the nucleus, β-catenin activated the TCF/LEF complex and the transcription of target genes including cyclin D1, c-myc [22], both of which were important oncogenes. The interaction of PFTK1 with CCNY was reported to corporately mediate phosphorylation of LRP6 [14], which promoted more specifically the Wnt/β-catenin pathway [11]. We found that the overexpression of PFTK1 raised the expression of β-catenin, c-myc and cyclinD1, but the expression of DKK1 and Axin2, as negatively regulators of Wnt signaling, were increased in the overexpression of PFTK1 cells (**Figure 7**). Previous study indicated that TCF/LEF-mediated DKK1 and Axin2 expression would form a feedback loop to prevent hyperactivity of Wnt signaling [12, 19] and DKK1 was highly expressed in a variety of cancers, including lung cancer [23]. Therefore, we speculated that the hyperactivity of Wnt/β-catenin signaling might contribute to the uncontrolled cell growth and tumorigenesis in NSCLC.

In conclusion, we first discovered that PFTK1 promoted cell proliferation by controlling the cell cycle in NSCLC cells and was an independent prognostic factor for overall survival in patients with NSCLC. Meanwhile, our results indicated that PFTK1 participated in the Wnt/

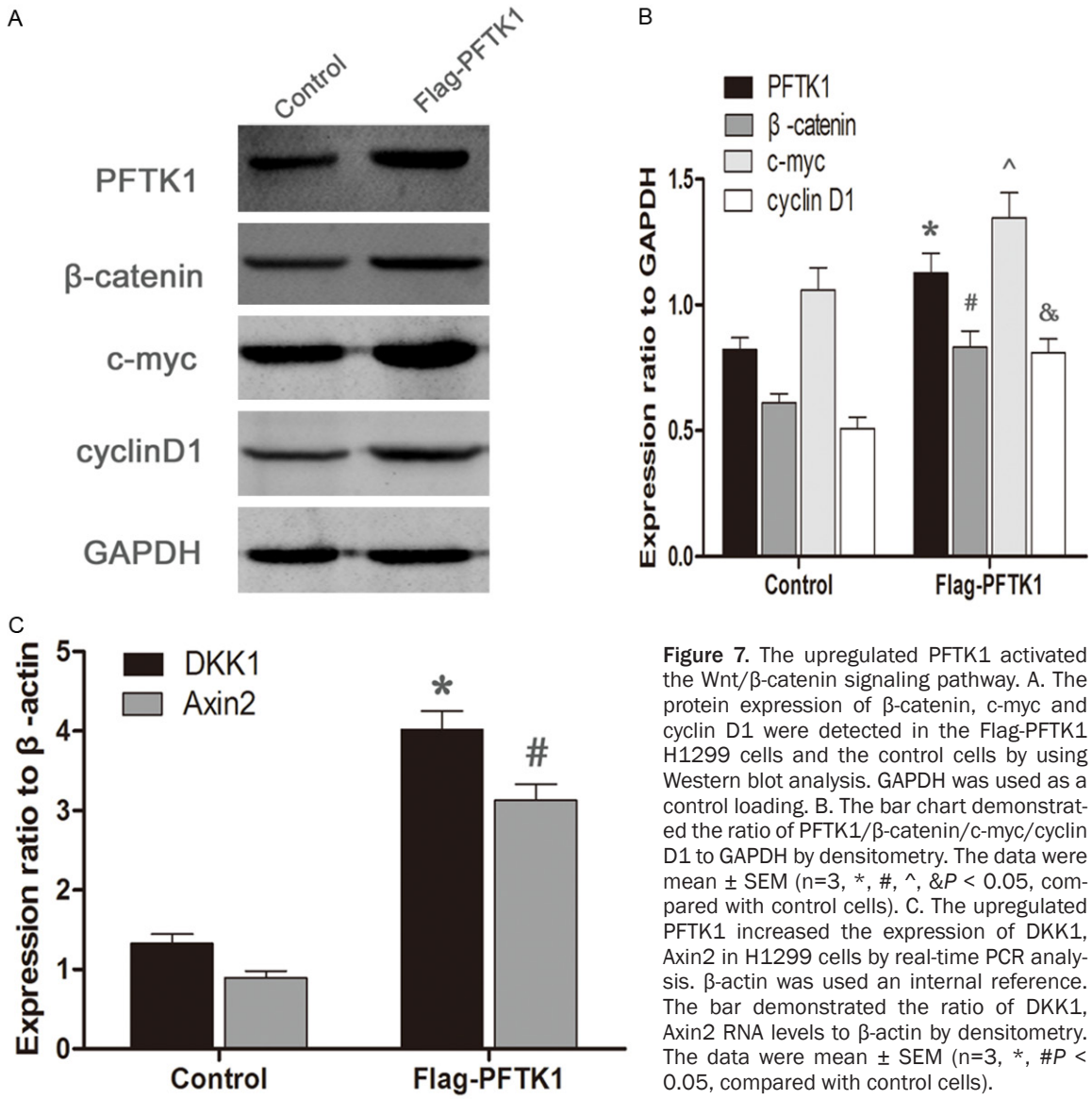

Figure 7. The upregulated PFTK1 activated the Wnt/β-catenin signaling pathway. A. The protein expression of β-catenin, c-myc and cyclin D1 were detected in the Flag-PFTK1 H1299 cells and the control cells by using Western blot analysis. GAPDH was used as a control loading. B. The bar chart demonstrated the ratio of PFTK1/β-catenin/c-myc/cyclin D1 to GAPDH by densitometry. The data were mean ± SEM (n=3, *, #, ^, &$P < 0.05$, compared with control cells). C. The upregulated PFTK1 increased the expression of DKK1, Axin2 in H1299 cells by real-time PCR analysis. β-actin was used an internal reference. The bar demonstrated the ratio of DKK1, Axin2 RNA levels to β-actin by densitometry. The data were mean ± SEM (n=3, *, #$P < 0.05$, compared with control cells).

β-catenin signaling pathway in NSCLC and PFTK1 might through this pathway regulate cell proliferation. This investigation might provide a new strategy for targeting therapy.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 8150-1975), University Science Research Project of Jiangsu Province (15KJB310013), Nantong Science and Technology Project (MS12015103).

Disclosure of conflict of interest

None.

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References

[1] Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000; 103: 311-320.

[2] Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001; 61: 3986-3997.

[3] Cho S, Park TI, Lee EB, Son SA. Poor Prognostic Factors in Surgically Resected Stage I Non-small Cell Lung Cancer: Histopathologic and

Immunohistochemical Analysis. Korean J Thorac Cardiovasc Surg 2012; 45: 101-109.
[4] Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006; 127: 469-480.
[5] Davidson G, Niehrs C. Emerging links between CDK cell cycle regulators and Wnt signaling. Trends Cell Biol 2010; 20: 453-460.
[6] Davidson G, Shen J, Huang YL, Su Y, Karaulanov E, Bartscherer K, Hassler C, Stannek P, Boutros M, Niehrs C. Cell cycle control of wnt receptor activation. Dev Cell 2009; 17: 788-799.
[7] Espey DK, Wu XC, Swan J, Wiggins C, Jim MA, Ward E, Wingo PA, Howe HL, Ries LA, Miller BA, Jemal A, Ahmed F, Cobb N, Kaur JS, Edwards BK. Annual report to the nation on the status of cancer, 1975-2004, featuring cancer in American Indians and Alaska Natives. Cancer 2007; 110: 2119-2152.
[8] Essers J, Theil AF, Baldeyron C, van Cappellen WA, Houtsmuller AB, Kanaar R, Vermeulen W. Nuclear dynamics of PCNA in DNA replication and repair. Mol Cell Biol 2005; 25: 9350-9359.
[9] Gu X, Wang Y, Wang H, Ni Q, Zhang C, Zhu J, Huang W, Xu P, Mao G, Yang S. Upregulated PFTK1 promotes tumor cell proliferation, migration, and invasion in breast cancer. Med Oncol 2015; 32: 641.
[10] He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science 1998; 281: 1509-1512.
[11] He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development 2004; 131: 1663-1677.
[12] Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002; 22: 1172-1183.
[13] Ji L, Li H, Gao P, Shang G, Zhang DD, Zhang N, Jiang T. Nrf2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer. PLoS One 2013; 8: e63404.
[14] Jiang M, Gao Y, Yang T, Zhu X, Chen J. Cyclin Y, a novel membrane-associated cyclin, interacts with PFTK1. FEBS Lett 2009; 583: 2171-2178.
[15] Liu D, Finley RL Jr. Cyclin Y is a novel conserved cyclin essential for development in Drosophila. Genetics 2010; 184: 1025-1035.
[16] Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004; 20: 781-810.
[17] Lv L, Wan C, Chen B, Li M, Liu Y, Ni T, Yang Y, Liu Y, Cong X, Mao G, Xue Q. Nemo-like kinase (NLK) inhibits the progression of NSCLC via negatively modulating WNT signaling pathway. J Cell Biochem 2014; 115: 81-92.
[18] MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009; 17: 9-26.
[19] Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, Sugano S, Akiyama T. DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. Oncogene 2004; 23: 8520-8526.
[20] Pang EY, Bai AH, To KF, Sy SM, Wong NL, Lai PB, Squire JA, Wong N. Identification of PFTAIRE protein kinase 1, a novel cell division cycle-2 related gene, in the motile phenotype of hepatocellular carcinoma cells. Hepatology 2007; 46: 436-445.
[21] Polakis P. Wnt signaling and cancer. Genes Dev 2000; 14: 1837-1851.
[22] Sampson EM, Haque ZK, Ku MC, Tevosian SG, Albanese C, Pestell RG, Paulson KE, Yee AS. Negative regulation of the Wnt-beta-catenin pathway by the transcriptional repressor HBP1. EMBO J 2001; 20: 4500-4511.
[23] Sheng SL, Huang G, Yu B, Qin WX. Clinical Significance and Prognostic Value of Serum Dickkopf-1 Concentrations in Patients with Lung Cancer. Clin Chem 2009; 55: 1656-1664.
[24] Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. Genes Dev 2004; 18: 2699-2711.
[25] Shu F, Lv S, Qin Y, Ma X, Wang X, Peng X, Luo Y, Xu BE, Sun X, Wu J. Functional characterization of human PFTK1 as a cyclin-dependent kinase. Proc Natl Acad Sci U S A 2007; 104: 9248-9253.
[26] Sun T, Co NN, Wong N. PFTK1 interacts with cyclin Y to activate non-canonical Wnt signaling in hepatocellular carcinoma. Biochem Biophys Res Commun 2014; 449: 163-168.
[27] Swenson KI, Farrell KM, Ruderman JV. The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in Xenopus oocytes. Cell 1986; 47: 861-870.
[28] Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999; 398: 422-426.
[29] Tripathi V, Shen Z, Chakraborty A, Giri S, Freier SM, Wu X, Zhang Y, Gorospe M, Prasanth SG, Lal A, Prasanth KV. Long Noncoding RNA MALAT1 Controls Cell Cycle Progression by Regulating the Expression of Oncogenic Transcription Factor B-MYB. PLoS Genet 2013; 9: e1003368.
[30] Vijayachandra K, Higgins W, Lee J, Glick A. Induction of p16ink4a and p19ARF by TGF-beta1 contributes to growth arrest and senescence response in mouse keratinocytes. Mol Carcinog 2009; 48: 181-186.

[31] Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development 2006; 133: 4993-5000.
[32] Willert K, Nusse R. Beta-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 1998; 8: 95-102.
[33] Yang T, Chen JY. Identification and cellular localization of human PFTAIRE1. Gene 2001; 267: 165-172.
[34] Yue W, Zhao X, Zhang L, Xu S, Liu Z, Ma L, Jia W, Qian Z, Zhang C, Wang Y, Yang X. Cell Cycle Protein Cyclin Y Is Associated With Human Non-Small-Cell Lung Cancer Proliferation and Tumorigenesis. Clin Lung Cancer 2011; 12: 43-50.
[35] Zeng X, Huang H, Tamai K, Zhang X, Harada Y, Yokota C, Almeida K, Wang J, Doble B, Woodgett J, Wynshaw-Boris A, Hsieh JC, He X. Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. Development 2008; 135: 367-375.