ORIGINAL ARTICLE

mTOR and PTEN expression in non-small cell lung cancer: analysis by real-time fluorescence quantitative polymerase chain reaction and immunohistochemistry

Liang Wang · Wentao Yue · Lina Zhang ·
Xiaoting Zhao · Yue Wang · Shaofa Xu

Abstract

Purpose   To detect the expression of mammalian target of rapamycin (mTOR) and PTEN in non-small cell lung cancer (NSCLC), and explore their role in the prognosis of patients with NSCLC.

Methods   Samples of cancer tissues and normal lung tissues from 78 patients with NSCLC were examined for expression of mTOR and PTEN by real-time polymerase chain reaction and immunohistochemistry. The differences in mTOR and PTEN expression were compared by Student's t test. A Cox regression model was used to analyze the relationship between the influencing factors and the prognosis. Kaplan–Meier survival curves and the log-rank test were used to analyze the progression-free survival.

Results   The mTOR expression in NSCLC tissues was significantly higher than that in normal lung tissue, while the levels of PTEN expression in NSCLC tissue were significantly lower than that in normal lung tissues ($P < 0.05$). No significant correlations were observed between the mTOR and PTEN expressions and the patients' age, sex, pathological type, differentiation, lymph node metastasis, or distant metastasis. The only correlation was with the T stage. The Cox regression analysis showed that mTOR and PTEN expression had an important impact on the patient prognosis.

Conclusions   The absence of and/or a low expression of PTEN and activated mTOR may play an important role in the development of NSCLC, and may represent new prognostic biomarkers for a poor prognosis in patients with NSCLC.

Keywords   Non-small cell lung cancer · Prognosis · mTOR · PTEN

L. Wang · S. Xu (✉)
Department of Thoracic Surgery, Beijing Chest Hospital,
Affiliated to Capital Medical University, Beijing 101149,
People's Republic of China
e-mail: xushaofa@126.com

W. Yue (✉) · L. Zhang · X. Zhao · Y. Wang
Department of Cellular and Molecular Biology,
Beijing Chest Hospital, Affiliated to Capital Medical University,
Beijing 101149, People's Republic of China
e-mail: yue.wentao@gmail.com

Introduction

Lung cancer is the leading cause of tumor-specific death, and approximately 80–85% of lung cancers are classified as non-small cell lung cancer (NSCLC). Surgical resection remains the most effective treatment for NSCLC, but the 5-year survival after curative resection is only 20–30% [1]. Therefore, novel therapeutic strategies are urgently needed to improve this poor prognosis. A series of molecular markers for the early diagnosis and treatment of NSCLC have been identified, including p53, EGFR, AKT, and mammalian target of rapamycin (mTOR), among others. The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR/ PTEN signaling pathways constitute important mediators of tumor growth and proliferation. In this pathway, mTOR, a 289-kDa protein serine/threonine kinase, was first identified as the cellular target of rapamycin [2]. mTOR belongs to the PI3K-related family of kinases, which includes the ataxia–telangiectasia-related protein and DNA-dependent protein kinase, and plays an important role in checkpoint regulation of the cell cycle. The phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor gene that functions as a dual-specificity phosphatase in the PI3K/AKT/mTOR pathways

[3]. It was demonstrated that PTEN could downregulate the PI3K/AKT/mTOR pathway via its lipid phosphatase activity, which places PTEN into a mechanistically critical position. Absent and/or low expression of PTEN may result in increased mTOR activity. The important role of the PTEN suppressive function had been confirmed in many different human cancers. Tumors with the absent and/or low expression of PTEN have greater proliferative activity, growth, and survival [4]. In this study, we conducted an exploratory analysis to detect the expression of mTOR and PTEN in NSCLC patients. We also determined the different expression of mTOR and its negative regulator, PTEN, to determine whether they have any significant impact on the prognosis of patients with NSCLC.

Materials and methods

Patients and tissue samples

In this study, samples of fresh lung cancer tissues and paired normal lung tissues (5 cm away from the malignant tissue) were obtained from 78 patients with NSCLC, who underwent complete resection at Beijing chest hospital between May 2006 and March 2007. All tissue samples were fresh frozen in liquid nitrogen and stored at $-80°C$ until use, and were confirmed by pathological examinations. Patients who had recurrent NSCLC or received chemoradiotherapy before resection were excluded from the study. The detailed demographic and clinicopathologic characteristics of the patients are listed in Table 1, based on the 2002 International Union Against Cancer TNM classification. All of the patients were divided into two groups (Table 2): group A included samples with positive expression of mTOR and negative expression of PTEN, and group B included samples with positive expression of PTEN and negative expression of mTOR. The clinical end point used was the time to progression-free survival (PFS).

RNA isolation and reverse transcription

Total RNA was isolated from fresh tissue samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 2 μg of RNA were reverse transcribed into single-strand cDNA in 20 μl of reaction buffer using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT)15 (Promega) as a primer.

Real-time fluorescence quantitative polymerase chain reaction

The specific trans-intronic primers used for conventional polymerase chain reaction (PCR) were designed according

Table 1 Clinicopathological characteristics of the patients and the gene expression

Characteristics	Cases	P	
		mTOR	PTEN
Age (years)		0.471	0.273
≤60	37		
>60	41		
Sex		0.810	0.974
Male	66		
Female	12		
Smoking status		0.281	0.347
Smoker	34		
Non-smoker	44		
Pathological type		0.235	0.124
Squamous	44		
Adenocarcinoma	34		
Differentiation		0.765	0.777
Well	6		
Moderate	51		
Poor	21		
T stage		0.034*	0.012*
T1–T2	25		
T3–T4	53		
Lymph node metastasis		0.468	0.335
No	55		
Yes	23		
Distant metastasis		0.496	0.958
No	71		
Yes	7		

* $P < 0.05$

Table 2 Main clinical features in groups A and B

Clinical features	Cases (78)	
	Group A (51)	Group B (27)
Pathological type		
Squamous	28	16
Adenocarcinoma	23	11
T stage		
T1–T2	8	17
T3–T4	43	10
Lymph node metastasis		
No	32	23
Yes	19	4
Distant metastasis		
No	45	26
Yes	6	1

to the National Center for Biotechnology Information RefSeq, and were: for mTOR, forward primer 5′-CGC TGT CAT CCC TTT ATC G-3′ and reverse primer 5′-ATG CTC

AAA CAC CTC CAC C-3′; for PTEN, forward primer 5′-ACC AGG ACC AGA GGA AAC CT-3′ and reverse primer 5′-GCT AGC CTC TGG ATT TGA CG-3′. Real-time fluorescence quantitative PCR reactions were performed on an ABI 7500 real-time fluorescence quantitative PCR system (Applied Biosystems, Foster City, CA, USA). The expression of mTOR and PTEN were analyzed using 10 μl SRBY Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μl with 2 μl of cDNA. The thermal cycle conditions for mTOR and PTEN were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The uniform amplification of the products was verified by analyzing the melting curves of the amplified products, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for each sample. All reactions were carried out in triplicate. The relative expression of the mRNA was calculated with the following formula:

$$Ratio = 2^{\Delta cT}$$

in which

$$\Delta cT = cT_{GAPDH} - cT_{PTEN,mTOR}.$$

Immunohistochemistry

To verify the protein expression of mTOR and PTEN, we performed immunohistochemistry for all specimens. The polyclonal rabbit anti-PTEN antibody (PAD: PN37) was purchased from Zymed Laboratories (San Francisco, CA, USA). The mTOR (Ser473) antibodies were from Cell Signaling (Beverly, MA, USA). Reagents for immunohistochemistry were purchased from BioGenex (San Ramon, CA, USA). Immunohistochemistry was performed using the streptavidin–biotin complex method on a BioGenex i6000 automated staining system based on protocols provided by the manufacturer (BioGenex). The primary antibodies were diluted 1:50. The secondary antibody was purchased from BioGenex and was ready to use without dilution. Slides were scanned with a Nikon Eclipse microscope (E800) using a MetaMorph Imaging System (Molecular Devices, Sunnyvale, CA, USA). A semiquantitative assessment of protein expression was used to score PTEN and mTOR in lung tissue. The intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), or 3(strong). The extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%), according to the percentage of cells stained positive for each protein. The sum of the intensity and extent scores was used as the final score (0–7). Tissue specimens having a final score >2 were considered positive. Final scores of 2–3 were considered +, 4–5, ++; and 6–7, +++. mTOR was considered to be overexpressed if the final score was at

least ++, and PTEN was considered to be expressed if the score was at least + [5].

Statistical analysis

The statistical analysis was performed using the SPSS software program version 13.0 (SPSS, Chicago, IL, USA). The data were presented as the means ± estimated standard deviation. Fisher's exact test and Student's *t* test were used to compare the clinicopathological characteristics of the patients (patient's sex, age, tumor size, etc.). A Cox regression model was used to analyze the relationship between the influencing factors and the prognosis. The Spearman correlation coefficients were calculated to estimate the correlation between the data on the expression levels of mTOR and PTEN. The chi-square test was used to compare the results of real-time PCR and immunohistochemistry. Kaplan–Meier survival curves and the log-rank test were used to analyze the univariate distribution for PFS.

Results

The level of mTOR mRNA was markedly higher in NSCLC tissues than in normal tissues

The clinical and pathological data of the 78 NSCLC patients are displayed in Table 1. In this study, we detected the expression of the mTOR gene in the paired tumor tissues and normal lung tissues by real-time fluorescence quantitative PCR. A significant difference in mTOR expression was observed between the tumor tissues and non-tumor tissues ($P < 0.05$) (Fig. 1), and there was a
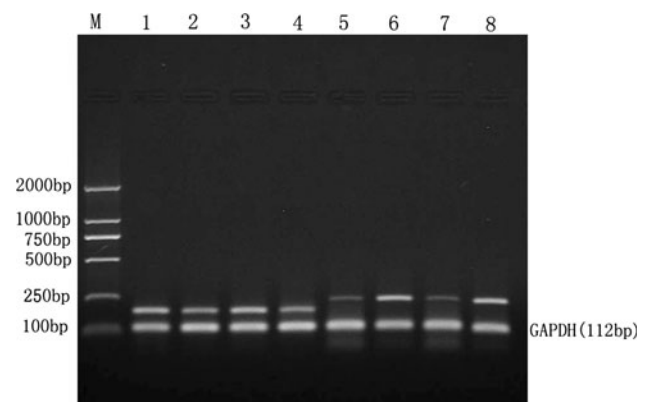

Fig. 1 Expression of mTOR and PTEN mRNA. *Lanes 1, 3, 5, 7* lung cancer. *Lanes 2, 4, 6, 8* normal lung tissues. *Lanes 1–4* mTOR (193 bp). *Lanes 5–8* PTEN (232 bp). *M* molecular weight marker, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

twofold difference between the two groups (2.20 vs. 1.13) (Fig. 2a, c). mTOR was upregulated in 60 tumor tissues compared with the matched non-tumor tissues.

The level of PTEN mRNA had an inverse correlation with mTOR expression in NSCLC tissues

To explore tumor suppressor genes in this pathway, we focused on the tumor suppressor gene PTEN. The 78 pairs of matched NSCLC specimens were analyzed to detect PTEN mRNA. In comparison with the non-tumor counterparts, tumor tissues expressed a significantly lower level of PTEN ($P < 0.05$) (Fig. 1), and there was a threefold difference between the two groups (1.48 vs. 4.10) (Fig. 2b, d). The expression of the PTEN gene in tumor tissues was significantly lower than that in normal tumor lung tissue. In addition, PTEN was present in a greater percentage of patients in the early stage than that in the advanced stage, although no statistically significant difference was found. A statistically significant inverse correlation was observed between mTOR and PTEN, with high expression of mTOR correlating with low expression of PTEN (54/78 cases).

With the progression of tumor development, the expression level of PTEN decreases [6].

Expression of PTEN and positive mTOR in NSCLC determined by immunohistochemistry

All NSCLC tissues and normal tissues were evaluated for mTOR and PTEN protein expression by an immunohistochemical analysis. PTEN was found to be positive in 26.9% (21/78) of the tumor tissue samples, mainly in the nuclei of tumor cells, and was obviously lower in normal tissues (90%, $P < 0.01$). Positive mTOR expression was noted in 43.5% (36/78) of tumor tissue samples, mainly in the cytoplasm of tumor cells, and was obviously higher in normal tissues (10%, $P < 0.01$) (Table 3; Fig. 3). The high expression of mTOR appears to negatively correlate with the absent or low expression of PTEN in NSCLC tissues, as indicated by a correlation test ($r = 0.05$).

When comparing the mRNA levels from real-time PCR data and the protein expression from immunohistochemistry data, no significant differences were observed for PTEN between the two for both the lung cancer and normal

Fig. 2 Overexpression of mTOR and low expression of PTEN in non-small cell lung cancer (NSCLC). **a** The relative expression of mTOR in NSCLC tissues and in normal non-tumor tissues ($n = 78$); the mTOR expression was obviously higher in tumor tissues than in normal non-tumor tissues. **P < 0.01.* **b** The relative expression of PTEN in NSCLC tissues and in normal non-tumor tissues ($n = 78$); the PTEN expression was obviously lower in tumor tissues than in normal non-tumor tissues. **P < 0.01.* *T* lung cancer; *N* normal lung tissues. **c** Scatterplot of the relative expression of mTOR in NSCLC tissues and in normal non-tumor tissues ($n = 78$). **d** Scatterplot of the relative expression of PTEN in NSCLC tissues and in normal non-tumor tissues ($n = 78$)
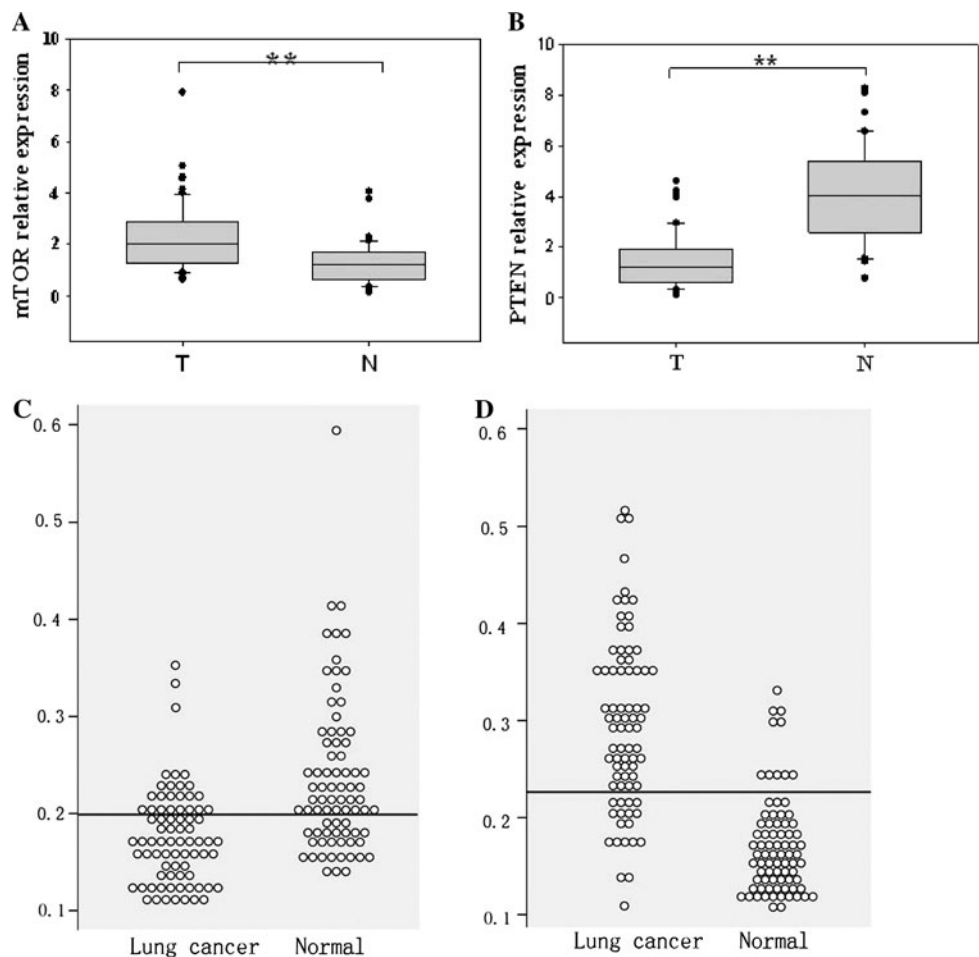
Table 3 Protein expression of mTOR and PTEN

Groups (based on histology)	No.	mTOR					PTEN				
		−	+	++	+++	% Positive	−	+	++	+++	% Positive
Normal tissues	78	69	6	1	2	11.5	22	21	20	15	71.8
Tumor tissues	78	42	15	18	3	43.5	57	8	11	2	26.9

Fig. 3 Expression of the mTOR and PTEN proteins in NSCLC (×400). **a** Positive expression of the p-mTOR protein in NSCLC. **b** Positive expression of the PTEN protein in NSCLC. **c** Negative expression of the p-mTOR protein in NSCLC. **d** Negative expression of the PTEN protein in NSCLC
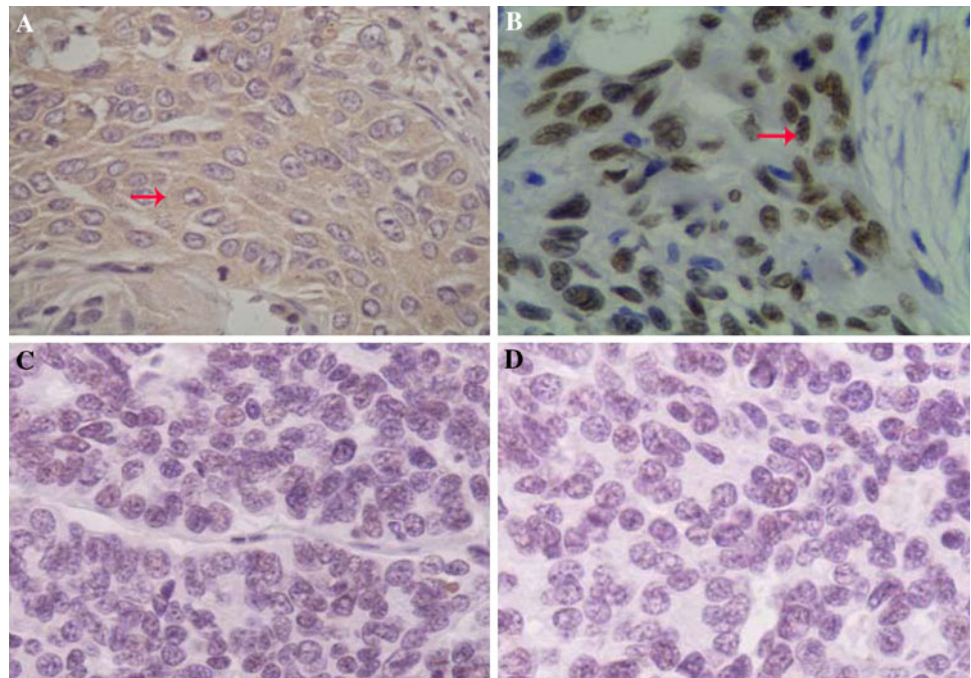
control groups (Fig. 4a; $z = 0.132$, 0.0774, $P > 0.05$). For mTOR, no significant differences were observed for the normal control group (Fig 4b; $z = 0.028$, $P > 0.05$), but in the lung cancer samples, the mRNA expression was higher than the protein expression ($z = 4.0246$, $P < 0.05$).

Relationship between clinicopathologic parameters, gene expression, and survival

This study showed that there was no significant relationship between the mTOR or PTEN gene expression levels and the patients' age, sex, pathological type, or differentiation (Table 1). However, there was a significant relationship between the patients' T stage and the expression level of PTEN or mTOR. The expression of PTEN in T1–2 stage NSCLC was significantly lower than in T3–4 stage NSCLC. However, the opposite was true for mTOR. The absent and/or low expression of PTEN and the overexpression of mTOR may be events related to advanced-stage NSCLC. According to our findings, there were 51 cases in group A and 27 in group B. The median PFS for group A was 8.05 (±4.36) months, and that for group B was 20.03 (±2.65) months. In group A there were 24 patients (47.1%,

24/51) with a poor prognosis, including relapse, metastasis, or death. However, in group B there were only 7 patients (25.9%, 7/27) with a poor prognosis (Fig. 5). The log-rank test showed that the patients in group B had a markedly longer PFS than the patients in group A ($P < 0.05$).

Analysis of the relationship between mTOR and PTEN expression and the prognosis in NSCLC analyzed by a Cox regression model

Before Cox analysis, we transformed each factor for the data into different levels (0 and 1). The mTOR level in NSCLC tissues and in normal tissues was also transformed into two levels (this classification rule was according to the scatterplot of the relative expression of mTOR in NSCLC tissues and in normal tissues shown in Fig. 2c). Similarly, the PTEN in NSCLC tissues and in normal tissues was also transformed into 0 and 1 according to Fig. 2d. The results of the Cox regression analysis showed that among these influencing factors, mTOR and PTEN had an important impact on the prognosis of NSCLC. Interestingly, for mTOR in tumors, the regression coefficient was 1.189 and its risk ratio was 3.285, which indicated that mTOR in
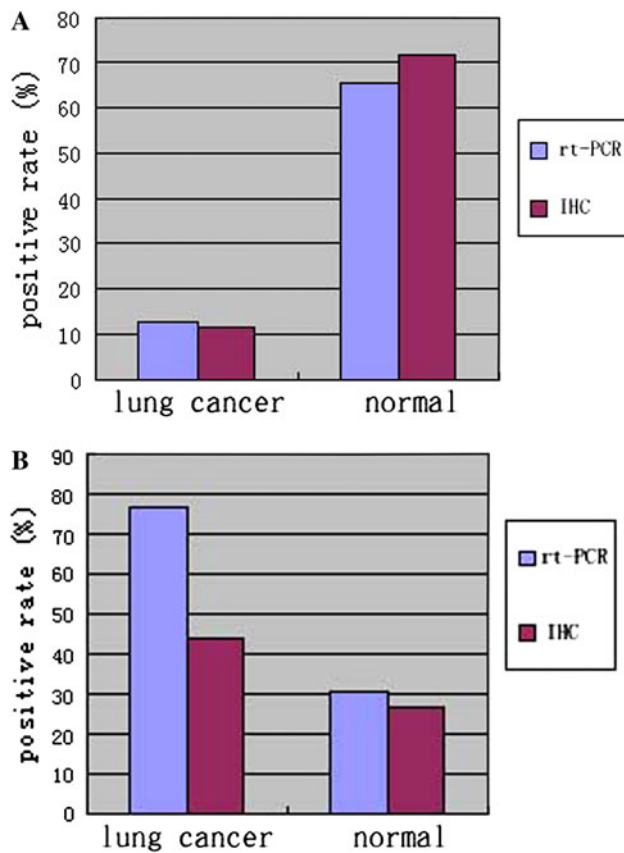
Fig. 4 Comparison of the positive rates of mTOR and PTEN messenger RNA (mRNA) and protein expression in the two groups. **a** PTEN; **b** mTOR. *rt-PCR* real-time polymerase chain reaction, *IHC* immunohistochemistry

Fig. 5 The survival curves of groups A and B based on mTOR and PTEN expression ($n = 78$). *PFS* progression-free survival

Table 4 Results of Cox regression analysis

Variables	Regression coefficient	Standard error	Relative risk	P
PTEN	−1.121	0.446	0.326	0.012*
mTOR	1.189	0.312	3.285	0.000*

* $P < 0.05$. The factors with $P > 0.05$ were not presented in this form, because they were minor factors in terms of the prognosis

NSCLC was a factor predicting a poorer prognosis. By contrast, PTEN in tumors was associated with a better prognosis, its regression coefficient being −1.121, and its risk ratio was 0.326. According to these parameters, we concluded that high expression of mTOR and low expression of PTEN in NSCLC could increase the risk of a poor prognosis, while low mTOR and high PTEN in NSCLC could decrease the risk. These results were concordant with the real-time PCR and immunohistochemistry results shown above (Table 4).

Discussion

Non-small cell lung cancer is one of the most common malignant tumors worldwide. The main treatment methods include surgery, chemotherapy, and radiotherapy. About 85% of patients with NSCLC have a poor prognosis, and the 5-year survival rate is still <15% [2]. Therefore, early diagnosis and early treatment is critical for NSCLC. On the basis of current evidence, the main prognostic factor is the *p*-TNM stage. With the development of molecular biology,
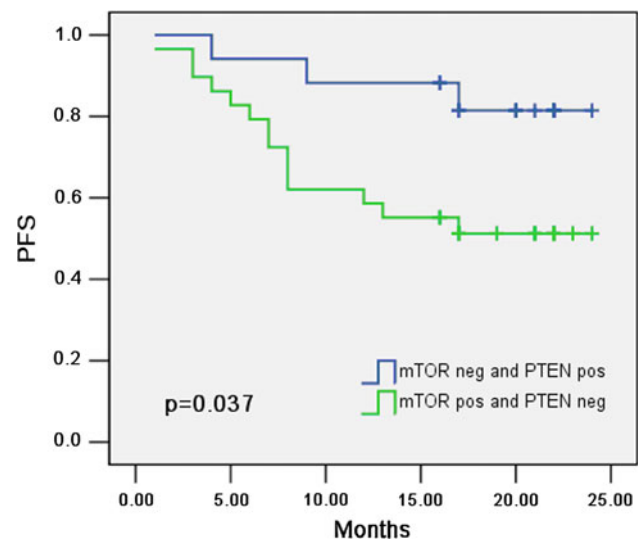
tumor markers may now be of some help with the diagnosis and treatment of NSCLC. From other research reports, mTOR has a critical role in apoptosis, cell growth, proliferation, differentiation of tumor cells, survival, and tumorigenesis [7].

As a negative regulatory factor in the PI3K/AKT/mTOR pathway, PTEN encodes a phosphatase that catalyzes the reverse reaction of PI3K [8]. On one hand, PI3K can be activated by growth factors and can activate mTOR directly. On the other hand, AKT can activate mTOR by dephosphorylating phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-triphosphate (PIP3). As a result, the PI3K/AKT/mTOR signaling pathway would be activated if PTEN was absent and/or expressed at a low level. Absent and/or low expression of PTEN allows for overactivity of the PI3K/AKT/mTOR pathway, inducing the upregulation of mTOR and its downstream signaling pathways [3].

It has been reported that the absent and/or low expression of PTEN occurs in various kinds of malignancies, and this is related to carcinogenesis [9]. In this study, we detected mTOR and PTEN expression in NSCLC by real-time fluorescence quantitative PCR and immunohistochemistry. No significant relationship was observed

between the mTOR and PTEN gene expression levels and the patients' age, sex, pathological type, differentiation, lymph node metastasis, and distant metastasis. However, there was a significant association with the T stage. At the same time, our results confirmed that mTOR was expressed more highly in tumor tissues than in normal tissues, whereas the opposite was true for PTEN. This suggested that the absent and/or low expression of the tumor suppressor PTEN, in combination with overexpression of the mTOR oncogene, might be an important step in NSCLC development, which is similar to the previous reports [7, 8]. Furthermore, we explored the impact of PTEN and mTOR on the prognosis of NSCLC patients by real-time fluorescence quantitative PCR and immunohistochemistry. The results showed that the patients with overexpression of mTOR and absent and/or low expression of PTEN had a poorer PFS. The results indicated that PTEN and mTOR are important determinants of the prognosis in patients with NSCLC. This suggested that absent and/or low expression of PTEN in tumors may enhance the growth and invasion of the tumor. Moreover, the absent and/or low expression of PTEN is likely to be a late event in the development of tumors. More recently, the consequences of aberrantly expressed mTOR and PTEN on tumor behavior have started to be understood, and the status is increasingly recognized as an important prognostic indicator for cancer [10, 11]. Lim et al. reported that activated mTOR was an adverse prognostic factor in patients with biliary tract adenocarcinoma [12]. As a critical downstream target of AKT activation, mTOR has been considered as a target for cancer therapy. The analysis of mTOR and PTEN in NSCLC patients may represent a valuable tool in elucidating tumor development and selecting optimal treatments. However, the conclusions of this study might be limited because of the small number of cases examined, and our future studies will need to expand the population of patients with a longer follow-up to study the relationship between these markers and the aggressive and recurrent behavior of NSCLC.

In summary, our study demonstrated that mTOR and PTEN might play a significant role in NSCLC, and showed that mTOR and PTEN were predictive factors for the prognosis of NSCLC. The level mRNA expression of mTOR and PTEN, determined using real-time fluorescence quantitative PCR, in NSCLC samples may prove to be a valuable diagnostic and prognostic tool in NSCLC patients. The present results indicated that PTEN and mTOR might be important determinants of prognosis in patients with NSCLC.

References

1. Spira A, Ettinger DS. Multidisciplinary management of lung cancer. N Engl J Med. 2004;59:225–49.
2. Toloza EM, Damico TA. Targeted therapy for non-small cell lung cancer. Semin Thorac Cardiovasc Surg. 2005;17:199–204.
3. Pal SK, Figlin RA, Reckamp KL. The role of targeting mammalian target of rapamycin in lung cancer. Clin Lung Cancer. 2008;9:340–5.
4. Hay N, Sonenberg N. Upstream and downstream of mTOR. Gene Dev. 2004;18:1926–45.
5. Steelman LS, Bertrrand FE, McCubrey JA. The complexity of PTEN: mutation, marker and potential target for therapeutic intervention. Expert Opin Ther Targets. 2004;8:537–50.
6. Han W, Ming M, He TC, He YY. Immunosuppressive cyclosporin A activates AKT in keratinocytes through PTEN suppression: implications in skin carcinogenesis. J Biol Chem. 2010; 285:11369–77.
7. Rosner M, Hanneder M, Siegel N, Valli A, Fuchs C, Hengstschläger M. The mTOR pathway and its role in human genetic diseases. Mutat Res. 2008;659:284–92.
8. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. Science. 1997;275: 1943–7.
9. Iyoda A, Hiroshima K, Moriya Y, Yoshida S, Suzuki M, Shibuya K, et al. Predictors of postoperative survival in patients with locally advanced non-small cell lung carcinoma. Surg Today. 2010;40(8):725–8.
10. Conde E, Angulo B, Tang M, Morente M, Torres-Lanzas J, Lopez-Encuentra A, et al. Molecular context of the EGFR mutations: evidence for the activation of mTOR/S6K signaling. Clin Cancer Res. 2006;12:710–7.
11. Herberger B, Puhalla H, Lehnert M, Wrba F, Novak S, Brandstetter A, et al. Activated mammalian target of rapamycin is an adverse prognostic factor in patients with biliary tract adenocarcinoma. Clin Cancer Res. 2007;13:4795–9.
12. Lim WT, Zhang WH, Miller CR, Watters JW, Gao F, Viswanathan A, et al. PTEN and phosphorylated AKT expression and prognosis in early-and late-stage non-small cell lung cancer. Oncol Rep. 2007;17(4):853–7.