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Immunoreactive Prostate-Specific Antigen Levels in Female and Male Breast Tumors and Its Association With Steroid Hormone Receptors and Patient Age

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Prostate-specific antigen (PSA) is believed to be a highly specific marker for normal or cancerous prostatic tissue. We recently found that immunoreactive PSA (IR-PSA) is present in 30% of breast tumor cytosols (from 525 breast cancer patients). In this paper we analyzed a new series of 750 breast tumor cytosols, obtained from 744 women and six men, for IR-PSA. The positivity rates in the old and new series were very similar (~30%). Combining the two series of breast cancer patients, we examined the associations between IR-PSA and estrogen (ER) or progesterone (PR) receptors, or patient age. We found that IR-PSA positivity rate declines with age. PSA-positive tumors were highly associated with either ER-positive or PR-positive tumors alone. However, analysis in a subset of tumors that combine the two receptors, ER(-)/PR(-), ER(+)/PR(-), ER(-)/PR(+), and ER(+)/PR(+), revealed that IR-PSA was only associated with PR, and no relationship was found between IR-PSA and ER. We speculate that the presence of IR-PSA in breast cancer may be associated with the PR action and that the association between PSA and ER is indirect due to the known association between ER and PR. As five of the six male breast tumors were found negative for IR-PSA, it is suggested that androgen may not be involved in the presence of IR-PSA in breast tumor. In conclusion, we confirmed that IR-PSA is associated with the presence of PR, is present in 30% of breast tumors, and propose that IR-PSA may be used as a new biochemical marker for prognosis, and/or treatment of breast cancer.

KEY WORDS: prostate specific antigen; breast cancer; steroid hormone receptors; prognostic indicators in breast cancer.

Introduction

Prostate-specific antigen (PSA) is a glycoprotein produced almost exclusively by the epithelial cells of the prostate and is currently used as a tumor marker for the diagnosis and monitoring of prostate cancer (1-3). Production of PSA by tumors other

than those of prostatic origin is an extremely rare event (4). However, using a highly sensitive immunofluorometric procedure for PSA (5), we found that about 30% of breast tumors contain immunoreactive PSA (IR-PSA) (6). In addition, we found that this IR-PSA in breast tumors is associated with steroid hormone receptors and occurs preferentially in younger patients and early stage cancer.

PSA production by cancer cell lines other than those from normal or cancerous prostatic tissue has not, to our knowledge, been reported so far in the literature. We recently demonstrated that the breast cancer cell line T-47D can be induced by a variety of steroid hormones and tamoxifen to produce IR-PSA (H. Yu and E.P. Diamandis, unpublished observations).

In this paper we analyzed a new series of 750 breast tumor cytosols for IR-PSA using our highly sensitive immunofluorometric procedure (5). We initiated this undertaking for several reasons. First, we wanted to reproduce the earlier finding of IR-PSA in breast tumors in a subsequent series of patients. Second, we had the opportunity to study six tumors from male patients, and third, we were able to conduct a more reliable statistical analysis in subgroups of patients stratified by age or receptor status. We also used the Abbott IMx PSA assay to confirm our finding of IR-PSA in breast cancer cytosols. We now present evidence that the progesterone receptor is associated with IR-PSA appearance in breast tumors and demonstrate that the PSA-positivity rate progressively declines with age.

Materials and methods

PATIENTS WITH BREAST CANCER

We studied a new series of primary breast cancers that were collected from 744 females and six males

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at hospitals collaborating in the Ontario Provincial CEA/Steroid Receptor program. The breast tumor tissue was stored in liquid nitrogen immediately after surgical resection, transported to the laboratory, and subsequently stored at -70°C until extraction was performed ($\sim 1-2$ weeks). Approximately 0.5 g of tumor tissue was weighed out, fragmented with a hammer if necessary, and pulverized in a Thermo-vac tissue pulverizer at liquid nitrogen temperature. The resulting powder was transferred into 50-mL plastic tubes along with 10 mL of extraction buffer (Tris-HCl, pH 7.40, 10 mmol/L with ethylenediaminetetraacetic acid 1.5 mmol/L, sodium molybdate 5 mmol/L). The suspended tissue powder was solubilized on ice with a single 5-s burst of a Polytron homogenizer. The particulate material was pelleted by centrifugation at $105,000 \times g$ for 1 h. The intermediate layer (cytosol extract) was collected without disturbing the lipid or particulate layers. Protein concentration of the cytosol extract was determined by the Lowry method. The remainder of the extracts were stored at -70°C until analysis (up to 3 months). In-house studies have shown that IR-PSA in cytosol extracts was stable for at least 4 months in these conditions. Results were expressed as ng IR-PSA/mg total protein.

MEASUREMENTS

For quantitative analysis of estrogen and progesterone receptors (ER, PR) we used the Abbott enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL, USA). The kits were used according to the manufacturer's instructions. Analysis for IR-PSA by time resolved immunofluorometric assay was performed as described elsewhere (5,6). PSA was also measured using the Abbott IMx (Abbott Laboratories) as previously reported (7).

PSA ASSAY CALIBRATION

The calibration of PSA assays is currently arbitrary because of the lack of a recognized international reference preparation (8). Frequently, manufacturers use serum-based or synthetic matrix-based standards that are calibrated in such a way that the sera will give similar PSA values measured by the Hybritech's Tandem-R PSA assay, a widely used and FDA-approved method. Another problem with the PSA assay calibration is the existence of various forms of serum PSA (5). In this study, we used seminal plasma PSA standards prepared in 6% (w/v) bovine serum albumin. Our standards are identical to those used in the Abbott IMx PSA method. Other PSA methods that employ different standards may give different PSA values as measured by our assay or the Abbott IMx method.

STATISTICAL ANALYSIS

The associations between IR-PSA and steroid hormone receptors or patient age were examined using

the chi-square (χ^2) test, and the strength of these associations were expressed in odds ratio (an estimate of likelihood ratio) and its 95% confidence interval. All statistical parameters used in the data analysis were calculated through a computer software SAS (SAS Institute Inc., Cary, NC, USA).

Results

In our previous study, we used an IR-PSA cutoff level of $0.05 \mu\text{g/L}$. The mean and median total protein concentration of all tumor extracts were identical, 1.64 g/L . Thus, the equivalent cutoff expressed as ng/mg would be $0.030 \text{ ng/mg protein}$. We have transformed all IR-PSA data from $\mu\text{g/L}$ used in our previous study (6) to ng/mg protein by dividing the IR-PSA $\mu\text{g/L}$ value with the individual protein concentration in each sample.

Using the above cutoff level, we found that 29% of tumors in the first series (6) and 32% of tumors in the new series were IR-PSA-positive. When we combined the two series, the positivity rate for IR-PSA is 30%. The frequency distribution of IR-PSA levels in the combined series, involving 1275 tumors, is shown in Figure 1. Numerical data describing the distributions of PSA, ER, and PR receptors and patient age are shown in Table 1. The values of the three biochemical markers are not normally distributed.

Among the 750 new samples, there were 37 specimens that contain a relatively high level of IR-PSA, and because these levels are measurable by commercially available PSA method, we remeasured the 37 samples with the Abbott IMx PSA method. The results are shown in Figure 2. An excellent correlation and agreement was found between our method and the Abbott method.

No linear correlation was found between IR-PSA and ER or PR levels, either in the whole population studied, or in the 386 IR-PSA positive samples, or in the 40 most highly positive IR-PSA samples. We observed positive linear correlations between ER and PR ($r = 0.40$; $p < 0.0001$), and between ER or PR and patient age ($r = 0.38$ and 0.11 , respectively; $p < 0.0001$). These correlations have also been seen in another study (9).

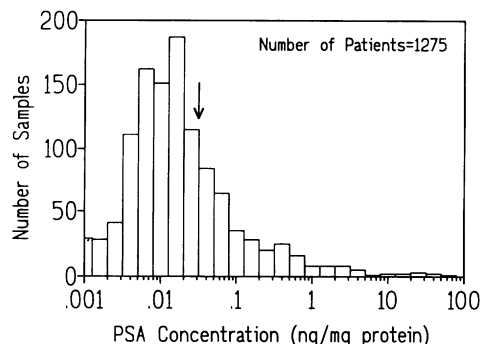


Figure 1 — Frequency distribution of 1275 breast tumors in terms of PSA content. The threshold for IR-PSA positivity is $0.03 \text{ ng/mg protein}$, shown by an arrow.

TABLE 1
Percentile Values for PSA, Estrogen, and Progesterone Receptors and Patient Age in 1275 Breast Tumor Cytosolic Extracts

Percentile	PSA (ng/mg)	ER (fmol/mg)	PR (fmol/mg)	Age (Years)
5th	0	0	0	37
10th	0	1	1	42
25th	0.006	7	4	49
50th	0.015	60	40	61
75th	0.038	193	204	71
90th	0.16	354	388	78
95th	0.59	432	486	83

In accordance with our previous report (6), we found a significant association between the presence of IR-PSA and the presence of either ER or PR alone (Table 2). Setting the steroid hormone receptor threshold at 10 fmol/mg instead of 5 fmol/mg, we did not find any significant changes in the strength of the association (data not shown).

In order to examine which of the two receptors studied is most likely to relate to IR-PSA, we analyzed the presence of IR-PSA in association with the status of both receptors, that is, dividing receptor status into four subgroups that include: ER(+) and PR(+); ER(+) and PR(-); ER(-) and PR(+); and ER(-) and PR(-). The results of this analysis are shown in Table 3. The presence of IR-PSA was significantly associated with the PR-positive tumors as the odds ratios were all substantially high regardless of whether or not the ER is present. No relationship was found between the presence of IR-PSA and ER-positive tumors when the PR was negative.

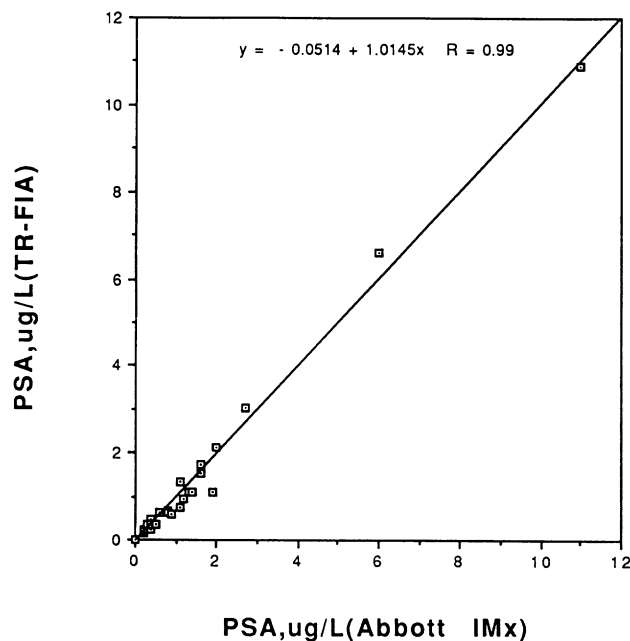


Figure 2 — Correlation between PSA levels measured by the time resolved immunofluorometric assay (TR-FIA) (5) and the Abbott IMx method for 37 breast tumor cytosols.

TABLE 2
Relationships Between Estrogen and Progesterone Receptors and PSA Immunoreactivity

Receptor Status ^a	IR-PSA(+)	IR-PSA(-)	p Value
ER(+), 991	330 (33.3%)	661 (66.7%)	
ER(-), 284	56 (19.7%)	228 (80.3%)	<0.001
PR(+), 910	326 (35.8%)	584 (64.2%)	
PR(-), 365	60 (16.4%)	305 (83.6%)	<0.001

^a Cutoff level for both receptors was 5 fmol/mg protein.

The results did not change when a receptor threshold of 10 fmol/mg was used.

The positivity rates of IR-PSA, ER, and PR by age are shown in Figure 3. IR-PSA-positive rate decreased with age, but the proportion of steroid hormone receptor-positive tumors tends to increase with age. The data for IR-PSA in six male breast tumors is shown in Table 4. Of the six men, only one patient had a IR-PSA-positive tumor, and all but one had both ER and PR receptors.

Discussion

In our first report (6), we studied 525 breast tumor cytosols, and found that about 30% of them contain immunoreactive PSA. Experiments using HPLC and the Western Blot method both demonstrated that this IR-PSA has a molecular weight identical to that of seminal PSA. Although the results from our various studies strongly suggest that the immunoreactive PSA in the breast cancer cells seems identical to the seminal PSA, a final proof of PSA has to come from the protein sequencing of the IR-PSA isolated and purified from the breast cancer cytosols.

We now report an analysis of IR-PSA in a new series of breast tumor cytosols, consisting of 750 samples. The positivity rates for IR-PSA in the two series were very similar when using a cutoff level of 0.030 ng of PSA/mg of total protein. The two groups were merged in this study to provide a relatively large size of samples (1275 breast cancer patients) for statistical analysis.

We have previously shown that high levels of IR-PSA in breast tumor extracts can be measured by two commercial PSA kits (6), namely the Hybritech Tandem-R™ assay (Hybritech Inc., San Diego, CA, USA) and the IRMA-Count™ PSA kit (Diagnostic Products Corp., Los Angeles, CA, USA). In this study, we also compared our PSA results with another widely used automated PSA assay (Abbott IMx). The results are in excellent agreement. We should emphasize that commercial PSA kits incorporate PSA standards optimized to measure PSA in serum. These standards may vary between manufacturers by as much as 100%, which was found in our cross-comparison experiments (H. Yu and E.P. Diamandis, unpublished data). Thus, the absolute IR-PSA values in breast tumor cytosols, which seems to contain only free PSA, may also vary between methods. Our own standards used in this and

TABLE 3
Relationships Between PSA Immunoreactivity and Estrogen and Progesterone Receptors

Receptor Status ^a	Number of Patients	IR-PSA(+) Samples (%)	Odds Ratio (95% Confidence Interval)	p Value
ER(-), PR(-)	226	32 (14)	1.00	
ER(+), PR(-)	139	28 (20)	1.53 (0.88-2.67)	0.13
ER(-), PR(+)	58	24 (41)	4.28 (2.25-8.14)	<0.001
ER(+), PR(+)	852	302 (35)	3.33 (2.23-4.96)	<0.001

^a Cutoff level for both receptors was 5 fmol/mg protein.

the previous study are the same as those of the IMx assay, and the agreement between the results of our method and IMx is excellent.

Currently, our method (5) seems to be the only method with sufficient sensitivity to reliably quantify low levels of IR-PSA in breast tumor cytosols. This sensitivity enabled us to set a cutoff point, which is easily and precisely measured by our method, at 0.05 µg/L of IR-PSA. This cutoff level, when it is expressed as ng of IR-PSA/mg of total protein to compensate for differences in the amount of cells extracted, is equivalent to 0.030 ng/mg of protein. Use of other PSA methods with sensitivity lower than that of our assay will probably result in a lower positive rate of IR-PSA in breast tumor cytosols.

Our data indicate that IR-PSA is associated with expression of the progesterone receptor (Table 3). The apparent close association between ER and IR-PSA (Table 2) is probably due to the known relationship between ER and PR (9,10). ER mediates the production of the PR, and the two receptor concentrations correlate with each other. If the PR mediates the PSA production, it is conceivable that an association between ER and PSA would also exist even if the ER is not directly involved. These data

are in accord with the results of our tissue culture studies using the breast cancer cell line T-47D that possesses ER, PR, AR, and GR receptors (11). We found in these experiments that estrogens do not mediate IR-PSA production but progestins as well as androgens and glucocorticosteroids do (unpublished observations). Because these last three receptors utilize the same hormone response element (HRE) which is different from the HRE of the estrogen receptor (2), the PSA gene may be under the control of the HRE of the progestin/glucocorticosteroid/androgen receptor in breast cancer.

The role of the androgen (AR) and glucocorticosteroid (GR) receptor in IR-PSA gene regulation in breast cancer is unknown because we did not measure these two receptors in the breast tumor cytosols. Previous reports have shown that the AR and GR are present in many breast cancer cell lines (11) and tumors (9,13). As PSA production is induced by androgens in the prostate (13), we expected to see high IR-PSA expression by breast tumors in males if androgens were the major mediating ligands. The failure to demonstrate an increased level of IR-PSA in five of the six male breast tumors (Table 4) suggests that androgens may not be important mediating ligands for the appearance of IR-PSA in breast cancer.

The percentage of IR-PSA-positive tumors progressively declines with age. This decline continues even when the receptor positivity rate increases sharply after menopause. This discrepancy between IR-PSA and receptors associated with age may be explained as receptors alone do not regulate the transcription of genes. Steroid hormone receptor must bind to its ligand forming ligand-receptor

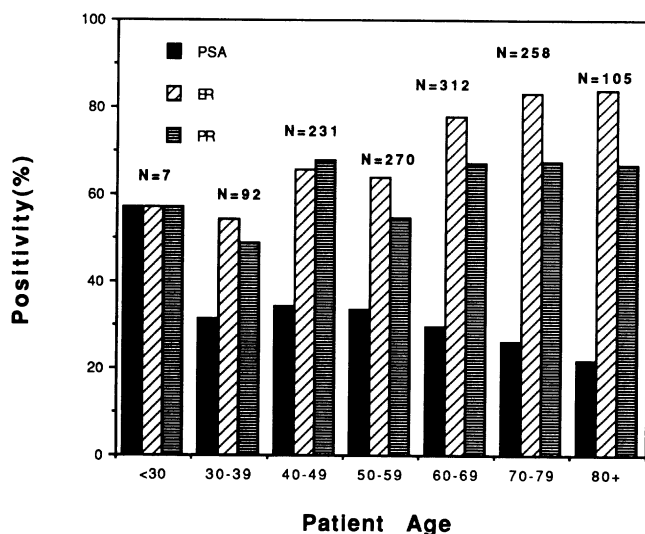


Figure 3 — Percentage of IR-PSA-positive tumors and estrogen receptor-positive and progesterone receptor-positive tumors in patients by age.

TABLE 4
PSA and Steroid Hormone Receptor Levels in Six Male Breast Tumors

Patient	IR-PSA (ng/mg)	ER (fmol/mg)	PR (fmol/mg)	Age (Years)
A	<0.010	89	85	51
B	<0.010	0	2	68
C	<0.010	370	350	80
D	0.016	72	5	76
E	0.021	415	387	83
F	0.041	267	116	71

complex, and then interacts with HRE to regulate gene transcription. PSA production may be mediated by steroid hormones released from the ovaries. As the ovarian function declines with age and ceases after menopause, the production of IR-PSA also goes down with age. For those who still produce IR-PSA at an older age, adrenal production of the putative steroids may be the source of ligand supply. The role of central mechanisms involving hypothalamic hormones and the pituitary glycoprotein hormones, such as LH and FSH, in the regulation of the putative ligands is unknown, but may be worth studying in the future.

Whether the IR-PSA production by breast tumors is associated with breast cancer initiation or progression is unknown. However, we have found that IR-PSA presence is preferentially associated with early stages of breast cancer (6), which may suggest that IR-PSA is a potentially favorable prognostic indicator in breast cancer. The association between IR-PSA and PR further supports this hypothesis as PR is a known favorable prognostic indicator. The presence of IR-PSA may also indicate the presence of functional PR receptors because it has been believed that PR is an indicator of functional ER receptors. After all, the presence of IR-PSA in breast cancer may thus be regarded as a marker of functional ER and PR receptors. Because only a fraction of patients with positive ER or PR respond to endocrine treatment, we speculate that IR-PSA may have a value of predicting which patients would respond to endocrine treatment that targets the estrogen and/or the progesterone receptor. The value of IR-PSA as a prognostic marker in patients with breast cancer needs further exploration. We are currently investigating if tumors can be visualized by utilizing their PSA content. This would be an important application because it is known that no female tissue contains PSA. Finally, our tissue culture study (unpublished observation) provides evidence that progestins are functionally related to IR-PSA production. The culture system may offer opportunities for screening agents that can be used to control or eliminate breast cancer cells because we found that tamoxifen, a widely used antiestrogen agent, can induce PSA production *in vitro*.

We conclude that our finding of IR-PSA presence in breast tumors may have potential application for breast cancer prognosis, selection of therapy, and design of new treatments.

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