Clinical significance of serum and tumor tissue endostatin evaluation in operable non-small cell lung cancer

MING-MING HU[1], YING HU[1], HAI-QING ZHANG[2], WEN-YUN JIA[1], ZHE QIAN[1], YUAN YANG[1] and BAO-LAN LI[1]

Departments of [1]General Medicine and [2]Pathology, Beijing Tuberculosis and Thoracic Tumor Research Institute/ Beijing Chest Hospital, Capital Medical University, Beijing 101149, P.R. China

Received June 7, 2014; Accepted July 15, 2014

DOI: 10.3892/br.2014.319

Abstract. Endostatin, as the most potential antiangiogenic factor, is a naturally occurring fragment of collagen XVIII in bloodstream capable of inhibiting tumor growth and metastasis. This study was conducted to explore the clinical value of endostatin in serum and tumor tissue in patients with operable non-small cell lung cancer (NSCLC). ELISA and immunohistochemistry were applied to detect the expression of endostatin in serum and tumor tissue in 105 patient-matched operable NSCLC patients. The serum level of endostatin was significantly higher in NSCLC patients than healthy individuals (P=0.0018). Cases with poorer differentiation showed a higher endostatin serum level (P=0.008). There was no significant correlation between tumor tissue expression and clinical parameters, such as TNM stage, differentiation degree, histological type and lymph node invasion status. A stronger expression of endostain in tumor tissue was associated with a higher serum level (r=0.223). The univariate and multivariate analyses with Cox proportional hazards model for overall survival showed that tumor stage and node status were independent prognostic factors, whereas neither endostatin levels in serum nor in tumor tissue showed potential in predicting the long-term survival of operable NSCLC patients. In conclusion, the results observed in the present study did not support the prediction of overall survival in operable NSCLC based on the expression levels of endostatin in serum and tumor tissue.

Introduction

Non-small cell lung cancer (NSCLC) is responsible for a large number of mortalities in China as compared to other forms of cancer. Recent advances in therapy and diagnosis, notwithstanding, the prognosis of NSCLC patients remains poor. The 5-year survival rate is only 15%. The TNM staging system is considered the optimum received prognostic index for lung cancer (1). Nevertheless, patients with the same post-surgical stage exhibited marked variability in recurrence and overall survival. However, additional information on molecular biology is necessary to explain this intricate phenomenon. This may assist in the identification of patients with a particular favorable or unfavorable outcome, thus allowing the selection of subgroups for adjuvant treatment, or generation of follow-up strategies.

Angiogenesis, the formation of new vasculature, is essential to tumor growth and progression (2). As in the case of many other malignances, lung cancer is angiogenesis-dependent. The emergence of tumor angiogenesis is thought to be the result of a shift in balance between positive (proangiogenic) and negative (antiangiogenic) regulators of angiogenesis in tumor (3,4). A correlation between tumor angiogenesis and prognosis has been reported for some malignant tumors, including NSCLC. Endostatin, a 20-kDa internal fragment, generated from collagen XVIII by a proteolytic process, capable of inhibiting endothelial cell proliferation and inducing endothelial cell apoptosis, is a potent angiogenesis inhibitor (5). Findings of recent studies have shown that the administration of endostatin suppresses the growth of primary and metastatic lesions in tumor-bearing animal models (6-10). A recombinant endostatin (Endostar), expressed and purified in *Escherichia coli* with an additional nine-amino acid sequence, was approved by the State Food and Drug Administration of China in 2010 for the treatment of NSCLC.

Elevated circulating endostatin level has been observed in a variety of malignancies (11-13). Additionally, no definite conclusion has been reached regarding the clinical value of endostatin expression in tumor patients. It was previously documented that tumor patients with poor survival have higher endostatin (11,13,14). By contrast, no association was found between the endostatin level and patient prognosis (12,15). In the present study, we attempted to clarify the prognostic value of endostatin expression in serum and tumor tissue, respectively. The secondary objective was to analyze the correlation between endostatin expression in serum and tumor tissue and to analyze the relationship between the endostatin expression and various clinical parameters in NSCLC patients.

Correspondence to: Professor Bao-Lan Li, Department of General Medicine, Beijing Tuberculosis and Thoracic Tumor Research Institute/Beijing Chest Hospital, Capital Medical University, 97 Beimachang Road, Tongzhou District, Beijing 101149, P.R. China
E-mail: libaolan1109@163.com

Key words: non-small cell lung cancer, endostatin, angiogenesis, prognosis

Patients and methods

Study population. The retrospective study comprised 105 patients who underwent surgery for pathologically staged I-IIIA NSCLC between 2007 and 2008. NSCLC was diagnosed historically in excised tumor tissues and staged according to the TNM-7 classification system. Patient age range was 36-84 years (median, 62 years). Tumor samples included 56 squamous cell carcinoma, 40 adenocarcinomas, 8 adenosquamous cell carcinomas and 1 larger cell carcinoma. Approximately half (43.8%) of the patietns were stage I and 72.4% were current or former smokers. At the time of diagnosis, 32.4% of the patients developed lymph node invasion. The main demographic and clinicopathological factors are shown in Table I. No patients received induced chemotherapy or radiotherapy prior to surgery. Patients with a postoperative survival of ≥60 days were included to remove the bias of perioperative death. Patients with a positive resection margin were excluded from this study. Serum samples were taken from these patients for the circulating endostatin assay and 93 tumor samples out of the 105 patients were available for the immunohistochemical examination. Blood samples from 48 healthy volunteers matched by gender and age were selected as controls. All the subjects received necessary information with regard to the study and consent was obtained. This study was approved by the Ethics Committee of Capital Medical University.

Survival time was calculated from the date of resection until the last date of contact or date of death. At the final analysis time, 54 patients succumbed to the disease during the follow-up period, 48 patients survived, while 1 case was censored in the first year and 2 cases censored in the third year following surgery. The median follow-up period for all the subjects was 55 months (range, 3-77 months).

Blood samples and assays. Peripheral venous blood was collected in commercially available EDTA tubes (Greiner Bio-One GmbH, Kremsmunster, Austria) prior to surgery, then centrifuged at 3,000 x g for 15 min to obtain the serum aliquots and stored at -80˚C until further assay. The endostatin ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to determine the circulating endostatin concentration step by step according to the manufacturer's instructions. The minimum detection limit was 23 pg/ml. Serum samples required a 50-fold dilution. In brief, 96-well plates were coated with the anti-endostatin mouse monoclonal antibody, then 100 μl assay diluent was added to each well, followed by 50 μl diluted serum sample incubated for 2 h at room temperature on a horizontal orbital microplate shaker (IKA, Guangzhou, China) set at 500 rpm. After washing four times with 400 μl washing buffer, each well was saturated by 200 μl of endostatin conjugate and incubated for 2 h at room temperature on the shaker. The washing procedure was repeated as above, then 200 μl of substrate solution was added to each well and incubated for 30 min on the benchtop in the dark. Stop solution (50 μl) was subsequently added to each well and the color in the wells changed from blue to yellow. The optical density of each well was determined within 30 min, using a microplate reader (Bio-Rad, Hercules, CA, USA) set to 450 nm. All the determinations were performed in duplicate.

Table I. Clinicopathologic characteristics of the 105 NSCLC patients.

Characteristics	No. (%)
Age, years (range)	62 (36-84)
Gender	
Male	87 (82.9)
Female	18 (17.1)
Smoking status	
Current or ever	76 (72.4)
Never	29 (27.6)
Pathologic stage	
I	46 (43.8)
II	22 (21.0)
IIIA	37 (35.2)
Tumor stage	
T1	31 (29.5)
T2	56 (53.3)
T3	16 (15.2)
T4	2 (1.9)
Node invasion	
Absent	71 (67.6)
Present	34 (32.4)
Histological type	
AC	40 (38.1)
SCC	56 (53.4)
ASC	8 (7.6)
LCC	1 (1.0)
Differentiation	
Well	1 (1.0)
Moderate	88 (83.8)
Poor	16 (15.2)
Resection margin	
Negative	10 (9.6)
Positive	95 (90.4)
Surgery type	
Segment lobectomy	4 (3.8)
Lobectomy	79 (75.2)
Pneumonectomy	22 (21.0)

NSCLC, non-small cell lung cancer; AC, adenocarcinoma; SCC, squamous cell carcinoma; ASC, adenosquamous carcinoma; LCC, large-cell carcinoma.

Immunohistochemical analysis. For immunohistochemical analysis, 93 patient-matched formalin-fixed paraffin-embedded sections (4 μm) of tumor samples obtained from 105 patients were selected for this study. Sections were mounted on saline-coated slides, dewaxed in xylene, rehydrated through graded alcohols and antigen was retrieved using citric acid buffer (0.01 M and pH 6.0) in a pressure cooker. Endogenous peroxidase activity and non-specific binding sites were blocked with 3% hydrogen peroxide and normal rabbit serum

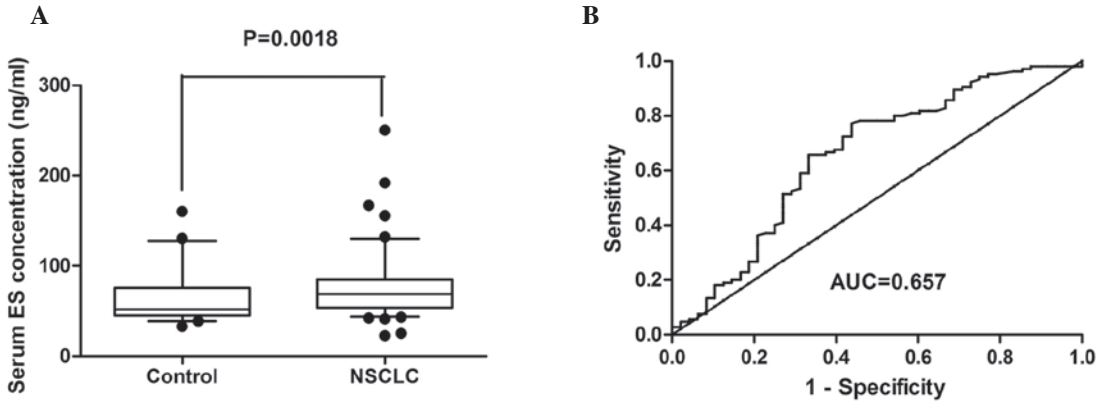

Figure 1. (A) Comparison of preoperative serum endostatin (ES) between controls (n=48) and non-small cell lung cancer (NSCLC) patients (n=105). (B) Receiver operating characteristic curve analysis of serum ES from control subjects and NSCLC patients. AUC, the area under the curve.

(ZhongShan Golden Bridge Biotechnology Co., Beijing, China), respectively, prior to the incubation with the primary antibody (1:400; Bioss Biotechnology Ltd., Beijing, China) at 4˚C overnight. The slides were then sequentially incubated with a second biotinylated antibody and streptavidin-biotin-peroxidase complex (Boster Biological Technology, Wuhan, China). Color development was performed with diaminobenzidine. Sections were counterstained with hematoxylin. The primary antibody was omitted in the negative controls. Known positive controls were used according to the manufacturer's instructions.

Two investigators without access to the clinical and pathologic data evaluated the result of immunohistochemical staining. A score was established corresponding to the multiplication of the percentage of positive cell (0, negative; 1, <25%; 2, 26-50%; and 3, >50% positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, high). Slides were observed under five random high-power fields and the mean scores were calculated. Samples with scores between 0 and 3 were considered as negative or weakly positive (N/W), scores between 3+ and 6 as moderately positive (M) and scores between 6+ and 9 as strongly positive (S).

Statistical analysis. Statistical analysis was performed using SPSS software system (SPSS for windows, version 16.0; SPSS, Inc., Chicago, IL, USA). Due to the skew distribution of serum endostatin, the data were displayed as medians (interquartile range). A comparison between groups of independent samples was made using the Mann-Whitney U test or Kruskal-Wallis test. The association of categorical dichotomized variables was detected using the χ^2 test. The relationships between parameters were examined using Pearson's correlation analysis. To assess the diagnostic performance of measuring endostatin, receiver operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was analyzed. Cox proportional hazards regression model was used in the univariate and multivariate analyses to investigate the relative influence of variables on overall survival. As the tumor stage and node invasion status were associated significantly with TNM stage, the TNM stage in the multivariate analysis, recognized as a prognostic predictor, was excluded. P<0.05 indicated statistically significant differences.

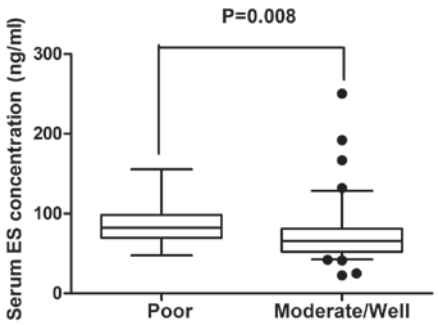

Figure 2. Concentration of serum endostatin (ES) in the groups with poor or well/moderately differentiated.

Results

Serum concentration of endostatin. The serum level of endostatin in lung cancer patients [68.5 (53.5-84.6 ng/ml)] was significantly higher than that in healthy controls [51.9 (45.1-76.0 ng/ml)] (P=0.0018). The comparison between the two groups was illustrated in Fig. 1A.

Diagnosis value of serum endostatin. To evaluate the power of serum endostatin to discriminate NSCLC between healthy controls, the ROC curve was plotted (Fig. 1B). The AUC was 0.657 (95% CI: 0.558-0.756, P=0.002). The best cut-off value with respect to the highest Youden-index was 53.0 pg/ml, with a sensitivity of 77.1%, specificity of 56.3%, positive predictive value of 79.4% and negative predictive value of 47.1%. The ROC analysis demonstrated that it is inadequate for clinical application for the early detection of NSCLC.

Correlation of preoperative serum endostatin and clinicopathological characteristics. Tumor samples with poor differentiation showed a much higher endostatin concentration of 82.4 (69.65-98.28 ng/ml), compared with well and moderately differentiated ones [65.7 (52.10-81.10 ng/ml)] (Fig. 2). There was no notable correlation of serum endostatin levels of TNM stage, histological type, node invasion or tumor stage.

Immunohistochemical analysis. In these 93 tumor tissue samples, endostatin location was detected mainly in the

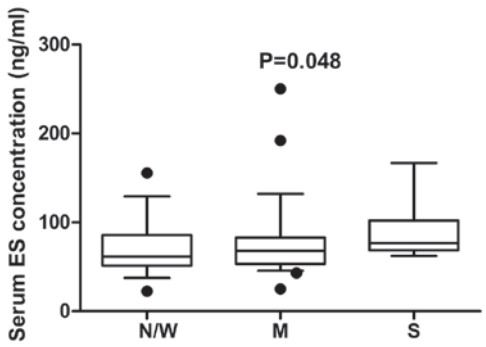

Figure 3. Concentration of serum endostatin (ES) in the groups with strongly positive (S), moderately positive (M) and negative or weakly positive (N/W) endostatin immunostaining.

cytoplasm of tumor cells. Moderately positive immunohistochemical staining was present in 59 subjects, a weakly positive score was identified in 23 subjects and a strongly positive score in 11 subjects. The results did not demonstrate any relationship between endostatin expression and various clinicopathological factors, including tumor differentiation status, histological type, tumor stage, lymph node invasion and TNM stage.

Association between serum endostatin and tumor immunohistochemical expression. A significant correlation was detected between circulating endostatin and tumor sample immunohistochemical staining score in 93 serum and tumor samples (r=0.223, P=0.032). The stronger immunohistochemical reactivity in tumor tissue had a statistically higher serum endostatin concentration compared with samples with a weaker immunohistochemical staining based on the Kruskal-Wallis test (Fig. 3).

Analysis of overall survival. To assess the prognostic value of serum endostatin, a step-wise method providing the optimal separation between a high and low group was applied to identify the optimum cut-off point. The study population was divided into two groups by each 10% increase of patients with a relatively lower serum ensostatin level. The relationship between the increasing cut-off level and overall survival time is presented in Table II. As the optimum cut-off point was selected as 90.3 ng/ml, the 105 patients were divided into two groups: 80% of the population, with a lower serum endostatin (≤90.3 ng/ml), demonstrated a marginally higher risk than the remaining 20% patients with an endostatin concentration of >90.3 ng/ml (P=0.053). We used 90.3 ng/ml as the cut-off value in the subsequent multivariate analysis.

As a continuous variable, serum endostatin levels inversely correlated with overall survival time, although there was no statistical significance (P=0.093). No correlation was found between endostatin expression in tumor samples with overall survival as a continuous or categorical variable (Table III). As the serum endostatin concentration was associated with the tissue immunohistochemical score, two models were developed to evaluate the prognostic value of factors including gender, age, tumor differentiation, histological type, tumor stage and lymph node invasion status, using the Cox proportional hazards regression model. Tumor stage and node status retained their independent significance in the multivariate and univariate analyses. Patients with a higher serum or immunohistochemical score indicated increased survival advantage, however, the difference was not statistically significant (Table IV).

Table II. Results of the univariate, proportional hazard analyses with serum endostatin.[a]

Cut-off point (%)	Endostatin level (ng/ml)	HR (95% CI)	P-value
20	50.84	1.379 (0.725-2.623)	0.328
30	56.12	1.247 (0.701-2.216)	0.453
40	62.54	1.024 (0.592-1.770)	0.933
50	68.50	1.008 (0.591-1.720)	0.976
60	73.04	1.213 (0.694-2.122)	0.498
70	80.96	1.819 (0.937-3.530)	0.077
80	90.30	2.315 (0.990-5.415)	0.053
Continuous		0.991 (0.981-1.001)	0.093

[a]Step-wise method was used to predict overall survival time among the 105 patients. HR, hazard ratio; CI, confidence interval.

Table III. Results of the univariate and proportional hazard analyses with endostatin expression in tumor tissue.[a]

Scores	HR (95% CI)	P-value
N/W vs. M, S	1.325 (0.704-2.494)	0.384
M vs. S	1.037 (0.433-2.487)	0.935
N/W vs. S	1.361 (0.516-3.588)	0.533
N/W vs. M	1.306 (0.683-2.498)	0.420
N/W	1.370 (0.520-3.607)	
M	1.041 (0.434-2.496)	0.682
S	Reference	
Continuous	0.944 (0.832-1.072)	0.377

[a]Grouped by immunohistochemical score level predicting overall survival time among 93 patients. HR, hazard ratio; CI, confidence interval; N/W, negative or weakly positive; M, moderately positive; S, strongly positive.

Discussion

Focus on the development of antiangiogenic strategies for anticancer therapy has led to the identification of numerous endogenous inhibitors of angiogenesis (16). Endostatin is generated naturally by elastase activity in EOMA murine hemangioendothelioma cells. In addition, matrix metalloproteinases, cathepsins and elastase have been reported to contribute to the production of endostatin (11,17,18). The recombinant endostatin, modulated by Chinese investigators, has manifest efficacy and safety in the treatment of advanced NSCLC patients and refractory malignant ascites of gastrointestinal cancer (19,20). Nevertheless, the specific molecular mechanism has not been elucidated.

Table IV. Results of univariate and multivariate proportional hazard analysis for predictors of overall survival time among the study population.

	Univariate		Multivariate	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Survival analysis model 1				
Age (years)	0.800 (0.469-1.365)	0.413		
≤62				
>62				
Gender				
Male	1.035 (0.520-2.057)	0.923		
Female				
Differentiation	1.322 (0.646-2.705)	0.446		
Poor				
Well/moderate				
Histological type	1.409 (0.825-2.408)	0.209		
Non-SCC				
SCC				
Tumor stage				
T1	0.286 (0.129-0.633)	0.007	0.284 (0.128-0.629)	0.008
T2	0.455 (0.235-0.880)		0.455 (0.258-0.977)	
T3/T4				
Node invasion				
Absent	0.412 (0.240-0.705)	0.001	0.404 (0.235-0.696)	0.001
Present				
Serum endostatin level	2.315 (0.990-5.415)	0.053		
Survival analysis model 2[a]				
Age (years)	0.800 (0.469-1.365)	0.413		
≤62				
>62				
Gender				
Male	1.035 (0.520-2.057)	0.923		
Female				
Differentiation	1.322 (0.646-2.705)	0.446		
Poor				
Well/moderate				
Histological type	1.409 (0.825-2.408)	0.209		
Non-SCC				
SCC				
Tumor stage				
T1	0.286 (0.129-0.633)	0.007	0.308 (0.134-0.707)	0.02
T2	0.455 (0.235-0.880)		0.579 (0.284-1.182)	
T3/T4				
Node invasion				
Absent	0.412 (0.240-0.705)	0.001	0.397 (0.224-0.705)	0.002
Present				
Tumor cell endostatin				
N/W	1.370 (0.520-3.607)	0.682		
M	1.041 (0.434-2.496)			
S				

[a]Survival analysis model was adjusted for the same factors as in survival analysis 1, including age, gender, differentiation, histological type, tumor stage and node invasion. HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma; N/W, negative or weakly positive; M, moderately positive; S, strongly positive.

Investigators have previously reported that endostatin exerts antiangiogenic effects by blocking vascular endothelial growth factor (VEGF)-induced tyrosine phosphorylation of KDR/Flk-1 of endothelial cells (21). Dong *et al* (22) and Brideau *et al* (23) found that endostatin inhibits lymphangiogenesis by downregulating the tumor expression of VEGF-C. By contrast, the osteopontin-related mechanism may be mediated in endostatin antitumor activity (24). These data indicate that endostatin remains to be adequately elucidated.

The association of serum endostatin with various clinical factors demonstrated that tumor cells with poor differentiation had a much higher serum endostain concentration. A possible explanation includes that, tumor cells with advanced histological grade, because of a higher amount of nourishment consumption required to maintain rapid proliferation, need more proangiogenic factors such as VEGF and PIGF to stimulate endothelial cell migration and sprouting (25,26). Consequently, to regulate angiogenesis, the more negative factors were released in the bloodstream from the tumor cells. As an important antiangiogenic member, the tumor releases more endostatin, attempting to recover the balance. To the best of our knowledge, the present is the first study to identify the association between serum endostatin and tumor cell differentiation.

In this study, we investigated the endostatin expression in serum and tumor tissue in operable patients with NSCLC and their significance in predicting patient prognosis, respectively. Elevated circulating endostatin concentration in patients with malignancies compared with healthy controls has been corroborated by a growing body of evidence (11-13), which was consistent with our findings. By contrast, circulating concentration was not enhanced in head and neck squamous cell carcinoma and hepatocellular carcinoma (14,27). The prognostic utility of serum endostatin concentration in NSCLC remains controversial. In the present study, a higher serum endostatin, as a continuous variable, exhibited longer overall survival time. For practical significance, we dichotomized the endostatin level by increasing the cut-off value step by step, to optimize the cut-off point, as demonstrated in other studies (28,29). When 90.3 ng/ml, the optimum cut-off value in the univariate analysis, was selected in the multivariate analysis, patients with higher endostatin lost survival advantage over those with lower endostatin levels. The findings of this study were in accordance with those of two studies on hepatocellular carcinoma and cervical cancer (12,15). By contrast, a higher endostatin level was found to correlate with poor prognosis in previous studies (11,13).

Immunohistochemistry was used to investigate the location of endostatin and the effects to survival time. Endostatin is expressed by various cell types, from stroma components to tumor cells (30,31). Guenther *et al* (32) used *in situ* hybridization to demonstrate that collagen XVIII was expressed in colorectal cancer stroma cells and ovarian cancer cells. Cytoplasm staining in NSCLC was reported, which is consistent with our observation (33). Among the available data presented, the potential prognostic role of endostatin expression in tumor tissue remains unclear due to the discordant results. Previously, higher collagen XVIII in tumor was found to be associated with worse prognosis in several malignancies (34). However, there were contradictory results (35,36). No correlation was found between endostatin expression in tumor tissue and NSCLC patient prognosis in a study from Korea, which was confirmed by our observation (37). Following a comparison of every two groups of three, no difference was identified in the univariate and multivariate survival analyses. These inconsistent results were probably due to differentiation in staining protocols, antibody sources, different scoring methods and threshold. The antibody used in our study recognized the amino acid in carboxyl terminus (1581-1680), thus it did not distinguish between endostatin cleaved from its precursor collagen XVIII and the endostatin portion of the intact collagen XVIII or its related proteolytic fragments.

Diagnostic biomarkers for lung cancer are lacking and there are no guidelines for NSCLC management, which incorporates the use of biomarkers. Determination of serum NSE and CYFRA21-1 is widely used for establishing the diagnosis of NSCLC. However, due to its low sensitivity and specificity, new diagnostic biomarkers for NSCLC should be identified. Therefore, in the present study, we investigated the potential of endostatin as a diagnostic biomarker for NSCLC. However, the ROC analysis did not result in a significant cut-off point with a reasonable degree of sensitivity and specificity to discriminate patients from healthy controls. This observation was supported by earlier studies as well (12,15). A combination of endostatin with other tumor markers may increase the diagnostic ability.

In addition, we examined the association between circulating endostatin and tissue expression in tumor samples. Although the correlation index was relatively weaker, we found that a stronger endostatin expression correlates with higher serum levels of endostatin, suggesting that serum endostatin originated partially from tumor cells. Our findings are in concordance with the result found in NSCLC by Iizasa *et al* (34). However, in 12 patient-matched cases, serum endostatin concentration manifested no significant association with immunohistochemical staining, although this maybe due to the small sample size (11).

In conclusion, no obvious association was observed between endostatin expression in serum and tumor tissue with overall survival in NSCLC. The reasons for this are that: i) Our study is a retrospective study with a relatively small sample size, which may decrease the power of the test. Prospective studies with larger sample size are therefore needed to confirm the effects of endostain on long-term survival. ii) The angiogenetic process is regulated by a number of angiogenetic factors through several signaling pathways, including VEGF, angiopoietin, DLL4, notch, Tie, PDGF, HGF, angiogenin and endoglin. Although important, endostatin is not able to affect survival in NSCLC.

Acknowledgements

We would like to thank Jin Guo, School of Public Health, Capital Medical University for excellent advice with statistical analysis and helpful discussions with regard to the results. We also thank all patients for providing blood and tissue samples.

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