

RESEARCH ARTICLE

Serum Pleiotrophin Could Be an Early Indicator for Diagnosis and Prognosis of Non-Small Cell Lung Cancer

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Abstract

Aims: Pleiotrophin (PTN), an angiogenic factor, is associated with various types of cancer, including lung cancer. Our aim was to investigate the possibility of using serum PTN as an early indicator regarding disease diagnosis, classification and prognosis, for patients with non-small cell lung cancer (NSCLC). **Methods:** Significant differences among PTN levels in patients with small cell lung cancer (SCLC, n=40), NSCLC (n=136), and control subjects with benign pulmonary lesions (n=21), as well as patients with different pathological subtypes of NSCLC were observed. **Results:** A serum level of PTN of 300.1 ng/ml, was determined as the cutoff value differentiating lung cancer patients and controls, with a sensitivity and specificity of 78.4% and 66.7%, respectively. Negative correlations between serum PTN level and pathological differentiation level, stage, and survival time were observed in our cohort of patients with NSCLC. In addition, specific elevation of PTN levels in pulmonary tissue in and around NSCLC lesions in comparison to normal pulmonary tissue obtained from the same subjects was also observed (n=2). **Conclusion:** This study suggests that the serum PTN level of patients with NSCLC could be an early indicator for diagnosis and prognosis. This conclusion should be further assessed in randomized clinical trials.

Keywords: Pleiotrophin - non-small cell lung cancer - diagnosis - prognosis

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Introduction

Pleiotrophin (PTN), a secretory growth factor, is also known as heparin affinity regulatory peptide (Laaroubi et al., 1995), heparin binding growth associated molecule (Hampton et al., 1992), heparin binding growth factor-8 (Milner et al., 1989), and heprin binding neutrophilic factor (Kovesdi et al., 1990), due to its high affinity to heparin. PTN shares 50% homogeneous amino acid sequence with midkine (MK), and these two consist the family of heparin binding growth factors (Kovesdi et al., 1990). PTN is highly consistent among different species, which was reported to present in insects, chicken, mouse, cows, fishes, frogs and human (Tsutsui et al., 1991; Englund et al., 2006).

The overexpression of PTN through either autocrine or pericrine is associated with various metastatic human cancers, including breast, prostatic, ovarian, pancreatic and lung cancer, as well as melanoma, choriocarcinoma, and glioblastoma (Schulte et al., 1996; Jager et al., 1997; Lu et al., 2005). It was reported that PTN promoted cell mitosis, migration and chemotaxis (Kadomatsu et al., 2004). Usually, serum PTN in patients with metastatic tumors were easily detected (Kadomatsu et al., 2004), and were highly consistent with the expression of PTN in tumor tissues (Souttou et al., 1998).

However, clinical studies concerning the overexpression of PTN in lung cancer were limited. In 1997, Jager et al. used RT-PCR to profile the PTN expression in 26 lung cancer cell lines, including 12 small cell lung cancer (SCLC) cell lines and 14 non-small cell lung cancer (NSCLC) cell lines, with normal human bronchial epithelial cells as control. It was revealed that PTN mRNA was expressed in 9 SCLC and 3 NSCLC cell lines (Jager et al., 1997). In a later clinical study with small sample size, the same group reported that 87% SCLC patients and 63% NSCLC patients demonstrated elevated serum PTN levels, leading to a 10-fold increase of serum PTN levels in lung cancer patients compared with healthy control subjects, suggesting a potential role could be played by serum PTN levels in early diagnosis and chemo response of lung cancer (Jager et al., 2002). Furthermore, it was demonstrated in our previous studies that knockdown of PTN gene could effectively reduce the transcription and expression of PTN in H446 cells, inhibit cell growth, induce apoptosis and further affect the expression of genes associated with H446 cells reproduction and migration, using a PTN specific lentivirus RNA interference vector constructed by our group (Yu et al., 2010).

However, the potential role of serum PTN levels in early diagnosis of lung cancer, especially NSCLC, a main type of lung cancer, still requires further investigation.

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Patients and Methods

Patients and benign control subjects

Plasma samples were collected from 176 patients (114 male, 62 female, mean age 63.66±8.68 years) with histologically confirmed lung cancer registered at department of respiration or thoracic surgery in the second affiliated hospital of Soochow University from 2008-2013, among whom 136 patients were diagnosed with NSCLC (76 cases of squamous cell carcinoma and 60 cases of adenocarcinoma), and 40 others were diagnosed with SCLC. Twenty-two of the NSCLC patients were staged as I, 14 as II, 49 as III and 51 as IV. Also, 42 of the NSCLC patients were classified as low-differentiated (or undifferentiated), 56 as median-differentiated and 38 as highly-differentiated. As for the SCLC patients, 24 cases were classified as limited diseases (LD) and 16 cases as extensive diseases (ED), according to the stage criteria of the veteran's administration lung cancer study group (VALSG). All the lung cancer patients were followed up by telephone every six month. Six patients with NSCLC and 3 patients with SCLC were lost in follow-up. Plasma samples obtained from twenty-one patients with benign pulmonary diseases (9 cases of inflammatory pseudotumor, 7 cases of tuberculoma, 3 cases of hamartoma and 2 cases of sarcoidosis) during the same period served as control (12 male, 9 female, mean age 59.90 ± 18.14 yrs).

ELISA for PTN serum levels

Blood samples (3.5 ml each) from the ulnar vein of patients with either lung cancer or benign pulmonary diseases were collected and stored at 4°C prior to ELISA analysis. The samples were centrifuged for 10 min at 3000 r/min at 4°C to isolate serum. PTN levels were determined using the human PTN ELISA kit strictly according to the manufacturer's manual (Shanghai BlueGene Biotech CO., LTD, Shanghai, China).

RT-PCR and western blot for PTN mRNA and protein expression, respectively

The primer of PTN was designed based on its gene sequence as: 5'-TATGTTCCACAGGTGACATC-3' (sense primer) and 5'-AGAGGACGTTTCCAACCTCAA-3' (anti-sense primer). β -actin was used as internal control with primer sequences of 5'-AGCGAGCATCCCCAAAGTT-3' (sense primer) and 5'-GGGCACGAAGGCTCATCATT-3' (anti-sense primer). The amplification fragments of PTN and β -actin were 550 and 285 bp, respectively. Intratumor specimens, with corresponding peritumor specimens and normal pulmonary tissues (used as control) were resected from surgical subjects suffering squamous cell carcinoma or adenocarcinoma (one of each) with total RNA extracted and reverse transcribed into cDNA using Revertaid First Stand cDNA Synthesis Kit, according to the manufacturer's instruction (Fermentas, Canada). The amplified PCR were determined by 1% agarose gel electrophoresis.

Subsequently, western blot was used to determine the PTN protein levels in the obtained surgical specimens. Protein samples were extracted the homogenized lung specimens with RIPA lysis reagent with the total concentration determined by BCA assay. Protein samples were then separated by 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane by 2 h 300 mA constant current, followed by saturation with 5% skim milk powder solution for another 2 h. anti-PTN (1: 100, Abcam, USA) and anti- β -actin (1: 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies were added and incubated overnight at 4°C. A peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added successively and incubated for another hour at room temperature. The protein levels of PTN and β -actin were then detected using HRP-ECL (Millipore, USA).

Statistical Analysis

The data obtained was presented as mean±standard deviation (SD), with statistical analyses performed with SPSS (version 11.0). Differences with $p < 0.05$ were considered statistically significant. The comparisons of mean PTN serum levels among different pathological

Table 1. Summary of Clinco-Pathological Data and Serum PTN Levels of Recruited Subjects with Statistical Comparisons

Group	Number	Serum PTN level (ng/ml)	Statistical comparison
Lung cancer patients	176	462.51±184.19	F=13.93, $p < 0.01$, compared with control
Gender			t=1.10, $p > 0.05$
Male	114	472.90±177.02	
Female	62	440.27±198.53	
Pathological classification			$\chi^2=105.349$, $p < 0.01$
squamous cell carcinoma ^a	76	367.03±164.68	a versus c, $\chi^2=193$, $p < 0.01$
adenocarcinoma ^b	60	423.76±136.19	b versus c, $\chi^2=118$, $p < 0.01$
Small cell lung cancer ^c	40	679.37±101.78	b versus c, $\chi^2=1826$, $p < 0.05$
Non-small cell lung cancer		136	398.73±151.51
Differentiation levels			F=18.02, $p < 0.01$
Low ^d	42	501.77±122.51	
Medium ^e	56	342.08±144.49	d versus e, d versus f, $p < 0.01$
High ^f	38	368.35±135.05	e versus f, $p > 0.05$
Stages			$\chi^2=105.35$, $p < 0.01$
I	22	168.99±46.61	
II	14	275.14±57.27	
III	49	386.85±72.31	
IV	51	543.18±79.21	
Control subjects	33	292.25±92.52	

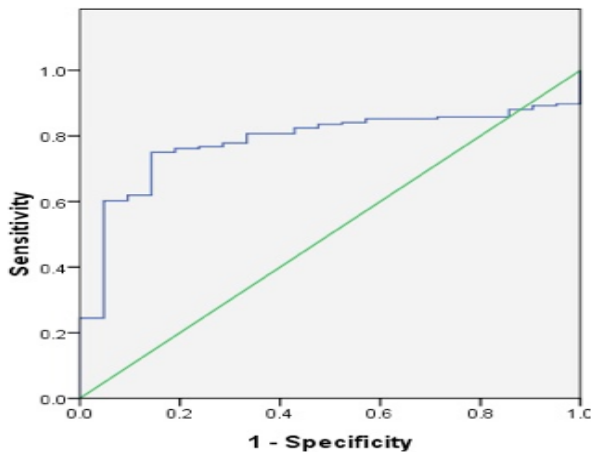


Figure 1. Receiver Operating Characteristic Curve (ROC) Indicating the Sensitivity and Specificity of the Chosed Cutoff in Differentiating Patients with

types/subtypes of lung cancers, genders and stages of SCLC, as well as lung cancers *versus* benign control were performed with student's test. Receiver operating characteristic curve (ROC) was employed to determine the threshold between the PTN levels of lung cancer patients and benign control subjects. Linear correlation analysis was used to determine the relationship between age and PTN levels. The relationship between differentiation levels and PTN was established by analysis of variance. The correlations between stages of NSCLC and PTN were analyzed using Cruskal-Wallis rank sum test, while the relationship between PTN and different pathological classifications were analyzed by Cruskal-Wallis and Mann-Whitney rank sum test. The survival analysis of both SCLC and NSCLC groups was performed by Cox proportional hazards regression with Enter method for variable selection.

Results

Elevated PTN serum levels in both SCLC and NSCLC patients

As listed in Table 1, the overall average serum PTN levels of patients with lung cancer was 462.51 ± 184.19 ng/ml, which was significant higher than that of patients with benign pulmonary diseases (292.25 ± 92.52 ng/ml), $p < 0.01$. As shown in Figure 1, serum PTN level of 300.12 ng/ml was determined as a cutoff value between lung cancer patients and controls, with a sensitivity and specificity of 78.4% and 66.7%, respectively, using ROC approach. Serum PTN levels of NSCLC patients were determined to be 398.73 ± 151.51 ng/ml, which was significantly lower than that of SCLC patients (679.37 ± 101.78 ng/ml), $p < 0.01$, and consistent with previous reports. No apparent correlation of serum PTN levels in lung cancer subjects with either ages ($r = 0.075$, $p > 0.05$) or genders ($p > 0.05$) of patients were observed.

Correlations of elevated PTN serum levels with pathological subtypes, differentiation grades and TNM stages in NSCLC patients

Besides the significantly different average PTN

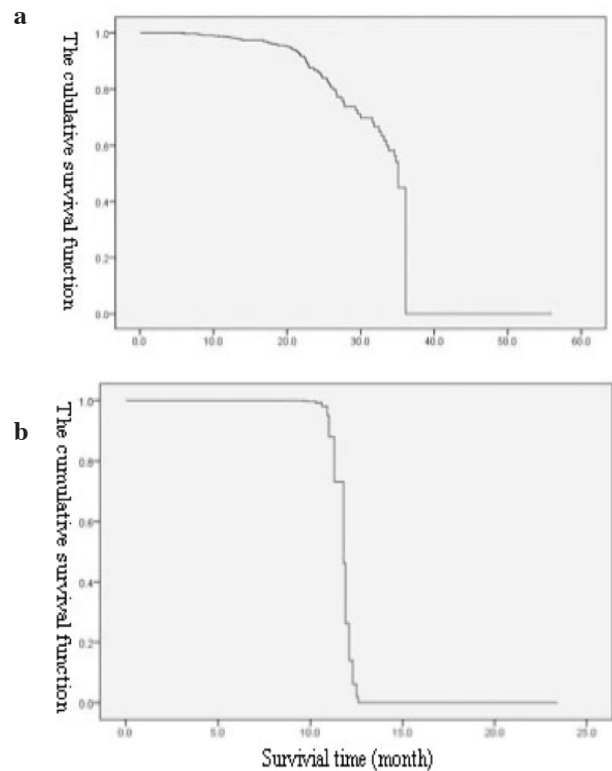


Figure 2. Survival Function at the Mean of Covariates: (a) Non-small cell lung cancer patients; (b) Small Cell Lung Cancer Patients

serum levels observed between NSCLC and SCLC patients, further classification of NSCLC into squamous cell carcinoma and adenocarcinoma revealed that serum PTN levels significantly varied among different subtypes ($p < 0.05$). Furthermore, correlations between serum PTN levels and differentiation grades of NSCLC were also demonstrated ($p < 0.01$). The serum PTN levels of low, medium and high differentiation group were 501.77 ± 122.51 , 342.08 ± 144.49 and 368.35 ± 135.05 ng/ml, respectively, with significant differences spotted between low and medium/high differentiation groups ($p < 0.01$). Moreover, Significantly different serum PTN levels were observed for NSCLC patients at different TNM stages ($p < 0.01$). Similarly, significant differences were also observed between LD and ED groups of SCLC ($p < 0.01$), which were consistent with literature reports (Jager et al., 2002).

Correlations of elevated PTN serum levels with survival times in both NSCLC and SCLC patients

Cox proportional hazard regression analysis revealed that PTN serum level was an independent risk factor associated with the survival times of NSCLC patients (Figure 2a). The relative risk (RR) value of serum PTN was 1.006, suggesting that the relative risk of survival time decreasing in NSCLC patients was 1.006% when serum PTN concentration increased by 1%. As shown in Figure 2b, similar correlation was also demonstrated in SCLC patients with the RR value calculated to be 1.051.

Specific elevation of PTN mRNA and protein levels in intratumor regions in NSCLC patients

As demonstrated in Figure 3a, the semi-quantitative

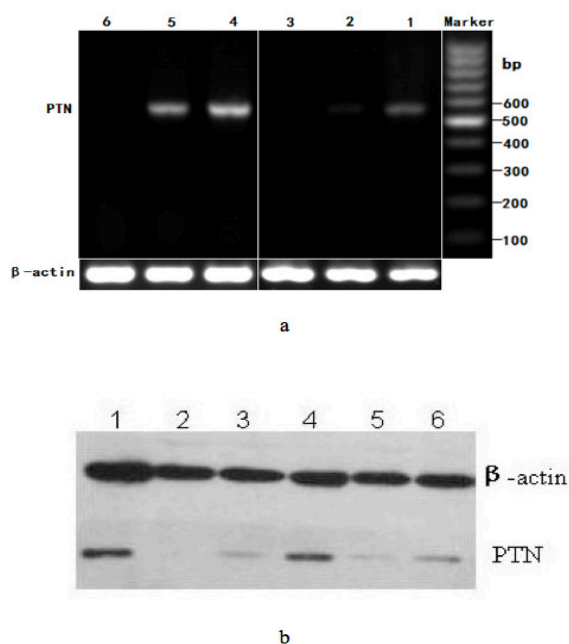


Figure 3. Pleiotrophin Expression in Intratumor (1, 4), Peritumor (3, 6) and Normal (2, 5) Specimens Obtained from a Squamous Cell Carcinoma Patient (1, 2, 3) and a Adenocarcinoma Patient (4, 5, 6): (a) Pleiotrophin mRNA Expression, Amplification Products of Semi-quantitative RT-PCR Shown in Agarose Gel Electrophoresis; (b) Pleiotrophin Protein Expression by Western Blotting

PCR analysis revealed that there was no apparent PTN mRNA expression in the normal tissues obtained from either squamous cell carcinoma and adenocarcinoma patients. On contrary, mRNA expression levels in the intratumor specimens from both squamous cell carcinoma and adenocarcinoma patients appeared to be highly overexpressed, compared with either normal tissues or peritumor specimen, which also overexpressed PTN mRNA (Figure 3a).

Similar pattern of PTN expression on the protein level was confirmed by western blotting. As shown in Figure 3b, the intensity of the bands representing the PTN protein levels in intratumor specimens obtained from patients suffering the two subtypes of NSCLC was the highest, followed by peritumor specimens and normal tissues. The lack of visible band for normal tissues suggested that normal pulmonary tissues did not expression PTN protein, which was consistent with the RT-PCR results.

Discussion

Lung cancer is a leading cause of cancer-related mortality throughout the world (Organization et al., 2014). In China, lung cancer related mortality rate increased by 464.84% in the past three decades (National et al., 2010; Xie et al., 2014), probably due to the dramatic increased rate of cigarette smoking (Zhang et al., 2003), as well as aggravated air pollution (Pruss et al., 2006).

Early diagnosis is very important. And in this area, one consideration is to develop biomarkers that allow early diagnosis possible. Based on previous results, overexpression PTN could be detected in a wide variety of human cancers, and is believed to be proto-oncogenic.

In-vitro and in-vivo experiments confirmed that elevated expression of PTN could increase the malignant grade of tumor cells, whereas knockdown of PTN can effectively inhibit tumor cell growth, migration and invasion (Schulte et al., 1996; Jager et al., 1997; Kadomatsu et al., 2004; Lu et al., 2005). Current strategies of target therapy towards PTN genes and its receptors include interferences of ribozymes and RNA, monoclonal antibody, etc. (Czubayko et al., 1994; Grzelinski et al., 2006; Papadimitriou et al., 2009). Therefore, PTN, as a potential molecular target of cancer therapy, has become a focus of research.

In this study, elevated serum PTN levels both for SCLC and NSCLC patients were confirmed in a relative large scale clinical investigation, especially for NSCLC. A higher serum PTN level in SCLC patients compared with NSCLC ones demonstrated here is consistent with the results reported early (Jager R et al., 2002; Ji et al., 2014). The change of PTN level between lung cancer group and control was much smaller than previously reported, which could be explained by the different nature of control group (subjects with benign pulmonary diseases *versus* healthy subjects, as well as the large proportion of NSCLC patients in the current study. Furthermore, since patients with benign pulmonary diseases were used as control, instead of healthy subjects, this study provided a much more realistic and plausible criterion to distinguish lung cancer patients from benign diseases such as inflammatory pseudotumor, which might actually be challenging for early diagnosis of lung cancer. Moreover, this is the first study revealed that serum PTN levels correlated with pathological subtypes in NSCLC patients, which was further confirmed in tumor specimens of NSCLC subjects by both RT-PCR and western blot analysis. In addition, the relative large number of NSCLC patents recruited in the current study allowed more detailed correlation analysis between NSCLC patient serum PTN levels and TNM stages/differentiation levels. All the results suggested that serum PTN could serve as an early indicator for NSCLC diagnosis, in terms of both initial disease confirmation and monitoring of disease progression.

By continuously following patient status through telephone interviews, we were able to investigate whether the serum PTN level at diagnosis was an independent risk factor for prognosis of lung cancer patients. The positive correlation between serum PTN levels and survival times for patients with both NSCLC and SCLC demonstrated in this current study suggested that serum PTN levels could serve as an early indicator for diagnosis and prognosis of lung cancer. In conclusion, this study suggests that serum PTN level of patients with NSCLC could be an early indicator for their diagnosis and prognosis. But this conclusion should be confirmed by randomized clinical trials.

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