OPN-a Splicing Variant Expression in Non-small Cell Lung Cancer and its Effects on the Bone Metastatic Abilities of Lung Cancer Cells *In Vitro*

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Abstract. *Background: Osteopontin (OPN) is known to be involved in the development of certain cancers, including non-small cell lung cancer (NSCLC). However, its role in tumour progression remains unclear. The present study investigated the expression and biological impact of the OPN variant, OPN-a in NSCLC. Materials and Methods: OPN-a splicing variant expression in human NSCLC tissues was analyzed by real-time qPCR and immunohistochemistry (IHC), respectively. The impact of OPN-a on cellular functions of lung cancer cells was also evaluated. In addition, an in vitro model was developed for the assessment of interactions between lung cancer cells and bone tissue. Results: The expression of OPN-a was higher in lung cancer tissues compared to normal controls. OPN-a promoted the malignant phenotypes of A549 cells by enhancing cell-adherent abilities to bone tissues, which could be mediated by the interaction with the cell surface receptor αvβ3 integrin. Conclusion: OPN-a may represent a bone metastatic factor in human lung cancer, as well as a potential therapy target.*

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Lung cancer is one of the most common causes of malignancy-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for >80% of primary lung cancers. Despite improvements in traditional treatments (such as surgical resection, chemotherapy, and radiotherapy), the prognosis for lung cancer patients is still poor with a 5-year overall survival being less than 20% because of high-frequency metastasis (1). Bone is one of the most common sites for metastasis of lung cancer, and approximately 30-40% of lung cancer patients show bone metastasis (2). Patients with bone metastasis experience distressful symptoms including bone pain, pathologic fractures, spinal cord and nerve compression, hypercalcemia and decreased mobility, that can seriously affect patients' life quality. Until now the underlying molecular mechanisms that lead to development of bone metastasis in NSCLC are still unclear. Therefore, studying the mechanisms of NSCLC progression and finding more effective biomarkers for an early diagnosis of bone metastasis are crucial for the treatment of this invasive malignancy.

Human osteopontin (OPN), a member of the "small integrin binding ligand N-linked glycoproteins" (SIBLINGs) family, is a 41-75 kDa extracellular matrix phosphoprotein expressed in multiple tissues (3-5). Also, OPN is most abundant in bone (6). During bone remodeling, OPN is produced by the osteoclasts thus contributing to their attachment to the mineral matrix. Previous studies have suggested that OPN expression is particular high in various primary tumors, including lung cancer (7). Furthermore, OPN overexpression is significantly associated with a high incidence of metastasis (8). In bone metastases, OPN seems

to mediate the interaction between the carcinoma and the bone surface (9). However, the molecular mechanism of OPN tumorigenicity in NSCLC, especially in bone metastasis, is not completely understood.

As a secreted protein with an arginine-glycine-aspartic (RGD) motif and serine-valine-valine-tyrosine-glutamate-leucine-arginine (SVVYGLR) sequence, OPN can also modulate cell behavior in autocrine or paracrine manors *via* their interaction with cell surface receptors such as integrins (10, 11). Integrins are a family of transmembrane adhesion receptors, formed by two subunits named α and β, that interact through non-covalent bindings. As one of the most common integrin heterodimer, αvβ3 integrin has been identified as a critical receptor for OPN in many biological processes (12). Recent findings indicate that binding and activation of αvβ3 integrin by OPN may promote the motility, adhesion and apoptosis of malignant cells (13, 14), while the potential biological roles of OPN and αvβ3 in the pathogenesis of NSCLC bone metastasis have not been fully elucidated.

Several isoforms of OPN transcripts have been identified, including OPN-a (full-length form), OPN-b (lacking exon 5), and OPN-c (lacking exon 4). Among the three OPN isoforms, OPN-a is the most highly expressed in lung cancer cell lines and lung tumors (15). In the present studies, we sought to investigate the protein levels of OPN-a in NSCLC patient tissue and examine the association of this molecule with clinical outcomes. We then examined the biological functions of OPN-a in human lung cancer cell line A549 after *in vitro* regulation of OPN-a expression. Furthermore, we investigated the interaction of OPN-a and αvβ3 integrin in tumorigenesis and the roles of these two components in the progression of bone metastasis in NSCLC.

Materials and Methods

Cell lines and human lung cancer specimen. A549, a human lung adenocarcinoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were routinely maintained with Dulbecco's modified Eagle medium (DMEM)-F12 supplemented with 10% fetal calf serum and 1x penicillin/streptomycin. A total of 182 lung tissue samples (92 were normal lung tissue and 90 were lung cancer tissue) from Peking University Hospital and another 10 fresh-frozen NSCLC tissues along with matched normal tissues from Xuanwu Hospital of Capital Medical University were obtained from NSCLC patients who received curative resection. These tissues were collected immediately after surgical resection and stored at -80°C until use. Clinicopathological factors, including age, sex, histological types of tumors, TNM stage, and lymph node metastasis were recorded in the patients' database.

RNA preparation and real-time quantitative polymerase chain reaction (qPCR). RNA extraction, reverse transcription (RT) and qPCR were performed as described previously. OPN-a qPCR primers as follow: sense: 5'-ACAACAAATACCCAGATGCT-3'; antisense: 5'-GTCATTGGTTTCTTCAGAGG-3'. Z-sequence on qPCR primers is 5'-ACTGAACCTGACCGTACA-3', which is complementary to the universal Z probe used for the QPCR (TCS Biological Ltd., Oxford, UK). Real-time QPCR conditions were 95°C for 15 min, followed by 60 cycles at 95°C for 20 s, 55°C for 30 s and 72°C for 20 s.

Immunohistochemical (IHC) staining. Frozen sections of lung tissues were cut at a thickness of 4 μm. After incubation with 5% Bull Serum Albumin blocking solution for 30 min, the sections were probing with the OPN antibody (1:200) (sc-10591, Santa Cruz, CA, USA) together with a negative control without primary antibody. Following extensive washing, sections were incubated for 30 min with the relevant secondary antibody (ZSGB Biotechnology, Beijing, China). Finally, Section colour was developed with the diaminobenzidine (DAB) chromogen (Cell Signal Technology, Danvers, MA, USA) and then were observed using light microscopy (BX43, Olympus, Tokyo, Japan).

Construction of OPN plasmids and cell transfection. OPN-a cDNA was cloned into GV141 vector by Genechem Company (Shanghai, China) to establish the OPN-a expression construct. The shRNA plasmid targeting OPN was constructed by Genechem Company (Shanghai, China) using vector GV248. A549 cells were transfected with OPN-a construct and anti-OPN-shRNA transgene using NeofectTM DNA transfection reagent (Neofect Biotech, Beijing, China). OPN expression was verified by western blotting after cells were transfected for 48 h.

Western blotting. To detect the expression level of OPN in the NSCLC cells, confluent cells were pelleted and then lysed using a lysis buffer. These proteins were separated using 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on to nitrocellulose membrane. Membrane was treated with 5% milk to block non-specific proteins prior to probing with anti-OPN primary antibody (1:500) (sc-10591, Santa Cruz) and anti-GAPDH antibody (1:1,000) (sc-32233, Santa Cruz), followed by peroxidase conjugated secondary antibodies. Protein bands were visualized using a chemiluminescence (ECL) detection system (Millipore, Watford, Hertfordshire, UK).

In vitro cell growth assay. Cells were plated into 96-well plates at density of 2,000 cells/well. The cell viability was then evaluated by a nonradioactive cell counting kit (CCK8, Dojindo, Kamimashiki-gun, Kumamoto, Japan) assay according to manufacturer's instruction. Briefly after incubation for 0, 1, 2, 3 and 4 days respectively, the cell viability was determined at a wavelength of 450 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

Wounding/migration assay. The cells were seeded at a density of 6×10^5 per well into a 12-well plate and allowed to reach confluence. The monolayer of cells was then scraped with a fine gauge needle to create a wound. The movement of cells to close the wound was recorded using an inverted microscope. Images were captured at the time intervals of 0, 6, 12 and 24 h.

Cell matrix adhesion assay. A 96-well plate was precoated with 5 μg/well of Matrigel (BD Biosciences, Oxford, UK) and allowed to dry. Following rehydration by serum-free medium,

$2×10^4$ cells were seeded to each well. After 40 min of incubation, non-adherent cells were washed off using PBS buffer. The remaining cells were fixed with 4% formalin, stained with 0.5% (weight/volume) crystal violet and quantified under a microscope.

In vitro invasion assay. Transwell inserts (upper chamber) with 8μm pore size were coated with Matrigel and air dried. Following rehydration, cells were seeded at a density of $2×10^4$ per insert and allowed to invade for 2 days. After incubation, cells that had migrated through the matrix and adhered to the other side of the inserts were fixed in 4% formalin, stained with 0.5% (weight/volume) crystal violet, and counted under a microscope.

Interaction with bone matrix. The femurs were extracted from 6-month-old minipigs, then after being sterile with 75% alcohol, the femur heads were made into bone slices with diameter of 20 mm and thickness of 4 mm. In order to obtain better cultivation conditions for accurate observation, the bone slides were polished by an apparatus to produce a smooth surface (Figure 4A-1). A549 cells were pre-stained with a fluorescent indicator according to its instruction and then seeded onto these bone sections (Figure 4A-2). After incubation of A549 cells on the bone sections for 1 h, non-adherent cells were washed with PBS. The remaining cells were fixed with 4% formalin and were qualitatively assessed by a fluorescence microscope (Nikon, ECLIPSE, Ti-U, Japan).

Statistical analysis. Statistical analysis was performed using the SPSS software package (Version 19.0 SPSS, Chicago, IL, USA). Briefly, the Normal-distributed data were assessed using non-paired (two-sided) Student's *t*-test (for two groups), one-way ANOVA test (for multiple groups). While Data did not follow normal distribution were assessed using Mann-Whitney *U*-test or Kruskal-Wallis test. OPN mRNA values obtained in the QPCR study are given as median transcript copy number per 50 ng of RNA±SD. A *p*-value <0.05 was defined as statistically significant.

Results

Association of OPN-a expression with clinical features of NSCLC tissue. The expression pattern of OPN-a was detected in 182 tissue specimens from NSCLC patients using QPCR. The associations between clinicopathologic features and the expression levels of OPN-a were shown in Table I. Statistical analysis indicated that OPN-a mRNA in primary lung cancer tissues was significantly higher than that in the paired normal tissues (p=0.036). Furthermore, the elevated level of OPN-a appeared to be more frequent in samples with high tumour stage, positive lymph node metastasis and high TNM stage, although statistical analysis did not show significance among these groups (Table I).

In addition, the expression pattern of OPN at the protein level showed the similar results in the IHC assay. NSCLC specimens showed markedly higher cytoplasmic protein expression of OPN, while the paired normal tissues consistently displayed weaker or undetectable immunostaining (Figure 1).

Table I. *Relationship between OPN-a expression and clinical pathological parameters in tissue from lung cancer patients.*

Variable	N	Median (IQ range)	*p*-Value
Tissue paired samples			
Paired tumor	81	866 (135,15764)	0.036
Paired normal	81	67 (9,465)	
Smoking history			
Yes	51	1612 (51,10394)	0.97
No	27	378 (177,16851)	
Histology			
Adenocarcinoma	41	1612 (46,29340)	0.48
Squamous carcinoma	30	1135 (205,10779)	
T stage			
T1	10	371 (118,11627)	1.00
T4	12	1670 (25,9767)	
Lymphy node status			
N0	36	463 (26,15621)	0.65
N2+3	41	1727 (208,14189)	
TNM staging			
TNM1	24	440 (36,17492)	0.95
TNM2+3+4	53	1404 (177,14189)	
Differentiation			
High-Medium	5	1612 (638,19793)	
Medium	36	434 (118,16063)	0.083
Medium-Low	14	682 (17,4675)	0.34
Low	11	3955(165,47452)	0.16

Effects of OPN-a on in vitro functions of A549 cells. The expression level of OPN was considerably increased in the OPN-a-overexpressing cells (Figure 2A) and reduced OPN expression markedly in the OPN-knockdown cells in A549 (Figure 2F). Compared to control groups, overexpression of OPN-a remarkably promoted malignant phenotype of human A549 cells, including cell proliferation, migration, adhesion and invasion. (p<0.01, respectively) (Figure 2B, C, D and E), whereas knockdown of OPN expression resulted in a significant reduction in growth ability of A549 cells (Figure 2G) (p<0.05). Cell adhesion, invasion and migration were also significantly inhibited respectively (p<0.01) (Figure 2H, I and J).

Effects of potential interaction between OPN and αvβ3 integrin on the malignant phenotypes of A549 cells. By depleting OPN with a specific antibody adding in medium, cell growth, migration, adhesion, and invasion were dramatically inhibited over 85%, 60%, 60% and 70% respectively, compared with the control groups (adding IgG- neu Ab) (p<0.01) (Figure 3). The similar results were observed that when the integrin αvβ3-neutralizing antibody was added to the culture medium of A549 cells, the cell growth, migration, adhesion and invasion were reduced to 70%, 50%, 40% and 45% respectively, compared to the control groups (adding IgG-neu Ab) (p<0.01) (Figure 3).
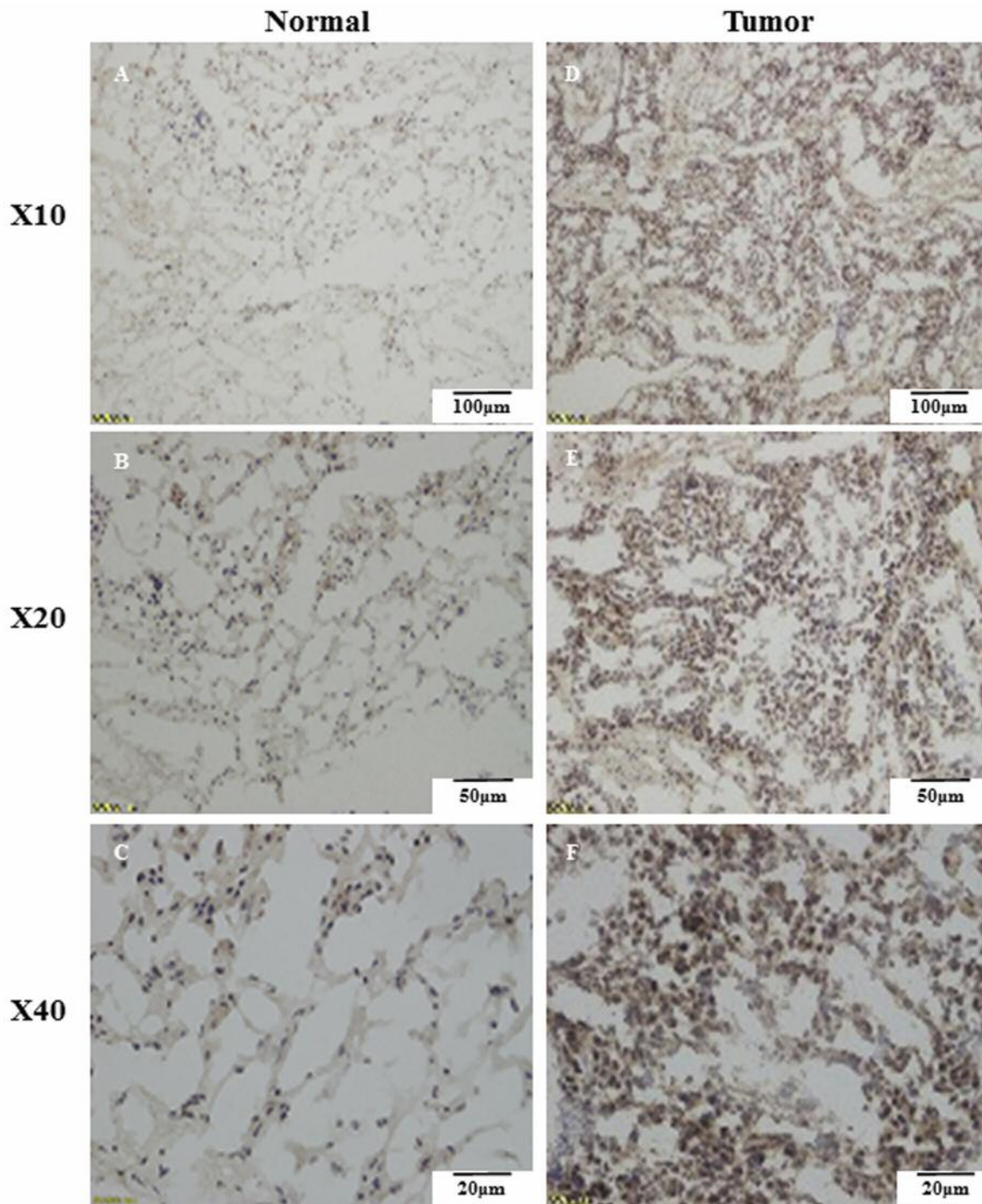
Figure 1. *Representative IHC staining of OPN in normal and lung carcinoma tissue sections. A, B, C: Normal tissue were OPN negative (SP×100, 200, 400). D, E, F: Strong OPN cytoplasmic staining in lung cancer cells (SP×100, 200, 400).*

The results suggested that secreted OPN played the main roles in the promotion of the malignant phenotypes of A549 cells, and mostly dependent on the interaction with αvβ3 integrin receptor.

Effects of interaction between OPN and αvβ3 integrin on A549 cells attachment to bone matrix. Overexpression of OPN-a significantly promoted the cell adherence to the bone slices (*p*<0.01) (Figure 4B), while knockdown of OPN significantly
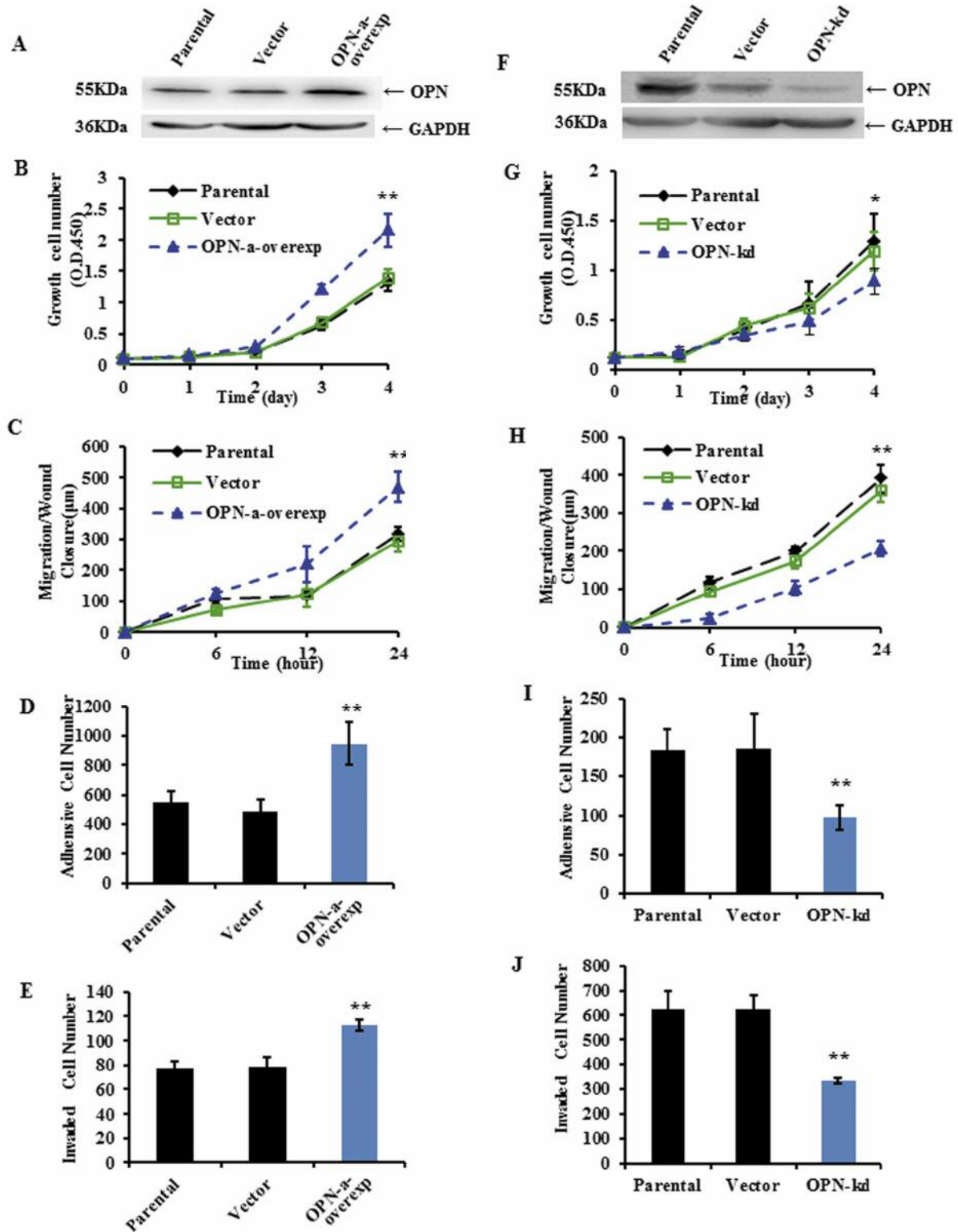
Figure 2. *Functional analysis of OPN-a in A549 cells. A: Validation of ectopic overexpression of OPN in A549 cells by western blotting. B: OPN-a overexpression promoted A549 cell growth over the experimental time points compared to the control cells. C: OPN-a overexpression in A549 promoted cellular migration. D: OPN-a overexpression enhanced cell matrix adhesion compared with control. E: Significant increase of in vitro Matrigel invasion in OPN-a-overexpressing cells. F: Knockdown of OPN in A549 cells as showed by western blotting. G, H, I, J: Knockdown of OPN in A549 cells reduced the cellular growth, migration, adhesion and Matrigel invasion, respectively. *p<0.05, **p<0.01.*
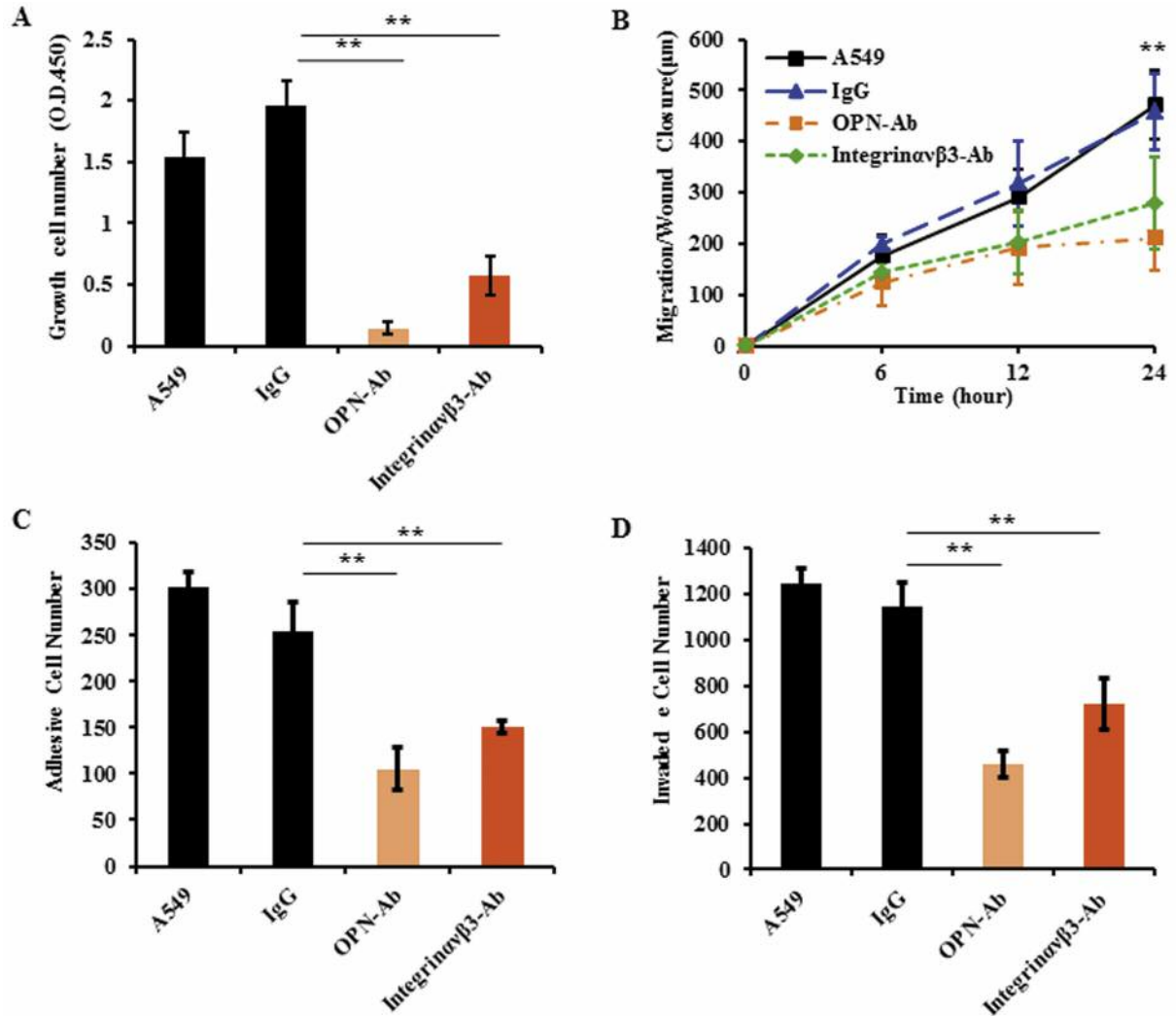
Figure 3. *Involvement of both secreted OPN and αvβ3 integrin in functions of A549 cells. We supplemented the same concentration (20 μg/ml) of OPN-neutralizing antibody and αvβ3 integrin-neutralizing antibody to the media of cultured A549 cells respectively and investigated the properties of A549 cells. Functions of A549 cells including growth (A), migration (B), adhesion (C) and invasion (D) were dramatically inhibited in OPN-neu Ab and αvβ3 integrin-neu Ab groups respectively compared to WT and IgG- neu Ab control groups (p<0.01). In addition, the blocking effects of the OPN-neu Ab were more significant than those groups with the integrin αvβ3-neu Ab (p<0.01).*

inhibited the A549 cells attachment to bone slices ($p<0.01$) (Figure 4C). Following incubation of cells with OPN-neutralizing antibody and αvβ3 integrin-neutralizing antibody respectively, the adhesion properties of A549 cells to bone matrix were strongly inhibited and the blocking effect by OPN antibody was stronger than that by αvβ3 integrin antibody (compared with control group, $p<0.01$, respectively) (Figure 4D).

Discussion

OPN is one of the known factors that have been considered to be correlated with the progression and severity of certain cancer types, including lung, breast, colon and prostate (16-19).

It has been reported that high OPN expression in the primary tumor is associated with early metastasis and poor clinical outcome. In the present study, we have showed that OPN-a is significantly overexpressed in NSCLC tissues when compared to matched normal lung tissues. Additionally, we focused on the role of OPN-a in mediation of malignant phenotype of NSCLC cells. Our present results demonstrate that overexpression of OPN-a is linked to the elevation of growth, adhesion, motility and invasion properties of A549 cells *in vitro*. On the contrary, when OPN was silenced, there was a significant reduction of the cellular growth, adhesion, motility and invasion properties of the A549 cells. Taken together, these results demonstrate the pro-cancer role of OPN-a in A549 cells.
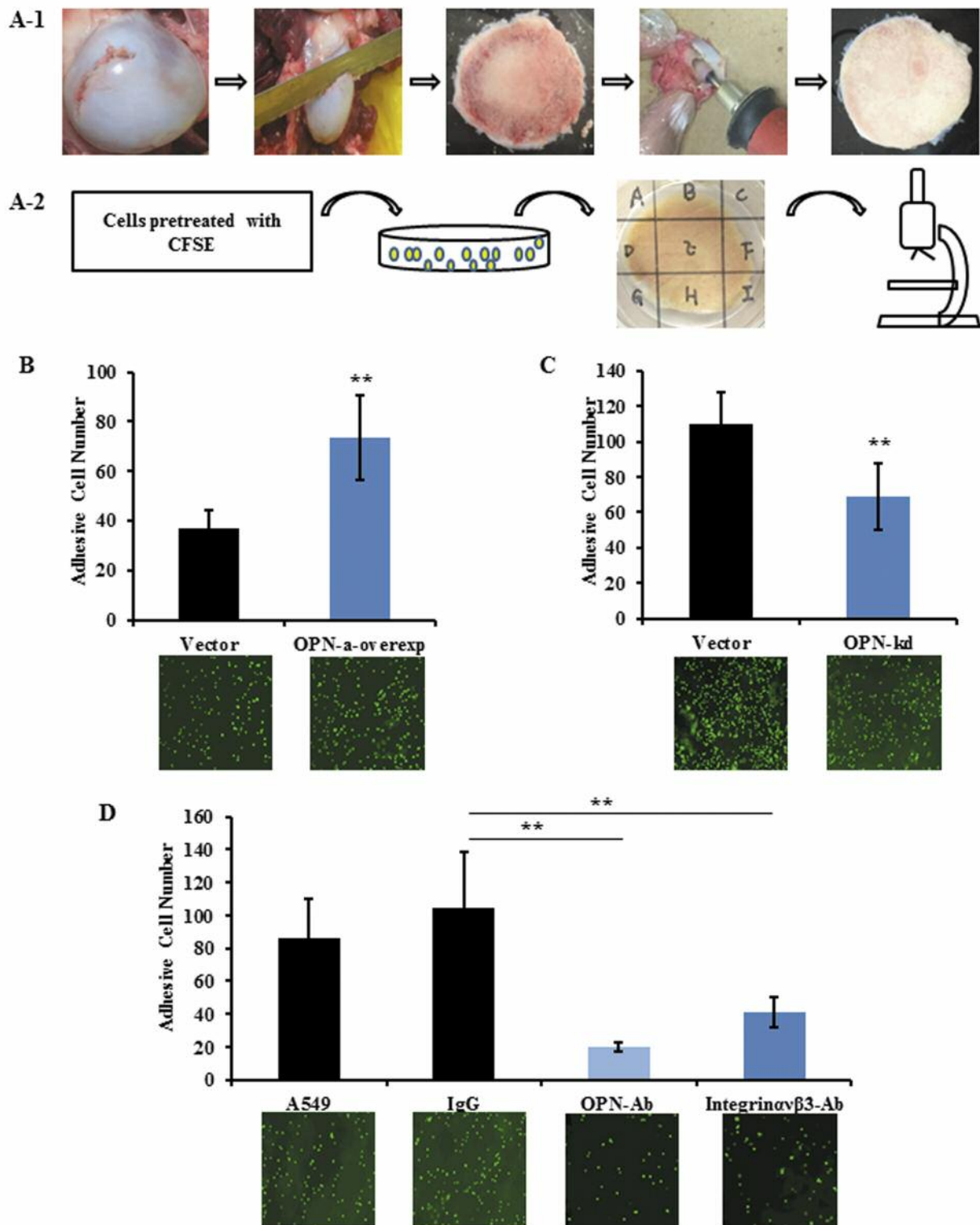
Figure 4. *Effect of OPN and αvβ3 integrin on adhesion of A549 cells to bone matrix. A-1, A-2: The femur heads from minipigs were cut into bone slices with diameter of 20mm and thickness of 4mm. And bone slices were polished by a related tool for a smooth surface. The fraction of attached cells was stained by a fluorescent indicator and then and visually counted. B, C: OPN could specifically affect adhesion of A549 cells to bone slices. D: By depleting OPN with antibody adding in medium, the secreted OPN-induced cell attachment to bone matrix was significantly inhibited in A549. After the interaction between secreted OPN and membrane αvβ3 integrin was blocked, the OPN-induced cell malignant phenotype was partially inhibited.*

OPN has been mainly studied as a secreted protein involved in cancer progression and metastasis. For example, studies show OPN is often present at high levels in the circulation of patients with metastatic cancers and in tumors with increased metastatic potential (8, 20). However, recent reports indicate that intracellular OPN can also play specific roles inside the cells, such as cell motility, cytoskeletal rearrangement, and mitosis by physical interaction with polo-like kinase-1 (Plk-1) (21, 22). In order to determine the significance of the secreted OPN (sOPN) in lung cancer progression and metastasis, the effects of sOPN on the malignant phenotypes of A549 cells were investigated by adding the OPN-neutralizing antibody in the A549 cell media. Blocking of sOPN resulted in reduced cellular growth, migration, adhesion and invasion suggesting the actual roles of sOPN in the promotion of the malignant phenotypes of A549 cells.

$\alpha v \beta 3$ Integrin was also considered in our research because it is the main membrane receptor of sOPN and an important heterodimer in integrin family. At the stages of cancer progression, $\alpha v \beta 3$ integrin is activated and then interacts with OPN, thus providing the necessary signals for the adhesion, migration, and cell survival. For example, cancer cells with aberrant $\alpha v \beta 3$ integrin expression can increase the chemotactical migration ability towards OPN (23). When OPN binds to the $\alpha v \beta 3$ integrin on cell surface, breast cancer cells are induced a great resistance to apoptosis (24). In our study, the results confirmed that although the blocking is not as efficient as that of OPN antibody, the $\alpha v \beta 3$ integrin antibody also remarkably inhibits the malignant properties of A549 cells. Thus, OPN appears to function in promoting the malignant phenotypes of A549 probably through its interaction with $\alpha v \beta 3$ integrin receptor.

The mechanisms that regulate the movement of the primary tumour to the bone have not been elucidated as yet, however the interaction between cancer cells and bone matrix is thought to be an early step in bone metastasis formation. It has been suggested that OPN and $\alpha v \beta 3$ integrin interaction may mediate many of the activities necessary for this initial step in the bone metastasis of malignancies. A number of studies showed that overexpression of OPN were determinative of a high incidence of bone metastases in breast cancer (25). Likewise, in prostate cancer, activation of $\alpha v \beta 3$ integrin allows cancer cells to adhere and migrate to bone matrix at early stages of skeletal metastasis (26). In order to understand the effects of OPN and $\alpha v \beta 3$ integrin on early steps bone colonization, we assessed A549 cells attachment (after OPN overexpression and knockdown in cells, respectively) to minipig bone slices. Interestingly, we observed that the A549 cells following OPN silencing displayed a significant inhibition of bone matrix attachment. In contrast, over expression of OPN-a in A549 cells increased their preferential migration to the bones. Moreover,

after treated with OPN antibody or $\alpha v \beta 3$ integrin antibody, A549 cells showed a strikingly reduced attachment ability to bone slices. Thus, the results suggest that when lung cancer cells invade in bone, the binding of OPN and $\alpha v \beta 3$ integrin could play a vital role in tumour cell adhesion at bone matrix. We established a new model of bone metastasis formation with pig bone for the first time, which presents certain advantages including simple operation and better simulation. However, this model could still have space to be improved, especially because the operation on the bone slices may destroy the bone marrow endothelial cells, thus disrupting the interactions between the cancer cells and the bone microenvironment.

Collectively, our results have clearly demonstrated the clinical value of OPN-a in human non-small cell lung cancer as a potential target for therapy and a potential prognostic factor. The study has also revealed the importance of OPN-a in the aggressiveness of lung cancer cells with a particular relevance to bone metastasis related cell functions of lung cancer cells. Presently, we are developing a test to evaluate the circulating levels of OPN in patients with lung cancer in order to establish if plasma levels of OPN can also be a prognostic/diagnostic factor for these patients and hopefully to develop a clinically deliverable test in the regards. Indeed, circulating OPN in patients with breast cancer (27), liver cancer (28), colorectal cancer (29), squamous cell carcinoma (30), ovarian cancer (31) and in non-cancer related medical conditions such as systemic lupus erythematomatus (32) and in autoimmune encephalomyelitis (33) have been reported to have clinical relevance. In lung cancer, this has also been tested, plasma OPN prior and after radiation therapy has been suggested to be an additional prognostic marker (34). We view this type of test, if developed successfully, would make it a convenient and reliable tool in the clinical settings. However, significant challenges exist including deciphering the variant and cleavage OPNs in the circulation (35). Furthermore, we are currently exploring the expression pattern of the OPN protein, both other as a family and for other variants, namely OPN-b and OPN-c, in lung cancer and explore the biological significant of these variant, alone or in combination in the disease progression of NSCLC. Finally, it would be reasonable suggestion that OPN-a should be continuously explore in the context of bone metastasis given the intimate link between OPN-a and the bone metastatic models using here. We are currently identifying a large clinical cohort of lung cancer with bone metastasis information and hopefully to further establish this link in a clinical setting.

In conclusion, our data suggest that higher levels of OPN-a are associated with poorer clinical outcome of lung cancer patients. The *in vitro* cell function results indicate that OPN-a acts as an oncogene in lung cancer cells. Moreover, $\alpha v \beta 3$ integrin is involved in the OPN-a-mediated malignant behaviors of lung cancer cells. The interaction of OPN and

αvβ3 integrin may play a vital role in adhesion of lung cancer cells to the bone matrix, which is probably one of the early steps of the bone metastasis in lung cancer. Therefore, OPN-a may have potential clinical applications as a biomarker for prognosis and diagnosis, and a novel therapeutic target in lung cancer.

Acknowledgements

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