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ORIGINAL ARTICLE – CANCER RESEARCH

Osteopontin is a novel prognostic biomarker in early-stage non-small cell lung cancer after surgical resection

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Abstract
Purposes Osteopontin (OPN), an extracellular matrix-secreted phosphorylated glycoprotein, has been reported overexpressed in many solid tumors. As an important part of lung cancer, the high recurrence of non-small cell lung cancer (NSCLC) also attracted great attention of scientists.
Methods In this study, we investigated the expression of OPN and the relationship with prognosis of NSCLC patients. We measured the expression of OPN among 163 NSCLC samples by immunohistochemical method and compared the expression of these 28 matched cDNA between tumor and peritumoral tissue by real-time polymerase chain reaction.
Results We demonstrated that the percentages of positive OPN expression is 66.8 % and OPN expression in tumor site was much higher than the tissue adjacent to carcinoma ($p = 0.0046$). By further analysis, we found that OPN expression was significantly correlated with poor prognosis of NSCLC. Moreover, for early-stage patients, OS and DFS rates of OPN (−) group were significantly higher than OPN (+) group. For advanced-stage patients, OPN expression was only associated with OS rates.
Conclusions These results suggest that OPN is commonly expressed in NSCLC and may guide the evaluation of prognosis with NSCLC, especially for early-stage patients.

Keywords Osteopontin · NSCLC · Prognosis · Immunohistochemistry

Introduction

Lung cancer is the most common cancer in the world; approximately 80–85 % of cases are non-small cell lung cancer (NSCLC). The overall 5-year survival rate for lung cancer is 18 % (Siegel et al. 2014). Even for patients with early-stage disease who undergo surgical resection, the postoperative recurrence rate is higher than other types of cancer (Chansky et al. 2009). Our understanding of possible factors correlated outcome is still quite limited. While gender, age, differentiation, and TNM staging are the most important clinical factors (Hoffman et al. 2000), NSCLC patients with similar clinical factors can have difference in recurrence rates. This suggests that there is a significant biological heterogeneity and complexity of these tumors. Thus, it is essential to identify novel and useful biological tumor markers that might more accurately establish the prognosis of different patients and allow better comprehensive therapy for high-risk ones.

OPN is an extracellular matrix-secreted phosphorylated glycoprotein, which also called the transformation-related protein phosphatase. It was first found by Senger in the epithelial cell strain of malignant transformation (Senger et al. 1979). There is evidence, suggesting that osteopontin (OPN), a chemokine-like, calcified ECM-associated protein, may play an vital role in determining the metastatic potential of

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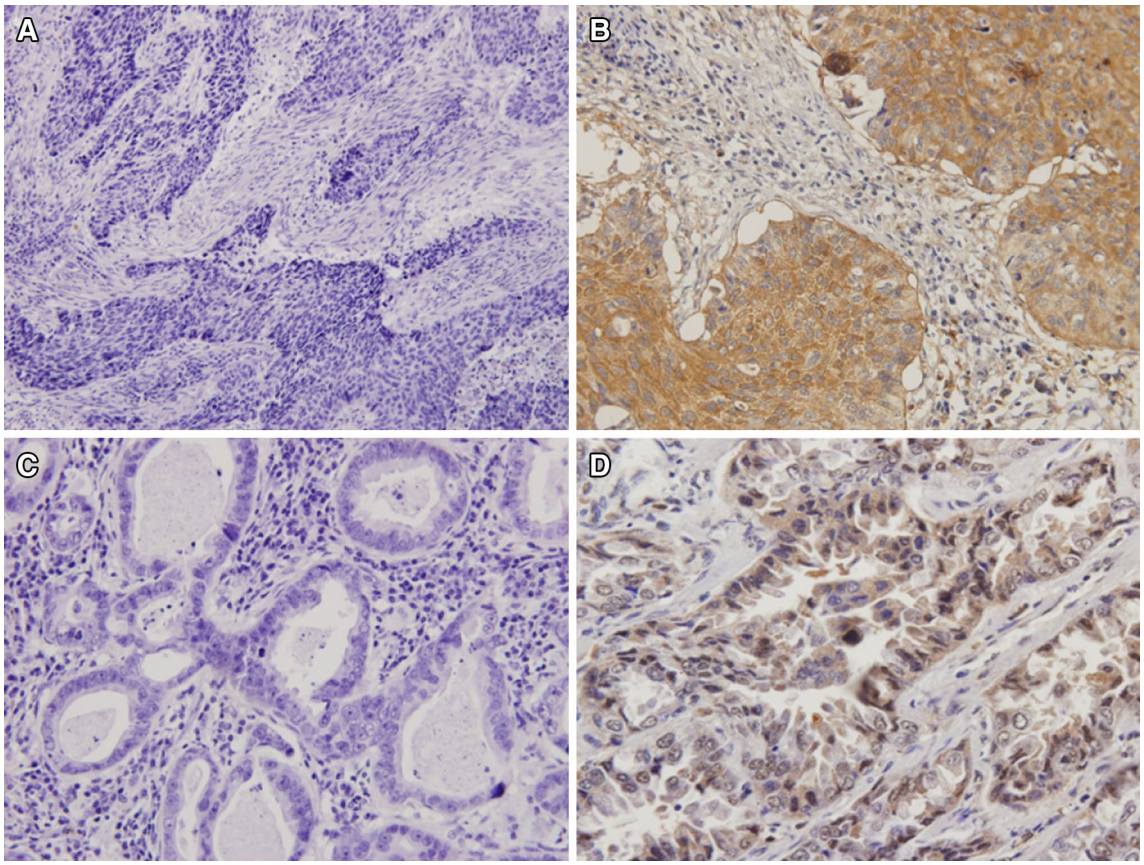

Fig. 1 Osteopontin (OPN) expression in NSCLC cancerous tissues (SP × 400). **a**, **c** Negative expression of squamous cell carcinoma and adenocarcinoma; **b**, **d** positive expression of squamous cell carcinoma and adenocarcinoma

various cancers (Rangaswami et al. 2006). OPN promotes cell adhesion and migration by binding to the receptors $a_V\beta3$ integrins and CD44 (Denhardt and Guo 1993). OPN overexpression has been demonstrated in many human tumors, including carcinoma of breast, lung, liver, gastric, prostate, colon, and ovaries, as well as mesotheliomas and others tumors (Wai and Kuo 2008). While various studies have evaluated the relationship between OPN expression and prognosis among different types of cancer (Rudland et al. 2002; Bramwell et al. 2006; Conway et al. 2009; Hui et al. 2008), the impact of OPN on NSCLC outcome remains unclear (Jin et al. 2012; Hu et al. 2005). In the present work, we measured OPN expression in 163 cases of NSCLC and 28 matched tumor and peritumoral tissue and then evaluated the correlation between OPN and patient prognosis.

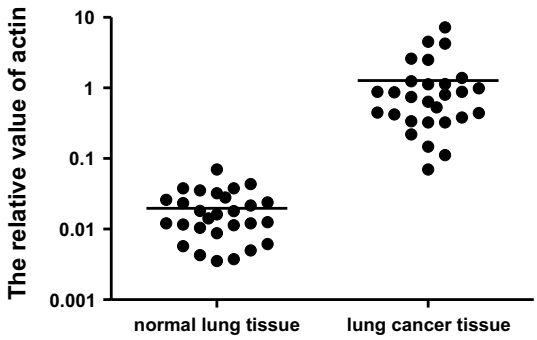

Fig. 2 Osteopontin (OPN) expression in 28 matched NSCLC cancer tissue and normal lung tissue ($t = 2.955$, $p = 0.0049$)

Materials and methods

Patients and tissue sample

Non-small cell lung cancer carcinoma tissues samples were studied from 163 patients who underwent complete pulmonary resection and systematic lymph node dissection (surgical resection) between 2004 and 2009 at the Cancer Institute and Hospital of Tianjin Medical University (China). Tumor differentiation was graded according to the Edmondson–Steiner grading system (Edmondson and Steiner 1954). Patients were classified according to the seventh edition of the International Union against Cancer (UICC) TNM staging. There were 104 males and 59 females, with ages ranging from

Table 1 Relationship between osteopontin and clinicopathologic factors of patients

Variables	OPN (−) Number (%)	OPN (+) Number (%)	Total (%)	χ^2	*p* value
Gender				0.287	0.592
Male	36 (34.6)	68 (65.4)	104 (63.8)		
Female	18 (30.5)	41 (69.5)	59 (36.2)		
Age (years)				0.185	0.667
<60	17 (30.9)	38 (69.1)	55 (33.7)		
≥60	37 (34.3)	71 (65.7)	108 (66.3)		
Smoking status				1.285	0.257
Never smoked	15 (27.2)	40 (72.8)	55 (33.7)		
Smoker	39 (38.9)	69 (61.1)	108 (66.3)		
Histologic subtype				8.179	0.004
Squamous cell carcinoma	39 (42.4)	53 (57.6)	92 (56.4)		
Adenocarcinoma	15 (21.1)	56 (78.9)	71 (43.6)		
Lymph node metastasis				6.235	0.013
Have	21 (24.4)	65 (75.6)	86 (52.8)		
No	33 (42.9)	44 (57.1)	77 (47.2)		
Numbers of involved nodes				18.932	<0.001
<4	52 (42.3)	71 (57.7)	123 (75.5)		
≥4	2 (5.0)	38 (95.0)	40 (24.5)		
Metastasis				1.921	0.166
No	49 (35.3)	90 (64.7)	139 (85.3)		
Yes	5 (25.0)	18 (75.0)	24 (15.7)		
TNM stages				13.068	0.001
I	17 (38.6)	27 (61.4)	44 (27.0)		
II	21 (50.0)	21 (50.0)	42 (25.8)		
III–IV	16 (20.8)	61 (79.2)	77 (47.2)		
Lymph node staging				7.175	0.028
N0	33 (43.4)	43 (56.6)	76 (46.6)		
N1	8 (28.6)	20 (71.4)	28 (17.2)		
N2	13 (22.0)	46 (78.0)	59 (36.2)		
Tumor recurrence				20.641	<0.001
No	25 (62.5)	15 (37.5)	40 (24.5)		
Yes	29 (23.6)	94 (76.4)	123 (75.5)		

35 to 78 years (median age 62 years). Disease-free survival (DFS) and overall survival (OS) were calculated as the period from surgery until the date of disease recurrence or of death, respectively. All patients were followed up until October 31, 2013, and 54 patients were still alive at the close of the study.

Expression of OPN in NSCLC cancerous tissue

Immunohistochemical examination to detect OPN expression was performed with monoclonal anti-mouse OPN antibody at 1:100 dilution (SC-73631, Santa Cruz, CA). All the sections were routinely deparaffinized and rehydrated; then, the sections were rinsed in phosphate-buffered saline (PBS, pH = 7.4) and subsequently were treated for antigen retrieval. Sections were treated in saline sodium buffer (pH = 6.0) in an autoclave sterilizer. After cooling at room temperature for 30 min, the sections were rinsed in PBS and then immersed in 3 % H_2O_2 for 20 min to block the endogenous peroxidase activity. After being rinsed in PBS, the sections were incubated with normal bull serum albumin at 37 °C for 30 min to reduce nonspecific hydrophobic interactions. After interaction with OPN antibody overnight at 4 °C, the sections were rinsed in PBS and then incubated with secondary antibodies (Maxin, Fujian) and rinsed in PBS again. Nuclei were counterstained blue with hematoxylin. The sections were then dehydrated, made transparent, and covered with coverslips and sealed with neutral gum. PBS without the primary antibody was used as negative control.

Two pathologists blinded to the clinical data adjudicated whether the tumor tissue was positive for OPN expression.

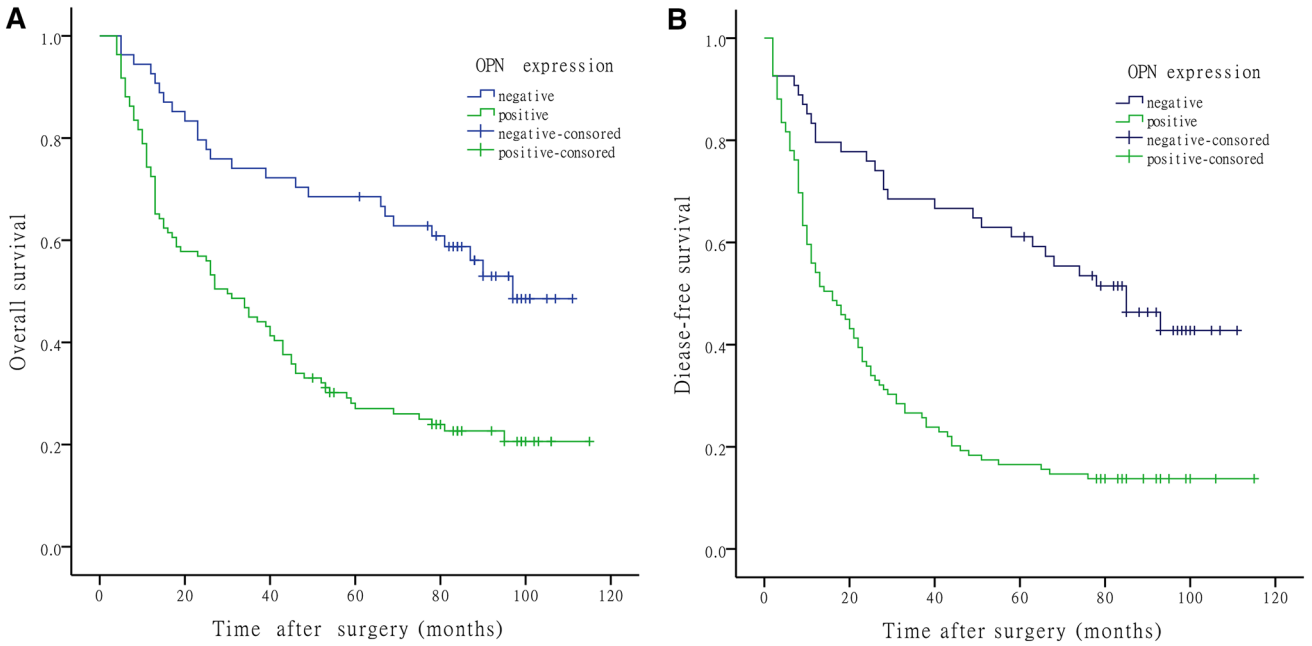

Fig. 3 Prognostic significance of osteopontin (OPN) expression was assessed by Kaplan–Meier method and log-rank test. **a** Comparisons of overall survival (OS) between OPN (−) group and OPN (+) group ($p < 0.001$, $\chi^2 = 18.805$). **b** Comparisons of disease-free survival (DFS) between OPN (−) group and OPN (+) group ($p < 0.001$, $\chi^2 = 26.412$)

Expression was quantified as the percentage of tumor cells with cytoplasmic immunoreactivity and staining intensity (0, negative; 1, weak; 2, intermediate; and 3, strong) by counting at least 1,000 cancer cells (100 cells in 10 HPF) for each section (0, none; 1, <10 %; 2, 10–30 %; and 3, >30 %). A mean score exceeding 3 was defined as positive (Zhang et al. 2001).

Real-time quantitative PCR analysis

Twenty-eight matched cDNA samples of tumor site and the peritumoral tissue from the 163 patients were selected randomly. The levels of OPN expression were detected using quantitative polymerase chain reaction (PCR). PCR was performed using 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Samples were assayed in 20 μl reaction mixture containing 2 μl cDNA, 0.8 μl of 10 μM PCR Forward Primers and Reverse Primer, 10 μl of 2X SYBR Premix Ex Tap II master mixes (TaKaRa Biosystems, Japan), and 2 μl of molecular grade H_2O. β-ACTION was used as a normalization control. The amplifications were performed for 40 cycles with annealing at 95 °C for 5 s and 60 °C for 34 s. Samples were run in triplicate including negative controls. Relative quantification ($2-\Delta\Delta CT$ method) was performed to determine the change in gene expression levels.

Statistical analysis

Data analysis was carried out using 20.0 SPSS software (SPSS Inc, Chicago, IL, USA). Categorical variables were analyzed with the Chi-square test. Univariate survival analysis was performed by modeling Kaplan–Meier survival curves and the log-rank test used to evaluate the statistical significance of differences in survival distributions. Multivariate analysis was tested by Cox proportional hazard model with patients surviving at the study end censored. Results were considered statistically significant if $p < 0.05$.

Results

OPN expression in NSCLC tissues

There were 104 male patients and 59 female patients with a mean age of 61.6 years (range 35–78 years; median age 62 years). There were 92 cases (56.4 %) of squamous cell carcinoma and 71 cases of adenocarcinoma (43.6 %). There were 44 TNM stage I, 42 stage II, 54 stage III, and 23 stage IV patients. A total of 123 (75.4 %) patients relapsed during follow-up. At the end of the follow-up, 54 patients (33.13 %) were alive, while 109 (66.87 %) had died.

Table 2 Univariate analyses of clinicopathologic factors associated with OS and DFS

Variables	5-Year overall survival rate			5-Year disease-free survival rate		
	Number (%)	χ^2	*p* value	Number (%)	χ^2	*p* value
Histologic subtype		5.518	0.019		7.757	0.005
Squamous cell carcinoma	38 (41.3)			31 (33.7)		
Adenocarcinoma	16 (22.5)			9 (12.7)		
OPN expression		18.805	<0.001		26.412	<0.001
Negative	29 (53.7)			25 (46.3)		
Positive	25 (22.9)			15 (13.8)		
Lymph node metastasis		34.310	<0.001		27.401	<0.001
Have	13 (15.1)			10 (11.6)		
No	41 (53.2)			30 (39.0)		
TNM stages		49.434	<0.001		44.620	<0.001
I	24 (54.5)			17 (38.6)		
II	21 (48.8)			17 (39.5)		
III–IV	9 (11.8)			6 (7.9)		
Metastasis		39.249	<0.001		32.963	<0.001
No	52 (37.4)			39 (28.1)		
Yes	2 (8.3)			1 (4.2)		
Lymph node staging		48.834	<0.001		41.635	<0.001
N0	41 (53.9)			30 (39.5)		
N1	8 (28.6)			6 (21.4)		
N2	5 (8.5)			4 (6.8)		
Numbers of involved nodes		18.506	<0.001		11.960	0.001
<4	49 (39.8)			35 (28.5)		
≥4	5 (12.5)			5 (12.5)		
Smoking status		3.529	0.060		3.510	0.061
Never smoked	15 (27.3)			10 (18.2)		
Smoker	39 (36.1)			30 (27.8)		
Gender		1.995	0.158		0.770	0.380
Male	37 (35.6)			27 (26.0)		
Female	17 (28.8)			13 (22.0)		

Table 3 Multivariate analysis of factors associated with OS and DFS

Variable	Hazard ratio (95 % CI)	*p* value*
OS		
Lymph node staging	1.603 (1.134–2.264)	0.007
TNM stages (I vs II vs III–IV)	0.443 (0.227–0.864)	0.017
OPN expression	2.408 (1.515–3.828)	<0.001
DFS		
Lymph node staging	1.527 (1.092–2.139)	0.013
TNM stages (I vs II vs III–IV)	0.409 (0.205–0.817)	0.011
OPN expression	2.553 (1.657–3.932)	<0.001

CI confidence interval, *OPN* osteopontin, *TNM* tumor-node-metastasis

* *p* value was calculated by Cox proportional hazards regression model

Osteopontin staining was seen mainly in the cytoplasm. Among the 163 patients, positive OPN expression was observed in 66.87 % (109/163) case of NSCLC cancerous tissue (Fig. 1). The percentages of positive OPN expression of squamous cell carcinoma and adenocarcinoma were 57.6 % (53/92) and 78.9 % (56/71), respectively ($p = 0.004$). OPN expression was much higher in tumor versus adjacent tissue by real-time PCR ($p = 0.0046$, $t = 2.955$) as shown in Fig. 2.

Relationship between OPN expression and clinicopathological data

The associations between clinicopathologic features and the expression of OPN levels are shown in Table 1. The

Table 4 Relationship between OPN and clinicopathologic factors of early stage (TNM I–II) NSCLC patients

Variables	OPN (−) Number (%)	OPN (+) Number (%)	Total (%)	χ^2	*p* value
Gender				0.000	1.000
Male	26 (44.8)	32 (55.2)	58 (66.6)		
Female	13 (44.8)	16 (55.2)	29 (33.4)		
Age (years)				0.023	0.879
<60	10 (43.5)	13 (56.5)	23 (26.4)		
≥60	29 (45.3)	35 (54.7)	64 (73.6)		
Smoking status				0.073	0.787
Never smoked	8 (42.1)	11 (57.9)	19 (21.8)		
Smoker	31 (45.6)	37 (54.4)	68 (78.2)		
Histologic subtype				3.077	0.079
Squamous cell carcinoma	29 (51.8)	27 (48.2)	56 (64.4)		
Adenocarcinoma	10 (32.3)	21 (67.7)	31 (35.6)		
Lymph node metastasis				0.254	0.621
Have	31 (46.3)	36 (53.7)	67 (77.0)		
No	8 (40.0)	12 (60.0)	20 (23.0)		
Numbers of involved nodes				1.772	0.183
<4	39 (47.0)	44 (53.0)	83 (95.4)		
≥4	0 (0)	4 (100)	4 (4.6)		
Lymph node staging				0.533	0.465
N0	31 (46.3)	36 (53.7)	67 (77.0)		
N1	7 (36.8)	12 (63.2)	19 (21.8)		
Tumor recurrence				18.347	<0.001
No	25 (71.4)	10 (28.6)	35 (40.2)		
Early recurrence ≤3 years	7 (20.6)	27 (79.4)	34 (39.1)		
Late recurrence >3 years	7 (38.9)	11 (61.1)	18 (20.7)		

percentages of OPN positive samples among the TNM stage I–II and III–IV patients were 55.8 % (48/86) and 79.2 % (61/77), respectively ($p = 0.002$). OPN expression in adenocarcinoma was significantly higher than that in squamous cell carcinoma ($p = 0.004$) and was associated with numbers of involved nodes ($p < 0.001$), lymph node staging ($p = 0.028$), stages of TNM ($p = 0.001$), and recurrence of statue ($p < 0.001$), but not with gender, age, smoking, and metastasis status.

Prognostic significance of OPN expression in NSCLC patients

For the entire study population, the 5-year OS and DFS rates were 39.9 % and 31.3 %, respectively. The 5-year OS rate in patients in the OPN (−) group was significantly higher than that of OPN (+) group ($p < 0.001$, $\chi^2 = 18.805$). Moreover, the difference of 5-year DFS rate among them was also significant ($p < 0.001$, $\chi^2 = 26.412$; Fig. 3). Univariate analysis showed that lymph node staging, number of involved nodes, TNM stage, OPN expression, and histologic subtype (adenocarcinoma) were unfavorable prognostic factors for OS and DFS (Table 2). Furthermore, multivariate analysis found that lymph node staging, TNM stages, and OPN expression were independently prognostic factors for OS and DFS ($p < 0.05$; Table 3).

Clinicopathologic characteristics and prognostic significance of OPN expression for patients with the TNM stage I–II NSCLC

Clinicopathologic features of patients with the TNM stage I–II NSCLC are summarized in Table 4. OPN expression was correlated with tumor recurrence after surgical resection ($p < 0.001$, $\chi^2 = 18.347$) in the TNM stage I–II NSCLC, but

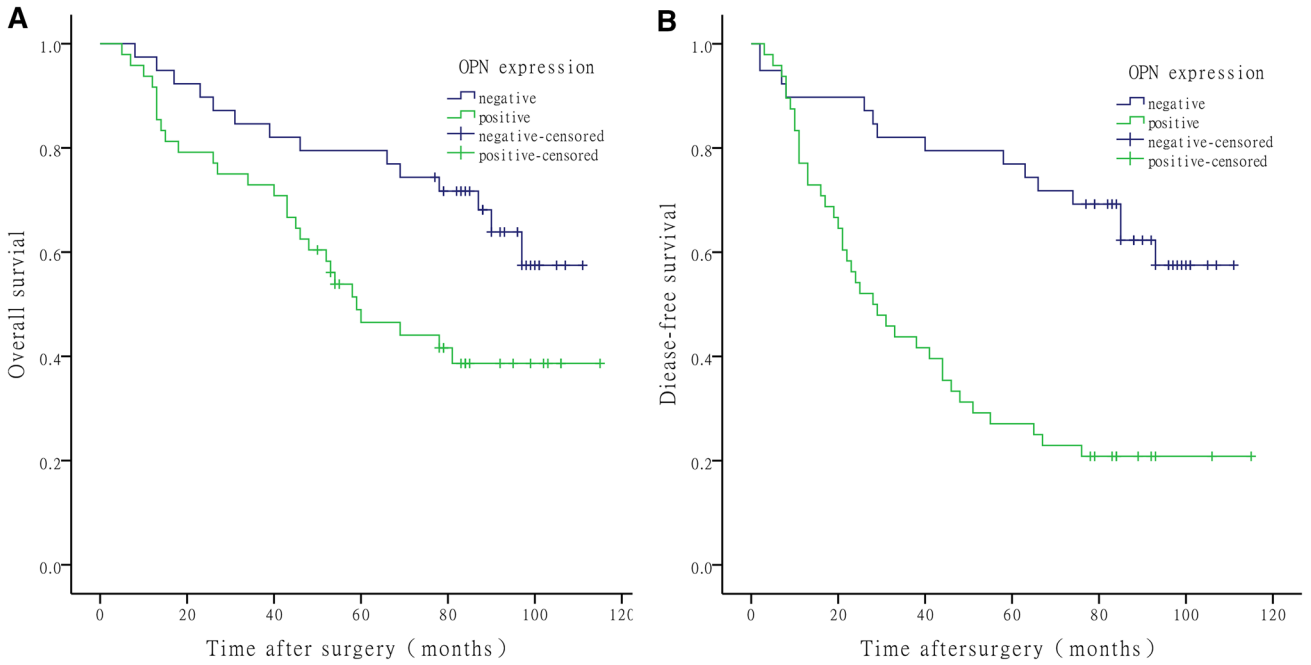

Fig. 4 Prognostic significance of osteopontin (OPN) expression in patients with the TNM stage I–II (NSCLC) was assessed by Kaplan–Meier method and log-rank test. **a** Comparisons of OS between OPN (−) group and OPN (+) group ($p = 0.012$, $\chi^2 = 6.282$). **b** Comparisons of DFS between OPN (−) group and OPN (+) group ($p < 0.001$, $\chi^2 = 18.844$)

not with histologic subtype, lymph node metastasis, numbers of involved nodes, and lymph node staging.

For the patients with the TNM stage I–II NSCLC, the 5-year OS and DFS rates were 59.7 % (52/87) and 49.4 % (43/87). The 5-year OS rate in the OPN (−) group was significantly higher than that of the OPN (+) group ($p = 0.012$, $\chi^2 = 6.282$). There is also difference of 5-year DFS rate between the OPN (−) group and the OPN (+) group ($p < 0.001$, $\chi^2 = 18.844$; Fig. 4).

For patients with the TNM stage III–IV NSCLC, the 5-year OS and DFS rates were 17.1 % (13/76) and 10.5 % (8/76), respectively. OPN expression was associated with 5-year OS rate between the OPN (−) group and OPN (+) group ($p = 0.046$, $\chi^2 = 3.976$), but were not correlated with 5-year DFS rate ($p = 0.147$, $\chi^2 = 2.105$).

Discussion

In this study, we measured the expression of OPN in 163 NSCLC patients using IHC method and detected the expression of OPN in 28 cases paired of NSCLC tumor tissues versus adjacent tissues by real-time PCR and verified that OPN is highly expressed in cancerous tissues. In the present patient cohort, the positive OPN expression was 66.8 %, and we found that OPN expression was not only associated with histologic subtype, numbers of involved nodes, and the statue of recurrence, but also closely related to the prognosis of different stage patients with NSCLC.

Osteopontin is commonly overexpressed in many solid cancers and contributes to tumor formation and progression (El-Tanani 2008). Previous studies have also found a correlation between plasma OPN, tumor burden, and poor prognosis in patients with cancer metastasis (Jin et al. 2012; Pan et al. 2003; Mack et al. 2008). However, OPN expression used as a single biomarker for NSCLC prognosis, especially for the TNM stage I–II, was not assessed (Coppola et al. 2004). In our study, the percentages of OPN positive samples among the TNM stage I–II and III–IV patients were 55.8 % (48/86) and 79.2 % (61/77), respectively. Moreover, for patients with the TNM stage I–II NSCLC, OPN expression was significantly associated with lower OS rate and DFS rate, while for patients with the TNM stage III–IV, OPN expression was associated with lower OS rate, but was not correlated with DFS rate. These results suggest that the statue of OPN expression to predict the prognosis of patients with early stage of NSCLC is more important than advanced-stage patients.

Cancer progression depends on an accumulation of genetic and epigenetic modifications and is regulated by multiple cell signaling molecules (Bogenrieder and Herlyn 2003). Transcriptional regulation of OPN is complex and involves multiple pathways, including AP-1, Myc, v-Src, Runx/CBF, TGF-B/BMPs/Smad/Hox, and Wnt/β–catenin/

APC/GSK–3β/Tcf-4 (Wai and Kuo 2008). OPN may be one of important molecules to promote tumor progression, assess recurrence risk, and predict the prognosis of patients (Anborgh et al. 2010; Johnston et al. 2008). Recently, Sun et al. (2013) reported that OPN was an independent and unfavorable predictor for OS and DFS in NSCLC. Their results agree with our own, and furthermore, our study documented that OPN expression was remarkably associated with increased risk of the recurrence after resection for early stage of NSCLC patients. Combined with other commonly recognized tumor markers, OPN can offer more effective and more accurate information about early disease progression and prognosis for NSCLC patients (Weber 2011).

In conclusion, our study suggest that OPN expression in NSCLC tumor tissue may be an effective predictor of prognosis, especially the risk of recurrence and prognosis of early stage of NSCLC patients. It may also provide guidance for individualized treatment patients at high risk of recurrence and potentially reduce tumor recurrence and prolong survival time. As a preliminary study, we plan to make a further research so as to explore the mechanism of OPN promote tumor metastasis on the basis of this study.

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Conflict of interest No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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