RESEARCH ARTICLE

Expression and Clinical Significance of Osteopontin in Calcified Breast Tissue

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Abstract

Osteopontin (OPN) is an integrin-binding protein, believed to be involved in a variety of physiological cellular functions. The physiology of OPN is best documented in the bone where this secreted adhesive glycoprotein appears to be involved in osteoblast differentiation and bone formation. In our study, we used semi-quantitative RT-PCR of osteopontin in calcification tissue of breast to detect breast cancer metastasis. The obtained data indicate that the expression of osteopontin is related to calcification tissue of breast, and possibly with the incidence of breast cancer. The expression strength of OPN by RT-PCR detection was related to the degree of malignancy of breast lesions, suggesting a close relationship between OPN and breast calcification tissue. The results revealed that expression of OPN mRNA is related to calcification of breast cancer tissue and to the development of breast cancer. Determination of OPN mRNA expression can be expected to be a guide to clinical therapy and prediction of the prognosis of breast cancer patients.

Keywords: Osteopontin; bone - RT-PCR detection - breast cancer

Asian Pacific J Cancer Prev, **13 (10)**, 5219-5223

Introduction

Osteopontin (OPN), also known as bone sialoprotein I (BSP-1 or BNSP), early T-lymphocyte activation (ETA-1), secreted phosphoprotein 1 (SPP1), and Rickettsia resistance (Ric)(Reinholt et al., 1990; Wang and Denhardt, 2008), is a human gene product, which is also conserved in other species. Osteopontin is a sibling glycoprotein that was first identified in 1986 in osteoblasts. OPN is a highly negatively charged, extracellular matrix protein that lacks an extensive secondary structure (Vetrone et al., 2009). The OPN gene is composed of 7 exons, 6 of which contain coding sequence (Kiefer et al., 1989; Young et al., 1990). OPN was first implicated in malignancy by in vitro studies detecting increased levels of OPN expression after cell transformation (Senger and Perruzzi, 1985; Senger et al., 1989) and from the observation that tumor cells with high metastatic potential had increased OPN expression. As discussed, OPN binds to several integrin receptors including $\alpha4\beta1$, $\alpha9\beta1$, and $\alpha9\beta4$ expressed by leukocytes. These receptors have been well-established to function in cell adhesion, migration, and survival in these cells. Therefore, recent research efforts have focused on the role of OPN in mediating such responses (Uaesoontrachoon et al., 2008). OPN gene transcription in bone tissue is regulated by the interaction between transactivating factors and vitamin D3 responsive elements (Staal et al., 1996).

In our study, we used semi-quantitative RT-PCR of osteopontin in calcification tissue of breast to detect breast cancer metastasis. The result showed that the expression of OPN m RNA in malignant calcified tissue and lymph node metastases was not significantly different; the expression in benign calcified tissue was lower than that of the first two; the expression of adjacent tissues was tiny and that of normal tissue was little. These data suggest that the expression of osteopontin is related to calcification tissue of breast, and possibly with the incidence of breast cancer. It is a viable means to detect the breast cancer with the expression of osteopontin and helpful to guide clinical treatment.

Materials and Methods

Materials

KOD FX polymerase, all PCR reagents and enzymes for molecular cloning were purchased from Takara. Trizol were purchased from Invitrogen. The E.coli JM109 and BL21 used for cloning and protein expression were both held in our laboratory collection. His-select Ni-Chelating affinity gel column used for protein purification was purchased from Novagen.

Academic samples were from Changzheng Hospital, Second Military Medical University, and the patients are 128 cases of female breast disease from November 2002 to July 2003 (Table 1). All samples were divided into five groups: Breast cancer group, 41; Paraneoplastic group, 41; Lymphoid transformation group, 9; Benign breast

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Table 1. General Information About the Lesions

Lesion characteristics	Number of cases	Pathological nature Benign	Malignant	Age <45	≥45
Calcification lesions	106	70	36	70	36
Non-calcification lesions	22	17	5	10	12
Total	128	87	41	80	48

lesions, 87; Normal mammary gland, 87 according to Lesion characteristics.

RNA isolation and cDNA synthesis of osteopontin

Total RNA of samples was extracted using the Trizol method, DNA was removed from RNA by scavenger of DNA. The extraction of total RNA was identified and isolated by agarose gel electrophoresis to check the integrity.

First-strand cDNA synthesis was carried out with a PrimeScript RT reagent Kit (Takara, Japan) Perfect Real Time using Oligo (dT) primers and total RNA as the template. Reaction condition was as follows: 65 °C 5 min, then chilling on ice 5 min, 37 °C 15 min, 42 °C 15 min, 50 °C 15 min, 85 °C 5 s. The PCR specific primers for osteopontin were designed according to osteopontin sequence published in GenBank. The two specific primers used were as follow: osteopontin-F: ATGCCTACCTCTGCTCCCCTCGTC;osteopont in-R:TTAGACGCTGACCTCGACACGACCC. PCR amplification was performed in 20μL reaction mixture containing 1.6 μL of cDNA, 10 μL of 2×PCR buffer, 2 μL of 2 mM dNTPs, 0.4 μL each of 10 μM forward and reverse primers, 0.4 μL of KOD FX. The reaction conditions comprised an initial denaturation of 2min at 94°C, followed by 42 cycles of 98 °C for 10 s, 68 °C for 30 s and 68 °C for 2 min, with a final extension at 68 °C for 10 min. After checking the product on an analytical 1% agarose gel, the 1.9 kb band was then extracted from the gel by Gel Extraction Kit (solarbio).

Cloning and sequencing of the PCR products

The purified PCR product from five samples was then ligated into vector pIJ2925 at 16 °C overnight. The pIJ2925-OPN vector was transformed into competent JM109 E. coli cells and plated on LB-ampicillin/IPTG/X-gal plates followed by incubation at 37 °C for about 20 h. The resulting colonies were screened by colony PCR using the gene specific oligonucleotide primers. The plasmid DNA was purified from overnight culture using the rapid plasmid DNA mini-prep kit (solarbio) and the presence of insert was verified by EcoR I and Pst I, Sac I, Xho I, Mun I restriction digestion of purified recombinant plasmid respectively.

Sub-cloning of OPN into expression vector pET28a$^+$

The OPN gene was amplified from pIJ2925-OPN vector using the gene specific forward and reverse primers: OPN-NdeI: GGGAATTCCATATGCCT ACCTCTGCTCCCCTCG; OPN-EcoRI:CGGAATTC TTAGACGCTGACCTCGACACGAC. The primers containing the restriction enzyme sites in order to generate the Nde I and EcoR I sites in the PCR product. After transformation, the OPN gene was cloned into vector pET28a$^+$ to get expression vector pET28a$^+$-OPN.

Expression and purification of OPN in E. coli

To purify the OPN protein, the large amount of pellet obtained as described above, was resuspended in 1×Ni-NTA Buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, 10% glycerin, pH 8.9). The cell suspension was sonicated as described above. One milliliters of the supernatant was applied onto a column filled with His-select Ni-Chelating affinity gel pre-conditioned with 1×Ni-NTA Buffer. The elution was performed with 5 mL of 1×bind buffer containing 50 mM imidazole and then 5 mL of 1×bind buffer containing 100 mM imidazole and then 5 mL of 1×bind buffer containing 300 mM imidazole and then 5 mL of 1×bind buffer containing 500 mM imidazole. The purity of recombinant OPN was analyzed on SDS-PAGE.

Semi-quantitative PCR analysis

The primers for semi-quantitative PCR analysis were 5'-AGCAACCGAAGTTTTCACTCC-3' and 5'-TAATACGACTCACTATAGGGG-3'. The primers for β-actin were 5'-TTCCAGCCTTCCTTCCTGGG-3' and 5'-TTG CGCTCA GGAGGAGCAAT -3'. PCR amplification was performed in 20 μL reaction mixture: dNTP (each 10 mmol /L) 1 μL; Buffer mixture 2 μL; Upstream primer (50 μm/L) 1 μL; Downstream primer (50 μm/L) 1 μL; cDNA 1 μL; Taq DNA polymerase 0.3 μL; Free water 14.7 μL. The reaction conditions comprised an initial denaturation of 2min at 94 °C, followed by 22 cycles of 94 °C for 10 s, 58 °C for 15 s and 72 °C for 15 s. The samples were checked on an analytical 1.8% agarose gel.

Statistical analysis

All results were expressed by x±s, and the different significance was test by t-test. P <0. 05 was statistically significant.

Results

Isolation and sequencing of OPN

The total RNA was extracted and was pure without genome DNA pollution. The full length of OPN cDNA was amplified from total RNA with RT-PCR using gene specific primers corresponding to the cDNA. The product of cDNA was cloned into pIJ2925 vector and then digested

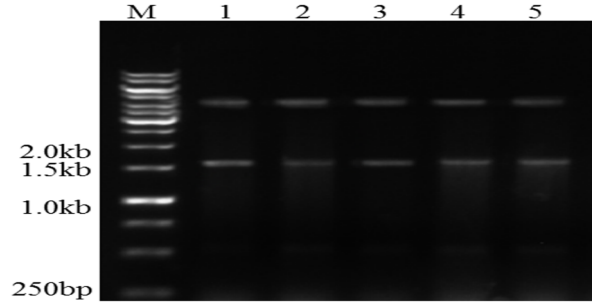

Figure 1. Digestion Verification of Plasmids M, 1kb Ladder; 1-5, Breast Cancer Group; Paraneoplastic Group; Lymphoid Transformation Group; Benign Breast Lesions; Normal Mammary Gland

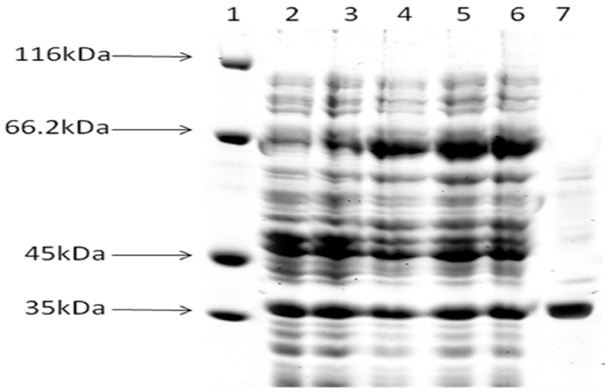

Figure 2. SDS-PAGE of Recombinant Protein1, Protein Molecular Weight Marker 2-6, Breast cancer Group; Paraneoplastic Group; Lymphoid Transformation Group; Benign Breast Lesions; Normal Mammary Gland; 7, The Protein Purified with Ni^{2+} Chelating Chromatography Column

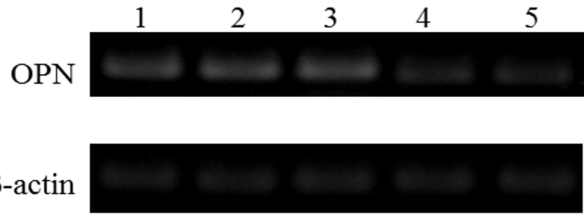

Figure 3. Semi-quantitative PCR Analysis1-5, Breast Cancer Group; Paraneoplastic Group; Lymphoid Transformation Group; Benign Breast Lesions; Normal Mammary Gland

with EcoR I and Pst I. After agarose gel electrophoresis, a 1.7kb DNA fragment was detected in 5 samples, which verified that the pIJ2925-OPN vectors were constructed successfully (Figure 1).

The cDNA insert was then sequenced. Nucleotide sequence corresponding 1716 bp was then compared with the nucleotide sequences deposited in the GenBank database using the BLAST program on the NCBI Blast server, and no mutations were found. The open reading frame of OST consisted of coding region of 1716 nucleotides and the deduced amino acid sequence have a calculated molecular weight of 33kDa.

Expression and purification of OPN

The isolated DNA was amplified with forward and reverse primers containing the restriction sites Nde I at 5' and EcoR I at 3' (see Materials and methods) for inserting them in the $pET28a^+$ vectors which has been previously used for the expression of proteins. The presence of OST cDNA fragment was verified by Nde I and EcoR I restriction digestion. The recombinant $pET28a^+$-acl1 plasmid, encoding OPN fused with the 34 amino acid extra N-terminal sequence MGSSHHHH HHSSGLVPRGSHMASMTGGQQMGRGS, containing a his6 tag, was used for heterologous expression and purification of the protein in the E. coli BL21 by Ni^{2+} chelate chromatograph column.

The cell lysates obtained were separated in the soluble and the insoluble fractions by centrifugation. The whole extracts, soluble and insoluble fractions of cell lysates were analyzed by SDS-PAGE. As shown in Figure 2, based

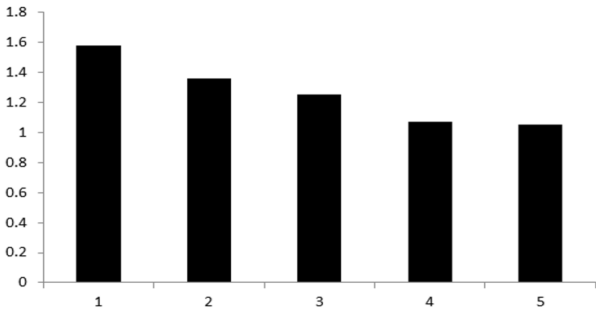

Figure 4. Ratio of OPN/β-actin 1-5, Breast Cancer Group; Paraneoplastic Group; Lymphoid Transformation Group; Benign Breast Lesions; Normal Mammary Gland

on the molecular weight of OPN protein, the expression whose value was about 33 kDa was notably visible on SDS-PAGE following. The purification of the recombinant OPN was achieved in a single chromatographic step on Ni^{2+} chelate chromatograph column with gradient elution of 100 mM imidazole, 300 mM imidazole and 500 mM imidazole. Fractions from the affinity column containing purified OPN were pooled together and analyzed by SDS-PAGE. SDS-PAGE analyses showed a single protein band of 37 kDa (+his tag peptide) that represent the molecular weight of the OPN fused to vector specific fusion histidine tag peptide.

Expression of OPN in different tissue

The expression of OPN m RNA in malignant calcified tissue and lymph node metastases was not significantly different; the expression in benign calcified tissue was lower than that of the first two; the expression of adjacent tissues was tiny and that of normal tissue was little (Figure 3).

The value of OPN/β-actin in breast cancer group, paraneoplastic group, lymphoid transformation group, benign breast lesions and normal mammary gland were1.58±0.10, 1.36±0.14, 1.25±0.13, 1.07±0.10 and 1.05±0.11 respectively (Figure 4).

The difference between calcified tissue and benign calcified tissue, lymph node metastases and benign calcified tissue, benign calcified tissue and paraneoplastic breast tissue, benign calcified tissue and normal breast tissue, the differences were significant (both $P < 0.05$). The difference between calcified tissue with lymph node cancer, adjacent milk Gland tissue and normal breast tissue was not statistically significant ($P > 0.05$).

Discussion

Osteopontin is a secreted phosphoprotein that has been implicated as an important mediator of tumor metastasis and has been investigated for use as a biomarker for advanced disease and as a potential therapeutic target in the regulation of cancer metastasis (Wai and Kuo, 2008). Osteopontin mediates the molecular mechanisms which determine metastatic spread, such as prevention of apoptosis, extracellular matrix proteolysis and remodeling, cell migration, evasion of host-immune cells and neovascularization (Wang and Denhardt, 2008; El-Abbadi et al., 2009). OPN regulates JAK2/STAT3

signaling cascade that ultimately controls apoptosis and breast tumor growth have been confirmed in vitro and in vivo experimental (Behera et al., 2010). OPN acts as a marker and the level of serum OPN in patients with breast, and is one of the most abundantly expressed genes in metastatic cancers (Rangaswami et al., 2006). Although tremendous efforts have been made in understanding the mechanism of OPN-regulated breast tumor progression, the signaling pathways and the cross talk among multiple pathways involved in regulating this process have not been fully characterized.

Breast milk is the physiological secretions, rich in the OPN. OPN in the presence of breast cancer may reflect the distortion of the normal process of lactation OPN production. The function of OPN in breast milk is not clear, but it inhibits hydroxyapatite crystal growth (Baht et al., 2008). Inhibition of calcification needs the phosphorylation of molecular (Mizobuchi et al., 2009), and milk contains a highly phosphorylated forms of osteopontin PPD (Saad et al., 2008). Some researchers (Baht et al., 2008) thought OP inhibited growth and deposition of hydroxyapatite crystals as the role of OPN in ductal carcinoma. The expression of OPN is related to breast calcification tissue, A ltundag et al (Catteau et al., 2012) found the calcification of breast cancer patients was related to the expression level of OPN with X-ray. In the current study, the correlation between the expression of osteopontin and the tumor size, stage, and grade was investigated separately. Expression of osteopontin increased with the tumor size and the clinical stage.

Osteopontin is not a specific bone protein and its mRNA has been demonstrated in skin, kidney (Baughman and Bader, 1977) and neuronal cells in the brain and inner ear (Nomura et al., 1988). Karen and his coworkers have further demonstrated expression of osteo-pontin mRNA in a variety of cell types in human bone and cartilage (Merry et al., 1993). Osteopontin expression was not dependent on attachment to bone, since high levels of osteopontin mRNA were detected in many osteoclasts distant from the bone surface, and the entire population of osteoclasts in the osteoclastoma tissue expressed osteopontin mRNA. The expression of osteopontin by osteoblasts was also dependent on maturation stage of the cell. Osteoblasts actively secreting osteoid expressed high levels of osteopontin (Bruder et al., 1997). In contrast, quiescent lining cells demonstrated very little expression. This expression profile is similar to many matrix components including osteonectin (Bianco et al., 1988).

The expression of bone matrix proteins such as osteo-pontin by osteoclasts highlights the potential of this cell type to synthesize products other than those directly involved with the dissolution of the matrix. The results indicated that the expression of OPN was related to calcification tissue of breast and deterioration of breast lesions. Semi-quantitative RT-PCR showed that the expression of OPN m RNA in malignant calcified tissue and lymph node metastases was not significantly different; the expression in benign calcified tissue was lower than that of the first two; the expression of adjacent tissues was tiny and that of normal tissue was little. The expression strength of OPN by RT-PCR detection was related to its degree of malignancy of breast lesions, suggesting that the close relationship between OPN and breast calcification tissue. Therefore, various concentrations of OPN might regulate these cellular functions depending on the degree of post-translational modification of the protein, the types of cell lines used and the sources from which it is obtained All data reveals that OPN can be used as one of the predictors of breast cancer from the perspective of molecular biology. OPN can be used as one of the predictors of breast cancer from the perspective of molecular biology. Expression of OPN mRNA is related to calcification of breast cancer tissue and to the development of breast cancer. Determination of OPN mRNA expression can be expected to be a guide to clinical therapy and prediction of the prognosis of breast cancer patients.

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