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Expression of P8 Protein in Breast Carcinoma; an Inverse Relationship with Apoptosis

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Abstract. *Background: P8 is a transcription factor and its expression is elevated in response to apoptotic stimuli. However, p8 in human carcinoma tissue has not been investigated in depth. In this study, we investigated p8 expression in breast carcinoma. Materials and Methods: We immunohistochemically investigated p8 expression in 50 cases of breast carcinoma, including 7 non-invasive ductal carcinomas. Results: High expression of p8 was observed in 60% of cases, which was inversely related to tumor size and UICC stage. All non-invasive ductal carcinomas diffusely expressed p8. Furthermore, in tumors of 2cm or larger, the p8 expression was inversely linked to the apoptotic index. Conclusion: These results suggest that p8 plays a role in the early phase of breast carcinoma progression and especially in tumors of larger size, acting as an inhibitor of apoptosis of breast carcinoma cells.*

Apoptosis, or programmed cell death, presents typical characteristics such as nuclear condensation, nucleosomal condensation and membrane convolution (1). This phenomenon occurs in cells that are not functionally required or have even become harmful to the body and, together with cell proliferation, plays a significant role in the maintenance of homeostasis. Therefore, abnormalities in the mechanisms regulating apoptosis are related to various diseases including cancer (2).

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Previous studies indicated that acceleration of apoptosis is directly linked to the progression of cancer in various sites (3-9), including the breast. Studies of apoptosis-related genes and/or proteins should be meaningful to evaluate the characteristics of a tumor. The *p8* gene was identified by Mallo *et al.* and Vassuer *et al.* as a transcription factor which is significantly related to cell growth (10, 11). To date, two functions of p8 have been identified. One is stimulation of the mitotic activity of the cell, and the other is the inhibition of apoptosis, because its expression is induced when cells are under stress, such as when they are receiving pre-apoptotic stimuli (10). The *p8* gene was mapped to chromosome 16p11.2, which is a region frequently amplified in breast carcinoma (12), suggesting that p8 expression is related to carcinoma growth. However, no studies have been performed regarding p8 expression in this carcinoma.

We therefore investigated p8 expression in breast carcinoma, the most common carcinoma of the endocrine organs, in order to confirm whether p8 protein is linked to its clinical characteristics, including apoptosis.

Materials and Methods

Tissue specimens. Tissue specimens of breast carcinomas were obtained from 50 patients who underwent surgery. They consisted of 7 non-invasive ductal carcinomas, 2 invasive lobular carcinomas, and 41 invasive ductal carcinomas. Four non-invasive ductal carcinoma patients underwent surgery in the Department of Surgical Oncology, Osaka University, Japan, between 2001 and 2002, while the remaining patients underwent surgery in the Department of Surgery, Kuma Hospital, Japan, between 1999 and 2003. This project was approved by the ethics committee of each hospital and written informed consent was obtained from the participating patients. For immunohistochemistry, tissues were fixed with 10% formalin and paraffin-embedded.

Antibodies. A polyclonal antibody against p8 was produced by immunizing New Zealand White rabbits with an oligopeptide corresponding to amino acids 62-82 of human p8 as an immunogen. The specificity of this antibody has already been confirmed in a previous report (11). A polyclonal anti-ssDNA antibody was purchased from Dako (Copenhagen, Denmark). They were applied as primary antibodies at a concentration of 1:250 and 1:400, respectively. Monoclonal antibodies against Ki-67 (clone MIB-1), p27 and Her-2 were purchased from Ylem (Rome, Italy), Neomarkers (Fremont, CA, USA), and Dako (Copenhagen, Denmark), respectively. They were applied at a concentration of 1:50, 1:50 and 1:250, respectively

Immunohistochemistry. Briefly, 4-μm-thick tissue sections were dewaxed and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min. For antigen retrieval for immunohistochemistry of Ki-67, p27 and Her-2, sections were immersed in 0.03mol/L citrate buffer (pH6.0) and incubated at 95˚C for 40 min. After rinsing in phosphate-buffered saline pH 7.2 (PBS), 10 % bovine serum (Wako, Osaka, Japan) was applied for 20 min to block non-specific reactions. The sections were then incubated with the primary antibody overnight at 4˚C. After rinsing in PBS, they were treated with peroxidase-labelled anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan) for 30 min. The peroxidase reaction was visualized by incubating the sections with 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 0.05M Tris buffer with 0.01% hydrogen peroxide (Nichirei). The sections were counterstained with hematoxylin. Sections for the negative control were prepared using rabbit immunoglobulins instead of the primary antibody.

Immunohistochemical evaluation. We regarded the cells as positive for p8 when immunoreactivity was clearly observed in their nuclei and/or cytoplasms. The immunoreactivity of p27, ssDNA and Ki-67 was predominantly observed in the nuclei. We calculated the labelling index (LI) for each case by counting cells expressing these proteins per 1000 cells at least. The immunostaining results for p8 were graded based on its LI as follows: 0, 0%; 1, >0% and ≤25%; 2, >25% and ≤50%; 3, >50% and ≤75%; and 4, >75%. This method was identical to that used in a previous study (13). Cases graded as 3 or 4 were classified as high for p8, whereas those lower than 3 were classified as low. We regarded cases as high group for p27 when they were graded as 2 or more. We classified cases as having a high cell proliferating activity when Ki-67 LI was greater than 10%, and as having a high apoptotic index when ssDNA LI was greater than 10%. Furthermore, we regarded cases as showing a high expression of Her-2 when more than 10% of carcinoma cells displayed weak to moderate or strong complete membrane staining.

Statistical analyses. We employed the Chi-square test and Fisher's exact test to analyze the relationship between p8 expression and histological type. *P* values less than 0.05 were regarded as statistically significant.

Results

In the normal mammary gland, p8 expression was observed in glandular epithelial and myoepithelial cells, but they were always graded as 0 or 1 and classified as low (Figure 1a). P8 immunoreactivity could be seen in the nuclei and/or cytoplasm of breast carcinoma cells (Figure 1b). Furthermore, weak p8 signals could also often be observed in stromal cells such as fibroblasts. Of the 50 cases of breast carcinoma, 30 (60.0%) were classified as high for p8 expression. An inverse relationship between p8 and apoptotic index was observed ($p=0.0089$) (Table I). Then we investigated the relationship for tumors of 2cm or larger and those smaller than 2 cm. An inverse relationship could be found in tumors of 2 cm or larger, but not in tumors smaller than 2cm (Table I).

Table I. *Relationship between p8 expression and apoptotic index of 50 cases of breast carcinoma.*

	P8 expression		
	High	Low	Total
Apoptotic index			
High	9	14	23
Low	21	6	27
		$p=0.0089$	
Tumor size > 2cm			
High	2	9	11
Low	8	5	13
		$p=0.0472$	
Tumor size < 2cm			
High	7	5	12
Low	13	1	14
		Not significant	

Table II shows the correlation between p8 expression and other clinicopathological features. P8 was highly expressed in all 7 non-invasive ductal carcinomas we examined, whereas 20 of the 43 cases (46.5%) of invasive ductal or lobular carcinomas were classified as the low group. P8 expression was significantly decreased in cases demonstrating large tumor ($p=0.0200$) and advanced stage ($p=0.0086$). However, we could not establish any relationship between p8 expression and cell proliferating activity or the expression of p27. Furthermore, no relationships were determined between p8 expression and other parameters such as age, menopause, histological grade (14), lymph node metastasis, the status of steroid receptors and Her-2 expression.

Discussion

To date, p8 mRNA expression has been observed in human organs such as the liver, prostate, ovary, colon, thyroid, spinal cord, trachea and adrenal gland (11). In this study, we found that p8 was occasionally expressed in glandular

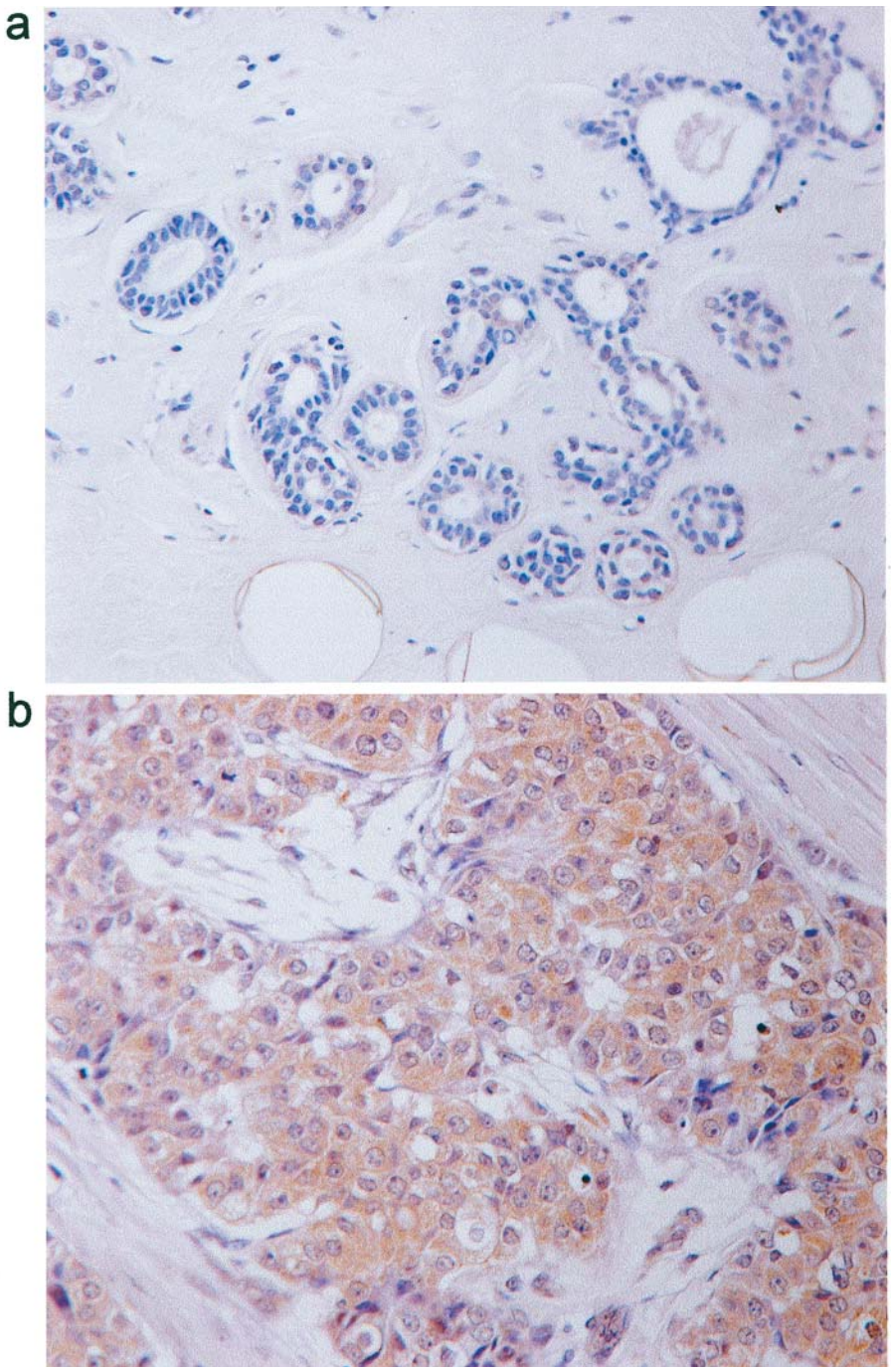

Figure 1. *a. P8 expression in normal mammary gland, graded as 1.*
b. P8 expression in breast carcinoma. The LI was greater than 75% and graded as 4. Original magnifications; x750

epithelial cells, indicating that it also plays a constitutive role in the breast.

Although p8 is a transcription factor, which should be localized mainly in the nuclei, we found that p8 immunoreactivity could be observed in the cytoplasms as well as the nuclei of breast carcinoma cells. Similar findings were also obtained in thyroid papillary carcinoma (13) and pancreatic carcinoma (15, 16). This may be due to overproduction of this protein, or its transportation from the nucleus, for some reason, by a mechanism such as acetylation. Further investigation is needed to elucidate the significance of this phenomenon.

Table II. *Relationship between p8 expression and other clinical features of 50 cases of breast carcinoma.*

	P8 expression		
	High	Low	Total
Histology			
Non-invasive ductal	7	0	7
Others	23	20	43
		$p=0.0328$	
Cell proliferating activity			
High	5	9	14
Low	25	11	36
		*N.S.	
P27 expression			
High	21	9	30
Low	9	11	20
		N.S.	
Age (years)	53.4 + 11.3	53.0 + 9.8	
		N.S.	
^Menopause			
Pre	12	6	18
Post	14	14	28
		N.S.	
Tumor size			
> 2.0 cm	10	14	24
< 2.0 cm	20	6	26
		$p=0.0200$	
**Histological grade			
III	4	7	11
I, II	19	13	32
		N.S.	
^Lymph node metastasis			
Present	7	8	15
Absent	19	12	31
		N.S.	
UICC Stage			
≥ II	10	15	25
I	20	5	21
		$p=0.0086$	
^Estrogen receptor			
Positive	19	16	35
Negative	7	4	11
		N.S.	
^Progesterone receptor			
Positive	18	13	31
Negative	8	7	15
		N.S.	
Her-2 expression			
High	14	6	20
Low	16	14	30
		N.S.	

*Not significant **Seven intraductal carcinomas were omitted
^Four cases unknown

In this study, we demonstrated that the p8 expression level was high in all cases of non-invasive ductal carcinoma, but was often decreased in invasive ductal carcinoma, regardless of the histological type (17). Furthermore, p8 expression was inversely linked to tumor size and stage. It may therefore be suggested that the p8 protein plays an important role predominantly in the early phase of breast carcinoma progression. A previous *in vitro* study has demonstrated that p8 is induced in cells, which are under stress and receiving pre-apoptotic stimuli (10), indicating that it may act by preventing cells from becoming apoptotic. Indeed, in tumors of 2cm or larger, p8 was inversely linked to the apoptotic index, suggesting that p8 inhibits apoptosis of breast carcinoma cells. Thus, we may regard p8 as a tumor survival factor, similar to heparin-binding type epidermal growth factor-like growth factor (HB-EGF), a ligand of EGF receptor and c-erbB-4 (18, 19). In tumors of less than 2cm in size, the p8 expression level was higher but was not significantly linked to apoptotic index. It is therefore suggested that p8 may have some important functions other than as an apoptotic inhibitor.

The mechanism of cell growth and apoptosis regulation by p8 seems to be different for different cells. In contrast to previous studies using pancreatic acinar cells and some carcinoma cells (10, 11), p8-deficient fibroblasts grew rapidly and were more resistant to chemotherapy-induced apoptosis (20). In breast carcinoma tissues, weak p8 immunoreactivity was occasionally observed in fibroblasts, but the significance is still unclear. As chemotherapy is one of the important strategies for breast carcinoma treatment, we should investigate p8 as a possible future therapeutic target for this carcinoma.

In summary, we demonstrated that p8 plays an important role in the early phase of breast carcinoma progression, especially in tumors of larger size, possibly inhibiting the response of carcinoma cells to apoptotic stimuli.

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