Original Article

Elevated Circulating Levels of Osteopontin Are Associated with Metastasis in Advanced Non-Small Cell Lung Cancer

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ABSTRACT

Objective: To investigate the relationship between postoperative metastasis and circulating levels of osteopontin in non-small cell lung cancer (NSCLC).

Methods: The expression of osteopontin mRNA were detected with RT-PCR technique. The circulating levels of osteopontin were measured through ELASA in 46 NSCLC cases that had not been received any anti-cancer treatment at the time of sampling. The tissues from fifteen patients with benign pulmonary diseases were studied as control group.

Results: The overall median mRNA expression level of osteopontin was approximately 70-fold higher in tumor tissues than in matched normal lung tissues (P<0.001). Over-expression of osteopontin mRNA was significantly associated with clinical stage (P=0.009). Advanced disease states had higher circulating level of osteopontin (stage I+II versus stage III+VI). In multivariate analysis, stage was the only independent factor influencing circulating levels of osteopontin. All patients were followed up for 12 months, 2 of the 46 patients with both osteopontin mRNA expression and elevated plasma osteopontin levels had local recurrence and 10 had distant metastasis. There was a significant difference in the osteopontin levels between metastasis group and non-metastasis group.

Conclusion: Preoperative plasma levels of osteopontin are significantly associated with post-operative metastasis in advanced NSCLC.

Key words: Osteopontin; Non-small cell lung cancer; Metastasis; Prognosis

INTRODUCTION

Lung cancer is currently the leading cause of cancer mortality worldwide. An estimated 1.2 million people are diagnosed annually with lung cancer and 1.1 million of them die from the disease[1]. Despite recent advances in detection and in surgical and medical treatments during the past two decades, the 5-year survival rate for the patients remains less than 15%[2]. Patients with lung cancer have a poor prognosis because of the high metastatic potential of the neoplasm.

Cancer progression depends on an accumulation of metastasis-supporting genetic modifications and physiologic alterations regulated by cell signaling molecules such as extra-cellular matrix (ECM) proteins. The latter contribute to the interaction among cancer cells and endothelial cells, which play a critical role in the development of local invasion and distant metastasis[3, 4]. One of the factors that have recently been shown to be linked to cancer development, progression, and metastasis in different malignancies is a multifunctional protein named osteopontin. Previous research suggests that osteopontin is a key extra-cellular molecule involved in tumor development and progression[5, 6]. In the current study, we investigated the relationship between circulating levels and mRNA expression of osteopontin and tumor metastatic status in NSCLC patients. We also analyzed the clinicopathologic variables and pathologic factors that may influence circulating osteopontin level.

MATERIALS AND METHODS

Patients and Clinical Data

Forty-six patients with NSCLC, who consecutively underwent radical surgical resection at the Department of Thoracic Surgery, Beijing Chao-Yang Hospital, Capital Medical University from January 2006 to December 2007, were retrospectively studied. All patients were pathologically diagnosed as NSCLC and had not received chemotherapy or radiotherapy before surgery. In course of

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preoperative examinations, all of the patients underwent routinely bone scintigraphy, brain MRI and abdominal ultrasound or CT scan as staging examinations. No detectable metastases in distant organs were present at the time of surgery. Radical surgery including mediastinal lymphadenectomy was performed in all cases.

Specimens and Blood Samples

Neoplastic specimens were removed from the tumor masses and then were formalin-fixed and paraffin-embedded for histologic analysis. The pathologic features of the samples were classified according to WHO histologic criteria, and tumor staging was conducted according to the International Union Against Cancer TNM classification[7].

Peripheral blood sample (4 ml) was taken prior to treatment by venipuncture and kept in a heparinized tube. Within 30 min of blood collection, the samples were centrifuged at 1,500 ×g for 10 min at 4℃ to separate the plasma and blood cell. The plasma sample was then stored at -8℃ till use.

The use of all of the human samples for this study were reviewed and approved by the Ethics Committee of the Beijing Chao-Yang Hospital, Capital Medical University.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) For Osteopontin mRNA Detection

Total RNA was extracted from tumor specimens and normal lung tissues were obtained during surgery according to the instruction of manufacturer [Tiangen Biotec (Beijing) Co. Ltd]. Proteinase K digestion time was 16 hours for each sample. After total RNA isolation, samples were kept at -8℃ until use. The integrity was checked with 1.5% agarose-gel electrophoresis. RT-PCR reaction was performed with 4 μl cDNA template in a total volume of 25 μl Amplification was optimized for each primer set. The following primers were used: β-actin 5′AAG AGA GGC ATC CTC ACC CT-3′; 5′TAC ATG GCT GGG GTG TTG AA-3′; Osteopontin 5'-CCC TGT GTT GGT GGA GGA TGT -3'; 5'-GAC AAC CAA GCC CTC CCA GA-3'. RT-PCR reaction was performed in duplicates in 96-well plates, for 3 min at 94℃ for initial denaturing, then 35 cycles at 94℃ for 15 s, at 52℃ for 15 s and 7℃ for 5 min. β-actin was used as reference genes.

Measurement of Plasma Osteopontin Concentration

The osteopontin levels in the plasma were measured with the osteopontin ELISA kit according to the manufacturer's instructions (R & D Systems, Inc. USA). Briefly, 1:5 diluted testing samples were incubated in the osteopontin antibody-precoated plate for 1 h at 37℃. Following washing, 100 μl of labeled osteopontin antibody solution was added into each well and incubated for 30 min at 4℃. After washing, tetramethyl benzidine was used as a coloring agent. Testing samples were detected on a plate reader, and the strength of coloring was proportional to the quantity of osteopontin.

Statistical Analysis

The *t* test for paired samples was applied to compare the mRNA expression levels in NSCLC tissue and normal lung tissue. The correlation between expression of OPN mRNA and other clinical variables (including histologic subtype, tumor staging, age, sex, and smoking status) was assessed using the χ^2 test and one-way ANOVA. The associations between plasma OPN expression and the risk of the postoperative metastasis of NSCLC were estimated by the χ^2 test and one-way ANOVA. The protein levels of OPN in the plasma and the risk of the postoperative metastasis of NSCLC were evaluated by using the t test. All statistical tests were two sided. Differences with P values <0.05 were considered statistically significant. Calculations were done with the Statistical Package for the Social Science version 11.5 (SPSS, Inc., Chicago, IL).

RESULTS

Patients Characteristics

The mean age of the 28 male and 18 female patients was 55.2±10.3 years (range, 33-77 years). The most common histologic type of tumor was adenocarcinoma (58.7%; 27 cases) and followed by squamous cell carcinoma (41.3%; 19 cases). With respect to tumor size, 14 (30.4%) cancers were classified as T1, 17(36.9%) were classified as T2, 9 (19.6%) were classified as T3, and 6 (13.0%) were classified as T4. Eleven (23.9%) patients showed no metastasis to the regional lymph node (N0). However, metastatic involvement of hilar lymph nodes (N1) was present in 14 (30.4%) patients; metastases to the mediastinal lymph nodes (N2) were observed in 16 (34.8%) cases; metastases to the contralateral mediastinal lymph nodes (N3) were observed in 5 (10.9%) cases. Regarding the tumor stage, there were 9 (19.6%) patients with stage I， 11 (23.9%) with stage II， 23 (50%) with stage III, and 3 (6.5%) with stage IV. Twelve (12/46, 26.1%) patients presented relapse during follow-up: 2 of them developed local recurrence, whereas 10 developed distant metastases (bone metastasis in 4 patients and brain metastasis in 6). The tissues from fifteen patients with benign pulmonary diseases were studied as control group. The clinical information was obtained by chart review (Table 1).

Table 1. Demographic Data

Variable	NSCLC	Control
Subjects No.	46	15
Age (y)	55.2±10.3	21±6
Male gender	28	10
Smokers	25	6
Histologic subtypes		
Adenocarcinoma	27	–
squamous cell carcinoma	19	–
Postoperative staging		
Stage I	9	–
Stage II	11	–
Stage III	23	–
Stage IV	3	–

Table 2. Comparison of osteopontin mRNA expression with different clinicopathologic characteristics from patients with NSCLC

	Case numbers	Expression number	$\bar{x}\pm s$	*P* value
Gander				
Males	28	17	0.366±0.389	
Females	18	10	0.274±0.339	0.493
Age				
<63 years	26	14	0.267±0.339	
>64 years	20	13	0.412±0.398	0.408
Smoking status				
Yes	25	13	0.349±0.409	
No	21	14	0.308±0.323	0.299
Histologic subtypes				
Adenocarcinoma	27	15	0.348±0.403	
SCC	19	12	0.304±0.322	0.423
Stage				
I	9	0		
II	11	5	0.061±0.116	
III	23	19	0.491±0.361	
IV	3	3	0.890±0.124	0.009

Table 3. Osteopontin concentration with age, gender, smoking status, clinical stage, histologic subtypes, from patients with NSCLC

	Case numbers	Expression number	$\bar{x}\pm s$	*P* value
Age				
<63 years	26	17	105.31±117.56	
>63 years	20	14	97.77±83.39	0.440
Gander				
Males	28	19	112.77±83.39	
Females	18	12	85.97±90.15	0.470
Smoking status				
Yes	25	18	102.61±92.76	
No	21	13	101.35±116.56	0.460
Histologic subtypes				
Adenocarcinoma	27	20	100.37±88.25	
SCC	19	11	104.40±123.74	0.480
Stage				
I	9	0		
II	11	7	39.95±73.41	
III	23	21	140.38±93.70	
IV	3	3	221.96±119.72	0.003

mRNA Expression of Osteopontin in the Lung Cancer Tissues and Control Tissues

Osteopontin mRNA expression was detectable in 27 of 46 (58.7%) tumor specimens and 7 of 15 (46.7%) control specimens. The overall median mRNA expression level of osteopontin represented significant higher in NSCLC tissues (0.498; range, 0.116-1.165) than that in control tissue (0.007; range: 0.003-0.013; $P<0.05$). It was approximately 70-fold higher in tumor tissues than in control tissues. Overexpression of osteopontin mRNA was significantly associated with clinical stage ($P=0.009$): patients with stage III or IV showed a significant higher osteopontin expression. On the other hand, there were no significant associations between osteopontin mRNA expressions and any of the following variables: gender ($P=0.49$), age ($P=0.41$), smoking status ($P=0.299$), and histologic subtypes ($P=0.42$). The mRNA expression of osteopontin in the various factors is summarized in Table 2.

Osteopontin Concentration Are Elevated in the Circulating Plasma of Patients with Advanced Disease Stage

The plasma osteopontin levels in stage I to II, stage III and stage IV patients were 39.95 ng/ml, 140.38 ng/ml, and 221.96 ng/ml, respectively. The difference was significant ($P<0.03$). To examine whether basic demographic characteristics of this study population influence plasma concentration of osteopontin, we performed univariate analysis on the effect of age, gender, smoking status,

histologic subtypes and osteopontin concentration. There were no significant associations between osteopontin expressions and any of the following variables: gender (P=0.47), age (P=0.44), histologic subtypes (P=0.48), and smoking status (P=0.46). Detailed data of the results are summarized in Table 3. These circulating plasma osteopontin concentrations were parallel with the mRNA expression of osteopontin in NSCLC.

Elevated Circulating Levels of Osteopontin Are Associated with Metastasis in Advanced Non-Small Cell Lung Cancer

Our finding, showing that the circulating level of osteopontin was elevated in the patients with post-operative metastasis, brought an interrogation whether osteopontin is a specific marker for metastasis in NSCLC. To verify the use of osteopontin as a marker for predicting post-operative metastasis in NSCLC, we compared the level of osteopontin between the groups of patients with and without post-operative metastasis.

Interestingly, we observed that a high circulating levels of osteopontin were one significantly unfavorable prognostic factor. In the patients with post-operative metastasis, the circulating levels of osteopontin were significantly higher than in those without metastasis (153.46 ng/ml versus 89.17 ng/ml, P=0.008). These mRNA expression of osteopontin in metastasis group (0.790 ng/ml versus 0.168 ng/ml, P=0.006) were parallel with the circulating plasma osteopontin concentrations (Figure 1 and Table 4).

Table 4. The mRNA expression of osteopontin and the circulating plasma osteopontin concentration in metastasis and non-metastasis patients

	Metastasis (n=12)	Non-metastasis (n=34)	*P* value
mRNA expression of osteopontin	0.790±0.242	0.168±0.249	0.006
Plasma levels of osteopontin concentration	153.460±63.48	89.170±23.980	0.008

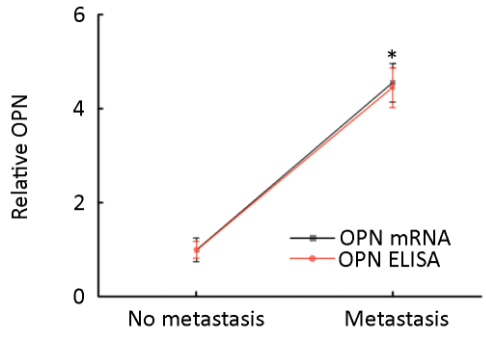

Figure 1. Comparison of mRNA expression of osteopontin and the circulating plasma osteopontin concentrations in metastasis group and non-metastasis group.

DISCUSSION

Osteopontin, an extracellular protein produced by osteoblasts, functions in various physiological and pathological processes including bone remodeling, cell-mediated immunity, maintenance of recon- figuration of tissue integrity during inflammatory processes, coronary restenosis, and tumor cell metastasis[8-13]. Osteopontin has long been implicated in the process of carcinogenesis, progression, and metastatic dissemination of several human tumors including lung cancer[14-22]. This study evaluated the association of circulating osteopontin levels with tumor progression using clinical samples from the patients who underwent surgical resection and mediastinal lymphadenectomy for their NSCLC and have taken no any anti-cancer therapy till time of sampling. mRNA expression of osteopontin was detected using resected lung cancer specimens. We analyzed the clinicopathologic variables and pathologic factors that may influence circulating osteopontin level. Moreover, we tried to give particular attention to the relationship between circulating levels and mRNA expression of osteopontin and tumor metastatic status, because a new prognostic factor might enable classification of such patients into different subsets corresponding to different risk of metastasis following surgery.

The present study analyzed 46 tumor samples of NSCLC and 15 control tissues. The results indicate that the mRNA expression of osteopontin were significantly higher in the tumor tissues than those found in the control tissues (patients with pulmonary benign diseases) (64.5% versus 27.9%, P<0.001). The finding suggests that the overexpression of osteopontin may play an important role in NSCLC.

As concerns the association between osteopontin expression and clinicopathologic variables, such as age, gender, histologic type, and staging, we observed statistically significant correlations between osteopontin expression and tumor staging. The fact that patients with advanced tumors (stage III to IV) showed a significantly higher mRNA expression of osteopontin than those with lower stage diseases (stage I to II)[17]. However, we did not describe significant differences between clinicopathologic variables, such as age, gender, and histologic type and osteopontin expression.

In the current study, the circulating levels of osteopontin were significantly higher in the patients with advanced diseases (stage III-IV) than those with low stage diseases (stage I-II). This could be in accordance with findings of Hu et al.[17]. In their study the circulating osteopontin level is higher in stage III+IV than stage I+II. Latest research confirms that low osteopontin serum levels were significantly associated with a favorable prognosis in advanced NSCLC both in the Japanese patients (JMTO LC 0004) and the US population (SWOG study S0003[23, 24]. Our results suggested that osteopontin is critically involved in the aggressive progression of NSCLC.

Further analysis of the follow-up data, with and without postoperative metastasis, showed a very significant difference between circulating levels of osteopontin detected from the two groups (P<0.001). Moreover, it was found that overexpression of osteopontin was remarkably associated with increased risk of metastasis. These data provide evidence that osteopontin accelerates metastasis in NSCLC.

The fact that the expression status of osteopontin implied that activation of osteopontin may be an early event in NSCLC metastasis. Previous studies have demonstrated that osteopontin plays important roles in tumor progression and metastasis. The results from an in vitro invasion study strongly support the hypothesis that osteopontin can activate the invasive potential of NSCLC cells[15]. Chang et al also reported that presence of metastasis influence the circulating levels of osteopontin[25].

In conclusion, the current findings suggest that a high osteopontin expression resulted as an unfavorable prognostic factor for metastasis of NSCLC. Osteopontin might serve as a predictor for dismal prognosis. Increased osteopontin levels in circulating plasma may be useful as a helpful clinical biomarker for diagnosing or monitoring the metastasis.

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