RESEARCH ARTICLE

TGF-β1 Protein Expression in Non-Small Cell Lung Cancers is Correlated with Prognosis

Ai-Li Huang[1,2], Shu-Guang Liu[1,2], Wen-Juan Qi[1,2], Yun-Fei Zhao[1,2], Yu-Mei Li[1,2], Bin Lei[1,2], Wen-Jie Sheng[1,2], Hong Shen[1,2]*

Abstract

To investigate the expression intensity and prognostic significance of TGF-β1 protein in non-small cell lung cancer (NSCLC), immunohistochemistry was carried out in 194 cases of NSCLC and 24 cases of normal lung tissues by SP methods. The PU (positive unit) value was used to assess the TGF-β1 protein expression in systematically selected fields under the microscope with Leica Q500MC image analysis. We found that the TGF-β1 PU value was nearly two-fold higher in NSCLC than in normal lung tissues (p=0.000), being associated with TNM stages (p=0.000) and lymph node metastases (p=0.000), but not to patient age, gender, smoking history, tumor differentiation, histological subtype and tumor location (P>0.05). Univariate analysis indicated that patients with high TGF-β1 protein expression and lymph node metastases demonstrated a poor prognosis (both p=0.000,). Multivariate analysis showed that TGF-β1 protein expression (RR = 2.565, p=0.002) and lymph node metastases (RR=1.874, p= 0.030) were also independent prognostic factors. Thus, TGF-β1 protein expression may be correlated to oncogenesis and serve as an independent prognostic biomarker for NSCLC.

Keywords: NSCLC - transforming growth factor β1 - quantitative analysis - prognosis

Asian Pac J Cancer Prev, **15 (19)**, 8143-8147

Introduction

Lung cancer has become the leading cause of cancer mortality in worldwide (Jemal et al., 2007), so scholars pay more and more attention to its diagnosis, treatment and prognosis. Non-small cell lung cancer (NSCLC) represents 80% of lung cancer. Previous study demonstrated that only 10% of all patients are long-term survivors for NSCLC. Now the main treatments for the patients with lung cancer are still surgical resection, chemotherapy and radiation therapy, but curative effect are not satisfactory. As targeted therapy has been put in used in clinical gradually, further investigating convert to studying new biomarkers which can regard as prognostic factors for targeted therapy in lung cancer.

TGF-β (transforming growth factor beta) is a member of multifunctional cytokine family that regulates cell proliferation, differentiation and extracellular matrix production (Jennings et al., 1998; Luwor et al., 2008). It was originally extracted from human platelet (Assoian et al., 1983) in 1983. Three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) were cloned in mammals encoded by different genes with 75-80% homology. TGF-β1 is one of major components of TGF-β signaling pathways, which inhibits the growth of normal epithelial cell in normal physiological conditions (Boyd et al., 1989). Notably, TGF-β1 plays a dual role in carcinogenesis. During the early stages of cancer, TGF-β1 functions as a tsumor suppressor by inhibiting cellular proliferation and thus promoting cellular differentiation or apoptosis (Blobe et al., 2000; Rich et al., 2001; Derynck et al., 2001; Siegel et al., 2003). In contrast, it acts as a tumor promoter to accelerate tumor progression and metastasis during the progressive stage of cancer. TGF-β initiates TGF-β signaling pathways by combining with cell surface receptors that contain three types. They are TGF- type I (TβRI), type II (TβRII), and type III (TβRIII), which were widely distributed in most cells. TGF-β1 protein was over expressed in carcinoma tissues of breast, prostate, lung, and colon than in each normal tissue, which are associated in part with loss of TβRII (Markowitz et al., 1995; Grady et al., 1998; Furuta et al., 1999).

Although TGF-β1 protein expression in pulmonary adenocarcinoma is significantly higher than in normal lung tissues by immunohistochemical method at the qualitative or semi-quantitative level, no studies have yet reported about the expression of TGF-β1 in NSCLC at quantitative level and in such large samples. In our study, we used immunohistochemical methods and image analysis to test the expression of TGF-β1 protein and measure the PU values, then analyzed its prognostic significance and assessed its clinical usefulness as a biomarker for NSCLC.

*[1]Department of Pathology, Nanfang Hospital, [2]Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China *For correspondence: shenhong 2010168 @163.com.*

Materials and Methods

Patients

The medical records of 194 patients diagnosed with NSCLC between 2003 and 2008 at the Department of Pathology, Nanfang Hospital, were reviewed retrospectively. All histologic diagnoses were confirmed by experienced pathologists. The clinicopathological characteristics of patients were summarized in Table 2. Twenty-one cases of normal lung tissues were served as control group. Informed written consent was obtained from all patients, and the study was approved by Southern Medical University Ethnics Committee.

Immunohistochemistry

Paraffin-embedded tissue sections (3μm thick) were deparaffinized in xylene and rehydrated in ethanol. The slides were subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0), followed by incubation in 3% hydrogen peroxide to block endogenous peroxidase activity. Slides were washed by PBS and incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal antibody to TGF-β1 (1:100; Biosis Biological Technology, Beijing, China). They were subsequently incubated with biotinylated secondary antibody (Zhongshan Golden Bridge Biological Technology, Beijing, China) in room temperature for 30 minutes at room temperature. The immunocomplexes were visualized using diaminobenzidine (DAB) as a chromogenic substrate (Zhongshan Golden Bridge Biological Technology). The sections which need qualitative analysis were counterstained with hematoxylin, others were not counterstained whey we conducted quantitative analysis.

Quantitative criterion

With Leica Q500 MC image analysis system (Leica Instruments Ltd, Germany), The 10 strongest dyeing visual field were selected and put into computer by microscopy at 40× magnification, then were converted to gray image. For normal lung tissues, 20 bronchial epithelium or glandular epithelial cells were tested in each visual field; as to the lung cancer, 20 positive cancer cells were tested in each visual field. Two hundred positive cells were measured in each specimen. An interactive method (Shen et al., 1993; Shen., 1994; Shen., 1995) was used to measure the gray level (Gα) of each positive cell. Background gray level (Gβ) was measured in the same field. The 'Drawing Area' mode of the Leica software was used to mark the outline of the positive nuclei. The average gray level of the 200 positive cells was recorded as Gα. The outline of a randomly chosen background was marked out, and the value of the average gray level was recorded as Gβ. According to the formula below, a positive unit (PU) of each positive cell was calculated. The average PU value of 200 positive cells was calculated using the equation:

$$PU = [(G\alpha - G\beta) \div G\ max] \times 100$$

Where G max equals 256.

Semi-quantitative criterion

TGF-β1 staining intensity and the number of positive cells were estimated using a four-tiered scoring system as described previously (Xue et al., 2011): negative (–), no staining at all; weak (+), weak staining regardless of positive cell percentages or moderate staining of ≤30% of cells; moderate (+ +), moderate staining of >30% of cells or strong staining of ≤50% of cells; strong (+++), strong staining of >50% of cells. Low expression of TGF-β1 was defined as (-) or (+), High expression was defined as (++) or (+++).

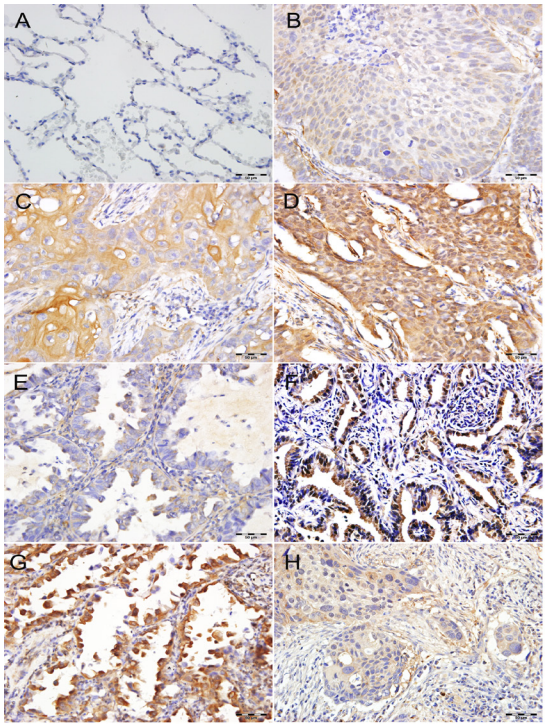

Figure 1. Expression of TGF-β1 in Normal Lung and NSCLC Tissues. **(A)** Negative TGF-β1 expression in alveolar epithelial cells of normal lung tissues. **(B)** Weak TGF-β1 expression in squamous cell carcinoma. **(C)** Moderate TGF-β1 expression in squamous cell carcinoma. **(D)** Strong TGF-β1 expression in squamous cell carcinoma. **(E)** Weak TGF-β1 expression in adenocarcinoma. **(F)** Moderate TGF-β1 expression in adenocarcinoma. **(G)** Strong TGF-β1expression in adenocarcinoma. **(H)** Moderate TGF-β1 expression in large cell lung cancer

Statistical analysis

All statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Independent-sample T Test was used for compare the differences between the two groups. One-way ANOVA was applied to overall comparison of means, Dunnett test was preformed for comparisons among group. Kaplan-Meier method was conducted to survival curves, which were compared with Log-rank test. Univariate and multivariate analyses were carried out by the Cox proportional hazards regression model. $p<0.05$ was considered statistically significant.

Results

The expression of TGF-β1 protein in NSCLC and normal lung tissues

TGF-β1 in NSCLC mainly located in cytoplasm (Figure

1). TGF-β1 positive cells were stained homogeneous brown granules, which were negative or weak expression in normal lung tissues. Of 115 NSCLC patients with five years of follow-up, 47 cases were low TGF-β1 expression (40.8%), 68 cases were high expression (59.2%).

The quantitive analysis of TGF-β1 protein PU value in NSCLC and normal lung tissues

The TGF-β1 protein PU value in NSCLC tissues (12.96±5.06) is significantly higher than in normal lung tissues (7.85±3.78), the difference was statistically significant (p=0.000) (Table 1).

Table 1.TGF-β1 Protein PU in NSCLC and Normal Lung Tissues (mean±SD)

Group	n	TGF-β1 PU	*p* value
Lung cancer tissues	194	12.96±5.06	0
Normal lung tissues	24	7.80±3.54	

Table 2. Relationships between TGF-β1 Protein PU and Clinicopathological Characteristics of Lung Carcinomas (mean±SD)

Variable	N	TGF-β1 PU	P value
Age			
≤60	111	12.77±4.81	0.549
>60	83	13.21±5.38	
Gender			
Male	138	12.97±4.99	0.939
Female	56	12.91±5.26	
Smoking story			
Never	88	13.18±4.92	0.571
Ever	106	12.77±5.19	
Lymph-node metastasis			
Negative	99	10.58±4.50	0
Positive	95	15.05±4.60	
Tumor location			
peripheral	148	12.80±4.99	0.448
Central	46	13.45±5.30	
TNM stage			
I-II	132	11.55±4.75	0
III-IV	62	15.87±4.44	
Tumor differentiation			
Poorly	61	13.28±4.97	0.504
Moderately	85	13.20±5.17	
Well	37	12.15±4.75	
Histology			
Squamous cell carcinoma	76	13.27±5.58	0.685
Adenocarcinoma	107	12.83±4.59	
Large cell lung cancer	11	11.97±5.90	

*The differentiation group consists of 76 squamous cell and 107 adenocarcinomas

The relationships between TGF-β1 protein PU value and clinicopathological characteristics

In 194 case of NSCLC, the TGF-β1 protein PU value was correlated with TNM stages and lymph node metastasis. As the stage became advanced, the TGF-β1 PU value was increased (p=0.000). TGF-β1 protein PU value was significantly higher in patients with lymph node metastasis (15.05±4.60) than in patients without lymph node metastasis (10.58±4.50) (p=0.000). There was no association between TGF-β1 protein PU value and patient's gender, age, smoking history, tumor differentiation, histological subtypes and tumor location (p>0.05). Data were shown in Table 2.

Correlations between TGF-β1 protein PU value and prognosis of NSCLC patients

According to Kaplan–Meier survival analysis, the prognosis was worse in patients with high expressed TGF-β1 than that patients with low expression (p=0.000, Figure 2). Upon the univariate analysis with the Cox proportional hazards regression model, TGFβ1 expression (p=0.000) and lymph node metastasis (p=0.000) were positively correlated with a poor prognosis. According to multivariable Cox regression model analysis, high expression of TGFβ1 and metastasis were identified as independent predictors of five-year survival rate for patients with NSCLC (p=0.002, p=0.030 respectively, Table 3).

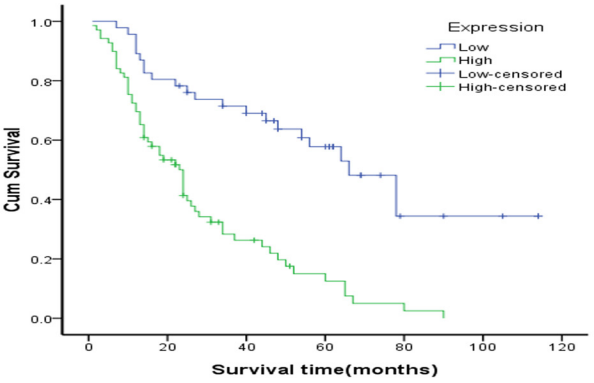

Figure 2. Kaplan-Meier Analysis of Five-Year Survival Rate in 115 NSCLC Patients. The prognosis of patients with high TGF-β1 expression was poorer than those with low TGF-β1 expression (p=0.000, Log rank test)

Table 3 Cox Proportional Hazards Regression Model Prognosis Analysis for 115 Non-Small Lung Cancer Patients

Variable	Univariate			Multivariate		
	HR	95%CI	*P*	HR	95%CI	*P* value
Age	1.135	0.731-1.761	0.573			
Gender	1.041	0.630-1.721	0.875			
Smoking story	0.969	0.623-1.507	0.889			
Lymph-node metastasis	3.11	1.921-5.035	0	1.874	1.061-3.309	0.03
Tumor location	1.398	0.840-2.328	0.198			
TNM stage	1.445	0.911-2.292	0.125			
Tumor differentiation	0.878	0.626-1.232	0.453			
Histology	0.824	0.610-1.115	0.21			
TGF-β1 expression	3.578	2.157-5.934	0	2.565	1.414-4.652	0.002

Discussion

Immunohistochemistry plays an important role in pathological study and diagnosis. Up to now, the traditional qualitative or semi-quantitative method based on area and intensity of protein expression is still widely used as immunohistochemical criterion. Meanwhile, even using the same criterion, the immunohistochemical result need to be evaluated by at least two experienced pathologists. The inevitable evident defects of the method such as much more subjectivity and lack of repeatablity and comparability may influence the validity of study and diagnosis. In order to overcome the above defects, a quantitative immunohistochemical method was developed. In our research, we used the positive unit (PU) value to reflect expresision intensity of positive cells in NSCLC and normal lung tissues. The PU value is presented as concrete numerical value. As PU value become higher, the corresponding protein expression intensity get stronger. Our previous studies have proved that this method can ensure the results more precisely and scientifically (Bai et al., 2009; Xu et al., 2013; Wan et al., 2013; Lei et al., 2013).

In our study, we found that the expression of TGF-β1 protein in NSCLC tissues were significantly higher than in the normal lung tissues. This result is in consistent with the previous studies (Takanami et al., 1994; Takanami et al., 1997). Although the specific mechanism is still not very clear, the high expression of TGF-β1 protein in NSCLC tissues may be related to reduction of TβR (TGF-β receptor) and abnormity of Smads protein. TGF-β ligand binding to TβR II receptor is attributed to the activation of TGF-β signalling pathway that lead to TβR I phosphorylation. Then, signals are transfered to a components of intracellular signaling known as Smads. Smads are divided into three types based on their functional properties, the receptor-regulated Smads (Smad1, 2, 3, 5, and 8), the common Smads (Smad4 and 4β), and the antagonistic Smads (Smad6 and 7). Upon activation of TβRI receptor, Smad2 and/or Smad3 are combined with the receptor and then are phosphorylated by the TβRI receptor. The phosphorylated Smad can forms a complex with Smad4. By interacting with DNA-binding proteins, Smad complexes positively or negatively regulate the transcription of target genes. Therefore, the abnormity of any members in TGF-β or Smad family are thought to play important roles in disrupting TGF-beta signaling. Kim. (1999) found decreased TGF-beta RII expression in NSCLC tissues, suggesting that decreased TβRII expression plays an important role in the carcinogenesis of lung cancer. Study (Yanagisawa et al., 2000) have shown that high frequent absence of Smad2 and Smad4 mutants changed the TGF-β signal transduction pathways and lead to the initiation of lung tumor.

We further studied the correlation between TGF-β1 protein expression and clinicopathological characteristics. TGF-β1 protein expression was associated with TNM stage of tumor. The higher is the expression of TGF-β1 protein, the more advanced stage for patients with NSCLC, suggesting TGF-β1 plays an important role in the progression of NSCLC. In addition, TGF-β1 protein expression was significantly higher in patients with lymph node metastasis than patients without lymph node metastasis, which is different from the study (Takanami et al., 1997) that there was no association between TGF-β1 protein expression and lymph node metastasis. We supposed that there may be three main reasons. Firstly, pulmonary adenocarcinoma tissues were chosen by Takamaka, whereas we select the NSCLC samples in our study. Secondly, semi-quantitative method was applied by Takamaka to evaluate the TGF-β1 expression, while we used quantitative method to analysis PU value of TGF-β1. Finally, The sample size in our study were larger than Takamaka's. Our results indicated that TGF-β1 protein overexpression is closely associated with lung cancer progression and metastasis. As we all know, TGF-β1 is one of growth factors that can regulate extracellular matrix composition and induces epithelial-to-mesenchymal transition (EMT) in alveolar epithelial cell (Kasai et al., 2005). During the EMT process, carcinoma cells lose cell-cell adherence junctions and transfer to fibroblast-like morphology, and become motile and invasive. Studies have indicated that tumor cells produce high levels of TGF-β1, which promotes the induction of EMT in cancer cells thus contributing to metastasis of tumor (Janda et al., 2002). Study have showd that the blockade of TGF-β signaling pathway using SiRNA can lead to suppression of cell proliferation, invasion and metastasis and induced cell apoptosis in lung adenocarcinoma (Xu et al., 2011), this study further proved that TGF-β1 is closely associated with the progression and metastasis of lung cancer from the opposite direction. PI3K/Akt and MAPK/Erk1/2 may be served as important regulators of TGF-β1-induced EMT process in human lung cancer (Chen et al., 2013)

This study found there were two factors influencing the prognosis: TGF-β1 expression and lymph node metastasis. The five-year survival rate in patients with low expression of TGF-β1 was higher than that in patients with over expression of TGF-β1. Several experiments found that inhibiting the expression of TGF-β1 show enhanced survival in some cancers. Wiedmann. (2005) have already indicated the therapeutic potential of antagonizing the TGF-β pathway in gastrointestinal cancer. Combining RIG-I activation with TGF-β1 silencing via bifunctional ppp-siRNA breaks tumor-mediated immunosuppresive mechanisms and confers potent antitumor efficacy in pancreatic cancer (Ellermeier et al., 2013). The expression of TGF-β1 might be suppressed by Cantharidinate and then prevent the development of colorectal cancer (Jie Ma et al., 2014). Furthermore, The prognosis of patients with lymph node metastasis was poorer than those without lymph node metastasis. Previous semi-quantitative study indicated that TNM stage was correlated with prognosis in lung adenocarcinoma. We considered that further research with larger sample size need to be conducted to confirm whether TNM stage or lymph node metastasis was associated with prognosis in NSCLC.

In summary, this is the first study showing the expression of TGF-β1 in NSCLC tissues from the quantitative point of view. High expression of TGF-β1 protein was corelated with progression, metastasis and poor prognosis of lung cancer patients, These findings

suggest that TGF-β1 protein expression can be used as an important indicator for evaluating the prognosis of patients with lung cancer, which have been proved as a usefull marker for progression and prognosis of breast cancer (Gregory Tripsianis et al., 2013). However, The molecular mechanism by which TGF-β1 associated with prognosis has not been elucidated, and whether suppressing the secretion of TGF-β1 or antagonizing TGF-β1' effect being a new strategy of antineoplastic treatment in lung cancer patients still needs to be studied.

Acknowledgements

This work was supported by a Grant (2010B060300001) from the Science and Technology Planning Project of Guangdong Province, People's Republic of China. All authors read and approved the final manuscript. There are no conflicts of interest relating to the publication of this manuscript.

References

Assoian RK, Komoriya A, Meyers CA, et al (1983). Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem*, **258**, 7155-60.

Bai X, Shen H, Zhou C, et a1 (2009). Expression of thyroid transcription factor-l (TTF—1)in lung carcinomas and its correlations with apoptosis and angiogenesis. *Clin Oncol Cancer Res*, **6**, 16-20.

Blobe GC, Schiemann WP, Lodish HF (2000). Role of transforming growth factor beta in human disease. *N Engl J Med*, **342**, 1350-8.

Boyd FT, Massague J (1989). Transforming growth factor-beta inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. *J Biol Chem*, **264**, 2272-8.

Chen XF, Zhang HJ, Wang HB, et al (2012). Transforming growth factor-beta1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. *Mol Biol Rep*, **39**, 3549-56.

Derynck R, Akhurst RJ, Balmain A (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet*, **29**, 117-29.

Ellermeier J, Wei J, Duewell P, et al (2013). Therapeutic efficacy of bifunctional siRNA combining TGF-beta1 silencing with RIG-I activation in pancreatic cancer. *Cancer Res*, **73**, 1709-20.

Furuta K, Misao S, Takahashi K, et al (1999). Gene mutation of transforming growth factor beta1 type II receptor in hepatocellular carcinoma. *Int J Cancer*, **81**, 851-3.

Grady WM, Rajput A, Myeroff L, et al (1998). Mutation of the type II transforming growth factor-beta receptor is coincident with the transformation of human colon adenomas to malignant carcinomas. *Cancer Res*, **58**, 3101-4.

Tripsianis G, Papadopoulou E, Romanidis K, et al (2013). Overall survival and clinicopathological characteristics of patients with breast cancer in relation to the expression pattern of HER-2, IL-6, TNF-α and TGF-β1. *Asian Pac J Cancer Prev*, **14**, 6813-20.

Janda E, Lehmann K, Killisch I, et al (2002). Ras and TGF cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol*, **156**, 299-313.

Jemal A, Siegel R, Ward E, et al (2007). Cancer statistics, 2007. *CA Cancer J Clin*, **57**, 43-66.

Jennings MT, Pietenpol JA (1998). The role of transforming growth factor beta in glioma progression. *J Neurooncol*, **36**, 123-40.

Kasai H, Allen JT, Mason RM, et al (2005). TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res*, **6**, 56.

Kim WS, Park C, Jung YS, et al (1999). Reduced transforming growth factor-beta type II receptor (TGF-beta RII) expression in adenocarcinoma of the lung. *Anticancer Res*, **19**, 301-6.

Lei B, Liu S, Qi WJ, et al (2013). PBK/TOPK expression in non-small-cell lung cancer: its correlation and prognostic significance with Ki67 and p53 expression. *Histopathology*, **63**, 696-703.

Luwor RB, Kaye, AH, Zhu HJ (2008). Transforming growth factor-beta (TGF-beta) and brain tumours. *J Clin Neurosci*, **15**, 845-55.

Ma J, Gao H-M, Hua X, et al (2014). Role of TGF-β1 in human colorectal cancer and effects after cantharidinate intervention. *Asian Pac J Cancer Prev*, **15**, 4045-8.

Markowitz S, Wang J, Myeroff L, et al (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science*, **268**, 1336-8.

Rich J, Borton A, Wang X (2001). Transforming growth factor-beta signaling in cancer. *Microsc Res Tech*, **52**, 363-73.

Siegel PM, Massague J (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer*, **3**, 807-21.

Shen H, Lu Y (1993). Study on quantitative method of immunochemical staining. *J Biomed Eng*, **10**, 281-4.

Shen H (1994). Study on quantitative method of intensity of immunohistochemical staining (II). *J Cell Mol Immunol*, **10**, 33-5.

Shen H (1995). Study on quantitative method of immunochemical staining (III). *Chin J Histochem Cytochem*, **4**, 89-92.

Takanami I, Imamura T, Hashizume T, et al (1994). Transforming growth factor beta 1 as a prognostic factor in pulmonary adenocarcinoma. *J Clin Pathol*, **47**, 1098-100.

Takanami I, Tanaka F, Hashizume T, et al (1997). Roles of the transforming growth factor beta 1 and its type I and II receptors in the development of a pulmonary adenocarcinoma: results of an immunohistochemical study. *J Surg Oncol*, **64**, 262-7.

Wan L, Li X, Shen H, et al (2013). Quantitative analysis of EZH2 expression and its correlations with lung cancer patients' clinical pathological characteristics. *Clin Transl Oncol*, **15**, 132-8.

Wiedmann MW, Caca K (2005). Molecularly targeted therapy for gastrointestinal cancer. *Curr Cancer Drug Targets*, **5**, 171-93.

Xu CC, Wu LM, Sun W, et al (2011). Effects of TGF-beta signaling blockade on human A549 lung adenocarcinoma cell lines. *Mol Med Rep*, **4**, 1007-15.

Xu XY, Lin N, Li YM, et al (2013). Expression of HAb18G/CD147 and its localization correlate with the progression and poor prognosis of non-small cell lung cancer. *Pathol Res Pract*, **209**, 345-52.

Xue YJ, Lu Q, Sun ZX (2011). CD147 overexpression is a prognostic factor and a potential therapeutic target in bladder cancer. *Med Oncol*, **28**, 1363-72.

Yanagisawa K, Uchida K, Nagatake M, et al (2000). Heterogeneities in the biological and biochemical functions of Smad2 and Smad4 mutants naturally occurring in human lung cancers. *Oncogene*, **19**, 2305-11.