J Korean Med Sci 1998; 13: 652-7
ISSN 1011-8934

Expression of Osteopontin and Osteonectin in Breast Cancer

Osteopontin (OP) and osteonectin (ON) are bone matrix proteins produced by mammary and other cancers. These proteins may play a role in tumor invasion and metastasis through integrin-mediated signal transduction. We evaluated expressions of OP and ON in 253 resected infiltrating ductal carcinoma of the breast, using immunohistochemical staining and follow-up data. OP and ON were detected 87.4% and 54.2% of 253 cases, respectively. The OP and ON positive staining were localized in the cytoplasm of carcinoma cells. OP and ON did not correlate with various clinicopathological parameters, such as age, lymph node involvement, tumor size, histologic grade, expression of p53 and estrogen receptor (ER). In the multivariate model, lymph node involvement and histologic grade were statistically significant prognostic factors. Assessed by a log rank test, the 5-year-survival rates of OP and ON positive groups and their negative groups were not statistically different. In conclusion, OP and ON immunopositivity of infiltrating ductal carcinomas of the breast provide no additional prognostic information in this study.

Key Words: *Breast neoplasms; Sialoglycoproteins, osteopontin; Osteonectin; Prognosis*

Youn Wha Kim, Yong-Koo Park, Juhie Lee, Suk Whan Ko,* Moon Ho Yang

Departments of Pathology and General Surgery*, College of Medicine, Kyung Hee University, Seoul, Korea

Received: May 4, 1998
Accepted: September 1, 1998

Address for correspondence
Youn Wha Kim, M.D.
Department of Pathology, College of Medicine, Kyung Hee University, #1, Hoiki-dong, Dongdaemun-gu, Seoul 130-701, Korea
Tel: +82.2-961-0543, Fax: +82.2-969-6958
E-mail: kimyw@nms.kyunghee.ac.kr
*The authors wish to acknowledge the financial support of Kyung Hee University made in 1997.

INTRODUCTION

Osteopontin (OP) and osteonectin (ON) are the most well-known bone phosphoproteins. OP is a 44-kDa phosphorylated glycoprotein with the amino acid sequence of Arg-Gly-Asp that elicits binding of integrin. It is associated with the transformation process, and is dramatically upregulated upon transformation of cells. OP shows high affinity binding to hydroxyapatite and appears to play a role in modulating mineralization of calcifying tissues. ON is a 38-kDa phosphorylated glycoprotein that shows high affinity binding to type I collagen. Although ON also shows high affinity binding to calcium and hydroxyapatite, ON is considered to be associated with tissue remodeling rather than calcification (1).

OP is expressed by a limited number of normal cells and tissues (including lactating mammary gland, developing bone, kidney, activated T cells and macrophages, and smooth muscle cells) (2). Because OP is known to have an adhesive function, it is the interesting possibility that OP may play a role in tumor cell invasion, metastasis, and processes in which adhesive interactions between tumor cells and extracellular matrix are critical (3). Recently, there have been reports of increased plasma levels of OP in human breast cancer (4), and of immunohistochemically increased OP expression in primary breast tumors on immunohistochemical staining (1, 3, 5, 6). The significance of increased OP expression (either plasma or tumor levels) in predicting the biological behavior of breast cancer is unknown at present. ON is produced by the osteoclast and can be regarded as a marker in the differentiation of bone-forming cells (7). Bellahcene and Castronovo (5) reported an analysis of both in situ and invasive carcinoma of the breast for ON immunoreactivity with high expression.

The ectopic secretion of OP and ON by breast cancers could provide a key in the understanding of the complex process that leads to hydroxyapatite crystallization in breast cancer as well as preferred bone homing of circulating mammary metastatic cells (5). The objective of present study is to evaluate the value of immunohistochemical expression of OP and ON as prognostic markers in infiltrating ductal carcinoma of the breast. Also expressions of OP and ON are compared to several clinicopathological parameters including p53 and estrogen receptor (ER).

MATERIALS AND METHODS

Two hundred and fifty-three cases of infiltrating ductal carcinoma of the breast were included in this study. All

patients with infiltrating ductal carcinomas underwent radical or modified radical mastectomy with axillary lymph node dissection from January 1983 to December 1996 at Kyung Hee University Hospital. The median age of the patients was 47.4 years (range, 21-77 years). One hundred and twenty-one tumors were located in the right breast (47.8%) and 132 were located in the left breast (52.2%). The authors reviewed all of the H-E slides and reclassified microscopic grading using Nottingham modification of the Bloom-Richardson system (8).

Immunolocalization was performed using a streptoavidin-biotin immunoperoxidase method, according to supplier's protocol (DAKO LSAB kit, Carpinteria, California). Briefly, paraffin-embedded sections were deparaffinized in xylene and dehydrated with graded ethanol. After quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 30 minutes and blocking reagents for 30 minutes. Primary anti-osteopontin monoclonal antibody (kindly provided by Dr. Fisher, Bone Research Branch, National Institute of Dental Research, NIH, USA), anti-osteonectin monoclonal antibody (Biodesign Company, Kennebunk, USA), anti-human p53 protein DO-7 (DAKO, Carpinteria, California), and anti-human estrogen receptor (DAKO, Carpinteria, California) was applied to the sections at a dilution of 1:100 and incubated in a moist chamber for 2 hours at room temperature. After washing with Tris-buffered saline (DAKO), a biotinylated link antibody was applied for 30 minutes, followed by streptavidin peroxidase for an additional 30 minutes. After washing out the excess complex, the localization of antibodies were visualized by incubating the sections for 10 minutes in 3,3'-diaminobenzidine tetrahydrochloride (Research Genetics, Huntsville, USA). Osteochondroma of the bone, as a positive control, was specifically stained with anti-OP and anti-ON antibodies. The location of immunostaining was mainly in the cytoplasms of the tumor cells with both antibodies.

The degree of cytoplasmic immunoreactivity of tumor cells for OP and ON was determined by three independent observers. The scoring system was as follows: absence of immunoreactivity in tumor cells (0); focal (<50% of the tumor cells) and weak cytoplasmic immunoreactivity (1+); focal (<50% of the tumor cells) and strong cytoplasmic immunoreactivity (2+); diffuse (>50 % of the tumor cells) and weak cytoplasmic immunoreactivity (3+); diffuse (>50% of the tumor cells) and strong cytoplasmic immunoreactivity (4+). All scores greater than 0 were interpreted as a positive result; a score of 0 was interpreted as a negative result. For the p53 protein and ER, the degree of nuclear immunoreactivity of tumor cells was considered as positive when positive cells were found in more than 5% of the tumor cells.

The OP and ON expressions in infiltrating ductal carcinoma of the breast were compared with various clinical and histologic features, including age, tumor size, histologic grade, axillary lymph node metastasis, and expression of p53 and ER. A follow-up of patients whose tumors were examined in this study is currently in progress. Fifty-two patients died, 153 alive and 38 were lost to follow-up or died by other disease (follow-up rate= 85.0%). Statistical analysis was done with chi-square test. By using the proportional hazards model of Cox, multivariate analysis was done on the factors said to affect the prognosis of patients with breast carcinoma. A Kaplan-Meier survival curve was constructed and assessed using the log rank test to compare the difference of survival among the subgroups.

RESULTS

Osteopontin was expressed in 221 of 253 infiltrating ductal carcinomas (87.4%) and ON was expressed in 137 of 253 cases (54.2%). The OP and ON positive staining was localized in the cytoplasm of carcinoma cells with granular or reticular in shape (Fig. 1, 2). Nuclear staining was not observed. In the areas of microcalcification of the tumor, the highest immunoreactivity for OP and ON was observed. In normal adjacent ductal epithelium and scattered macrophages, weak reactivity with anti-osteopontin and anti-osteonectin was seen. The number of positive cells and intensity of staining were variable.

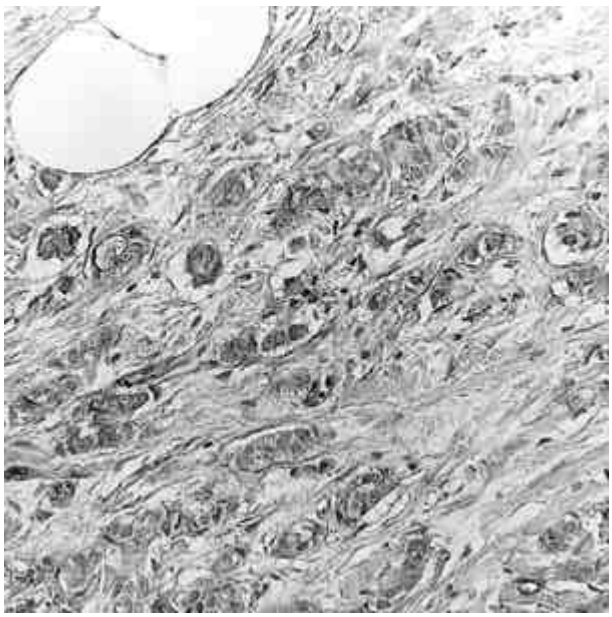

Fig. 1. Immunohistochemical finding; diffuse cytoplasmic osteopontin staining of breast cancer cells (×100).

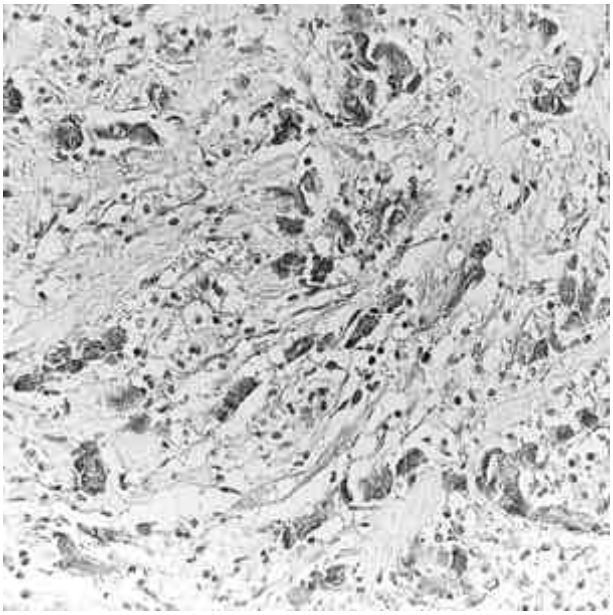

Fig. 2. Immunohistochemical finding; diffuse cytoplasmic osteonectin staining of the breast cancer cells (×100).

Mostly cases of the positive staining for OP and ON were grade 3 or 4.

The relationship between expression of OP and ON and several clinico-pathological parameters related to prognosis is shown in Table 1. OP expression did not correlate with age (p=0.74), lymph node involvement (p=0.07), histologic grade (p=0.48), expression of p53 (p=0.11), and ER expression (p=0.06). Tumors having less than 3cm in size expressed 81.0% of OP positivity and tumors having larger than 3cm in size expressed 91.5% (p=0.01). ON expression did not correlate with age (p=0.88), lymph node involvement (p=0.06), tumor size (p=0.18), histologic grade (p=0.81), and expression of p53 (p=0.32) and ER (p=0.21). The percentage of positive cytoplasmic staining, staining intensity, and distribution of positive areas did not correlate with prognostic factors.

Follow-up data of 250 cases was analysed (range, 3 months to 160 months). In the univariable analysis of prognostic factors of breast cancer, lymph node involvements (p=0.01) and histologic grade (p=0.04) were significantly correlated with prognosis (Table 2). However, multivariate analysis demonstrated lymph node involvements (ration of risk=4.02) and histologic grade (ration of risk=1.68) were significant prognostic factors in using the proportional hazards model of Cox (Table 3). Assessed by log rank test, the mean survival periods of the OP positive group and its negative group were 93 months and 39 months, respectively (p=0.91) (Fig. 3). The mean survival periods of the ON positive group and its negative group were 106 months and 54 months, respectively (p=0.09) (Fig. 4).

Table 1. Expression of osteopontin and osteonectin and clinicopathological features related to known prognostic factors

Clinicopathological findings	No. of cases (%)	No. of osteopontin positive (%)	p value*	No. of osteonectin positive (%)	p value*
Age (yr)			0.743		0.884
< 34	39 (15.4)	35 (89.7)		22 (56.4)	
35~50	118 (46.6)	104 (88.1)		62 (52.4)	
> 51	96 (38.0)	82 (85.4)		53 (55.2)	
Lymph node metastasis			0.073		0.068
Negative	113 (44.7)	94 (83.2)		54 (47.8)	
Positive	140 (55.3)	127 (90.7)		83 (59.3)	
Tumor size			0.014		0.184
< 3 cm	100 (39.5)	81 (81.0)		49 (49.0)	
> 3 cm	153 (60.5)	140 (91.5)		88 (57.5)	
Histologic grade			0.482		0.816
I	114 (45.0)	100 (87.7)		63 (55.2)	
II	115 (45.5)	102 (88.7)		60 (52.3)	
III	24 (9.5)	19 (79.2)		14 (58.3)	
p53 expression			0.116		0.326
Negative	180 (71.1)	161 (89.4)		101 (56.1)	
Positive	73 (28.9)	60 (82.2)		36 (49.3)	
Estrogen receptor			0.065		0.210
Negative	153 (60.5)	129 (84.3)		78 (51.0)	
Positive	100 (39.5)	92 (92.0)		59 (59.0)	

*$p<0.05$: tested by χ^2-test.

Table 2. Univariable analysis in 215 cases of breast cancer

Prognositic factor	Categories	β	S.E.	R.R. (95% C.I.)	p value
Age	34<, 35~50, 51	-0.19	0.19	0.82 (0.56~1.21)	0.31
Lymph node metastasis	negative, positive	1.28	0.38	3.60 (1.69~7.67)	0.01
Tumor size	<3 cm, >3 cm	0.48	0.30	1.62 (0.90~2.93)	0.11
Histologic grade	I, II, III	0.42	0.21	1.53 (1.02~2.28)	0.04
Osteonectin	negative, positive	-0.49	0.30	0.61 (0.34~1.09)	0.09
Osteopontin	negative, positive	0.05	0.52	1.01 (0.38~2.94)	0.91
p53	negative, positive	0.01	0.31	1.02 (0.56~1.86)	0.94
Estrogen receptor	negative, positive	-0.22	0.28	0.80 (0.46~1.40)	0.44

β. regression coefficient; S.E., standard error; R.R., ration of risk.
95% C.I.: 95% confidence interval.

Table 3. Multivariable analysis in 215 cases of breast cancer (Cox proportional hazard model)

Prognostic factor	Categories	β	S.E.	R.R. (95% C.I.)
Osteonectin	negative, positive	-0.51	0.30	0.60 (0.33~1.08)
Lymph node metastasis	negative, positive	1.39	0.39	4.02 (1.87~8.64)
Histologic grade	I, II, III	0.51	0.21	1.68 (1.11-2.54)

β, regression coefficient; S.E., standard error; R.R., ration of risk.
95% C.I.: 95% confidence interval.

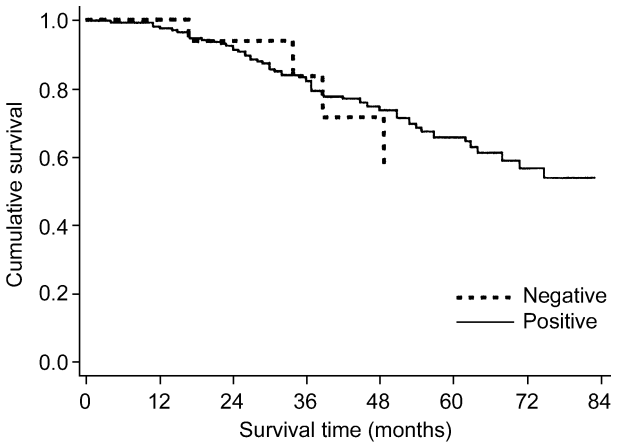

Fig. 3. Osteopontin expression in relation to survival in 215 patients with breast cancer. The curves were calculated by using the Kaplan-Meier method and compared with the log rank test.

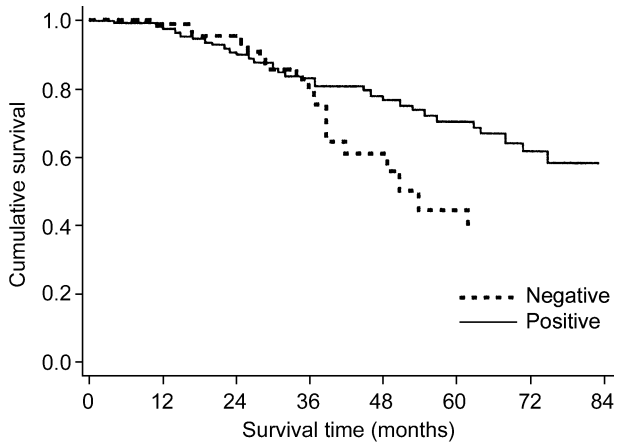

Fig. 4. Osteonectin expression in relation to survival in 215 patients with breast cancer.

DISCUSSION

Nearly 40% of malignant lesions of the breast are associated with the presence of radiologically detectable microcalcification. Little is known about microcalcification in mammary tissue but it is remarkable that they are always formed of hydroxyapatite crystals when associated with malignant breast lesions (5). An increased expression of the bone matrix proteins OP and ON has been documented suggesting that this may play a role in the bone homing of metastasis of breast carcinoma (5).

Bellahcene and Castronovo (5) evaluated the expression of OP and ON, using a immunoperoxidase technique, in 79 breast lesions including 28 benign and 51 cancerous specimens. They found that normal mammary tissue, adjacent to the lesions examined, generally expressed undetectable or vaguely detectable (0 or 1+) amounts of ON and OP. Benign breast lesions, including fibroadenoma and fibrocystic disease, were generally weakly stained (0 or 1+) with both ON and OP. Interestingly, the majority of in situ and invasive ductal carcinoma (80.0% and 88.0%, respectively) showed a strong expres-

sion (2 or 3+) for ON and OP. High expression of these proteins was associated with frequent microcalcification. Brown et al. (3) performed immunohistochemistry for OP on 10 cases of infiltrating ductal carcinoma, 2 cases of ductal carcinoma in situ, and three lymph nodes with metastatic breast cancer. They described that the tumor cells and nearby macrophages strongly stained for OP. Present study revealed weak reactivity with OP and ON was seen in normal adjacent ductal epithelium and scattered macrophages while positively stained carcinoma cells displayed strong immunoreactivity for OP and ON.

The significance of OP and ON expression in human cancers is unknown at present. A few studies including lung carcinoma (9), malignant astrocytoma (10), and esophageal carcinoma (11) were reported an association between elevated OP expression and poor prognosis of tumors. O'Malley et al. (6) reported that 26% (40/152) of breast cancer showed expression of OP and it was localized to the perinuclear region of the neoplastic cells. They showed OP positivity within the cancer cells to be significantly associated with decreased disease free and overall survival on univariate analysis. However, OP positivity of the cancer cells remained a significant predictor of decreased overall survival, not in disease free survival. Tuck et al. (2) reported that in the case of bilateral infiltrating mammary carcinomas of the same histologic type and grade, the tumor of the right breast was OP positive, whereas the tumor of the left breast was negative. The metastasis of right axillary lymph node, recurrence of chest wall, and distant metastases were seen. This finding showed that the association of increased OP expression with increased aggressiveness of breast cancer raises the possibility of a role for OP in tumor progression as well as the potential as a tumor marker in predicting clinical aggressiveness.

In this study, the expression of OP and ON in breast cancers was not affected by age, tumor size, histologic grade, lymph node involvement, and expression of p53 and ER. Also, expression of OP and ON is not correlated with prognosis and survival. O'Malley et al. (6) described expression of OP as a significant predictor of decreased survival rates in lymph node negative breast carcinomas. In present study, expression of OP in lymph node negative breast carcinoma was lower than lymph node positive cases (83.2% versus 90.7%). But, this is not statistically significant ($p=0.073$). In the case of ON expression, the result is same as OP expression (47.8% versus 59.3%, $p=0.068$). Present study revealed OP and expression of ON in infiltrating ductal carcinomas of the breast were 87.4% and 54.2%, respectively, and showed diffuse cytoplasmic staining. O'Malley et al. (6) has reported 26.0% of OP positive staining of the breast carcinomas, while Bellahcene and Castronovo (5) has reported 100% of OP positivity. Differences in the immunohistochemical staining positive rate among the studies may be resulted from the number of tumors examined or interpretation of positive number of tumor cells.

Brown et al. (3) described that OP protein of tumor cells and stroma were prominent at the invasive edge of tumors. In particular, the presence of OP in extracellular matrix of the tumor and on the surface of tumor cells interfacing with stroma suggest that OP participates in adhesive interactions at the tumor/host interface. Consequently, it raises the possibility that OP influences tumor cell invasion and metastasis. Also OP may be important function in macrophage adhesion and migration (3). OP and ON immunoreactivity were prominent at the invasive edge of tumors in present study.

This study is the first to examine the association between expression of OP and ON and prognosis in infiltrating ductal carcinoma of the breast. We found that there was no statistically significant correlation between the degree of OP and ON immunopositivity and prognostic factors. Further prospective clinical correlative studies are needed to evaluate the role of OP and ON as clinically relevant tumor markers.

REFERENCES

1. Hirota S, Ito A, Nagoshi J, Takeda M, Kurata A, Takatsuka Y, Kohri K, Nomura S, Kitamura Y. *Expression of bone matrix protein messenger ribonucleic acids in human breast cancers; possible involvement of osteopontin in development of calcifying foci. Lab Invest 1995; 72: 64-9.*
2. Tuck AB, O'Malley FP, Singhal H, Tonkin KS, Harris JF, Bautista D, Chambers AF. *Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. Arch Pathol Lab Med 1997; 121: 578-84.*
3. Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, Dvorak HF, Senger DR. *Osteopontin expression and distribution in human carcinomas. Am J Pathol 1994; 145: 610-23.*
4. Senger DR, Perruzzi CA, Gracey CR. *Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. Cancer Res 1988; 48: 5770-4.*
5. Bellahcene A, Castronovo V. *Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. Am J Pathol 1995; 146: 95-100.*
6. O'Malley FP, Harris JF, Doig GS, Kerkvliet N, Saad Z, Bautista D, Tonkin K, Chambers AF. *Osteopontin expression is an independent prognostic indicator in lymph node negative breast cancer. The 85th Annual Meeting of United States and Canadian Academy of Pathology 1996: 22A (Abstract only).*

7. Jundt G, Schultz A, Berghauser K-H, Fisher L, Gehron-Robey P, Termine JD. *Immunohistochemical identification of osteogenic bone tumors by osteonectin antibodies. Virchows Arch [A] 1989; 414: 345-53.*
8. Elston CW, Ellis IO. *Pathological prognostic factors in breast cancer. I. The value of histological grades in breast cancer, Experience from a large study with long-term follow-up. Histopathology 1991; 19: 403-10.*
9. Chambers AF, Wilson SM, Kervliet N, O'Malley FP, Harris JF, Casson AG. *Osteopontin expression in lung cancer. Lung Cancer 1996; 15: 311-23.*
10. Saitoh Y, Kuratsu J, Takeshima H, Yamamoto S, Ushio Y. *Expression of osteopontin in human glioma: its correlation with the malignancy. Lab Invest 1995; 72: 55-63.*
11. Casson AG, Wilson SM, McCart JA, O'Malley FP, Ozcelik H, Tsao MS, Chambers AF. *Ras mutation and expression of the ras-regulated genes osteopontin and cathepsin L in human esophageal cancer. Int J Cancer 1997;72:739-45.*