ANTICANCER RESEARCH 28: 4105-4110 (2008)

Osteopontin Expression Profiles Predict Pathological and Clinical Outcome in Breast Cancer

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Abstract. *Background: Osteopontin (OPN) overexpression in breast cancer has been associated with adverse pathological and clinical outcomes. In this study, the OPN expression profiles were examined in a cohort of breast cancer patients. Patients and Methods: RNA extraction and reverse transcription were performed on breast carcinomas (n=127) and normal tissues (n=33). Transcript levels were determined using real-time PCR. Results: The OPN-a levels decreased with increasing TNM stage and worse clinical outcome. The OPN-b levels increased with tumour grade and Nottingham Prognostic Index (NPI) stage, were higher in patients who died of breast cancer than in those who were disease-free after 10 years and predicted disease-free survival. The OPN-c expression was associated with tumour grade and poor prognosis. Furthermore, the expression levels predicted local recurrence, disease-free survival and bone metastases. Conclusion: OPN expression profiles are significantly associated with tumour grade, stage and patient prognosis in breast cancer. OPN-c is likely to be of particular utility as a prognostic marker and should be included in future validation studies.*

Osteopontin (OPN) is a phosphorylated glycoprotein secreted by several cell types, including those involved in bone turnover and cells of the immune system. Diverse physiological and pathological roles have been attributed to OPN (1). Three alternative splice variants of the *OPN* gene have been described: *OPN-a*, *OPN-b* and *OPN-c*. Alternative splicing occurs in a region of the molecule that is upstream of the central integrin binding domain and the *C*-terminal CD44-binding domain (2, 3). Whilst some studies have examined individual splice variants of the *OPN* gene, others have considered *OPN* as a single entity. OPN has been shown to be overexpressed in a variety of carcinomas, including breast, lung, colorectal, stomach, ovarian and melanoma. OPN expression levels are correlated with the stage of disease, particularly in breast cancer, and elevated blood levels have been identified in patients with metastatsis (4). Associations with poor prognosis and reduced survival appear to be independent of nodal metastasis and conventional prognostic indicators (5). Elevated OPN levels within the plasma are also associated with increased tumour burden and worse prognosis. Furthermore, following therapy, changes over time have been shown to reflect the outcome (6, 7).

Despite these strong associations, the functional role of OPN in carcinogenesis remains poorly defined. *In vitro* transfection studies have demonstrated the invasive potential and metastatic competence associated with the *OPN* gene (8, 9). *OPN* knockdown has been demonstrated to suppress the tumourigenicity of breast cancer cell lines which have invasive and metastatic capacity (10). Furthermore, the breast cancer metastasis suppressor gene (*BRMS1*) has been shown to down-regulate OPN (11). In a rat mammary model system, the tumour suppressor gene breast cancer 1, early onset (*BRCA1*) has also been shown to specifically repress OPN expression by selectively binding several OPN-activating transcription factors (12). OPN is known to interact with a variety of cell surface receptors, including several integrins and CD44, secreted proteases and growth factor/receptor pathways. These are likely to mediate the contributions to carcinogenesis, including cellular migration, the development of the invasive phenotype, increased metastasis, protection from apoptosis, interactions with immunological cells and the induction of angiogenic factors (13). OPN can lead to changes in the expression of numerous genes and regulate a series of signalling cascades through the activation of various kinases and transcription factors that ultimately control the expression of downstream effector genes. These have been shown to contribute to tumour progression and angiogenesis *in vitro* and in animal

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Key Words: Breast cancer, osteopontin, mortality, survival.

0250-7005/2008 $2.00+.40

Table I. *Clinical and pathological data.*

Parameter	Category	Number
Node status	Node-positive	54
	Node-negative	73
Tumour grade	1	24
	2	43
	3	58
Tumour type	Ductal	98
	Lobular	14
	Medullary	2
	Tubular	2
	Mucinous	4
	Others	7
TNM staging	1	70
	2	40
	3	7
	4	4
Outcome	Disease-free	90
	Alive with metastasis	7
	With local recurrence	5
	Died of breast cancer	16
	Died of unrelated disease	9

Note: missing values reflect discarded/uninterpretable values.

Table II. *Sequences for primers.*

Primers for *OPN-a*
5'-ACAACAAATACCCAGATGCT-3'
5'-ACTGAACCTGACCGTACACATTGGTTTCTTCAGAGGAC-3'
Primers for *OPN-b*
5'-ACAACAAATACCCAGATGCT-3'
5'-ACTGAACCTGACCGTACAGGACTTACTTGGAAGGGTCT-3'
Primers for *OPN-c*
5'-AAGTTCTGAGGAAAAGCAGA-3'
5'-ACTGAACCTGACCGTACACTTTCGTTGGACTTACTTGG-3'
Primers for *beta-actin*
5'-ATGATATCGCCGCGCTCGTC-3'
5'-CGCTCGGTGAGGATCTTCA-3'
Primers for *CK19*
5'-CAGGTCCGAGGTTACTGAC-3'
5'-ACTGAACCTGACCGTACACACTTTCTGCCAGTGTGTCTTC-3'

models (14-16). *In vitro* studies have demonstrated that OPN plays an important anti-apoptotic role and can mediate resistance to chemotherapeutics (17). OPN has also been suggested to be a key molecular player involved in the lymphatic metastasis of breast cancer, potentially by enhancing the establishment and/or persistence of tumour cells in the lymphatic system (18).

In this study, the expression profile of three *OPN* splice variants were assessed in a cohort of women with breast cancer. The *OPN* transcript levels were evaluated against established pathological parameters and clinical outcome over a 10-year follow-up period.

Patients and Methods

Patients and samples. Institutional guidelines, including ethical approval and informed consent, were followed. Breast cancer tissues (n=127) and normal background tissues (n=33) were collected immediately after excision during surgery and stored at –80˚C until use. A consultant pathologist examined haematoxylin and eosin-stained frozen sections to verify the presence of tumour cells in the collected samples. The normal tissue was derived from the background breast parenchyma of breast cancer patients within the study group. Medical notes and histology reports were used to extract the clinicopathological data (Table I). A customized database was established to record the data.

Materials. RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, UK). The PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Sigma-Aldrich. Custom made hot-start Master mix for quantitative PCR was obtained from Abgene (Surrey, England, UK) (19, 20).

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5-10 mm and kept for routine histological analysis. An additional 15-20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using a reverse transcription kit with an anchored olig (dT) primer supplied by Abgene, using 1 mg of total RNA in a 96-well plate. The quality of cDNA was verified using β-actin primers (Table II).

Quantitative analysis of osteopontins. The level of *OPN* transcripts from the above prepared DNA were determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously (20). The PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added. The product expands one intron. The primers used for each OPN are detailed in Table II. The reaction was carried out using Hotstart Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which had the Z sequence, 10 pmol of FAM (fluorogenic reporter dye, carboxyfluorescein) tagged probe (Intergen Inc.) and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (Bio-Rad Ltd, Hemel Hempstead, England, UK), which is equipped with an optic unit that allows real-time detection of 96 reactions, under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s and 72˚C for 20 s. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. The levels of

Table III. *Summary of OPN expression profiles for the overall cohort, followed by subgroup analysis for tumour specimens and benign specimens. Values represent the true copy number of mRNA transcripts and are expressed as mean (range, median).*

	Overall	Tumour	Benign
OPN-a	807 (0-18504, 1)	884 (0-18504, 1)	588 (0-7824, 2)
OPN-b	1199 (0-87723, 0)	1425 (0-87723, 0)	396 (0-4838, 0)
OPN-c	14 (0-563, 0)	12 (0-563, 0)	6 (0-71, 0)

OPN expression were then normalized against cytokeratin (CK) 19 expression already measured in these specimens, to correct for varying amounts of epithelial tissue between samples. The CK19 transcripts were quantified as previously reported (21) using primers detailed in Table II. With every PCR run, a negative control without a template and a known cDNA reference sample as a positive control were included.

Statistical analysis. The Mann-Whitney *U*-test and two-sample *t*-test were used for statistical analysis. The *OPN* transcript levels within the breast cancer specimens were compared to normal background tissues and analyzed against conventional pathological parameters and clinical outcome over a 10-year follow-up period. In each case, the true copy number was used for statistical analysis and hence samples were not classified as positive or negative. Statistical analysis was carried out using Minitab version 14.1 (Minitab Ltd. Coventry, England, UK) using a custom written macro (Stat 2005.mtw). For purposes of the Kaplan-Meier survival analysis, the samples were divided arbitrarily into two groups for each OPN splice variant: 'high transcript level' or 'low transcript level'. The cut-off was guided by the Nottingham Prognostic Index (NPI) value, with which the value of the moderate prognostic group was used as the dividing line at the start of the test. Survival analysis was performed using SPSS version 12.0.1 (SPSS Inc. Chicago, IL, USA).

Results

OPN-a. OPN-a was found to be expressed in both normal/benign breast tissue and breast cancer specimens (Table III). Although higher in the latter, this did not reach statistical significance. The expression levels decreased with increasing TNM stage (American Joint Committee on Cancer, AJCC Cancer Staging manual, 6th Edition 2002, Springer-Verlag, New York, USA) and this reached statistical significance when comparing stage 1 with stage 4 disease (mean copy number = 1174 *vs.* 1.83, p=0.02). Using the Mann-Whitney *U*-test, the levels seemed to decrease with increasing tumour stage and worse clinical outcome, however, this was only found to reach statistical significance when comparing NPI 1 to NPI 2 (median copy number = 12.6 *vs.* 0.0, p=0.029). The *OPN-a* levels were lower in patients who died of breast cancer than in those who were

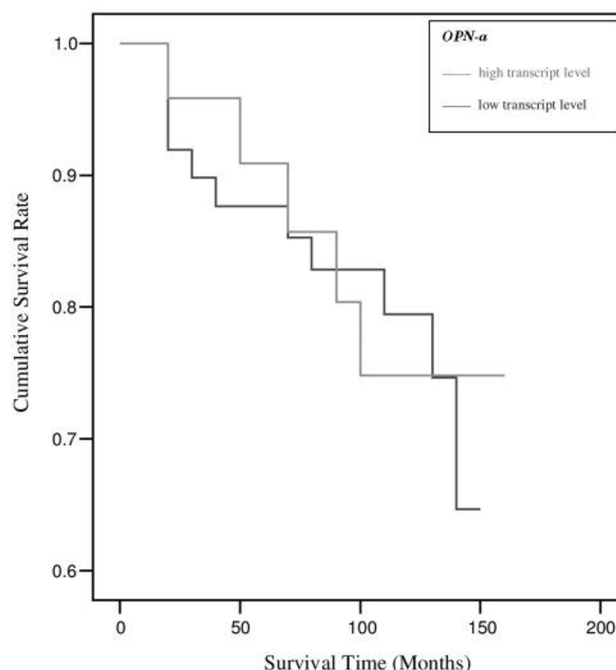

Figure 1. *Overall survival curve according to OPN-a (p=0.942).*

disease free (DF) after a median follow-up of 10 years, however this did not reach statistical significance (mean copy number = 910 *vs.* 193, p=0.078). The overall survival curve for women with tumours which were classified as having 'high levels' of *OPN-a* transcript was not found to differ significantly from that of their 'low level' counterparts, (Figure 1, p=0.942). The expression levels of *OPN-a* were found to be significantly higher in ductal tumours, however, no relationship with tumour grade or oestrogen receptor (ER) status was observed.

OPN-b. OPN-b showed a similar trend to *OPN-c* (Table III). The expression levels of *OPN-b* were found to increase with tumour grade (Bloom and Richardson criteria, Rosen's Breast Pathology, 2nd Edition 2001, Lippincott Williams & Wilkins, Philadelphia, PA, USA), NPI stage and tumours with poor clinical outcome. The difference reached statistical significance when comparing grade 3 tumours with grade 1 (median copy number = 0.1 *vs.* 0.0, p=0.02). The relationship approached significance when comparing grade 2 tumours with grade 1 (p=0.0535). Increased *OPN-b* levels were also found to be significantly associated with tumour stage, when comparing NPI 3 to NPI 1 (median copy number = 336.4 *vs.* 0.0, p=0.03) and NPI 2 (median copy number 336.4 *vs.* 0.0, p=0.04). The *OPN-b* levels were significantly higher in the patients who died of breast cancer than in those who were DF after a median follow-up of 10 years (median copy number = 332 *vs.* 0.0,

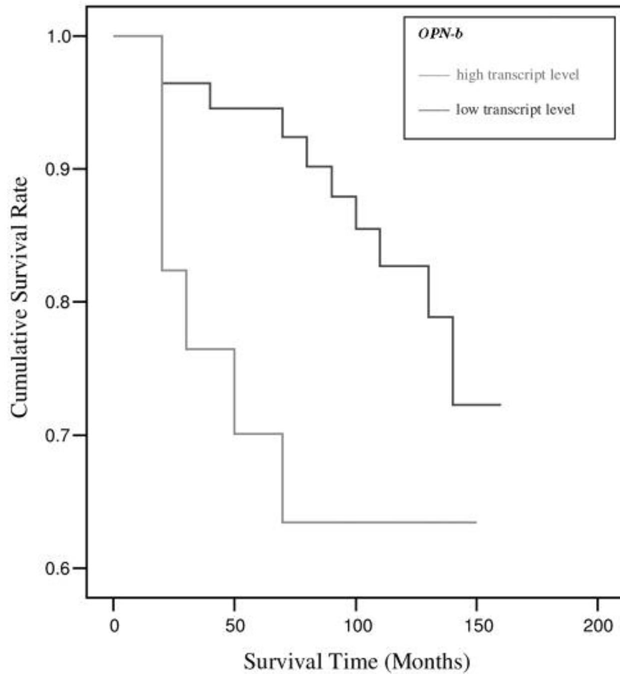

Figure 2. *Overall survival curve according to OPN-b (p=0.022).*

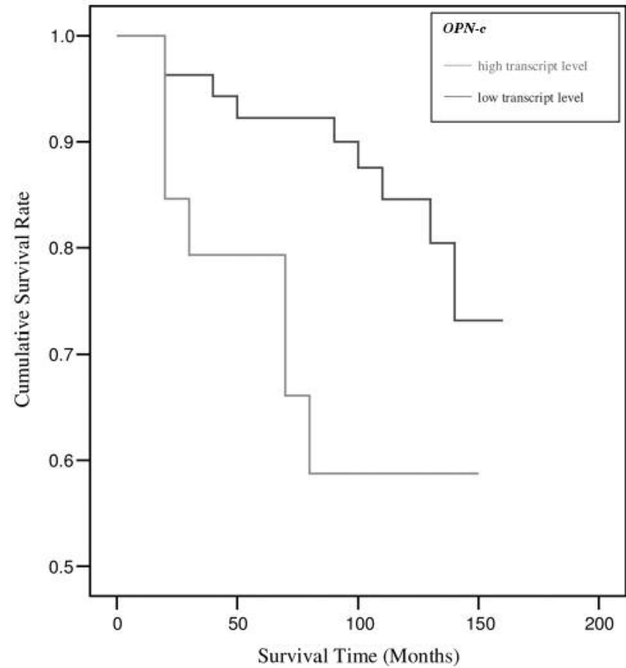

Figure 3. *Overall survival curve according to OPN-c (p=0.016).*

p=0.02). The expression levels of *OPN-b* mRNA significantly predicted disease-free survival (median copy number = 56.6 *vs*. 0.00, p=0.02). The overall survival curve for women with tumours which were classified as having 'high levels' of *OPN-b* transcript was found to differ significantly from that of their 'low level' counterparts (Figure 2, p=0.022).

OPN-c. *OPN-c* mRNA expression was higher in tumour samples compared with normal breast tissue (Table III), although this did not reach statistical significance (mean copy number = 12 *vs*. 6.2, p=0.45). The *OPN-c* mRNA expression was found to increase with increasing tumour stage and grade. Grade 3 tumours expressed significantly higher levels than grade 1 tumours (median copy number = 0.000 *vs*. 0.009, p=0.0091) and approached significance when compared with grade 2 tumours (median copy number = 0.00 *vs*. 0.01, p=0.0577). However, the association with tumour stage did not reach statistical significance. The expression levels of *OPN-c* mRNA significantly predicted local recurrence (p=0.03) and disease-free survival (median copy number = 0.34 *vs*. 0.00, p=0.0051). Furthermore in patients with ductal carcinoma, high *OPN-c* levels were associated with bone metastases (median copy number = 11.6 *vs*. 0.0, p=0.0262). The overall survival curve for women with tumours which were classified as having 'high levels' of *OPN-c* transcript was found to differ significantly from that of their 'low level' counterparts, Figure 3 (p=0.016).

Discussion

The first demonstration of OPN expression in breast cancer was by Brown *et al*. in 1994 (22) in their study which compared various human tumours to corresponding normal tissues. Differences in the extent of positivity between breast carcinomas and benign proliferative lesions were subsequently demonstrated by Bellahcene and Castronovo (23). The prognostic significance of OPN has been demonstrated in a cohort of 333 women with stage I-II breast cancer. OPN positivity of the primary tumour was found to be associated with high histological grade, staining for c-*erb*B-3 and p53. The percentage of carcinoma cells staining positive for OPN was also found to be associated with a progressive decrease in survival. After 19 years of follow-up, 94% of patients who were OPN negative were found to be alive (median survival 228 months) compared to only 26% of those classified as OPN positive (median survival 68 months) (5). Interestingly, another study has found that amongst women with node negative breast cancer, OPN positivity of the primary tumour was significantly associated with decreased survival (24). More recent studies have examined individual splice variants of the *OPN* gene and Mirza *et al*. reported that *OPN-c* could be a selective diagnostic and prognostic marker for human breast cancer (3). In their study, *OPN-c* mRNA was identified in 80% of breast carcinomas (16/20). By immunohisto-chemistry, 77% (43/56) of core biopsies from breast

carcinomas were positive and staining was found to increase with tumour grade. No positivity was identified within mammoplasty specimens with molecular analysis. However, 3 out of 69 normal breasts were found to have low levels of staining on immunohistochemistry. In contrast, *OPN-a* mRNA was found to some extent in all breast carcinomas and nearly all normal samples (21/22). *OPN-b* was identified at low levels in most of the breast carcinomas (18/20) and approximately one-quarter of normal breasts (6/22). The authors suggested that the molecular profile of *OPN* expression could provide a useful adjunct to traditional histological analysis and may be of greatest utility when used in conjunction with conventional markers including the oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (Her)-2 (3). The results of the present study are consistent with recent contributions to the literature describing the strong association of OPN with histopathological parameters and clinical outcome. In the present study, increased *OPN-b* and *OPN-c* expression were significantly associated with adverse pathological and clinical outcomes, both within the cohort and amongst purely ductal carcinomas. *OPN-b* and *OPN-c* were also associated with significant survival differences in the Kaplan-Meier curve. Interestingly, the pathological and clinical associations of *OPN-a* were found to be qualitatively inverse to those of *OPN-b* and *OPN-c*. It is noteworthy that *OPN-c* is the shortest splice variant and has been postulated to support breast tumour progression by conveying anchorage independence and inducing the expression of oxidoreductases (6-8). Further studies are required to elucidate the important functional role that *OPN-c* may have in the development of the malignant phenotype.

Limitations of the present study included the use of background parenchyma from breast cancer patients to provide ‘normal tissue’ for comparison. Ideally, such material should be derived from patients without breast cancer in order to avoid any ‘field change’ which may exist within cancer-bearing tissues. Although the sample size and follow-up period was substantial, it could be possible that a larger cohort may have influenced several results which approached, but failed to reach, statistical significance. Finally, in addition to the measurement of mRNA transcript levels, quantitative analysis of OPN protein expression should be undertaken to ensure concordance.

In addition to OPN expression within the primary tumour, OPN measurement within the blood has also been shown to be of prognostic utility. In a prospective study of 158 women with newly diagnosed metastatic breast cancer, high baseline OPN plasma levels were found to be significantly associated with shorter survival in a multivariate model incorporating standard prognostic factors. Furthermore, OPN increase >250 ng/ml at any time during the follow-up period was found to be the variable with the greatest prognostic value for poor survival (6). Singhal *et al*. (7) reported similar findings in their cohort of 70 women with known metastatic carcinoma and also demonstrated significant differences in OPN levels with increasing metastatic burden. The measurement of OPN in the blood or tumours of patients with cancer may provide valuable prognostic information and allow stratification of risk. Advances in molecular biology have significantly improved our understanding of the structure and function of OPN (13). Descriptive studies have confirmed that OPN is likely to be of diagnostic and prognostic utility in patients with breast cancer. Furthermore, in the light of recent mechanistic studies, there may also be potential for the functional suppression of OPN and novel OPN-based therapeutic manipulation (25).

Conclusion

Increased *OPN-b* and *OPN-c* expression profiles are significantly associated with adverse pathological and clinical outcomes in breast cancer. Of the three splice variants, *OPN-c* is likely to be of particular utility as a prognostic marker and should be included in future validation studies.

Conflicts of Interest

None declared.

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Received July 6, 2008
Revised September 12, 2008
Accepted September 30, 2008