

## Original Article

# Expressions of Osteopontin (OPN), $\alpha_v\beta_3$ and Pim-1 Associated with Poor Prognosis in Non-small Cell Lung Cancer (NSCLC)

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## ABSTRACT

**Objective:** To examine the expressions of osteopontin (OPN),  $\alpha_v\beta_3$  and Pim-1 in non-small cell lung cancer (NSCLC), and investigate their potential pathogenic roles in the development of NSCLC.

**Methods:** Immunohistochemistry was used to examine the expressions of OPN,  $\alpha_v\beta_3$  and Pim-1 in cohort (136 cases) of NSCLC samples and their adjacent normal lung tissue specimens. Statistical analysis was performed to evaluate the relationships among expressions of OPN,  $\alpha_v\beta_3$  and Pim-1 and their associations with patients clinicopathological parameters.

**Results:** The expressions of OPN and Pim-1 were predominantly observed in cytoplasm. The expression of  $\alpha_v\beta_3$  was mostly detected in cytoplasm and/or membrane. In NSCLC samples, the positive rates of OPN,  $\alpha_v\beta_3$  and Pim-1 expressions were 68.4% (93/136), 77.2% (105/136) and 57.4% (78/136), respectively. In normal lung tissues, in contrast, the positive rates of OPN,  $\alpha_v\beta_3$  and Pim-1 were 24.0% (12/50), 26.0% (13/50) and 16.0% (8/50), respectively. There were significant differences of the positive expression rates of OPN,  $\alpha_v\beta_3$  and Pim-1 between NSCLCs samples and normal lung tissues ( $P<0.01$ ). In addition, the positive expression of OPN,  $\alpha_v\beta_3$  and Pim-1 in NSCLCs samples was significantly associated with increased pathological grade, lymph node metastasis and advanced clinical stage ( $P<0.01$ ), and they were independent of other clinicopathological parameters ( $P>0.05$ ). Furthermore, a significantly positive correlation between the expression of OPN and  $\alpha_v\beta_3$  ( $r=0.38$ ,  $P<0.01$ ), OPN and Pim-1 ( $r=0.37$ ,  $P<0.01$ ), or  $\alpha_v\beta_3$  and Pim-1 ( $r=0.20$ ,  $P<0.05$ ) was evaluated in our NSCLC cohort.

**Conclusion:** OPN,  $\alpha_v\beta_3$  and Pim-1 proteins are frequently overexpressed in NSCLC, and they may play important roles in the development and/or progression of NSCLC.

**Key words:** NSCLC; OPN;  $\alpha_v\beta_3$ ; Pim-1; Immunohistochemistry

## INTRODUCTION

Primary bronchogenic carcinomas have the highest mortality rate of malignant tumors in the world. Among them, non-small cell lung cancer (NSCLC) accounts for 85%, most patients who have been already clinically diagnosed are in middle-advanced stage, and the 5-year survival rate is very low<sup>[1]</sup>. Thus, early diagnosis and early treatment are particularly important to improve the survival rate. It is now considered that various pathogenic factors were involved in the evolution of NSCLC.

Osteopontin (OPN) is a multifunctional secreted

phosphorylated glycoprotein, and it can promote cell chemotaxis, adhesion and migration, which mediate invasion and metastasis of tumor cells, and it is closely related to the occurrence, development, metastasis and prognosis of a variety of cancers<sup>[2,3]</sup>. Recent studies have indicated that OPN is involved in NSCLC progression and metastasis through interaction with its receptor,  $\alpha_v\beta_3$  integrin, and overexpression of OPN is associated with progression and poor prognosis of NSCLC<sup>[2,3]</sup>.  $\alpha_v\beta_3$  is a peptide of heparin binding factor and is an important molecule of integrin family.  $\alpha_v\beta_3$  can mediate adhesion of cell to cell and cell to matrix, regulate intracellular signaling pathways, and induce the activation of protein dissolving enzymes, thereby contributing to extracellular matrix and basement membrane degradation, and promoting invasion and migration of tumor cells<sup>[4]</sup>. Previous study

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found that  $\alpha v\beta 3$  is detectable in a variety of tumors, and is closely associated with the tumorigenesis and the degree of malignancy of tumor<sup>[5]</sup>. Other factors which are closely related with genes of cell cycle regulation and proliferation, including Pim-1 (coded by *pim-1* oncogene), are involved in tumorigenesis<sup>[6]</sup>. It has been reported that OPN acts through  $\alpha v\beta 3$  integrin, which in turn activates the FAK, PI3K, Akt, ERK, NF- $\kappa$ B and Pim-1 pathways, contributing to the migration of lung cancer cells<sup>[6]</sup>. Taken together, these results suggest OPN mediates migration in human lung cancer cells via the  $\alpha v\beta 3$  integrin, FAK, PI3K, Akt, ERK, NF- $\kappa$ B and Pim-1 signaling pathways. Therefore, we chose three factors of signaling pathways, OPN,  $\alpha v\beta 3$  integrin and Pim-1 in this study. To date, however, the expression dynamics of OPN,  $\alpha v\beta 3$  and Pim-1 in NSCLCs, and their potential biological roles in tumorigenesis of NSCLC have not been elucidated.

In the present study, immunohistochemistry was used to examine the expression dynamics of OPN,  $\alpha v\beta 3$  and Pim-1 in cohort (136 cases) of NSCLC samples and their adjacent normal lung tissue specimens. Next, the potential correlations among the 3 protein expressions and their associations with NSCLC patients' clinicopathological features were evaluated.

## MATERIALS AND METHODS

### Clinical Data

In this study, specimens were obtained from archived paraffin-embedded tissue sections of 136 patients with NSCLC at the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou from January 1st, 2009 to December 31st, 2010. A group of 50 normal lung tissue cases was conducted as control. In this cohort of NSCLC patients, 97 were men and 39 were women, with a median age of 60 years (range 30–82 years). According to the World Health Organization criteria of lung cancer published in 2004<sup>[7]</sup>, NSCLC patients were classified as follows: adenocarcinoma 72 cases, squamous cell carcinoma 40 cases, and other types 24 cases; well and moderately differentiated carcinoma 88 cases, and poorly differentiated carcinoma 48 cases. According to the staging standard of the TNM system of International Association for the Study of Lung Cancer<sup>[8]</sup>, the NSCLC patients were classified as stage I/II 96 cases, and stage III/IV 40 cases. In 136 NSCLC cases of the present study, 35 had lymph node metastasis.

### Protein Expression of OPN, $\alpha v\beta 3$ and Pim-1 in NSCLC

Immunohistochemical technique using streptavidin-peroxidase (SP) was employed for OPN,  $\alpha v\beta 3$  and Pim-1 detection. Mouse-anti-human monoclonal antibodies of OPN,  $\alpha v\beta 3$ , Pim-1 and SP kit were purchased from Maxim biological and technical company, Fuzhou, China; ready-to-use. All the sections were routinely

deparaffinized and rehydrated, then the sections were rinsed in phosphate-buffered saline (PBS, pH=7.4), subsequently were treated for antigen retrieval. Sections were treated in EDTA buffer (pH=8.0) in an autoclave sterilizer. After cooling at room temperature for 20 min, the sections were rinsed in PBS, then immersed in 3% H<sub>2</sub>O<sub>2</sub> for 15 min to block the endogenous enzymes. After being rinsed in PBS, the sections were incubated with normal goat serum at 37°C for 15 min to block nonspecific antibodies. After interaction with OPN antibody,  $\alpha v\beta 3$  and Pim-1 antibodies (monoclonal antibodies), the sections were rinsed in PBS, then incubated with biotinylated secondary antibodies and rinsed in PBS again. After interaction with streptavidin-horseradish peroxidase (HRP) and being rinsed in PBS, the sections were visualized by reaction with 3,3'-diaminobenzidine and counterstained with hematoxylin. They were then dehydrated, transparentized and covered with coverslips and sealed with neutral gum. PBS substituting the primary antibody was used as negative control.

### Judgment of Positive Result of OPN, $\alpha v\beta 3$ and Pim-1

The judgment that whether the tumor and the normal tissues were positive or not was performed by two pathology doctors. The positive expression of OPN was mostly in cytoplasm with brown-yellow staining,  $\alpha v\beta 3$  was mostly in cytoplasm and membrane with brown-yellow staining, and Pim-1 was mostly in cytoplasm with brown-yellow staining. A tumor or normal tissue in which more than 10% of cells were stained with those antibodies was recognized as being positive; while less than 10% was recognized as being negative.

### Statistical Analysis

Data were analyzed with computer aided SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test was used in comparing the expressions of OPN,  $\alpha v\beta 3$  and Pim-1 in NSCLC samples and in normal lung tissue specimens. The associations between the expressions of OPN,  $\alpha v\beta 3$  and Pim-1 and NSCLC patients' clinicopathological parameters were also evaluated by the Chi-square test. The correlation between two variables was evaluated by the Spearman rank correlation test. A value of  $P < 0.05$  was considered statistically significant.

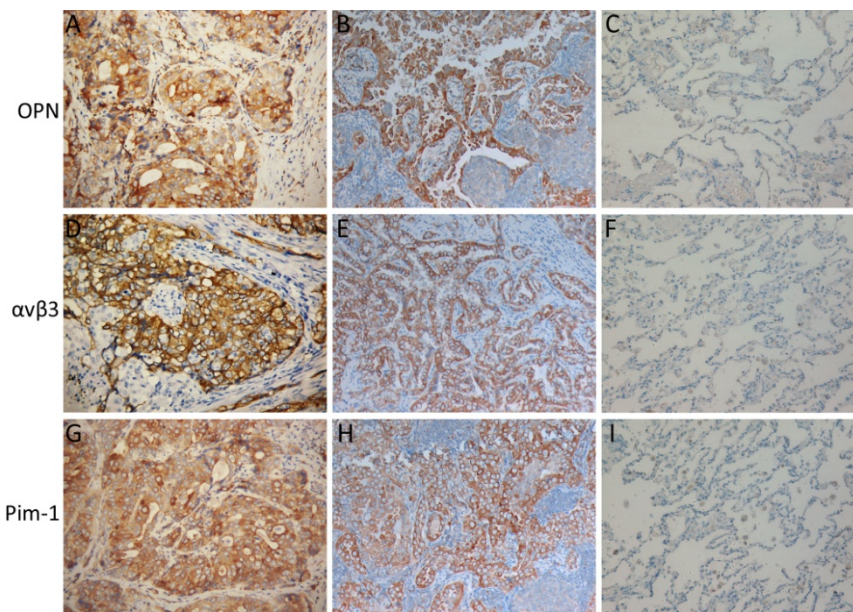
## RESULTS

### Relationship between Protein Expression of OPN and Clinicopathological Parameters in NSCLC

The expression of OPN in NSCLC tissues was predominantly a cytoplasmic pattern (Figure 1 A, B). In our study, 93 (68.4%) of 136 NSCLC cases showed positive expression of OPN, while the positive rate of OPN in normal lung tissues was 24.0% (12/50). There

was a significant difference of the expression of OPN between NSCLC and normal lung tissues ( $P<0.05$ ) (Table 1). Further correlation analysis demonstrated that the expression of OPN was significantly associated with

tumor differentiation degree, lymph node metastasis and clinical staging in NSCLC patients ( $P<0.01$ ), but was not correlated with other clinicopathological parameters studied ( $P>0.05$ ) (Table 2).



**Figure 1.** Protein expressions of OPN,  $\alpha_v\beta_3$  and Pim-1 in NSCLC. Protein expression of OPN is mostly in cytoplasm with yellow or brown-yellow staining. Positive expression of OPN was examined in a squamous carcinoma (A) and an adenocarcinoma (B) of the lung. In normal lung tissue, the expression of OPN was negative (C).  $\alpha_v\beta_3$  is mostly expressed in membrane or cytoplasm with yellow or brown-yellow staining.  $\alpha_v\beta_3$  is positive in another squamous carcinoma (D) and adenocarcinoma (E) of the lung. In normal lung tissue, the expression of  $\alpha_v\beta_3$  was negative (F). The expression of Pim-1 is predominantly a cytoplasmic pattern. Pim-1 is positive in a squamous carcinoma (G) and an adenocarcinoma (H) of the lung. In normal lung tissue, the expression of Pim-1 was negative (I) (SP  $\times 200$ ).

#### Relationship between Protein Expression of $\alpha_v\beta_3$ and Clinicopathological Parameters in NSCLC

In NSCLC, we observed that  $\alpha_v\beta_3$  protein was mostly expressed in membrane or cytoplasm (Figure 1 D, E). The positive rate of  $\alpha_v\beta_3$  expression in NSCLCs was 77.2% (105/136), which was significantly higher than that (26.0%, 13/50) in normal lung tissues ( $P<0.05$ ) (Table 1). In addition, the expression of  $\alpha_v\beta_3$  was also positively associated to NSCLC histopathological differentiation, lymph node metastatic status and clinical stage ( $P<0.01$ ) (Table 2).

#### Relationship between Protein Expression of Pim-1 and

#### Clinicopathological Parameters in NSCLC

The expression of Pim-1 was mostly observed in cytoplasm (Figure 1 G, H). In our NSCLC cohort, 78 (57.4%) of the 136 cases had positive expression of Pim-1. The positive rate of Pim-1 in normal lung tissues was 16.0% (8/50). There was a significant difference of Pim-1 expression between NSCLC and normal lung groups ( $P<0.05$ ) (Table 1). The expression of Pim-1 was significantly correlated to differentiation degree, lymph node metastasis and clinical staging in NSCLC ( $P<0.01$ ), while no correlations were observed between the expression of Pim-1 and other clinicopathological parameters studied ( $P>0.05$ ) (Table 2).

**Table 1.** The expression of OPN,  $\alpha_v\beta_3$  and Pim-1 between NSCLC and normal lung tissue

Groups	n	OPN (%)	$\alpha_v\beta_3$ (%)	Pim-1 (%)
NSCLC	136	93 (68.4)	105 (77.2)	78 (57.4)
Normal lung tissue	50	12 (24.0)	13 (26.0)	8 (16.0)

There was significant difference in the expression of OPN between NSCLC and normal lung tissue ( $\chi^2=29.29$ ,  $P<0.01$ ). There was significant difference in the expression of  $\alpha_v\beta_3$  between NSCLC and normal lung tissue ( $\chi^2=41.33$ ,  $P<0.01$ ). There was significant difference in the expression of Pim-1 between NSCLC and normal lung tissue ( $\chi^2=25.15$ ,  $P<0.01$ ).

#### Correlations among Protein Expression of OPN, $\alpha_v\beta_3$ and Pim-1 in NSCLCs

In our study, the potential correlations among protein expression of OPN,  $\alpha_v\beta_3$  and Pim-1 in NSCLC were further evaluated. Spearman rank correlation analysis showed that the expression of OPN was

positively correlated  $\alpha_v\beta_3$  expression in our NSCLC cohort ( $r=0.38$ ,  $P<0.01$ ) (Table 3). The expression of OPN in NSCLC was also positively correlated with Pim-1 expression ( $r=0.37$ ,  $P<0.01$ ) (Table 4). Furthermore, a significant positive correlation between expressions of  $\alpha_v\beta_3$  and Pim-1 was observed ( $r=0.20$ ,  $P<0.05$ ) (Table 5).

**Table 2.** Relationship between expressions of OPN,  $\alpha_v\beta_3$  and Pim-1 and clinicopathological parameters in NSCLC

Parameter	n	OPN (%)	P*	$\alpha_v\beta_3$ (%)	P*	Pim-1 (%)	P*
Sex							
Male	97	68 (70.1)	0.496	75 (77.3)	0.960	56 (57.7)	0.888
Female	39	25 (64.1)		30 (76.9)		22 (56.4)	
Age (year)							
<60	61	40 (65.6)	0.525	47 (77.0)	0.969	36 (59.0)	0.724
≥60	75	53 (70.7)		58 (77.3)		42 (56.0)	
Tumor size (cm)							
<3	51	34 (66.7)	0.739	38 (74.5)	0.562	28 (54.9)	0.654
≥3	85	59 (69.4)		67 (78.8)		50 (58.8)	
Lymph node metastasis							
Have	45	39 (86.7)	0.001	43 (95.6)	0.000	33 (73.3)	0.008
No	91	54 (59.3)		62 (68.1)		45 (49.5)	
Differentiation							
Well/moderate	88	51 (58.0)	0.000	60 (68.2)	0.001	37 (42.1)	0.000
Low	48	42 (87.5)		45 (93.8)		41 (85.4)	
Pathological typing							
Squamous carcinoma	40	26 (65.0)	0.854	31 (77.5)	0.960	22 (55.0)	0.837
Adenocarcinoma	72	50 (69.4)		55 (76.4)		43 (59.7)	
Other types	24	17 (70.8)		19 (79.2)		13 (54.2)	
Tumor location							
Peripheral	60	41 (68.3)	0.991	46 (76.7)	0.894	34 (56.7)	0.886
Central	76	52 (68.4)		59 (77.6)		44 (57.9)	
TNM stage							
I+II	96	57 (59.4)	0.000	68 (70.8)	0.006	47 (49.0)	0.002
III+IV	40	36 (90.0)		37 (92.5)		31 (77.5)	

\* Chi-square test

**Table 3.** Relativity of protein expressions of OPN and  $\alpha_v\beta_3$  in NSCLC

OPN	$\alpha_v\beta_3$		Total
	Positive	Negative	
Positive	82	11	93
Negative	23	20	43
Total	105	31	136

There are relationships to expressions of OPN and  $\alpha_v\beta_3$  in NSCLC ( $P<0.01$ ,  $\chi^2=20.10$ ), correlation coefficient,  $r=0.38$ .**Table 4.** Relativity of protein expressions of OPN and Pim-1 in NSCLC

OPN	Pim-1		Total
	Positive	Negative	
Positive	65	28	93
Negative	13	30	43
Total	78	58	136

There are relationships to expressions of OPN and Pim-1 in NSCLC ( $P<0.01$ ,  $\chi^2=18.91$ ), correlation coefficient,  $r=0.37$ .**Table 5.** Relativity of  $\alpha_v\beta_3$  and Pim-1 expressions in NSCLC

$\alpha_v\beta_3$	Pim-1		Total
	Positive	Negative	
Positive	62	43	105
Negative	16	15	31
Total	78	58	136

There are relationships to expressions of  $\alpha_v\beta_3$  and Pim-1 in NSCLC ( $P<0.05$ ,  $\chi^2=5.41$ ), correlation coefficient,  $r=0.20$ .

## DISCUSSION

It is known that malignant transformation of cells needs changes of gene phenotype and angiogenesis. OPN is considered to be the most important factor. OPN is an extracellular matrix-secreted phosphorylated glycoprotein which is also called the transformation-related protein phosphatase, and it was found by Senger, et al.<sup>[9]</sup> for the first time in the epithelial cell strain of malignant transformation. In recent years, studies have confirmed that overexpression of OPN can promote tumor growth, invasion and/or metastasis<sup>[3]</sup>.

Previously, it was reported that the expression of OPN was 68.8% in NSCLC<sup>[10]</sup>. Other groups reported that OPN expression was associated with tumor growth, tumor staging, and lymph node invasion of patients with NSCLC<sup>[11]</sup>. In the present study, the expression of OPN protein was detected in 68.4% of NSCLC, while only 24.0% of normal lung tissues expressed OPN protein, suggesting that the positive expression of OPN may provide a selective advantage for the development of NSCLC. In our study, further statistical analysis showed that the expression of OPN was correlated closely to NSCLC's differentiation degree, lymph node metastasis and clinical staging, and it was independent

of other clinicopathological parameters. The positive expression of OPN was more frequently observed in poorly differentiated cancer, tumors with lymph node metastasis and/or tumors in more advanced clinical stage III/IV. A current study suggested that OPN can produce a marked effect by binding to receptors on endothelial cell membrane. After OPN binds to the receptors such as  $\alpha\beta3$  integrins, it can directly stimulate the differentiation and proliferation of lung cancer cells, and might be capable of regulating genesis and migration of lung cancer cells, increase vascular permeability, change extracellular matrix, induce angiogenesis, activate intracellular signaling pathways and promote the growth of NSCLC<sup>[12]</sup>. These data suggested that the increased expression of OPN may facilitate the development and/or progression of NSCLC and OPN might be used as a therapeutic target and useful biomarker for NSCLC.

The reason why OPN is highly expressed in NSCLC is that it is regulated by some transcription factors. Among them,  $\alpha\beta3$  and Pim-1 are more important. Previous studies have demonstrated that  $\alpha\beta3$  regulates the expressions of Pim-1 and VEGF, and mediates tumor angiogenesis through the signaling pathways of PI3K/AKT. In turn, they also promote the expression of OPN<sup>[13,14]</sup>. Recently, it has been reported that the overexpression of  $\alpha\beta3$  integrin on tumor is associated with an aggressive phenotype of several solid tumor types<sup>[15,16]</sup>. In this study, we further found that the expression rate of  $\alpha\beta3$  was 77.2% in NSCLC, and significantly higher than that (26.0%) in normal lung tissue, suggesting that  $\alpha\beta3$  might be involved in the development of NSCLC. In addition, the statistical analysis showed that the protein expression of  $\alpha\beta3$  was positively associated with NSCLC's differentiation degree, lymph node metastasis and clinical staging. These results further suggested that the expression of  $\alpha\beta3$  might be involved in the development of NSCLC, and closely related to the degree of malignancy, invasion and metastasis of NSCLC. Possible molecular mechanism why  $\alpha\beta3$  promotes the development of NSCLC is that  $\alpha\beta3$  integrin plays an important role in promoting angiogenesis, and adhesion of cell to matrix so that it enhances the development of NSCLC<sup>[17]</sup>. Thus,  $\alpha\beta3$  can be used as an important indicator to evaluate the malignant degree and invasion of NSCLC. These results also suggest that  $\alpha\beta3$  could be a surrogate marker in the diagnosis of NSCLC and the prediction of therapeutic response for treatment with anti- $\alpha\beta3$  antibody in NSCLC.

The gene, *pim-1*, is a potential oncogene, which is located on chromosome 6p21.2. The encoded protein of Pim-1 is a serine/threonine kinase<sup>[18]</sup>. Recent studies have confirmed that it is highly expressed in some malignant tumors<sup>[19,20]</sup>. It is involved in the regulation of proliferation, differentiation and apoptosis of lung

cancer cells<sup>[21]</sup>. In our present study, the positive expression of Pim-1 was detected in the majority of NSCLC, while in few normal lung tissues. This result was in consistent with the literature reported by Zhang, et al. and He, et al.<sup>[22, 23]</sup>. It has been documented that Pim-1 can promote G2/M process of cell cycle, activate growth, differentiation and proliferation of cells<sup>[6]</sup>. In addition, we also found that the expression of Pim-1 in NSCLC with poor differentiation, clinical stage of III and IV and lymph node metastasis was significantly higher than that in clinical stage of I and II and without lymph node metastasis. Based on the results mentioned above, collectively, we proposed that Pim-1 might also be involved in the tumorigenesis/progression of NSCLC.

In this study, Spearman rank correlation analysis showed that there was an obvious positive correlation of protein expression between OPN and  $\alpha\beta3$ , in which the higher the expression level of OPN, the higher the expression of  $\alpha\beta3$ . It was suggested that OPN acts through  $\alpha\beta3$  integrin, which in turn activates the FAK pathways, contributing to the overexpression of  $\alpha\beta3$ <sup>[24]</sup>. Other groups also revealed that OPN is involved in tumor growth and angiogenesis of lung cancer by upregulating vascular endothelial cell migration and proliferation via interacting with  $\alpha\beta3$  integrin<sup>[25]</sup>. Upregulation of OPN specifically activates the activity of integrin receptor  $\alpha\beta3$ , and may accelerate tumor angiogenesis, and induce activation of a variety of kinases such as Pim-1, PI3K, and AKT<sup>[13,14]</sup>. Therefore, when the expression level of OPN is higher, it can elevate the expressions of  $\alpha\beta3$  and Pim-1 through regulating signaling pathways. In NSCLC, the expression of OPN, Pim-1 and the expression of  $\alpha\beta3$ , Pim-1 are also positively correlated. Therefore, our results, together with others' findings, suggested that OPN plays a crucial role for tumor growth and/or progression of human lung cancer cells by interacting with  $\alpha\beta3$  integrin, and thus, regulating signaling pathways and activating Pim-1<sup>[13,14,18,24]</sup>, resulting in the overexpression of OPN,  $\alpha\beta3$  and Pim-1.

With regards to the molecular targets studied in this report, it was concluded that OPN can promote extracellular matrix degradation, inhibit apoptosis, and accelerate tumor angiogenesis in the process of invasive growth and metastasis. The other one, Pim-1 may promote cell cycle G2/M process<sup>[6]</sup>, and promote cell proliferation through enhancing the cell cycle promoter activity, such as Cdc25A and Cdc25C, so that the decline of homotypic adhesion, degradation of extracellular matrix and cell proliferation can be a vicious cycle and promote each other. This may indicate that a synergistic effect among the three working together might be capable of regulating migration of lung cancer cells and promote the occurrence, invasion and metastasis of NSCLC. The expressions of OPN,  $\alpha\beta3$  and Pim-1 may be early diadynamic criteria of NSCLC. Taken together,

our results suggested that targeting the interaction among OPN,  $\alpha\text{v}\beta 3$  and/or Pim-1 could be effective for future development of anti-angiogenic therapeutic agents for patients with NSCLC.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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