Original Paper

Osteopontin Knockdown Inhibits $\alpha_v\beta_3$ Integrin-Induced Cell Migration and Invasion and Promotes Apoptosis of Breast Cancer Cells by Inducing Autophagy and Inactivating the PI3K/Akt/mTOR Pathway

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Key Words

Osteopontin • Integrin $\alpha_v\beta_3$ • Cell migration • Cell invasion • Apoptosis • RNA interference • Phosphoinositide 3-kinase • Akt • mTOR

Abstract

Background: Osteopontin (OPN) is associated with tumor formation, progression and metastasis, and increased OPN levels have been associated with poor survival in breast cancer. We investigated the mechanisms responsible for OPN activity, and the relationships between OPN expression and clinical parameters in breast cancer. **Methods:** OPN mRNA and protein levels were compared in malignant and benign breast tumors by polymerase chain reaction (PCR) and immunohistochemistry, respectively, and levels in breast cancer cells were determined by PCR and western blotting. The effects of lentiviral-mediated knockdown of OPN on OPN and $\alpha_v\beta_3$ integrin expression, cell invasion and migration, autophagy and apoptosis were analyzed in MDA-MB-231 cells. **Results:** OPN expression increased with aggressiveness of breast cancer phenotype. OPN knockdown inhibited $\alpha_v\beta_3$ integrin expression in MDA-MB-231 cells, with subsequent inhibition of cell migration and invasion. Knockdown also inhibited the PI3K/Akt/mTOR pathway, promoted expression of the autophagy-related gene products LC3 and Beclin 1, and increased apoptosis. OPN expression was positively associated with tumor grade and lymph node metastasis. **Conclusion:** These results suggest that knockdown of OPN may inhibit breast cancer metastasis by regulating $\alpha_v\beta_3$ integrin expression and inducing autophagy and subsequent inhibition of PI3K/Akt/mTOR signaling, thus providing further insights into the complex mechanisms regulating tumor growth and metastasis.

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer-related mortality in women worldwide [1]. A better understanding of the molecular basis of breast cancer is thus essential to improve the prognosis of this disease. Osteopontin (OPN) is a matricellular protein that binds to cell surface receptors, including integrins and CD44, resulting in a wide range of effects [2]. OPN plays important physiological roles in bone remodeling [3], the immune response [4] and inflammation [5]. OPN is also well-known as a tumor-associated protein, and elevated OPN has been detected in a wide range of human tumors, such as breast [6, 7], prostate [8, 9], liver [10], cervical [11] and lung cancers [12, 13]. Overexpression of OPN has also been detected in tumor cells and tumor-infiltrating inflammatory cells in breast cancer [14], and elevated serum levels of OPN in breast carcinoma have been associated with reduced survival [15].

The roles of OPN in cancer are mediated by binding to a range of cell surface receptors, growth factor/receptor pathways, and proteases [16]. OPN has been shown to interact with several integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_8\beta_1$ and $\alpha_5\beta_1$ [17, 18], via the Arg-Gly-Asp (RGD) sequence, and with $\alpha_9\beta_1$, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ [19, 20] via the serine-valine-valine-tyrosine-glutamate-leucine-arginine (SVVYGLR) sequence. The interaction of OPN with cell surface receptors can in turn activate various signal transduction pathways, resulting in changes in gene expression and leading to alterations in cell behavior, including migration, invasion and apoptosis [21, 22]. However, although OPN is known to be associated with cell migration and invasion in breast cancer, the precise mechanisms responsible for this role remain unclear.

The phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway is well known to be involved in the control of migration, invasion and apoptosis in cancer [23, 24], and PI3K/Akt signaling has been shown to be activated by OPN [25]. Importantly, Akt signaling has also been shown to inhibit autophagy, and thus promote tumorigenesis [26]. However, the coordinated involvement of these factors in breast cancer has not yet been demonstrated.

In this study, we therefore analyzed the relationships between OPN and autophagy-related-gene expression in clinical benign tumor and breast tumor samples *in vivo*. We also compared the gene and protein expression levels of OPN in breast cancer cells *in vitro*, and investigated the effects of OPN knockdown on $\alpha_v\beta_3$, LC3 I/II and Beclin 1 expression. We further examined the effects of OPN knockdown on cell migration, invasion, apoptosis, and the molecular pathways involved in these processes. The results of this study will help to clarify the complex mechanisms whereby OPN may promote breast cancer, and thus identify potential targets for future drug development.

Materials and Methods

Patients and tissue samples

A total of 60 invasive ductal carcinoma breast tissue samples and 11 benign breast tumor tissue samples were obtained from patients who underwent surgical treatment at the Center Hospital of Nanyang City from May 2012 to March 2013, after obtaining written consent. All the patients were women, with a mean age of 51 years (range 34–77 years). None of the patients had received therapy before surgery. All the tissues were staged according to the American Joint Committee on Cancer (AJCC) breast cancer TNM staging system: stage I, n = 13; stage II, n = 21; and stage III, n = 26. Tissue samples were fixed in 10% formalin or stored in liquid nitrogen for subsequent experiments. OPN expression was analyzed in relation to patient age, clinical stage, tumor size, axillary lymph node status, and expression levels of estrogen receptor (ER), progesterone receptor (PR) and c-erb-B2.

Immunohistochemistry

Histological sections were cut and stained by immunohistochemistry (IHC), as described previously [27]. Antibodies to OPN, ER, PR, and c-erb-B2 were obtained from Abcam (Cambridge, MA, USA). Bound complexes were visualized with 3,3-diaminobenzidine and 0.003% H_2O_2 and nuclei were counterstained

Table 1. Primers used in real-time PCR

Target gene	Primer sequence	Product length
OPN	Forward: 5'-TCCAACGAAAGCCATGACCA-3'	226 bp
	Reverse: 5'-CTGTGGGGACAAGTGGAGTG-3'	
Integrin α_v	Forward: 5'-GAACCCCTTCCTCGGAAGCTC-3'	180 bp
	Reverse: 5'-CATCCCGAAGATAGGCGACC-3'	
Integrin β_3	Forward: 5'-GAACGGGACTTTTGAGTGTGG-3'	193 bp
	Reverse: 5'-CTTGCCGAAGTCGCTGCTAT-3'	
LC3 II	Forward: 5'-GAGAAGCAGCTTCCTGTTCTGG-3'	138 bp
	Reverse: 5'-GTGTCCGTTACCAACAGGAAG-3'	
Beclin 1	Forward: 5'-GGCTGAGAGACTGGATCAGG-3'	127 bp
	Reverse: 5'-CTGCGTCTGGGCATAACG-3'	
GAPDH	Forward: 5'-CACCATCTTCCAGGAGCGAG-3'	154 bp
	Reverse: 5'-AGAGGGGGCAGAGATGATGA-3'	

blue with Mayers' hemalum. The degree of immunostaining in the formalin-fixed, paraffin-embedded sections was evaluated independently by two observers, using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan), based on the observation of two sections per specimen and 10 fields per section, at a magnification of $\times 200$. Sections were scored according to the percentage of stained cells: <1%, negative (-); 1–5%, borderline ($\pm$); 5–25%, intermediate (+); 25–50%, moderate (++); 50–75%, strong (+++); 75–100%, very strong (++++). Cells were then classified as either negatively stained (-/ $\pm$) or positively stained (+/++/+++ /++++).

Cell cultures

Human breast cancer cell lines (MCF-7, MDA-MB-435S and MDA-MB-231) and spontaneously immortalized normal mammary epithelial cells (MCF-10A) were obtained from the American Type Culture Collection, and cultured as described previously [28]. Briefly, breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma). MCF-10A cells were cultured in mammary epithelium basal medium plus mammary epithelium growth medium (Invitrogen, Carlsbad, CA, USA). All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Quantitative real-time polymerase chain reaction analysis

RNA extraction, reverse transcription (RT) and quantitative real-time-polymerase chain reaction (QPCR) were performed essentially as described previously [29]. Briefly, total RNA was isolated from cells or tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RT was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Foster City, CA, USA). QPCR was performed using an ABI prism 7900 sequence detection system and SYBR green Rox Master Mix (Qiagen, Hilden, Germany). The primers used for QPCR are shown in Table 1. Fluorescent QPCR amplifications were performed under the following conditions: 95°C for 10 min; 40 cycles at 95°C for 15 s and 60°C for 45 s. Each sample was amplified in triplicate. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Products were quantified using the 2^{- $\Delta\Delta C_t$} method and expression was shown relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting

Total protein of cells or tissues was extracted using RIPA Extraction Reagents (Pierce, Rockford, IL, USA), as described previously [30]. The lysate protein concentration was estimated using a BCA protein assay kit (Pierce). Equal amounts of protein (20 μ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Piscataway, NJ, USA). The membrane was blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, then washed four times in TBST and incubated with the primary antibodies overnight at 4°C. Primary antibodies were purchased from Abcam

and used at the following dilutions: anti-OPN (1:1000); anti-integrin α_v (1:500); anti-integrin β_3 (1:1000); anti-LC3I/II (1:100); anti-Bec1 1 (1:400); anti-PI3K p85 α (1:1000); anti-Akt (phospho S473) (1:200); anti-Akt (1:500); anti-mTOR (phospho S2448) (1:500); anti-mTOR (1:500); and anti- β -actin (1:1000). After washing three times in TBST, membranes were incubated with secondary antibodies (goat anti-rabbit IgG heavy and light chain/horseradish peroxidase, 1:2000, Abcam) for 1 h at room temperature. β -Actin was used as a loading control. Blots were developed using the enhanced chemiluminescence procedure and exposed on photographic film. Immunoreactive bands were quantified using Image J analysis software (National Institutes of Health, Bethesda, MD, USA).

RNA interference

Small interfering RNA (siRNA) was transfected into breast cancer cells using lentiviral vectors, prepared as described previously [31]. The OPN-targeting oligo sequence was 5'-ACGAGTCAGCTGGATGACC-3' and the non-homologous control sequence was 5'-ATTGCGTTCGCAGTAATCT-3'. The siRNA sequences were designed and synthesized by Ambion (Austin, TX, USA). pLVTHM, pCMV and pMD2G plasmids were purchased from Invitrogen. siRNA sequences were subcloned into the MluI/ClaI sites of the pLVTHM vector. Lentiviral vectors were produced by transient transfection of 293T cells. Titers of lenti-OPN-siRNA vectors were calculated to be around 5×10^8 transducing units/ml. MDA-MB-231 cells were subsequently infected with control siRNA (con-siRNA) and OPN-siRNA lentiviruses.

Cell migration

The mobility of MDA-MB-231 cells was assessed by scratch wound assays, as described previously [32]. Cells infected with OPN-siRNA, con-siRNA, and mock-infected cells were grown to 80% confluence in 6-well plates at 37°C in a 5% CO₂ incubator. A wound was created by scratching the cells with a sterile 200 μ l pipette tip. The cells were then washed twice with phosphate-buffered saline to remove floating cells and incubated for 24 h. Cell migration into the wound surface was determined under an inverted microscope (LSM 510, Carl Zeiss, Oberkochen, Germany). Cells that migrated across the black lines were counted in five random fields in triplicate experiments.

Cell invasion assay

The abilities of mock, con-siRNA, and OPN-siRNA-infected MDA-MB-231 cells to pass through Matrigel-coated filters were measured in Transwell chamber invasion assays, as described previously [33]. Cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in serum-free DMEM medium. The cell suspension was added into each upper chamber of the transwell chamber. The bottom chamber contained medium with 10% FBS to serve as a chemoattractant. The chamber was incubated at 37°C in 5% CO₂ for 24 h. Non-invading cells on the upper surface of the filter were removed by scraping. Invaded cells on the lower surface were fixed in 10% formalin at room temperature for 30 min, stained with 0.05% crystal violet, and counted under a microscope.

Apoptosis detection

Apoptosis was detected by propidium iodide (PI) and annexin-V staining, as described previously [34]. MDA-MB-231 cells were seeded at 150,000 cells/35-mm cell culture dish, infected with lentiviral-mediated OPN-siRNA or con-siRNA, and grown for 48 h. The cells were then resuspended in binding buffer and stained with 5 μ l annexin V-fluorescein isothiocyanate and 5 μ l PI using an Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) and incubated for 15 min at room temperature in the dark. Flow cytometry was performed using a FACS 440 (BD Biosciences) with a 488 nm argon ion laser. All experiments were performed in triplicate. The lower left quadrant indicated viable cells, the lower right indicated early apoptotic cells, the upper right indicated late apoptotic cells, and the upper left quadrant indicated mechanically-injured cells.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 statistical software packages (SPSS Science, Chicago, IL, USA). All data were expressed as mean $\pm$ standard deviation based on triplicate experiments. Data for QPCR, western blot, and migration, invasion and apoptosis assays were analyzed using Student's *t*-tests. Relationships between OPN expression and clinicopathological characteristics was analyzed by χ^2

Fig. 1. OPN expression in clinical tumor samples. (A) Expression of OPN in benign tumor and breast cancer tissues determined by immunohistochemical staining. (B) OPN mRNA levels were notably increased in breast tumor tissues compared with benign tumor tissues. (C) OPN mRNA levels were significantly increased in breast cancer tissues from patients with axillary lymph node metastases. All values are given as the mean $\pm$ SD of three replicates. * $P < 0.05$, ** $P < 0.01$.

and Fisher's exact tests. Differences were considered to be statistically significant when the P value was < 0.05 .

Results

OPN expression in clinical tumor samples

We compared OPN expression in 60 invasive and 11 benign breast tumor samples by IHC and QPCR. OPN expression determined by IHC increased with increasing tumor stage (Fig. 1A), and OPN mRNA levels were significantly higher in invasive, compared with benign samples (Fig. 1B). Among the 60 breast cancer samples, 38 (63.3%) showed positive OPN staining by IHC and 22 (36.7%) showed weak or no staining. None of the benign samples were positive for OPN by IHC.

Positive OPN expression levels were significantly associated with increasing clinical stage, axillary lymph node metastasis and c-erb-B2 expression, but not with patient age, tumor size, or ER or PR expression (Table 2). OPN mRNA levels were significantly higher in tumor samples from patients with axillary lymph node metastases, compared with those without metastases (Fig. 1C).

Expression of autophagy-related markers in clinical tumor samples

mRNA expression levels of the autophagy-related markers LC3 II and Beclin 1 were both significantly higher in benign breast samples than in malignant breast tumors (Fig. 2A and B), as demonstrated by QPCR. The corresponding protein levels were also higher in benign compared with malignant samples, as demonstrated by western blotting (Fig. 2C).

Table 2. Patient characteristics and relationships between OPN expression and clinicopathological characteristics in breast cancer patients

Characteristic	Total (n=60)	OPN n (%)		χ^2 test P value	Fisher's exact test P value
		Negative	Positive		
Age (years)					
≥51	32	12 (37.5)	20 (62.5)	>0.05	>0.05
<51	28	10 (35.7)	18 (64.3)		
Clinical stage					
I	13	8 (61.5)	5 (38.5)	<0.001	<0.001
II	21	8 (38.1)	13 (61.9)		
III	26	6 (23.1)	20 (76.9)		
Tumor size					
≤2 cm	22	8 (36.4)	14 (63.6)	>0.05	>0.05
>2–≤5 cm	30	11 (36.7)	19 (63.3)		
>5 cm	8	3 (37.5)	5 (62.5)		
Axillary lymph nodes					
Metastasis	39	9 (23.1)	30 (76.9)	<0.001	<0.001
No metastasis	21	13 (61.9)	8 (38.1)		
ER expression					
Positive	27	10 (37.0)	17 (63.0)	>0.05	>0.05
Negative	33	12 (36.4)	21 (63.6)		
PR expression					
Positive	35	13 (37.1)	22 (62.9)	>0.05	>0.05
Negative	25	9 (36.0)	16 (64.0)		
c-erbB-2 expression					
Positive	33	10 (30.3)	23 (69.7)	<0.001	<0.001
Negative	27	12 (44.4)	15 (55.6)		

Fig. 2. Expression levels of LC3 I/II and Beclin 1 in clinical tumor samples. LC3 II (A) and Beclin 1 (B) mRNA levels in benign tumor and breast cancer tissues detected by QPCR. GAPDH was used as a control. (C) LC3 I/II and Beclin 1 protein levels were detected by western blot. β -Actin was used as a loading control for normalization. **P<0.01 vs benign tumor.

OPN expression in breast cancer cells

OPN mRNA and protein expression were significantly increased in breast cancer cells compared with normal breast cells, as demonstrated by PCR and western blotting, respectively. Both mRNA (Fig. 3A) and protein levels (Fig. 3B) increased with increasingly aggressive phenotype of breast cancer cells, in the order MCF-10A < MCF-7 < MDA-MB-435S < MDA-MB-231, though the differences between MDA-MB-435S and MDA-MB-231 cells were not significant.

Fig. 3. Expression level of OPN in MCF-10A, MCF-7, MDA-MB-435S and MDA-MB-231 cells. (A) OPN mRNA expression levels were normalized to GAPDH expression and depicted as fold-change relative to MCF-10A. (B) Representative results of protein expression analysis for OPN using western blot. β-Actin was used as a loading control. Quantification of protein levels was performed by normalization to β-Actin levels. All values are given as the mean ± SD of three replicates. *P<0.05, **P<0.01 vs MCF-10A.

Fig. 4. Validation of OPN knockdown. MDA-MB-231 cells were infected with con-siRNA or OPN-siRNA for 48 h. (A) OPN mRNA expression levels were detected by QPCR. GAPDH was used as a control. (B) OPN protein expression level were detected by western blot. β-Actin was used as a loading control for normalization. All values are given as the mean ± SD of three replicates. **P<0.01 vs mock. mock: without infected; con-siRNA: infected with lenti-con-siRNA; OPN-siRNA: infected with lenti-OPN-siRNA.

Validation of OPN knockdown

PCR and western blotting showed that OPN mRNA (Fig. 4A) and protein levels (Fig. 4B) were significantly reduced in OPN-siRNA-infected MDA-MB-231 breast cancer cells compared with mock-infected and con-siRNA-infected cells, confirming this as a suitable model for studying the effects of OPN knockdown. There were no significant differences in expression between mock- and con-siRNA-infected cells.

Effect of OPN knockdown on cell invasion and migration

MDA-MB-231 cell migration was significantly reduced by OPN knockdown (Fig. 5A), as determined by scratch assay. Similarly, cell invasion determined by Transwell migration was also significantly inhibited in OPN knockdown cells (Fig. 5B).

Effect of OPN knockdown on apoptosis

Apoptosis in MDA-MB-231 cells was greatly increased by OPN knockdown, represented by a significant increase in the percentage of annexin-V-positive cells demonstrated by flow cytometry (Fig. 6).

Effect of OPN knockdown on integrin, autophagy-related genes and PI3K/Akt/mTOR pathway

Expression levels of αV and β3 integrin subunits in MDA-MB-231 cells were significantly reduced by OPN knockdown at both mRNA (Fig. 7A) and protein levels (Fig. 7B), compared with mock-infected and con-siRNA-infected cells. There were no significant differences in expression between mock- and con-siRNA-infected cells. Protein expression levels of the autophagy-related genes LC3I/II and Beclin 1 were significantly increased by OPN knockdown

Fig. 5. Effect of OPN knockdown on cell invasion and migration. MDA-MB-231 cells were infected with con-siRNA or OPN-siRNA for 48 h. (A) Migration abilities of MDA-MB-231 cells were determined by scratch assay. (B) Invasion abilities of MDA-MB-231 cells were determined by Transwell assay. All values are given as the mean $\pm$ SD of three replicates. ** $P < 0.01$ vs mock.

Fig. 6. Effect of OPN knockdown on apoptosis. MDA-MB-231 cells were infected with con-siRNA or OPN-siRNA for 48 h. Cell apoptosis was determined by flow cytometric analysis with annexin-V/PI double-staining. ** $P < 0.01$ vs mock.

(Fig. 7C), as demonstrated by western blotting. PI3K, p-Akt and p-mTOR levels were also demonstrated by western blotting, and all significantly reduced in OPN-siRNA-infected MDA-MB-231 cells, compared with mock- and con-siRNA-infected cells (Fig. 7D).

Discussion

We demonstrated that OPN expression was increased in breast cancer, both *in vivo* and *in vitro*. OPN was positively associated with increasing stage and lymph node metastasis in clinical samples (Fig. 1), and with increasing aggressiveness in cancer cell lines (Fig. 3). The autophagy-related genes LC3 and Beclin 1 were also down-regulated in breast cancer tissues (Fig. 2). In terms of its cellular effects, OPN increased cell migration and invasion, and reduced apoptosis in highly aggressive MDA-MB-231 breast cancer cells, as confirmed by the reversal of these parameters in OPN knockdown cells (Figs. 5 and 6). Furthermore, OPN knockdown was associated with reduced expression of the integrin $\alpha_v\beta_3$ receptor (Fig.

Fig. 7. Effect of OPN knockdown on integrin and autophagy-related-gene expression. MDA-MB-231 cells were infected with con-siRNA or OPN-siRNA for 48 h. (A) Integrin α_v and β_3 mRNA levels were assessed by QPCR, and normalized to GAPDH expression. (B) Integrin α_v and β_3 , (C) LC3 I/II and Beclin 1, and (D) PI3K, p-Akt and p-mTOR protein expression levels were detected by western blotting. β -Actin expression was used as a loading control and for normalization. All values are given as the mean $\pm$ SD of three replicates. **P<0.01 vs mock.

7), which facilitates OPN binding to tumor cells, reduced activation of the PI3K/Akt/mTOR pathway (Fig. 7), which mediates cell migration and invasion, and increased expression of LC3 and Beclin 1, thus providing a mechanistic explanation for the role of OPN in breast cancer.

Despite progress in the understanding of breast cancer and the development of new therapies, breast cancer remains the second leading cause of cancer-related deaths in women worldwide, mostly due to metastatic disease [35]. A better understanding of the factors associated with breast cancer metastasis may therefore help in the development of more effective treatments.

OPN has been shown to be strongly associated with tumor progression [7, 9, 11, 36] and has been shown to cause metastasis in a rat breast cancer model [37]. In addition, high circulating levels of OPN have been reported in patients with metastatic breast cancer [27]. Accordingly, the current study confirmed that OPN expression increased with increasing aggressiveness of breast cancer cell lines. In addition, OPN expression was positively associated with c-erb-B2, which is known to be over-expressed in 20–30% of breast cancers, and to correlate with metastasis and poor prognosis [27, 38]. OPN expression also increased with increasing cancer stage, and was higher in breast cancer patients with lymph node metastasis.

Cell migration and invasion are essential characteristics of tumor metastasis. The results of this study showed that OPN knockdown reduced the migration and invasion of aggressive breast cancer cells, in accordance with previous studies that demonstrated a relationship between OPN expression and increased cell migration and adhesion [39–41]. OPN mediates

its effects by binding to a range of integrin receptors, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_9\beta_1$, of which $\alpha_v\beta_3$ is among the best characterized. $\alpha_v\beta_3$ was recently shown to be involved in the effects of OPN in the highly metastatic breast cancer cell line, MDA-MB-435, in contrast to two non-metastatic breast cancer cell lines, which used $\alpha_v\beta_1$ and $\alpha_v\beta_5$ integrins [42]. Liaw et al. [43] demonstrated that the adhesive and migratory effects of OPN in smooth muscle cells required the presence of $\alpha_v\beta_3$ integrin, and that OPN and integrin mRNA and protein levels were coordinately elevated. In the present study, OPN knockdown was associated with inhibition of $\alpha_v\beta_3$ integrin expression in MDA-MB-231 cells, suggesting that OPN may control the migration, invasion and survival of breast cancer cells not only through binding to be $\alpha_v\beta_3$ integrin, but also by regulating its expression levels.

PI3K regulates the cell cycle via phosphorylation of Akt and mTOR. This signaling pathway thus plays an important role in apoptosis and cancer, and has been shown to be hyperactive in over 70% of breast cancers [23]. Zheng et al. [44] demonstrated that $\alpha_v\beta_3$ binding to OPN activated the PI3K/Akt pathway, and that $\alpha_v\beta_3$ -mediated migration of prostate cancer cells required activation of this pathway. Akt activation has also been shown to be associated with migration and invasion of breast cancer cells [45] and to promote metastasis by reducing intercellular adhesion [46].

Autophagy is an important process that protects against tumorigenesis. Beclin 1 is essential for autophagy, but is phosphorylated and inactivated by Akt [26]. Given that the PI3K/Akt/mTOR pathway was inhibited by OPN knockdown in the current study, this further supports the importance of autophagy, as well as cancer cell migration and invasion, in OPN-mediated breast cancer and metastasis.

The current study found that 63.3% of clinical breast cancer samples were positive for OPN by IHC, and that expression levels were positively associated with increasing cancer stage and the presence of axillary lymph node metastasis. Rudland et al. [27] examined the prognostic significance of OPN in breast cancer and found that 66% of carcinoma samples were OPN-positive, while other studies [47, 48] have reported even higher levels.

This study had some limitations. Although OPN is known to interact with several integrin receptors, only integrin $\alpha_v\beta_3$ was examined in this study. In addition, further studies should be performed to assess the relationships between OPN expression and other clinical factors, such as p53 expression and survival.

Although previous studies have demonstrated the involvement of OPN and integrin $\alpha_v\beta_3$ binding in breast carcinogenesis, the current study not only confirmed the importance of OPN in promoting breast cancer progression and metastasis, but also provides detailed evidence for the mechanisms involved. OPN increases cell migration and invasion and inhibits apoptosis and autophagy by up-regulating and binding to integrin $\alpha_v\beta_3$, thus activating the PI3K/Akt/mTOR pathway. These results further our understanding of the complex mechanisms responsible for breast cancer metastasis, and highlight the existence of several potential new targets for the treatment of patients with metastatic breast cancer.

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References

- 1 Desantis C, Ma J, Bryan L, Jemal A: Breast cancer statistics, 2013. *CA Cancer J Clin* 2013.
- 2 Ailane S, Long P, Jenner P, Rose S: Expression of integrin and cd44 receptors recognising osteopontin in the normal and lps-lesioned rat substantia nigra. *Eur J Neurosci* 2013;38:2468-2476.
- 3 Denhardt DT, Noda M: Osteopontin expression and function: Role in bone remodeling. *J Cell Biochem Suppl* 1998;30-31:92-102.
- 4 Wang KX, Denhardt DT: Osteopontin: Role in immune regulation and stress responses. *Cytokine Growth Factor Rev* 2008;19:333-345.

- 5 Cho HJ, Kim HS: Osteopontin: A multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr Atheroscler Rep* 2009;11:206-213.
- 6 Anborgh PH, Mutrie JC, Tuck AB, Chambers AF: Pre- and post-translational regulation of osteopontin in cancer. *J Cell Commun Signal* 2011;5:111-122.
- 7 Chakraborty G, Jain S, Kundu GC: Osteopontin promotes vascular endothelial growth factor-dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms. *Cancer Res* 2008;68:152-161.
- 8 Forootan SS, Foster CS, Aachi VR, Adamson J, Smith PH, Lin K, Ke Y: Prognostic significance of osteopontin expression in human prostate cancer. *Int J Cancer* 2006;118:2255-2261.
- 9 Jain S, Chakraborty G, Kundu GC: The crucial role of cyclooxygenase-2 in osteopontin-induced protein kinase c alpha/c-src/ikappab kinase alpha/beta-dependent prostate tumor progression and angiogenesis. *Cancer Res* 2006;66:6638-6648.
- 10 Chen RX, Xia YH, Xue TC, Zhang H, Ye SL: Down-regulation of osteopontin inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving mmp-2 and upa. *Oncol Rep* 2011;25:803-808.
- 11 Kumar V, Behera R, Lohite K, Karnik S, Kundu GC: P38 kinase is crucial for osteopontin-induced furin expression that supports cervical cancer progression. *Cancer Res* 2010;70:10381-10391.
- 12 Zhao B, Sun T, Meng F, Qu A, Li C, Shen H, Jin Y, Li W: Osteopontin as a potential biomarker of proliferation and invasiveness for lung cancer. *J Cancer Res Clin Oncol* 2011;137:1061-1070.
- 13 Hu Z, Lin D, Yuan J, Xiao T, Zhang H, Sun W, Han N, Ma Y, Di X, Gao M, Ma J, Zhang J, Cheng S, Gao Y: Overexpression of osteopontin is associated with more aggressive phenotypes in human non-small cell lung cancer. *Clin Cancer Res* 2005;11:4646-4652.
- 14 Behera R, Kumar V, Lohite K, Karnik S, Kundu GC: Activation of jak2/stat3 signaling by osteopontin promotes tumor growth in human breast cancer cells. *Carcinogenesis* 2010;31:192-200.
- 15 Weber GF, Lett GS, Haubein NC: Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer* 2010;103:861-869.
- 16 Christensen B, Klaning E, Nielsen MS, Andersen MH, Sorensen ES: C-terminal modification of osteopontin inhibits interaction with the alphavbeta3-integrin. *J Biol Chem* 2012;287:3788-3797.
- 17 Rittling SR: Osteopontin in macrophage function. *Expert Rev Mol Med* 2011;13:e15.
- 18 Yokosaki Y, Tanaka K, Higashikawa F, Yamashita K, Eboshida A: Distinct structural requirements for binding of the integrins alphavbeta6, alphavbeta3, alphavbeta5, alpha5beta1 and alpha9beta1 to osteopontin. *Matrix Biol* 2005;24:418-427.
- 19 Ito K, Kon S, Nakayama Y, Kurotaki D, Saito Y, Kanayama M, Kimura C, Diao H, Morimoto J, Matsui Y, Uede T: The differential amino acid requirement within osteopontin in alpha4 and alpha9 integrin-mediated cell binding and migration. *Matrix Biol* 2009;28:11-19.
- 20 Lund SA, Wilson CL, Raines EW, Tang J, Giachelli CM, Scatena M: Osteopontin mediates macrophage chemotaxis via alpha4 and alpha9 integrins and survival via the alpha4 integrin. *J Cell Biochem* 2013;114:1194-1202.
- 21 Beausoleil MS, Schulze EB, Goodale D, Postenka CO, Allan AL: Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorigenicity, and metastasis. *BMC Cancer* 2011;11:25.
- 22 Stemberger C, Matusan-Ilijas K, Avirovic M, Bulat-Kardum L, Ivancic A, Jonjic N, Lucin K: Osteopontin is associated with decreased apoptosis and alphav integrin expression in lung adenocarcinoma. *Acta Histochem* 2014;116:222-229.
- 23 Grunt TW, Mariani GL: Novel approaches for molecular targeted therapy of breast cancer: Interfering with pi3k/akt/mtor signaling. *Curr Cancer Drug Targets* 2013;13:188-204.
- 24 Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M: Pi3k/akt signalling pathway and cancer. *Cancer Treat Rev* 2004;30:193-204.
- 25 Lin YH, Yang-Yen HF: The osteopontin-cd44 survival signal involves activation of the phosphatidylinositol 3-kinase/akt signaling pathway. *J Biol Chem* 2001;276:46024-46030.
- 26 Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J, Levine B: Akt-mediated regulation of autophagy and tumorigenesis through beclin 1 phosphorylation. *Science* 2012;338:956-959.
- 27 Rudland PS, Platt-Higgins A, El-Tanani M, De Silva Rudland S, Barraclough R, Winstanley JH, Howitt R, West CR: Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. *Cancer Res* 2002;62:3417-3427.

- 28 Potier M, Joulin V, Roger S, Besson P, Jourdan ML, Leguennec JY, Bougnoux P, Vandier C: Identification of sk3 channel as a new mediator of breast cancer cell migration. *Mol Cancer Ther* 2006;5:2946-2953.
- 29 Zhang X, Min J, Wang Y, Li Y, Li H, Liu Q, Liang X, Mu P: Rabex-5 plays an oncogenic role in breast cancer by activating mmp-9 pathway. *J Exp Clin Cancer Res* 2013;32:52.
- 30 Tsai PC, Chu CL, Chiu CC, Chang LS, Lin SR: Inhibition of src activation with cardiotoxin iii blocks migration and invasion of mda-mb-231 cells. *Toxicon* 2013;74:56-67.
- 31 Sun H, Yang F, Chu W, Zhao H, McMahon C, Li C: Lentiviral-mediated rnaï knockdown of cbfa1 gene inhibits endochondral ossification of antler stem cells in micromass culture. *PLoS One* 2012;7:e47367.
- 32 Pai HC, Chang LH, Peng CY, Chang YL, Chen CC, Shen CC, Teng CM, Pan SL: Moscatilin inhibits migration and metastasis of human breast cancer mda-mb-231 cells through inhibition of akt and twist signaling pathway. *J Mol Med (Berl)* 2013;91:347-356.
- 33 Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY: MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res* 2008;18:350-359.
- 34 Kim JB, Yu JH, Ko E, Lee KW, Song AK, Park SY, Shin I, Han W, Noh DY: The alkaloid berberine inhibits the growth of anoikis-resistant mcf-7 and mda-mb-231 breast cancer cell lines by inducing cell cycle arrest. *Phytomedicine* 2010;17:436-440.
- 35 Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J, Cristofanilli M, Dontu G, Bidaut L, Valero V, Hortobagyi GN, Yu D: Breast cancer metastasis: Challenges and opportunities. *Cancer Res* 2009;69:4951-4953.
- 36 Behrend EI, Craig AM, Wilson SM, Denhardt DT, Chambers AF: Reduced malignancy of ras-transformed nih 3t3 cells expressing antisense osteopontin rna. *Cancer Res* 1994;54:832-837.
- 37 Chen H, Ke Y, Oates AJ, Barraclough R, Rudland PS: Isolation of and effector for metastasis-inducing dnas from a human metastatic carcinoma cell line. *Oncogene* 1997;14:1581-1588.
- 38 Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, Borresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001;98:10869-10874.
- 39 Allan AL, George R, Vantighem SA, Lee MW, Hodgson NC, Engel CJ, Holliday RL, Girvan DP, Scott LA, Postenka CO, Al-Katib W, Stitt LW, Uede T, Chambers AF, Tuck AB: Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. *Am J Pathol* 2006;169:233-246.
- 40 Shevde LA, Samant RS, Paik JC, Metge BJ, Chambers AF, Casey G, Frost AR, Welch DR: Osteopontin knockdown suppresses tumorigenicity of human metastatic breast carcinoma, mda-mb-435. *Clin Exp Metastasis* 2006;23:123-133.
- 41 Chakraborty G, Jain S, Patil TV, Kundu GC: Down-regulation of osteopontin attenuates breast tumour progression in vivo. *J Cell Mol Med* 2008;12:2305-2318.
- 42 Tuck AB, Elliott BE, Hota C, Tremblay E, Chambers AF: Osteopontin-induced, integrin-dependent migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor (met). *J Cell Biochem* 2000;78:465-475.
- 43 Liaw L, Lindner V, Schwartz SM, Chambers AF, Giachelli CM: Osteopontin and beta 3 integrin are coordinately expressed in regenerating endothelium in vivo and stimulate arg-gly-asp-dependent endothelial migration in vitro. *Circ Res* 1995;77:665-672.
- 44 Zheng DQ, Woodard AS, Tallini G, Languino LR: Substrate specificity of alpha(v)beta(3) integrin-mediated cell migration and phosphatidylinositol 3-kinase/akt pathway activation. *J Biol Chem* 2000;275:24565-24574.
- 45 Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH: Twist transcriptionally up-regulates akt2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res* 2007;67:1979-1987.
- 46 Qiao M, Sheng S, Pardee AB: Metastasis and akt activation. *Cell Cycle* 2008;7:2991-2996.
- 47 Gillespie MT, Thomas RJ, Pu ZY, Zhou H, Martin TJ, Findlay DM: Calcitonin receptors, bone sialoprotein and osteopontin are expressed in primary breast cancers. *Int J Cancer* 1997;73:812-815.
- 48 Tuck AB, O'Malley FP, Singhal H, Harris JF, Tonkin KS, Kerkvliet N, Saad Z, Doig GS, Chambers AF: Osteopontin expression in a group of lymph node negative breast cancer patients. *Int J Cancer* 1998;79:502-508.