Journal of Clinical Laboratory Analysis 13:241–245 (1999)

Development and Evaluation of a Competitive Time-Resolved Immunofluorometric Assay for the Estrogen-Regulated Protein pS2

Rachel S. Rosenberg Zand,[1,2] David J.A. Jenkins,[1] and Eleftherios P. Diamandis[2,3]*

[1]*Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada*
[2]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada*
[3]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*

We have developed a competitive assay to measure the estrogen-regulated protein pS2. A monoclonal pS2 antibody (mAb) and a biotinylated pS2 peptide are used, with time-resolved fluorometry as a detection technique. The assay has a detection limit of 16 ng/mL and is precise (within-run and day-to-day CVs 3–12%). We used this assay to determine steroid hormone activity of six steroids in cell culture, both in terms of time course and dose response. pS2 concentrations in the tissue culture supernatant of the BT-474 breast carcinoma cell line were significantly higher when estradiol was the stimulating steroid. There was a significant time course and dose response observed for estradiol, but not for the other steroids. The availability of a sensitive, reliable, and convenient method for quantifying pS2 will allow for many research applications including the screening of natural and synthetic compounds for putative estrogenic activity. J. Clin. Lab. Anal. 13:241–245, 1999.

Key words: steroid hormone receptors; estrogen-regulated genes; pS2; estrogenic activity; immunofluorometric assay; time-resolved fluorometry

INTRODUCTION

pS2 is a secreted (1), estrogen-regulated protein (2), classified as a member of the trefoil family of proteins (3). It is expressed in normal tissue, primarily in the stomach (4,5), but is also found in normal breast epithelium and other sites (6,7). Like human intestinal trefoil factor (hITF) and human spasmolytic polypeptide (hSP), the other two family members, the functions of pS2 have not been completely resolved. However, current research is focusing on its motogenic capabilities and its role in tissue healing and repair (8–12).

pS2 was first discovered in MCF-7 breast cancer cells by virtue of its estrogen-controlled regulation (2). The presence of this protein in breast tumors has been shown to be a favorable prognostic indicator (13). It is associated in many studies with estrogen (ER) and progesterone receptors (PR) (13). pS2 has also been used in vitro to measure estrogenic activity of natural and synthetic compounds (14). To date, pS2 has been measured with Northern blot (15), immunohistochemistry (16), and with immunoradiometric (IRMA) assays (17). To our knowledge, only one IRMA-type assay is currently commercially available for this interesting protein.

The scarcity of quantitative assays for pS2 protein suitable for analysis of tissue culture supernatants and other biological samples prompted us to develop this method which we then applied to practical applications. By using the developed assay we here show the mode of regulation of pS2 protein by steroid hormones in the breast carcinoma cell line BT-474.

MATERIALS AND METHODS

BT-474 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). All steroids were from Sigma Chemical Co. (St. Louis, MO). Stock—10^{-3} M—solutions of steroids were prepared in absolute ethanol. The secondary antibody (sheep antimouse IgG, SAMIg) was purchased from Jackson ImmunoResearch (Westgrove, PA). The pS2 mouse monoclonal antibody (catalog no. MS-111-PABX) was obtained from NeoMarkers (Union City, CA). pS2 peptide was a gift from Dr. Atul Tandon. The sequence of the peptide, which represents 28 amino acids of the carboxyterminus of native pS2 is NH_2-KGCCFDDTVRGVPWCFYPNTIDVPPEEE.

Diflunisal phosphate (DFP) was synthesized in our labo-

*Correspondence to: Eleftherios P. Diamandis, M.D., Ph.D., F.R.C.P.C., Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. E-mail: ediamandis@mtsinai.on.ca

Received 10 May 1999; Accepted 31 May 1999

ratory. The stock solution of DFP was 0.01 mol/L in 0.1 mol/L NaOH. Alkaline phosphatase-labeled streptavidin (SA-ALP) was obtained from Jackson ImmunoResearch as a 1-g/L solution. Working SA-ALP solutions were prepared by diluting the stock solution 20,000-fold in a bovine serum albumin (BSA) diluent (described below). White, opaque, 12-well microtiter strips were obtained from Dynatech Labs (Alexandria, VA). The substrate buffer was a Tris buffer (0.1 mol/L, pH 9.1) containing 0.1 mol of NaCl and 1 mmol of $MgCl_2$ per liter. The substrate working solution (DFP, 1mmol/L in substrate buffer) was prepared just before use by diluting the DFP stock solution 10-fold in the substrate buffer. The SA-ALP diluent was a 60-g/L solution of BSA in 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g/L sodium azide. The wash solution was prepared by dissolving 9 g of NaCl, and 0.5 g of polyoxyethyenesorbitan monolaurate (Tween 20) in 1 L of a 10 mmol/L Tris buffer, pH 7.40. The developing solution contained 1 mol of Tris base, 0.4 mol of NaOH, 2 mmol of $TbCl_3$, and 3 mmol of EDTA per liter (no pH adjustment).

Instrumentation

A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, Ontario), was used to measure Tb^{3+} fluorescence in white microtiter wells. The procedure has been described in detail previously (18–20).

pS2 Standard Solutions

MCF-7 breast cancer cells were grown to confluency in 75-mL flasks with 50 mL of media (phenol-free RPMI with 10% fetal bovine serum, 10 mg/L insulin, 200 mmol/L L-glutamine (Gibco BRL, Gaithersburg MD). The flasks were stimulated with 10^{-8} mol/L estradiol and incubated for 8 days at 37°C, 5% CO_2. The supernatants were then harvested and pooled. This stock solution was calibrated as described below.

Procedures

Coating of microtiter plates with pS2 mAb

We coated polystyrene microtiter wells by incubating overnight 500 ng/100 µL per well of sheep-antimouse immunoglobulin (SAMIg) in a 50-mmol/L Tris buffer, pH 7.80. The wells were then washed six times with the wash solution and incubated for 2 hr with 100 µL per well of pS2 mAb diluted 1:4,000 (25 ng/100 µL) in 6% BSA. The strips were then washed another six times.

Biotinylation of pS2 peptide

Biotinylation was performed with sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-LC-Biotin) obtained from Pierce Chemical Co., Rockford, IL. Approximately 1 mg of NHS-LC-LC-Biotin was reacted with 200 µL of the peptide in 1 mL of a 0.5 M carbonate buffer, pH 9.1. After biotinylation for 1 hr at room temperature, the peptide was stored at 4°C and used without any further purification. The concentration of the stock biotinylated peptide solution was 200 µg/mL.

pS2 calibrators

MCF-7 supernatants were analyzed for pS2 concentration using an immunoradiometric kit (IRMA) (Cis-BIO, Gif-Sur-Yvette Cedex, France). pS2 calibrators of 0, 62, 125, 250, 500 and 1,000 ng/mL were prepared by diluting the standard stock in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of sodium azide per liter.

pS2 assay

Calibrators or samples (100 µL) were pipetted into coated microtiter wells and 50 µL of biotinylated peptide diluted 20,000-fold in 6% BSA was added (approximately 0.5 ng of peptide per well). The plates were incubated with mechanical shaking for 1 hr at room temperature and then washed six times. To each well we then added 100 µL of SA-ALP conjugate diluted 20,000-fold in the SA-ALP diluent (approximately 5 ng of conjugate per well), incubated for 15 min as described above, and then washed six times. To each well we then added 100 µL of the 1 mmol/L DFP working substrate solution and incubated for 10 min as described above. We added 100 µL of developing solution to each well, mixed by mechanical shaking for 1 min, and measured the fluorescence with the time-resolved fluorometer essentially as described elsewhere (21). The calibration curve and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

Tissue Culture Experiments With Cell Lines

BT-474 breast cancer cells, positive for estrogen, progesterone, and androgen receptors were grown to confluency in phenol-free RPMI media supplemented with 10% fetal calf serum, insulin, and L-glutamine at 37°C, 5% CO_2. They were then transferred to 24-well microtiter plates (Corning no. 25280), where they were grown to confluency in media containing charcoal-stripped fetal calf serum instead of the regular fetal calf serum. They were stimulated with either estradiol, aldosterone, dihydrotestosterone (DHT), dexamethasone, norgestrel, mibolerone at 10^{-7} M, or alcohol. They were incubated for 7 days, at which time their supernatants were harvested and measured for pS2 concentration.

Time Course Study

The same protocol as above was used, with the exception of the incubation period being 1, 3, or 5 days.

Dose-Response Study

BT-474 cells were grown and plated as above. Cells were then stimulated with each one of six steroids at 10^{-12}

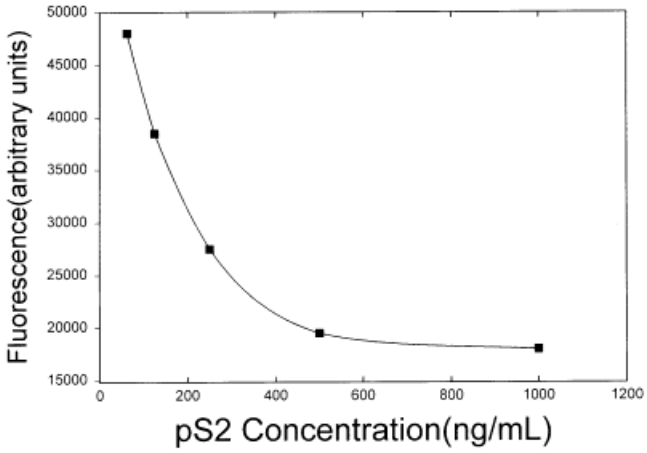

Fig. 1. Typical calibration curve of the pS2 assay.

to 10^{-7} M, or alcohol (control) and incubated for 7 days. At this time, supernatants were harvested and analyzed for pS2 concentration.

RESULTS

Antibody Selection and Assay Optimization

Two mouse monoclonal antibodies and one rabbit polyclonal antibody were initially evaluated to develop the pS2 immunofluorometric assay. Among all possible assay configurations, as described elsewhere (22), we found that indirect immobilization of the primary antibody and use of the monoclonal antibody described above, along with a one-step protocol, gave the best overall results. Other variables, including incubation times and amounts of reactants, were optimized with a previously published optimization strategy (22). The final conditions are described in "Materials and Methods."

Calibration Curve, Detection Limit, and Precision

A typical calibration curve of the proposed assay is shown in Figure 1. The detection limit, defined as the concentration of pS2 detected with a precision of 20%, is 16 ng/mL. Within-run and between-run precision was assessed at various pS2 concentrations of tissue culture supernatants between 30 and 300 ng/mL. CVs ranged from 3 to 12% within this range, which are typical for this type of assay.

Tissue Culture Experiments

In Figure 2 we present data showing conclusively that the pS2 protein expression is regulated by estrogens only. All other tested steroids had no detectable effect on pS2 regulation. Furthermore, as shown in Figure 3, pS2 secretion and accumulation into the tissue culture supernatant is time-dependent following stimulation with estradiol. The effect of estradiol was dose-responsive (Fig. 4). The effect of estradiol on pS2 upregulation is evident at estradiol concentrations equal or higher than 10^{-11} M.

DISCUSSION

We have developed a simple, convenient, and sensitive competitive immunoassay for quantifying pS2 protein. Unlike Northern blotting and immunohistochemistry, this method

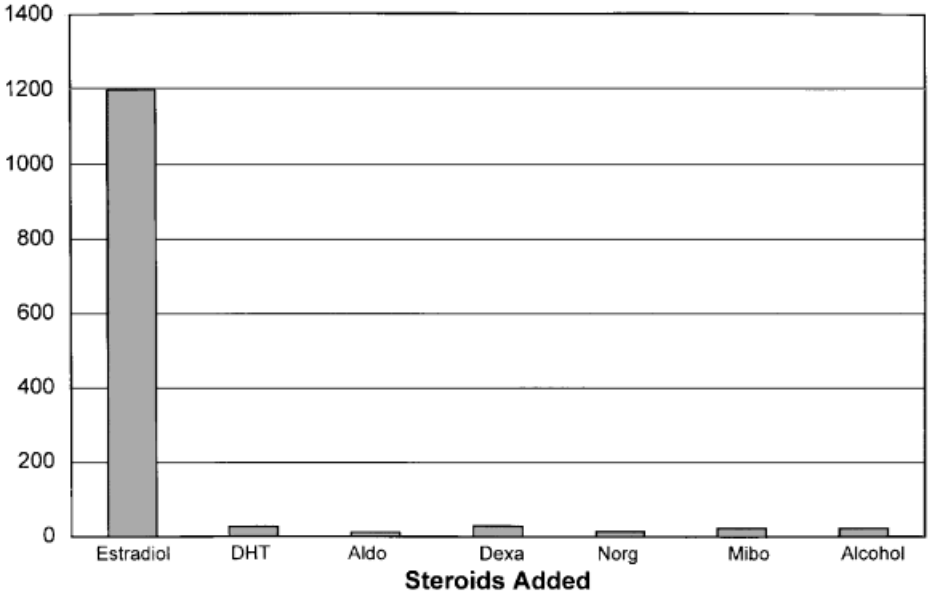

Fig. 2. pS2 production by six steroids and alcohol (control). All steroids were used at a concentration of 10^{-7} M. DHT, dihydrotestosterone; Aldo, aldosterone; Dexa, dexamethasone; Norg, norgestrel; Mibo, mibolerone.

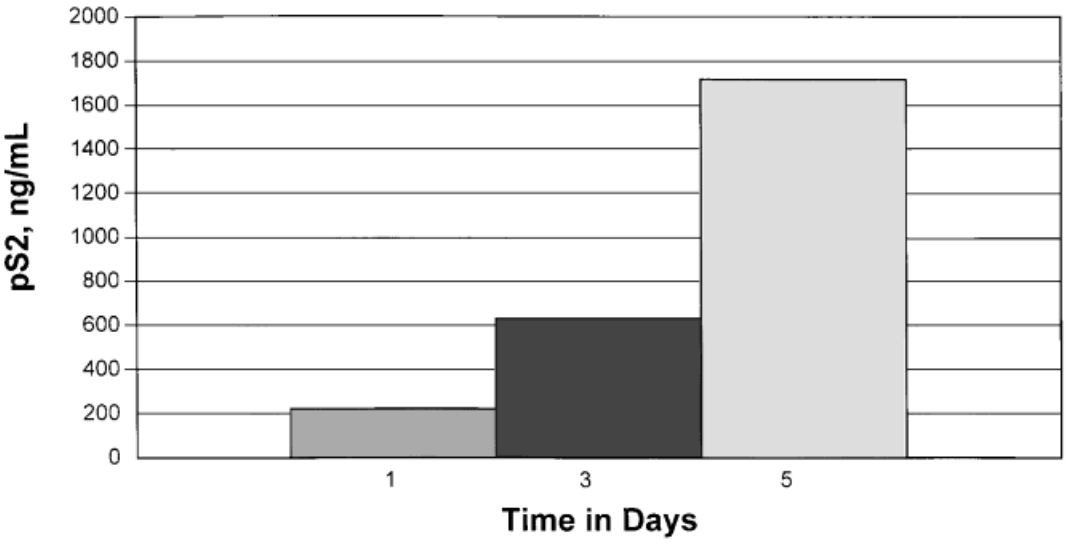

Fig. 3. Time course of pS2 production by estradiol at a concentration of 10^{-7} M. Tissue culture supernatants were analyzed after collection post-simulation, at the days specified.

allows for secreted or extracted pS2 to be measured in tissue culture supernatants and possibly in other fluids. The method is efficient because about 42 samples per plate can be analyzed in duplicate within 2–3 hours.

pS2 has been demonstrated here and in previous studies to be an excellent marker of estrogenic activity. Estradiol significantly upregulated pS2 production among all of the steroids tested. pS2 production was shown to be suppressed by dexamethasone in other studies (23), as measured by Northern blot. However, this was not evaluated in the present study. Furthermore, pS2 expression was shown here to be both time- and estradiol dose-dependent.

A number of clinical applications have already been realized for pS2. This protein is associated with ER and PR presence

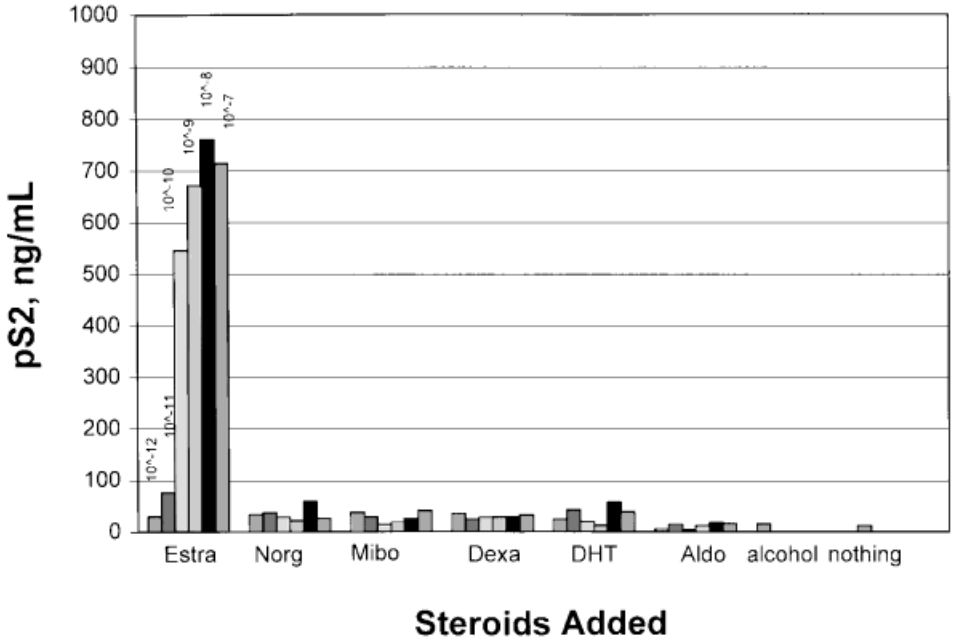

Fig. 4. Dose response of steroids at concentrations between 10^{-7} and 10^{-12} M. For abbreviations see Figure 2. Supernatants were analyzed 7 days poststimulation.

in breast tumors (13), it has been shown that pS2 is a predictor of response to tamoxifen treatment (24), and patients with pS2-positive breast tumors show greater relapse-free (13), progression-free, and overall survival rates (25,26). In patients with intermediate levels of ER and PR, pS2 has been able to predict tamoxifen effectiveness (27), allowing for therapy decisions to be made.

Research applications of pS2 analysis are now emerging in the literature. This protein has been used as a marker of estrogenic activity for phytoestrogens and xenoestrogens, compounds with estrogen-like structures found in plant foods and in the environment (15,17). We intend to use this assay for such applications.

In conclusion, we have described a simple, quantitative assay for pS2 protein and have applied it to study the mode of regulation of the pS2 gene by steroid hormones in the breast carcinoma cell line BT-474. Previously, we have shown that in breast cancer cell lines, the PSA gene is upregulated by androgens and progestins (28). The combined use of pS2 and PSA as markers of hormonal activity in BT-474 cells may allow us to assess the biological activity of natural and xenobiotic compounds which are currently considered as putative endocrine disruptors. These studies are now in progress.

REFERENCES

1. Kida N, Yoshimura T, Mori K, Hayashi K. Hormonal regulation of synthesis and secretion of pS2 protein relevant to growth of human breast cancer cells (MCF-7). Cancer Res 1989;49:3494–3498.
2. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res 1982;10:7895–7903.
3. Plaut AG. Trefoil peptides in the defense of the gastrointestinal tract. N Engl J Med 1997;336:506–507.
4. Suemori S, Lynch-Devaney K, Podolsky DK. Identification and characterization of rat intestinal trefoil factor: tissue- and cell-specific member of the trefoil protein family. Proc Natl Acad Sci U S A 1991; 88:11017–11021.
5. Rio MC, Bellocq JP, Daniel JY, et al. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. Science 1988;241:705–708.
6. Hanby AM, Poulsom R, Singh S, Elia G, Jeffery RE, Wright NA. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. Gastroenterology 1993;105:1110–1116.
7. Piggott NH, Henry JA, May FE, Westley BR. Antipeptide antibodies against the pNR-2 oestrogen-regulated protein of human breast cancer cells and detection of pNR-2 expression in normal tissues by immunohistochemistry. J Pathol 1991;163:95–104.
8. Dignass A, Lynch-Devaney K, Kindon H, Thim L, Podolsky DK. Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. J Clin Invest 1994;94:376–383.
9. Williams R, Stamp GW, Gilbert C, Pignatelli M, Lalani EN. pS2 transfection of murine adenocarcinoma cell line 410.4 enhances dispersed growth pattern in a 3-D collagen gel. J Cell Sci 1996; 109:63–71.
10. Playford RJ, Marchbank T, Calnan DP, et al. Epidermal growth factor is digested to smaller, less active forms in acidic gastric juice. Gastroenterology 1995;108:92–101.
11. May FE, Westley BR. Trefoil proteins: their role in normal and malignant cells. J Pathol 1997;183:4–7.
12. Wright NA, Poulsom R, Stamp GW, et al. Epidermal growth factor (EGF/URO) induces expression of regulatory peptides in damaged human gastrointestinal tissues. J Pathol 1990;162:279–284.
13. Foekens JA, Rio MC, Seguin P, et al. Prediction of relapse and survival in breast cancer patients by pS2 protein status. Cancer Res 1990; 50:3832–3837.
14. Rio MC, Bellocq JP, Gairard B, et al. Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. Proc Natl Acad Sci U S A 1987;84:9243–9247.
15. Hsieh CY, Santell RC, Haslam SZ, Helferich WG. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. Cancer Res 1998;58:3833–3838.
16. Jansen RL, Hupperets PS, Arends JW, et al. pS2 is an independent prognostic factor for post-relapse survival in primary breast cancer. Anticancer Res 1998;18:577–582.
17. Zava DT, Duwe G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr Cancer 1997;27:31–40.
18. Diamandis EP. Immunoassays with time-resolved fluorescence spectroscopy: principles and applications. Clin Biochem 1988; 21:139–150.
19. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. Anal Chem 1992;64:342–346.
20. Diamandis EP. Multiple labeling and time-resolvable fluorophores. Clin Chem 1991;37:1486–1491.
21. Ferguson RA, Yu H, Kalyvas M, Zammit S, Diamandis EP. Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. Clin Chem 1996;42:675–684.
22. Diamandis EP, Christopoulos TK, Khosravi MJ. Development of in-house immunological assays. In: Diamandis EP, Christopoulos TK, editors. Immunoassay. San Diego: Academic Press; 1996. p 555–568.
23. May FE, Westley BR. Expression of human intestinal trefoil factor in malignant cells and its regulation by oestrogen in breast cancer cells. J Pathol 1997;182:404–413.
24. Soubeyran I, Quenel N, Coindre JM, et al. pS2 protein: a marker improving prediction of response to neoadjuvant tamoxifen in post-menopausal breast cancer patients. Br J Cancer 1996;74:1120–1125.
25. Foekens JA, van Putten WL, Portengen H, et al. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol 1993;11:899–908.
26. Gion M, Mione R, Pappagallo GL, et al. PS2 in breast cancer—alternative or complementary tool to steroid receptor status? Evaluation of 446 cases. Br J Cancer 1993;68:374–379.
27. Foekens JA, Portengen H, Look MP, et al. Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer. Br J Cancer 1994;70:1217–1223.
28. Zarghami N, Grass L, Diamandis EP. Steroid hormone regulation of prostate-specific antigen gene expression in breast cancer. Br J Cancer 1997;75:579–588.