RESEARCH ARTICLE

Expression and Clinical Significance of mTOR in Surgically Resected Non-small Cell Lung Cancer Tissues: a Case Control Study

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Abstract

    Aims: Mammalian target of rapamycin (mTOR) is master regulator of the PI3K/Akt/mTOR pathway and plays an important role in NSCLCs. Here we characterized mRNA and protein expression levels of mTOR and its functional associated molecules including PTEN, IGF-1R and 4EBP1 in surgically resected NSCLCs. Methods: Fifty-four patients with NSCLCs who underwent pulmonary resection were included in current study. mRNA levels of mTOR, PTEN, IGF-1R, and 4EBP1 were evaluated by RT-PCR and protein expression of mTOR, PTEN, and IGF-1R by immunohistochemistry (IHC). Association of expression of the relevant molecules with clinical characteristics, as well as correlations between mTOR and PTEN, 4EBP1 and IGF-1R were also assessed. Results: The results of RT-PCR showed that in NSCLCs, the expression level of mTOR increased, while PTEN, 4EBP1 and IGF-1R decreased. Statistical analysis indicated high IGF-1R expression was correlated with advanced clinical stage (stage III) and PTEN expression was reversely associated with tumor size (P=0.16). The results of IHC showed mTOR positive staining in 51.8% of cases, while IGF-1R positive staining was found in 83.3% and loss of PTEN in 46.3%. Protein expression of mTOR was correlated with its regulators, PTEN and IGF-1R, to some extent. Conclusions: Abnormal activation of mTOR signaling, high expression of IGF-1R, and loss of PTEN were observed in resected NSCLC specimens. The poor expression agreement of mTOR with its regulators, PTEN, and IGF-1R, implied that combination strategy of mTOR inhibitors with other targets hold significant potential for NSCLC treatment.

Keywords: NSCLC - mTOR - PTEN - IGF - 1R - 4EBP1

Introduction

    Lung cancer is the leading cause of cancer-related deaths worldwide; non-small cell lung cancer (NSCLC), which includes squamous cell lung carcinoma, adenocarcinoma, large-cell lung carcinoma, accounts for more than 80% lung malignancy (Jemal et al., 2007). Despite recent advances in multimodality therapies, the prognosis for most patients with NSCLC is still unsatisfactory (Pfister et al., 2004). Recently, progress in the understanding of oncogenic kinase signaling pathways has provided better targets for developing effective therapeutic strategies, which may help to improve clinical outcomes.

    Mammalian target of rapamycin (mTOR) was first defined by Heitman et al. in 1991 and has attracted monumental scientific interest over the past few decade due to its broad involvement in many human cancers and other diseases (Heitman et al., 1991; Efeyan et al., 2010). mTOR is the master regulator of PI3K/Akt/mTOR pathway, and posses complex biological functions in the regulation of cell growth, proliferation, survival, as well as angiogenesis in response to the growth factors, hormones, energy supplement, and nutrients (Caron et al., 2010). Inappropriate activation of Akt/mTOR signaling, which results in unrestricted cancer cell proliferation and evasion of apoptosis, has been implicated in several tumor types (Shaw et al., 2006). Thus, the Akt/mTOR signaling pathway became an especially promising target for cancer therapy (Yuan et al., 2009). The mTOR inhibitor rapamycin and its analogues, such as temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus (AP23573), have been tested extensively in clinical trials for the past few years and have shown preliminary promise of efficacy in several tumor types, including NSCLC (Price et al., 2010; Tarhini et al., 2010; Witzig et al., 2011). However, the single-agent use of mTOR inhibitors for cancer therapy did not meet expectations (Dowling et al., 2009), while combination of an mTOR inhibitor with other anticancer agents was shown a better therapeutic efficacy in several clinical trials.

    mTOR signaling is an extensively complex network. Utilizing computational approaches combined with

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mathematical modeling techniques, Caron et al. constructed a comprehensive map of mTOR signaling which consists of hundreds of proteins (Caron et al., 2010). Insulin-like growth factor 1 receptor (IGF-1R) is one of positive regulators upstream of mTOR. The binding of IGF-1 to IGF-1R leads to activation of PI3K at the cell surface. Active PI3K then transmits signals to mTOR through the PI3K-Akt and Ras-raf pathways. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor commonly mutated or decrease in expression in many human cancers. PTEN has protein phosphatase activity and lipid phosphatase activity. It hydrolyzes the 3-phosphate on PIP3 to generate PIP2 thus negatively regulates PIP3-mediated PI3K/Akt/ mTOR signaling pathways (Jiang et al., 2009). Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and S6K1 are major mTOR downstream effectors, through which mTOR regulates dozens of gene expression and protein synthesis (Guertin et al., 2006). 4EBP1 appears to be involved in cell proliferation and cell cycle progression by selectively inhibiting the translation of mRNAs that encode proliferation-promoting and cell cycle regulatory proteins, while S6K mainly regulate cell size (Dowling et al., 2010). However, mTOR inhibitor treatment could only partly and variably inhibits 4EBP1 phosphorylation (Dowling et al., 2010), implying the existence of other regulatory pathway of 4EBP1, which was also suspected to be a reasonable explanation for failed rapamycin-based cancer therapies. In the current study, we investigated the expression of mTOR and the interaction of mTOR and its key regulatory and effective molecules, PTEN, IGF-1R, and 4EBP1 in NSCLC tissues.

Materials and Methods

Patients

The present study included 54 patients with NSCLC undergoing thoracotomy at the Surgical Department of Beijing Chest Hospital from April 2008 to December 2008. Resected lung tumor tissues with corresponding adjacent "tumor-free normal" tissues (served as controls) were collected and reviewed and diagnosed by experienced pathologists. Patients who had received primary neoadjuvant treatment in the form of either chemotherapy or radiation therapy were excluded from this study. Tumors were staged according to the tumor-node-metastasis (TNM) criteria of the International Union Against Cancer (IUCC, 1997 version) and histologically classified based on the World Health Organization guidelines after conventional hematoxylin and eosin morphologic examination. Clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all patients. The research was carried out according to the ethical standards laid down in an appropriate version of the 2000 Declaration of Helsinki.

RNA isolation and reverse-transcription PCR analysis

Fresh NSCLC specimens with corresponding matched normal tissue were collected following surgical dissection and flash frozen by immersion in liquid nitrogen. The

Table 1. Primer Sets in the Current Study

Gene	primers	Annealing temperature	Length
mTOR	Sense :5' CGCTGTCATCCCTTTATCG	56.5°C	193bp
	Antisense :ATGCTCAAACACCTCCACC		
PTEN	Sense: ACCAGGACCAGAGGAAACCT	55°C	232bp
	Antisense: GCTAGCCTCTGGGATTTGACG		
4EBP1	Sense: GCAATAGCCCAGAAGATAAGC	56.5°C	167bp
	Antisense: CAACGCCTGCCCAGTATGA		
IGF-1R	Sense: AACCCCAAGACTGAGGTGTG	62.5°C	171bp
	Antisense: TGACATCTCTCCGCTTCCTT		
β-actin	Sense: TCATCACCATTGGCAATGAG	62.5°C	154bp
	Antisense: CACTGTGTTGGCGTACAGGT		

mTOR, Mammalian target of rapamycin; PTEN, Phosphatase and tensin homolog deleted on chromosome 10; 4EBP1, 4E-binding protein 1. IGF-1R: Insulin-like growth factor 1 receptor

samples were then maintained in deepfreezer (-80°C) until use. For RNA isolation, frozen tissues were grinded using a precooled polytron homogenizer and subjected to TRIzol® Reagent. Total RNA was subsequently isolated according to the manufacturers' instructions. RNA quantification was determined spectrophotometrically (Nanodrop technologies, DE, USA), with RNA concentration subsequently used to carry out cDNA synthesis. RT-PCR was carried out using the SuperScriptTM Kit (Invtrogen). For each sample, 2 μg of total RNA was used to synthesize first strand cDNA at 42 °C 50 minutes and 70 °C 15 minutes. The cDNA products were stored at -20 °C. The primers for mTOR, PTEN, 4EBP1, IGF-1R gene amplification were designed with Primer 5.0 and Oligo dT 6.0 software. Primers' sequences for each gene were listed in Table1. House-keeping gene, β-actin, was co-amplified as internal control. PCR amplification was carried out for 40 cycles according to the parameters in Table 1, respectively. After amplification, 8 μL of each reaction products were subjected to electrophoresis on a 1.5% (w/v) agarose gel containing ethidium bromide. The gel images were scanned and analyzed using Alpha View image analysis software. The densitometric ratio of evaluated genes to β-actin was calculated.

Immunohistochemistry

The expression of mTOR, PTEN and IGF-1R protein was investigated by an immunohistochemic approach. Formalin-fixed and paraffin-embedded tissue blocks were cut into 5 μm-thick sections. The tumor tissue and matched normal tissue from same patient were placed onto the same section to ensure the identical experiment conditions. The sections were deparaffinized in xylene, and rehydrated in gradient alcohol and water. Antigen retrieval was preformed by microwaving in 0.01M citrate buffer (pH 6.0) at 90% power for 3 minutes followed by 30% power for 10 minutes, then cooling at room temperature for another 30 minutes. The sections were incubated with 3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase activity at room temperature. Following blocking to reduce nonspecific binding, the sections were incubated with primary antibodies overnight at 4°C. After immunohistochemical staining with the HistostainTM-Plus Kits, the sections were lightly counterstained with Hematoxylin. Nonimmune mouse IgG was used as negative control for the staining procedure.

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Table 2. mRNA Levels of mTOR, PTEN, IGF-1R, 4EBP1 in NSCLC and Adjacent Normal Specimens

	mTOR mean ± SD	PTEN mean ± SD	IGF-1R mean ± SD	4EBP1 mean ± SD
NSCLC specimens (n=54)	0.26±0.18	0.17±0.16	0.09±0.05	0.10±0.06
Normal specimens (n=54)	0.13±0.09	0.54±0.39	0.15±0.10	0.11±0.05
P value	0.00**	0.00**	0.00**	0.03*

**P<0.001, * P<0.05; mTOR, Mammalian target of rapamycin. PTEN: Phosphatase and tensin homolog deleted on chromosome 10; IGF-1R, Insulin-like growth factor 1 receptor; 4EBP1, 4E-binding protein 1; NSCLC, non-small lung carcinomas

Results of IHC for each protein expression were judged based on the staining intensity combined with percentage of cells with positive staining. For each tumor specimen, at least 3 tissue sections obtained from different areas were evaluated by 2 independent pathologists. 400–500 tumor cells were counted irrespective of the result of the staining reaction. The scoring was determined as following criterias. Averaged percentage of positive staining tumor cells <10%, scored as 0; 10-20%, scored as 1; 21-50%, scored as 2; >50% scored as 3. Staining intensity of tumor cells was determined on a traditional 0-3 scale system as 0 (no staining), 1(light brown), 2 (yellow brown), 3 (dark brown). The final score was determined by multiplying the intensity with percentage of positive cells. According to the semi-quantitative integral evaluation, PTEN and IGF-1R expression were divided into two groups as follows. When Score was < 2, the samples were defined as negative group, ≥ 2 as positive group. While for mTOR expression, score ≥ 1 as positive group, and score = 0 as negative group.

Statistical analysis

Statistical analysis was performed by SPSS 13.0 software. Comparison analysis between two groups was conducted by independent t-test, while multi-groups comparison was finished using one-way ANOVA. The correlation analysis adopted chi-square tests. P<0.05 was considered statistically significant.

Results

Patient characteristics

Totally 54 NSCLC patients were included in the current study. There were 43 males and 11 females with a median age of 59 years (range from 36 years to 86 years). Of the 54 patients, 19 patients had adenocarcinomas, 33 patients had squamous cell carcinomas, and 2 had other subtypes of NSCLC. Of the 54 patients, 19 patients were never or light smokers and the others were heavy or current smokers. 21 patients had TNM stage I disease (13 were stage IA, 8 were stage IB); 17 patients had TNM stage II disease (10 were stage IIA, 7 were stage IIB); and 16 patients had stage III disease (15 were stage IIIA, 1 was stage IIIB). The maximum tumor diameters of 20 patients were 0-3mm and 34 patients were more than 3mm. Tumor grade was classified based on the microscopic appearance of cancer cells. 2 patients were well-differentiated, 29 patients were medium-differentiated, and 18 were poor-differentiated. 18 patients had lymph node metastasis, and 36 had no evidence for lymph node metastasis. The histopathologic examination of the dissected regional lymph nodes revealed regional lymph node metastases
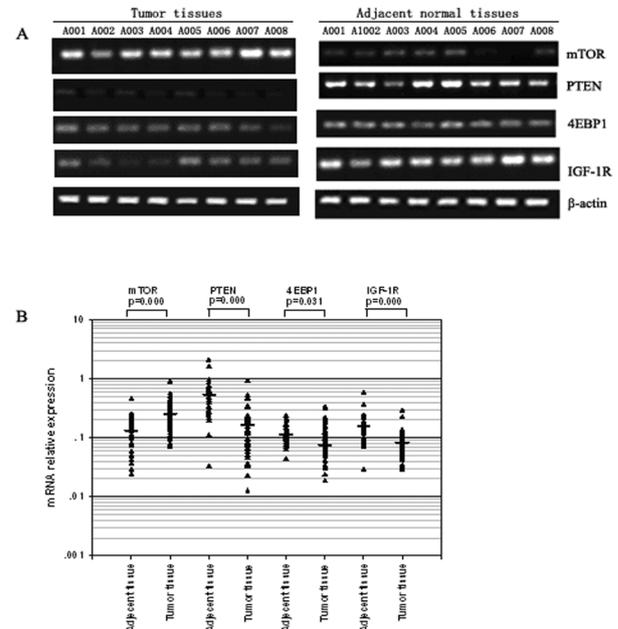

Figure 1. mRNA Expression of mTOR and Its Functional Associated Molecules in NSCLC. A. Representative cases of mRNA expression of mTOR, IGF-1R, PTEN and 4EBP1 in 8 matched surgical resected samples (A001-A008). β-actin was served as internal control. B. Graphical representation of mTOR, IGF-1R, PTEN and 4EBP1 mRNA expression in NSCLC tumor tissues and matched normal tissues. mRNA: messenger ribonucleic acid. mTOR: Mammalian target of rapamycin. NSCLC: non-small lung carcinomas. IGF-1R: Insulin-like growth factor 1 receptor. PTEN: Phosphatase and tensin homolog deleted on chromosome 10. 4EBP1: 4E-binding protein 1

in 18 cases.

mRNA levels of mTOR, PTEN, IGF-1R and 4EBP1 in NSCLC tissues

To investigate whether the NSCLC tumors differ from the adjacent normal tissues in PI3K/PTEN/mTOR signaling, we assessed the mRNA expression levels of mTOR, IGF-1R, PTEN and 4EBP1 on matched surgical samples from patients with NSCLC by RT-PCR. All 54 resected tumor specimens and corresponding normal tissue specimens were studied. The mRNA expression level was calculated based on the band density of evaluated genes to house-keeping gene β-actin. Examination of mTOR mRNA expression in a panel of NSCLC samples revealed an overexpression of mTOR in tumor samples, relative to matched normal controls (Figure 1A). Densitometric analysis confirmed these observations, with mTOR expression significantly (p<0.05) higher in tumor samples than in matched normal controls (0.255±0.175 and 0.133±0.09, respectively, Table 2). While PTEN, IGF-1R

Table 3. The Association of mTOR, PTEN, IGF-1R, and 4EBP1 mRNA Expression with Clinical Characteristics of NSCLCs

Characteristics	mTOR		PTEN		IGF-1R		4EBP1	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
Sex								
Male (n=43)	0.24±0.17	0.20	0.17±0.17	0.65	0.08±0.04	0.65	0.10±0.06	0.82
Female (n=11)	0.315±0.207		0.15±0.13		0.09±0.07		0.09±0.08	
Smoking history								
Current (n=35)	0.22±0.13	0.06	0.16±0.13	0.54	0.08±0.03	0.44	0.09±0.05	0.46
Never or rarely (n=19)	0.32±0.23		0.19±0.21		0.09±0.06		0.11±0.09	
Tumor size								
0-3cm (n=20)	0.27±0.19	0.61	0.11±0.08	0.02*	0.09±0.06	0.73	0.10±0.07	0.90
>3cm (n=34)	0.25±0.17		0.20±0.19		0.08±0.04		0.10±0.01	
Histology								
Squamous cell carcinoma (n=33)	0.22±0.13	0.08	0.17±0.13	0.52	0.08±0.04	0.49	0.09±0.04	0.12
Adenocarcinoma (n=19)	0.30±0.21		0.20±0.20		0.09±0.06		0.12±0.08	
Clinical stage								
Stage I, II (n=38)	0.24±0.16	0.21	0.16±0.16	0.51	0.08±0.04	0.04*	0.09±0.06	0.37
Stage III (n=16)	0.30±0.20		0.19±0.16		0.11±0.06		0.11±0.07	
Differentiation status								
Well-differentiated (n=2)	0.16±0.07	0.51	0.12±0.09	0.77	0.10±0.04	0.51	0.10±0.06	0.98
Medium-differentiated (n=29)	0.26±0.17		0.17±0.18		0.08±0.04		0.10±0.07	
Poor-differentiated (n=18)	0.27±0.21		0.19±0.15		0.10±0.06		0.10±0.07	
Lymph node metastasis								
Yes (n=18)	0.27±0.19	0.65	0.20±0.15	0.34	0.10±0.06	0.11	0.11±0.07	0.29
No (n=36)	0.25±0.17		0.15±0.17		0.08±0.04		0.09±0.06	

mTOR, Mammalian target of rapamycin; PTEN, Phosphatase and tensin homolog deleted on chromosome 10; IGF-1R, Insulin-like growth factor 1 receptor; 4EBP1, 4E-binding protein 1; mRNA, messenger ribonucleic acid; NSCLC, non-small lung carcinomas
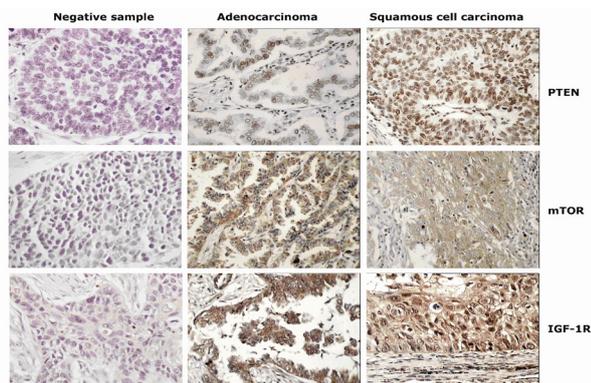

Figure 2. Examples of IHC Staining of mTOR, PTEN, and IGF-1R in Representative NSCLC Tissue. IHC: immunohistochemistry. mTOR: Mammalian target of rapamycin. PTEN: Phosphatase and tensin homolog deleted on chromosome 10. IGF-1R: Insulin-like growth factor 1 receptor. NSCLC: non-small lung carcinomas

and 4EBP1 mRNA expression were lower in NSCLC than that in non-tumorous tissue (Figure 1B).

Relationship between clinical characteristics and gene expression in NCSCL tissues

The univariate analysis was performed to assesse the association of their mRNA levels with clinical characteristics of NSCLC patients. There was no significant association of mTOR and 4EBP1 mRNA expression with clinical or pathologic characteristics. However, there was a tendency toward higher proportion of patients with poor-differentiation and advanced stage with high mTOR expression though it was not statistically significant (Table 3). IGF-1R expression was significantly

higher in the patients with later clinical stage (stage III) in comparison with that in early stage (stage I and II) patients. PTEN expression was reversely associated with the tumor size (P=0.016). PTEN mRNA expression was significantly lower in the patients with small size tumors (Table 3).

Relationship between clinical characteristics and protein expression in NCSCL tissues

Results of IHC staining were obtained from all recruited 54 patients. An overexpression of mTOR was observed in tumor section in 28 cases (51.8%), while 26 patients (48.2%) had very low or no mTOR expression. As illustrated in Figure 2, mTOR was mainly expressed in cytoplasma of tumor cells. PTEN negative immunoreactivity was noted in 25 (46.3%) NSCLC tumor tissues. Strongly cytoplasmic and membranous staining for IGF-1R were observed in most tumor specimens (45 cases, 83.3%), only 9 tumor samples (16.7%) were negative immunostaining of IGF-1R. Statistical analysis revealed that PTEN expression had no obvious correlation with clinical features. The positive mTOR and IGF-1R score were significantly associated with tumor size (P=0.038, P=0.028 respectively), with no significant association with other clinical characteristic, including gender, histology, smoking status and stage.

Relationship between mTOR protein expression and the expression of PTEN and IGF-1R

PTEN and IGF-1R play negative and positive regulation function on mTOR pathway, respectively. We therefore investigated the protein expression correlation between mTOR and the two upstream regulators in

NSCLC specimen. The chi-square tests revealed the kappa coefficient of agreement of mTOR vs. PTEN and mTOR vs. IGF1R were 0.146 and 0.051, respectively, indicating a poor agreement of protein expression of mTOR and two regulators.

Discussion

mTOR is an intracellular serine-threonine protein kinase. It is now considered a central regulator in a variety of cell events, including cell proliferation, growth, differentiation, and survival. Abnormal activation of mTOR was frequently found in many human cancers. Inhibitors of mTOR were therefore expected to be one of most promising therapeutic targets. Clinical trails demonstrated that the mTOR inhibitors, like rapamycin, temsirolimus, everolimus, have great anticancer activity in breast cancer, leukemia, lymphoma, hepatocellular carcinoma, pancreatic cancer, and NSCLC as well (Yuan et al., 2009). The clinical phase I data for everolimus and deferolimus have shown a signal of activity in NSCLC; the subsequent trail, however, indicated monotherapy of mTOR inhibitors was not promising (Milton et al., 2007). A rational combination of other anticancer agents might be an effective therapeutic strategy. Investigating the expression status of several key molecules of mTOR signaling network in NSCLC will be helpful to find potential pharmacological targets.

We firstly examined the expression of mTOR in NSCLC specimens. As expected, the mRNA expression level of mTOR in NSCLC tissues was much higher than that in adjacent normal tissue (0.255 ± 0.175 vs. 0.133 ± 0.090, $P<0.01$), indicating the abnormal activation of mTOR in NSCLC. Stratified analysis showed a higher mTOR expression in the patients with advanced stage and poor differentiation although it did not reach the statistical significance (Table 3). Immunohistochemistry results showed that 51.8% patients were mTOR positive. These data implied positive correlation of mTOR activation and disease progression. 4EBP-1, a translator inhibitor, is another major molecule of in the mTOR signal transduction pathway. Activated mTOR can directly phosphorylate 4EBP-1 and lead to lower inhibition of protein synthesis, which may account for the malignant cell growth (Wang et al., 2005). Our results of RT-PCR in surgically resected specimens showed that 4EBP-1 decreased significantly in NSCLC (Table 2). This suggested that the progress of malignant transformation and progression need to synthesize more protein. Our results concurred in essence with the findings of previous studies that mTOR and its substrates play an important role in the tumorigenesis and progression, and inhibition of mTOR pathway could be a promising approach to treat NSCLC (Balsara et al., 2004, Schmid et al., 2010).

The phosphatase and tensin homologue gene (PTEN) located on human chromosome 10q23, is one of the most commonly lost tumor suppressor genes in human malignances. PTEN protein expression is often lost in NSCLC, which leads to constitutive activation of PI3K/Akt/mTOR signaling. Iwanaga K et al. reported that loss of the PTEN suppressor gene is a critical secondary event in a mouse model of lung tumorigenesis (Iwanaga et al., 2008). Our data showed that the mRNA expression level in NSCLS was significantly decreased than in adjacent normal tissue (0.169 ± 0.161 and 0.536 ± 0.390 respectively, $P<0.01$). Immunohistochemical analysis indicated loss of PTEN protein was not uncommon in NSCLC (46.3%). Several lines of evidence suggested loss of PTEN expression were correlated with poor differentiation, metastasis, late stages, and thus were considered an independent poor prognostic factor for NSCLC patients (Tang et al., 2006). However, our study found that the expression level of PTEN had no obvious correlation with clinical features of NSCLC. The limited tumor sample size in this study might be the reason. There is evidence that downregulated PTEN expression would active mTOR signaling, thus contributes to lung carcinogenesis (Steck et al., 1997). In the present study, the chi-square test showed that the protein expressions of mTOR and PTEN in NSCLC were correlated to some extent ($P>0.05$), however, the poor agreement (kappa coefficient less than 0.4) implied that loss of PTEN and activation of mTOR were not completely correlated.

The IGF-1R is a transmembrane heterotetrameric protein. The binding of IGF-1R to insulin would positively regulated mTOR signaling through the PI3K/Akt pathway and was implicated in the progression of malignant diseases by promoting oncogenic transformation and cancer cell survival (Caron et al., 2010). In a retrospective study, Cappuzzo et al. reported that 76.4% surgically resected NSCLC were positive IGF-1R expression (Cappuzzo et al., 2010). Dziadziuszko's group reported 84% NSCLC samples showed IGF-1R immunopositive staining (Dziadziuszko et al., 2010). They also demonstrated the high level IGF-1R expression was significantly associated with squamous cell histology and late stage. Our study showed 43 samples (83.3%) were IGF-1R positive, and mRNA expression level of IGF-1R was significantly correlated with the clinical stage (Table 3), which is consistent with most published data. The increased IFG-1R expression in a set of NSCLC leads to the development of IGF1R targeting strategies. Currently, a number of IGF-1R inhibitors like CP-751,871, IMC-A12, are being examined in phase I to III clinical trails (Pollak, 2008). Although the IGF-1R is a well-documented upstream positive regulator of mTOR, our analysis found the protein expression of IGF-1R and mTOR were not well agreement. These findings raised the question whether the combination of IGF-1R and mTOR inhibitors in patients with NSCLC might be more effective. Several preclinical experiments had demonstrated synergistic antitumor activity with combination blockade of mTOR and IGF-1R signaling (Quek et al., 2011).

In summary, we have demonstrated the variation expression of mTOR and its upstream and downstream associated molecules in NSCLC, and analyzed the correlation of their expression with clinical characteristics and the expression agreement between them. These data would be helpful for exploring potential pharmacological targets for combination therapeutic strategy.

Acknowledgements

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