Expression of pS2, c-erbB-2, and Cathepsin D During the Menstrual Cycle in Human Breast Cancers

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Background: Many studies have addressed the effect of the timing of surgery for breast cancer relative to menstrual cycle phase, with conflicting results. Explanations for the possibility that survival could be altered by the appropriate timing of breast cancer surgery in humans remain speculative.

Methods: We examined the expression of three estrogen related proteins (c-erbB-2, cathepsin D, pS2) in the breast tumors from 69 premenopausal women sampled in different phases of the menstrual cycle. Data on S-phase fraction and hormone receptor expression were also analyzed. Immunohistochemical assays were used to measure the proteins of interest. S-phase fraction was determined by flow cytometry. Analyses were performed based on fraction of cells staining positive for the protein, density of stain, and a histoscore that combined both fraction of positive cells and density.

Results: We found no differences in c-erbB-2, cathepsin D, hormone receptor, or S-phase levels in tumors sampled in the follicular versus luteal phase, or perimenstrual versus periovulatory phase. The exception was pS2, which was expressed at greater levels during the luteal than during the follicular phase of the cycle (p < 0.01); but there was no difference in pS2 expression when the patients were classified as periovulatory versus perimenstrual.

Conclusions: Our findings do not support a variation in c-erbB-2, cathepsin D, S-phase fraction, or receptor expression as an explanation for the differences in breast cancer prognosis when surgery is timed by menstrual cycle phase. The finding that pS2 (an indicator of hormone sensitivity, and possibly better prognosis) is expressed at higher levels in tumor samples during the luteal phase suggests that the biologic profile of breast tumors may vary with the menstrual cycle and that these variations deserve further study.

Key Words: Breast cancer—Menstrual cycle—pS2—Cathepsin D—c-erbB-2.
petus for a randomized trial. The prognostic proteins we chose to measure were pS2, an estrogen-inducible protein that may be a favorable prognostic marker (18); cathepsin D, an estrogen-inducible proteolytic enzyme involved in the metastatic process (19,20); and c-erbB-2, a marker of poor prognosis (21-23), the expression of which in breast cancer cells is inhibited by estradiol (24,25). In addition, we examined the proportion of cells in S phase (S-phase fraction, SPF) as a marker of proliferation because this too is an acknowledged prognostic factor (26,27) that may fluctuate in breast cancer cells in response to ambient estrogen levels.

METHODS

Patient Population

All patients were seen at the Comprehensive Breast Care Program of the State University of New York Health Science Center at Syracuse. Inclusion criteria for the study were as follows: premenopausal women with regular menstrual cycles, a diagnosis of invasive breast cancer, date of last menstrual period prior to cancer biopsy known, and sufficient material remaining in paraffin blocks for study. Sixty-nine women met these criteria. They were divided into groups by menstrual cycle phase using two methods: first, into follicular and luteal phase groups using the known date of the last cycle, and the usual cycle length (ovulation being presumed to occur 14 days before the expected next period). Second, the patients were divided into periovulatory and perimenstrual groups, the periovulatory phase occupying days 7 to 20 of the cycle and the perimenstrual phase days 21 to 6. The usual length of the cycle was unknown for 10 women and was assumed to be 28 days. Medical records were examined to retrieve pathologic data such as TNM status, pathologic grade, or presence of residual disease in the mastectomy or reexcision specimen.

Immunohistochemistry

Tumor samples for analysis were obtained from paraffin blocks of the tumors. Blocks were obtained for the initial excisional biopsy specimens in all patients, although 26 of 69 patients (38%) had residual disease, usually microscopic, at the time of a subsequent definitive procedure. Paraffin sections were immunostained for pS2, cathepsin D, and c-erbB-2. Monoclonal antibodies to c-erbB-2, pS2, and cathepsin D (BioGenex, San Ramon, CA, USA) were used to label paraffin sections using the BioGenex MultiLink Super Sensitive HRP kit. After deparaffinization and hydrogen peroxide block, the sections were incubated as follows: mouse anti-pS2 (clone pS2.1) at 1:40 dilution for 2 h at room tempera-ture; mouse anti–cathepsin D (clone C5) at 1:40 dilution for 2 h at 37°C; and mouse anti-c-erbB-2 (clone CB11) at 1:40 dilution for 30 min at room temperature. Predicted biotinylated anti-immunoglobulins were applied for 30 min at room temperature, then labeled with prediluted horseradish peroxidase–conjugated streptavidin, followed by diaminobenzidine as a chromagen, and a hematoxylin counterstain. Negative controls were processed for each section, using control serum rather than primary antibody. Nonspecific staining was not seen on any of the negative control sections. Positive controls were run with each batch of processed slides and consisted of breast cancer sections that had stained strongly positive for the protein in question in previous experiments. Two sections from different blocks were processed on 12 patients, and immunostaining results were similar between two different sections in these patients. Positivity for all proteins studied was confined to epithelial cells. No stromal cell staining was identified (Fig. 1).

Evaluation of histochemical results was completely blinded to all information regarding the tumors; this included all identifying information, data on tumor stage, and, most importantly, data on menstrual cycle phase. Immunohistochemical findings were recorded as categories of percentage positive cells: 0, 0% to 5% cells stained; 1+, 5% to 25% cells stained; 2+, 26% to 50% cells stained; 3+, 51% to 75% cells stained; and 4+, 76% to 100% cells stained. The density of the stain was similarly recorded, ranging from 1+ to 3+. Scores for each of the proteins studied were calculated as the product of the two categorical scores (fraction of positive cells x stain density). The scores ranged from 0 to 12. To assess intraserver variability, 21 randomly chosen sections (10% of the total) were rescored, with blinding to the original score. The rescorer was concordant with the original assigned score in 19 sections. Two erbB-2 scores for positive cell fraction were reread as 0 rather than 1+ based on cytoplasmic rather than true cell membrane localization, and one pS2 score for stain density was reread as 3+ rather than 2+. The reported analyses are bases on the original scores, and not on the random rereadings.

Flow Cytometry

Tumor blocks were screened to identify tumor-rich areas; selected areas contained at least 30% tumor cells. Microdissection was not routinely performed. Fifty-micron thick paraffin sections were used to extract nuclei for propidium iodide staining and flow cytometry as previously described (28). Briefly, sections were deparaffinized, rehydrated, treated sequentially with pepsin, trypsin, and RNase, and stained with propidium iodide.