P7 Antigen Expression in Human Breast Cancer¹

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ABSTRACT

Purpose: Evaluate p7 expression in human breast cancer and determine whether chemotherapy and radiation therapy effect a change in p7 expression.

Experimental Design: Using a p7-specific monoclonal antibody with immunohistochemistry and Western immunoblot analyses to assess p7 expression in archival, frozen breast cancer specimens both before and after therapy.

Results: A novel 7 kDa protein (p7), originally identified in multidrug-resistant ovarian and breast cancer cell lines, was found to be expressed in 21 of 64 (32%) primary, unselected human breast cancer specimens by immunohistochemistry. P7 was observed in malignant cells but not in other types of cells in the breast tissue. Western blot analysis confirmed the 7 kDa polypeptide in p7-positive breast carcinomas identified by immunohistochemistry. P7 expression was significantly associated with breast cancers having distant metastasis and/or local recurrence (P = 0.027, Fisher's exact test). In addition, p7 expression was significantly increased in post-treatment breast cancer biopsy specimens compared with pretreatment breast cancer biopsy specimens in patients with locally advanced breast cancer after 5-fluorouracil chemotherapy and radiation therapy [2 of 15 (13%) pretreatment breast cancers compared with 8 of 15 (53%) post-treatment breast cancers; P = 0.016, McNemar's test].

Conclusions: These findings demonstrate that expression of p7 is associated with malignant tumor cells in primary breast cancers, especially those showing recurrent or metastatic disease. Its specific association with the malignant phenotype suggests it may have potential for novel target-based therapies. The markedly increased expression in patients with locally advanced disease after neoadjuvant therapy suggests a role for p7 in treatment outcome.

INTRODUCTION

Breast cancer is a major cause of morbidity and mortality in women in the United States (1). The process of tumor progression has been studied extensively (2–4), but research has not yet effected a significant change in understanding the process or in controlling tumor progression. There is a continuing need to identify new molecules that play a role in tumor development, progression, and treatment outcome of breast cancer patients (5, 6).

The p7 antigen, recognized by a Mab, ID7, was first identified in cancer cell lines (7). Previous in vitro data showed that its expression was limited to cancer cell lines but not cell lines derived from normal or fibroblast tissues tested (7). Additionally, its expression in ovarian cancer cell lines was increased after exposure to vinblastine or adriamycin treatment in vitro. A breast cancer cell line variant, MCF-7 variant, with high levels of p7 expression has been similarly obtained from MCF-7 cells after treatment with doxorubicin or vinblastine. This 7 kDa protein is presumably involved in the proliferation of p7-positive tumor cells because of specific inhibition of cell growth by Mab ID7 (8). In this study, we evaluated p7 expression in human breast cancer specimens, assessed its association with clinicopathologic variables, and evaluated changes in expression in paired biopsies from patients with locally advanced disease after treatment with 5-fluorouracil and radiation.

MATERIALS AND METHODS

Patient Information and Breast Cancer Specimens.

The expression of p7 was investigated in two groups of women with breast cancer. The first group consisted of 64 women diagnosed with breast cancer at Los Angeles County General Hospital between 1992 and 1996 who had surgery by either modified mastectomy (38 patients) or lumpectomy (26 patients). None of these patients received chemotherapy or radiation therapy before surgery. The median age of the 64 patients was 53 years (range, 34–83 years). Pathologic data, including histological type, tumor size, grade (Scarff, Bloom, and Richardson), and stage (T, N, M), were collected. The median clinical follow-up was 1.94 years and ranged from 0.38 to 3.24 years after initial surgery. The frozen specimens from those patients, in-
cluding tumors classified as T_1 to T_4, N_0 to N_3, and M_0 or M_1, were consecutively entered in the University of Southern California Breast Tissue and Tumor Bank. The University of Southern California Institutional Review Board reviewed and approved the collection and analysis of these tissues.

The second group of women with breast cancer consisted of 35 women who were enrolled in a neoadjuvant trial of 5-fluorouracil chemotherapy and radiation therapy to treat locally advanced disease (protocol no. 1B-93-3; Ref. 9). In this experimental protocol, sequential (pre and post-treatment) biopsies or specimens of the breast mass were obtained. From this trial, we evaluated 15 cases as paired samples which contained tumor cells in both the pre and post-treatment specimens. Neoadjuvant treatment involved preoperative continuous infusion of 5-fluorouracil (200 mg/m²/day) with radiotherapy (50 Gy at 2 Gy fractions) to the breast and regional lymph nodes. These patients had involvement of axillary lymph nodes without evidence of distant metastasis. The biopsies were frozen immediately after surgery and stored in liquid nitrogen until assays were performed. This group of women had a median age of 50 years (range of 31–64 years).

**Cell Lines.** The p7-positive ovarian cancer cell line OVCAR 4/Adr, breast cancer cell line MDA-MB-231, and p7-negative breast cancer cell line SKBR-3 were cultured, as described elsewhere, and used as controls for either immunohistochemistry or Western blot (7).

**1D7 Mab.** The production of 1D7 Mab, which recognizes p7, has been described previously (7). 1D7 antibody was used either as hybridoma supernatant or as purified immunoglobulin by precipitation with ammonium sulfate and DEAE-matrix chromatography.

**Immunohistochemical Detection of p7 in Human Breast Cancer.** The peroxidase antiperoxidase immunohistochemical technique was used to localize p7 in breast cancer specimens (10, 11). Cryostat sections (6 μm) were prepared and fixed with 4% formaldehyde at room temperature for 5 min. The sections were then washed in PBS and treated with 0.5% H2O2-PBS for 15 min to inactivate the endogenous peroxidase activity. 1D7 antibody (1:5 dilution of hybridoma supernatant or 5 μg/ml purified antibody) was applied to the sections for 1 h in 10% rabbit serum PBS. Specimens were washed in PBS three times for 3 min each. Rabbit antimouse IgG-bridging antibody (anti-H+L chains; Zymed, South San Francisco, CA) with a dilution of 1: 50 in 10% rabbit serum PBS was applied to the tissue section for 30 min. After washing, peroxidase mouse antiperoxidase antibody complexes (1:50 dilution) were applied for 30 min. 3,3'-diaminobenzidine was used to identify the sites of immunoprecipitate formation. The preparations were counterstained with ethyl green, mounted in Permount, and coverslipped. The section from OCT-embedded OVCAR-4/Adr cells was cut and stained as a positive control. Negative controls for each sample were performed as described above but with normal mouse immunoglobulins (Zymed) instead of primary antibody. The histology of each specimen was confirmed by review of one section stained with H&E. All slides were evaluated by two of the authors (X. Y. and M. F. P.) without knowledge of the clinical data. The pattern of staining was described as focal when <20% of tumor cells were immunostained or diffuse when ≥20% of tumor cells were immunostained. The intensity of staining was scored as: 0 = no staining, 1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining.

**Western Blot Analysis of p7.** Breast cancer tissues (~200 mg) were homogenized with a motor-driven homogenizer in 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), containing 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 150 mM NaCl, 2 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 10,000 × g for 20 min at 4°C. Supernatants were measured by spectrophotometry at A₂₈₀nm for protein quantitation and stored at −20°C until processed. Methanol-extracted proteins were similarly prepared with the following changes: breast cancer tissues or cells (washed twice with PBS) were homogenized in 0.5 ml of methanol and centrifuged at 10,000 × g for 20 min at 4°C. The supernatants were evaporated in a Speed-Vac (Savant, Farmingdale, NY) until a volume of 50 μl remained. Proteins were solubilized with SDS-containing sample buffer, and electrophoresis was carried out using 8–25% polyacrylamide gradient gels using PhastSystem Separation Unit. After electrophoresis, the proteins were transferred to nitrocellulose membrane using the PhastSystem Transfer Unit (Pharmacia, Piscataway, NJ). All subsequent steps were carried out at room temperature. Nitrocellulose was washed briefly with 0.05% Tween 20 PBS after the protein-transferring step and blocked with 10% rabbit serum in Tween 20 PBS solution for 1 h. The blot was then incubated for 1 h either with 10 μg/ml of the purified antibody in PBS containing 10% rabbit serum or 1D7 tissue culture supernatant. After washing, the nitrocellulose blot was incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (DAKO Corp., Carpinteria, CA) diluted at 1: 500 in 10% rabbit serum PBS and thereafter washed extensively. The immunoreactive proteins were detected by an enhanced chemiluminescence method as recommended by the manufacturer or by revealing the band with 3,3'-diaminobenzidine solution.

**Statistical Analysis.** Fisher’s exact test (two tails) was used to assess the association of p7 expression with distant metastases and local recurrence (presence of tumor cells in the ipsilateral breast >6 months after surgery) or other clinicopathologic variables. For the purpose of analysis, specimens with diffuse staining pattern were considered to be positive in primary breast cancer. McNemar’s test was used to assess changes in p7 expression, including both focal and diffuse staining patterns in paired pre and post-therapy samples in patients with locally advanced breast cancer.

**RESULTS**

**Effects of Various Fixatives on p7 Immunostaining.** To determine the optimal immunostaining conditions for 1D7 anti-p7 antibody, we first assessed the effects of various fixatives on p7 immunohistochemistry. We found that there were substantial differences in 1D7 antibody immunoreactivity with different fixatives tested. The strongest immunostaining was identified either with 4% formaldehyde or acetone fixation. No antibody immunoreactivity was identified with methanol or with 95% ethanol fixation. Only weak staining was seen with 50% ethanol fixation. The number of tumor cells stained with picric acid paraformaldehyde fixation was similar to that obtained with...
Fig. 1 Right panel, immunohistochemical analysis of p7 antigen in primary (A–C) and locally advanced (D and E) breast cancers. The expression pattern detected by Mab, 1D7, is either focal (A) or diffuse (C). Note the positive staining in invasive breast cancer cells but not in other histological architectures in the breast, such as stroma, stromal cells, benign lesions, lymphocytes, and/or macrophages (B). The change in p7 expression is demonstrated by immunohistochemistry in samples collected from pre- (D) and post- (E) radiation and 5-fluorouracil treatment in a patient with locally advanced breast cancer, showing increased p7 expression after treatment. Left panel, H&E-stained sections (A1–E1) corresponding to immunohistochemically stained sections (A–E) in the right panel.
formaldehyde or acetone fixation, but the intensity of staining in individual cells was not as strong as that with formaldehyde fixation. Thus, formaldehyde was selected as the fixative for the immunohistochemical staining.

Expression of p7 in Human Primary Breast Cancers. To determine whether p7 is expressed in human breast cancer, we examined 64 unselected primary invasive carcinomas of the breast. Among these untreated breast cancers, p7 was identified in 21 of the 64 (32%) specimens by immunohistochemistry using the p7-specific Mab 1D7. The focal pattern was found in 6 of the 64 (9%) tumors and the diffuse pattern was found in 15 of the 64 (23%) tumors (Fig. 1, A and C). This protein was localized in both the cytoplasm and plasma membrane of tumor cells of infiltrating ductal and lobular carcinomas. However, it was not found in adjacent benign breast ductal or lobular epithelium, stromal cells, endothelial cells of blood vessels, lymphocytes, or macrophages (Fig. 1B). Thus, p7 is expressed in a significant fraction of primary breast cancers and predominantly distributed in carcinoma cells but not in other types of cells, indicating its association with the malignant phenotype.

Association of p7 with Clinicopathologic Variables. We next analyzed p7 expression in association with clinical outcome or disease characteristics. In this series of tumors, 10 of the 55 (18%) p7-negative breast carcinomas and 5 of the 9 (56%) p7-positive breast carcinomas manifested either distant metastases or local recurrence (P = 0.027, Fisher’s exact test), suggesting a potential role of p7 in tumor spread. A trend for the presence of p7 in ductal and lobular carcinoma of the breast but not in other types of breast cancer (15 of 58, 26% versus 0 of 6, 0%) was noted (P = 0.322). Advanced disease stage (III/IV) was found in 5 of the 15 (33%) p7-positive cases but in only 7 of the 43 (16%) p7-negative cases (P = 0.279). There was also no statistically significant association of p7 expression with tumor size (P = 1), lymph node involvement (P = 1), estrogen or progesterone receptor status (P = 0.49), Her-2/neu expression (P = 0.34), or p53 protein expression (P = 1).

Changes in p7 Expression in Paired Biopsies Taken Before and After Neoadjuvant Treatment. To test whether p7 expression may be altered after radiation therapy and chemotherapy, the 15 paired frozen tumor specimens (see “Materials and Methods”) were examined for p7 expression. To facilitate comparison, frozen sections from each paired tumor specimen were always analyzed at the same time. The expression of p7 was identified in two (2 of 15, 13%) breast cancers before therapy and in eight (8 of 15, 53%) breast cancers after therapy (Table 1). p7 expression was markedly increased in terms of immunostaining intensity and percentage of tumor cells immunostained in one case (No. 7; Table 1) after radiation therapy and 5-fluorouracil chemotherapy (Fig. 1, D and E). In six other cases, the initial tissue samples were negative for p7, whereas the post-treatment specimens were positive for p7 expression. Finally, tumors from one case (No. 1) displayed similar p7 expression in both pre and post-treatment tissues. Overall, the change in p7 expression reached statistical significance (P = 0.016, McNemar’s test), suggesting a potential role for p7 in neoadjuvant treatment outcome. As shown in Fig. 1, D1 and E1, the size or morphology of these tumor cells after treatment is reduced or changed, but the localization of p7 did not change (Fig. 1, D and E).

Immunoblot Confirmation of 7 kDa Protein in Breast Cancer. To confirm that the immunohistochemical staining seen with 1D7 was specific for the 7 kDa p7 protein, proteins extracted from breast cancer tissues were subjected to Western blot analysis. Both detergent-solubilized breast tissue lysates (Fig. 2A) and methanol-extracted lysates (Fig. 2B) were analyzed. A Mr 7000 Dalton band was detected in samples from known p7-positive OVCAR4/Adr cells and breast cancers that stained positive for p7 by immunohistochemistry. As shown in Fig. 2A, the 7 kDa band was relatively weak in breast cancer samples (Lane 2) compared with positive control cells (Lane 1), and the signal in one case was absent in detergent-treated lysates (Lane 3). However, p7 bands were clearly observed in all methanol-extracted samples (Fig. 2B). These data indicate that p7 protein identified by immunohistochemistry with the use of Mab 1D7 has a molecular mass of 7 kDa. Using methanol to extract breast tissue provides a reliable means of obtaining p7.

To ensure the specificity of 1D7 antibody and validate the use of methanol-extraction to obtain 1D7 immunoreactive materials, equal numbers of MDA-MB-231 (p7 positive) and SKBR-3 cells (p7 negative) were subjected to extraction by methanol (see “Materials and Methods”). The 1D7 antibody solutions were absorbed with the cell extracts of MDA-MB-231 (10^8 cells) and SKBR3 (10^8 cells) human breast cancer cells, respectively, and were used for Western analysis. As shown in Fig. 2D, little signal was observed when 1D7 was treated with the methanol-extracted lysates from MDA-MB-231 cells, whereas a p7 band was identified, as expected, when 1D7 was incubated with the extracted lysates from SKBR-3 cells. Methanol extraction provided a reliable means of p7 detection by Western immunoblot in breast cancer tissue.

**DISCUSSION**

We have demonstrated the expression of a novel Mr 7 kDa protein in human breast cancer. Its expression was found in 21

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of 64 (32%) invasive breast carcinomas and associated with only the malignant carcinoma cells. This protein was found in 20 ductal breast carcinomas and 1 mixed ductal and lobular carcinoma. However, no immunostaining was found in breast cancer with other histologies, including mucinous and medullary carcinomas. These types are generally associated with a good prognosis because of less aggressive behavior and less tendency to metastasize (12). The expression of p7 was associated significantly with the distant metastases and local recurrences when ≥20% of the primary breast cancer tumor cells expressed p7.

Both focal and diffuse p7 immunostaining patterns were observed in breast cancer specimens. There are two possible explanations for focal expression of p7 in breast cancer. First, cancer cell populations in breast cancer may be derived from different origins. Some tumor cells may be inherently capable of expressing p7, whereas others may not, indicating heterogeneity of expression in breast tumors. The other possibility is that p7 may be present only at certain stages during the process of tumor progression or at certain phases of the cell cycle.

The proportion of breast cancers showing expression of p7 was increased from 13 to 53% after local breast radiation treatment and 5-fluorouracil chemotherapy. Whether the increased p7 expression was directly related to treatment or simply an epiphenomenon needs further study.

Western immunoblot analysis was used to confirm the immunohistochemical findings. With detergent-treated lysates of breast cancer tissue, we detected a relatively weak signal from p7-expressing specimens that displayed a diffuse staining pattern. For one p7-positive case, we were unable to detect the 7 kDa band by analyzing detergent-treated lysate, suggesting that p7 antigen may specifically or nonspecifically attach to some detergent nonsoluble components of the breast tissue during protein lysate preparation. Through the observations that methanol or ethanol can abolish the immunoreactivity of antibody 1D7, we speculated that either these solvents destroyed the epitope recognized by the antibody or these hydroxyl-containing solvents extracted p7. Our results demonstrated that these organic solvents extracted p7. The extracted p7 could be solubilized with SDS-containing sample buffer and transferred to nitrocellulose membrane, thus providing a reliable method for detection of p7 by immunoblot.

In summary, we have demonstrated that a 7 kDa protein is expressed in human breast cancer whose expression is associated with tumor spread in primary breast cancer and markedly increased in patients with locally advanced disease after neoadjuvant treatment. In addition, its association with tumor cells and not with normal cells makes it a potential target for antibody-based treatment or other types of target-based approaches. Further study of this novel molecule in breast cancer may lead to a better understanding of breast tumor biology that may provide new insights into determinants of treatment response and tumor metastasis.

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