Immunoradiometric Assay of pS2 Protein in Breast Cancer Cytosols

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We studied the ELSA-pS2™ immunoradiometric kit (CIS Bio International) for pS2 protein assay in breast cancer cytosols according to classic validation methods. In addition, we studied correlations between pS2, steroid receptors, and cathepsin-D assays. Repeatability (CV = 1.5% to 4.8%) and reproducibility (CV = 1.6% to 4.9%) were good. The results were linearly related to pS2 concentrations between 205 and 2200 ng/L; the detection limit was 40 ng/L. The accuracy of the assay was measured by assessing recovery; analytical recoveries were near 100% throughout the standard curve. The use of different compounds for cytosol preparation (Tris 10 mMol/L or phosphate 25 mMol/L, KCl 0.4 mol/L, bovine serum albumin 1 g/L) had no effect on pS2 results. pS2 was assayed in breast tumor cytosols from 197 postmenopausal and 92 premenopausal patients. The mean value was 24 μg/g of protein; the median and 25th and 75th percentiles were 6, 1, and 23 μg/g of protein, respectively. We observed a relation between concentrations of pS2 and those of estrogen and progesterone receptors, but there was no relationship between the concentrations of pS2 and cathepsin-D.

Additional Keyphrases: steroid receptors · cathepsin D

pS2 protein is a 60-amino acid polypeptide (7000 Da) (1), the synthesis of which is regulated by estrogens in human breast cancer (2). The pS2 gene has been isolated and cloned, and gene products have been studied previously (3, 4). pS2 protein is a marker of good prognosis and of responsiveness to hormone therapy in breast cancer patients, and may determine a subgroup of patients who might benefit from an adjuvant therapy (5). Here we present a technical evaluation of the immunoassay of pS2 in breast cancer cytosols (ELSA-pS2™; CIS Bio International, Gi6 Sur Yvette, France).

Materials and Methods

Reagents

The assay is based on an immunoradiometric two-site assay with a solid phase. pS2 protein is bound between two monoclonal antibodies directed against different epitopes. The first antibody (BC6) is coated on the ELSA solid phase (coated tube), whereas the second (BC4) is 125I-labeled and used as a tracer. The total activity of the kit is <370 kBq (10 μCi). pS2 standards contain highly purified pS2 protein from breast cancer cell lines, at concentrations close to 0, 200, 400, 800, 1400, and 2200 ng/L.

Cathepsin-D was assayed in breast cancer cytosols with an immunoradiometric method (ELSA-Cath-D; CIS Bio International). Estrogen receptors (ER) and progesterone receptors (PR) were assayed by the dextran-charcoal method (6, 7). Receptor values >10 pmol/g protein were considered positive.

Assay Procedure

The first step is the immuno-extraction phase. Add 200 μL of radiolabeled anti-pS2 antibody to 200 μL of standard or diluted cytosol in ELSA coated tubes, then incubate for 1 h with agitation at room temperature. After three washings, count the radioactivity of the tubes. For this study, we obtained radioactivity measures, standard curve drawing, and calculations of results with a Packard (Packard Instrument Co., Downers Grove, IL) coupled with an IBM™ personal computer, by using an automated iterative smoothed-spline-fit program.

Preparation of Samples

pS2 assays were performed with breast cancer cytosols obtained from tumor samples homogenized in 10 mmol/L Tris·HCl buffer, pH 7.4, as previously described for steroid receptor assays (6, 7). The total protein concentration, determined by the method of Lowry et al. (8), was adjusted to ~1 g/L by dilution of the cytosol with Tris buffer. pS2 was then assayed in a 41-fold dilution of this cytosol with the kit diluent. At this final dilution, >95% of pS2 concentrations were read within the standard curve (sample results expressed in μg/g of protein).

Evaluation Methods

Within-run and between-run precisions of the standard curve were measured by the distribution of values for all the standards for nine different curves (determined during one month for between-run precision). Detection limits were calculated as the theoretical amount of pS2 corresponding to the mean + 3SD result for the zero standard.

Within-run precision for tumor cytosols (TC) were obtained with four different samples, assayed 10 times each, on the same day. Between-run precision was calculated for the control cytosol of the kit and for three different TC, assayed 10 times each in 10 different assay series, and 26 TC were assayed twice in different series.

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3 Nonstandard abbreviations: ER, estrogen receptors; PR, progesterone receptors; TC, tumor cytosols; and BSA, bovine serum albumin.
Linearity was studied by serial dilution of TC between 80- and 2560-fold. Analytical recovery was studied by overloading three TC with increasing amounts of standards and calculating the percentages recovered.

Influence of Cytosol Preparation for pS2 Immunoassay

Fifteen breast cancer cytosols were pulverized in liquid nitrogen, separated into two aliquots each, homogenized with either 10 mmol/L Tris·HCl buffer or 20 mmol/L phosphate buffer, and then assayed. The influence of KCl (0.4 mol/L) and of added amounts of protein (bovine serum albumin (BSA), 1 g/L) was also studied.

Five cytosols were assayed either directly, or after freezing and thawing five times each, to study the stability of pS2 protein after storage of tumors in liquid nitrogen.

Tumor Material

This study was performed with a group of 289 patients with operable primary breast cancer, at the Centre Francois Baclesse. pS2 was assayed before treatment in samples from 197 postmenopausal patients and from 92 premenopausal patients. Tumor size was < 5 cm (T1 + T2) for 71% of the patients, nodal status was positive for 59% of patients, and metastasis at time of diagnosis was observed in 8% of patients.

Statistical Analysis

Correlations were calculated between concentrations of pS2 and cathepsin-D for 224 patients, and between two different between-assay series of 26 cytosols, by unweighted regression analysis.

Results

Precision

Table 1 summarizes the within-run and between-run precisions for the standard curve and for breast cancer cytosols. For within-run precision, CVs were very low, between 1.9% and 4.8% for the different segments of the curve, and between 1.2% to 2.5% for cytosols. For between-run precision, CVs were between 1.6% and 4.9% for standards and for breast cancer cytosols, except for the zero standard (16.6%).

A typical standard curve is shown in Figure 1, with values (B/B0 %) from Table 1. The detection limit of the assay was 40 ng/L. The correlation obtained for 26 breast cancer cytosols assayed twice in different series was $B = 1.01A - 19$ ng/L ($r = 0.988$), where A and B represent the first and the second assay, respectively.

Linearity

We studied the linearity by serial dilutions (30-fold, 160-fold, . . . 2560-fold) of three different cytosols. The results agreed with predicted values (measured values were 98% ± 4.4% of expected values, n = 15) and showed that linearity was observed throughout the standard curve, for pS2 values ranging from 113 to 2433 ng/L.

<table>
<thead>
<tr>
<th>pS2 concn, ng/L</th>
<th>Mean B/B0 %</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td><strong>Standard curve</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 = 0</td>
<td>1.32</td>
<td>4.8</td>
</tr>
<tr>
<td>B2 = 205</td>
<td>6.97</td>
<td>4.3</td>
</tr>
<tr>
<td>B3 = 400</td>
<td>16.98</td>
<td>2.1</td>
</tr>
<tr>
<td>B4 = 800</td>
<td>38.00</td>
<td>1.8</td>
</tr>
<tr>
<td>B5 = 1400</td>
<td>71.21</td>
<td>1.6</td>
</tr>
<tr>
<td>B6 = 2200</td>
<td>100</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Breast cancer cytosols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A = 201</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>B = 922</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>C = 1221</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>D = 1849</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>E = 224</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Ctrl = 710</td>
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<td></td>
</tr>
<tr>
<td>G = 787</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>H = 1792</td>
<td>4.9</td>
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</tbody>
</table>

Fig. 1. Typical standard curve of the ELSA pS2 immunoradiometric assay

Analytical Recovery

Recoveries obtained with cytosols overloaded with increasing amounts of standard (0, 100, 200, 400, 700, and 1100 ng/L) were between 94% and 125% (mean 103%, SD 5%, n = 14).

pS2 Stability

The stability of the pS2 protein was observed by studying the resistance of the molecule to freezing and
thawing five times before assaying; pS2 results after freezing (B) were not modified in comparison with controls (A), and the regression obtained was $B = 1.01A + 11$ ng/L ($r = 0.997$, $n = 5$).

Effect of Buffer, KCl, or BSA

Usually, either Tris·HCl (10 mmol/L) or phosphate (25 mmol/L) buffers are used for breast cancer cytosol preparation, sometimes with KCl (0.4 mol/L) and BSA (1 g/L) added to the medium for better extraction of DNA-bound receptors.

In this study, we pulverized 17 breast cancer samples in liquid nitrogen, separated them into two aliquots, and homogenized them in either Tris or phosphate buffer. The regression curve obtained between Tris results (B) and phosphate results (A) was $B = 1.12A - 3$ ng/L ($r = 0.999$), and results by both methods for contents ranging from 0 to 284 ng/L were very similar.

The effect of KCl (0.4 mol/L) and of BSA (1 g/L) was studied with 10 breast cancer cytosols. Identical results were obtained in the presence or absence of either KCl or BSA, and showed that this pS2 assay was unaffected by different medium conditions.

pS2 Concentrations in Breast Cancer

The mean pS2 concentration in 289 cytosols from breast cancer patients was 24 µg/g of protein, the median was 6 µg/g of protein, and the 25th and 75th percentiles were 1 and 23 µg/g of protein, respectively. The distribution of values is presented in Figure 2. As shown in Figure 3, there was no correlation between pS2 and cathepsin-D ($r = 0.199$).

ER, PR, and pS2 results were obtained for 281 of 289 patients. To study the relation between ER, PR, and pS2 positivity, we used two cutoff values for pS2: 6 µg/g of protein (median) and 10 µg/g of protein (arbitrary); the cutoff for ER and PR was 10 pmol/g of protein. The distribution of pS2 positivity is shown in Table 2: 146 of 281 (52%) and 113 of 281 (40%) patients were pS2-positive according to the cutoff values of 6 and 10 µg/g of protein, respectively. Among pS2-positive tumors, 81% to 83% were also ER positive, and 88% to 93% were PR positive. In tumors containing both ER and PR, 75% to 79% of tumors were pS2 positive.

Discussion

In this technical evaluation of the ELSA-pS2 kit, we found the kit easy to handle. The within-run and between-run precisions were always <5%. Recovery tests were good, even beyond the standard range (200–2200 ng/L). Given the variety of cytosol preparation techniques, it was important to show that these techniques would not influence the assay (possible bias for multicenter studies). The composition of buffer, total protein concentrations, or presence of KCl seem not to interfere with the pS2 results. The pS2 protein concentration is not influenced by freezing and thawing. There is a lack of correlation between cathepsin-D and pS2, for which the prognostic values are opposite (high concentrations of cathepsin-D correspond to a poor prognosis).

The estrogen-induced characteristics of pS2 are confirmed by the strong relation between steroid receptors (ER and PR) and pS2 concentrations, but further studies are necessary to assess more precisely the cutoff value of pS2 that best predicts hormone responsiveness.

**Table 2. Relation Between pS2 Positivity and Estrogen Receptor (ER) and Progesterone Receptor (PR) Positivity**

<table>
<thead>
<tr>
<th>ER</th>
<th>PR</th>
<th>pS2 cutoff: 6 µg/g</th>
<th>pS2 cutoff: 10 µg/g</th>
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<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>pS2 -</td>
<td>pS2 +</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>53</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>59</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135</td>
<td>146</td>
</tr>
</tbody>
</table>

* Number of patients in each class. Receptor cutoff: 10 pmol/g of protein.

**Fig. 2. Distribution of pS2 concentrations in 274 breast cancer cytosols**

Median, 6 µg/g of protein; 25th and 75th percentiles, 1 and 23 µg/g of protein, respectively

**Fig. 3. Correlation between pS2 and cathepsin-D in 224 breast cancer cytosols**

$y = 0.252x + 37.48$

$r = 0.199$

References


Using Mutagenic Polymerase Chain Reaction Primers to Detect Carriers of Familial Defective Apolipoprotein B-100
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Familial defective apolipoprotein (apo) B-100 is a genetic trait characterized by an Arg→Gln substitution in position 3500 of the apo B sequence. This genetic defect is associated with greatly increased concentrations of plasma cholesterol and may thus increase the risk of developing premature atherosclerotic disease. We describe here the use of mutagenic polymerase chain reaction primers, which greatly facilitate identification of carriers of this mutation. Moreover, we demonstrate that this method may also be used for determining the phase between two polymorphic sites. Using apo B-100 as an example, we located on different chromosomes the defect in codon 3500 and a mutation in codon 3611, which produces another Arg→Gln change in the encoded apo B-100 amino acid sequence, in two probands heterozygous for both mutations.

Additional Keywords: heritable disorders · risk of atherosclerosis · DNA probes · phase determination between polymorphic sites

Apolipoprotein B-100 (apo B-100), a single polypeptide chain containing 4536 amino acid residues, represents the protein moiety of low-density lipoproteins (LDL). Its gene, mapped on the short arm of chromosome 2 (1), has an approximate length of 43 kilobases (kb), and consists of 29 exons (2). Apo B-100 mediates the clearance of LDL from plasma by binding to the LDL receptor (3). From experimental data, Knott et al. (4) have proposed that the region in apo B that mediates the binding of LDL to this receptor is located between residues 3000 and 4000.

Recently, a single-nucleotide mutation in codon 3500 in exon 26 of the apo B gene (CGG to CAG) was described (5). This mutation, which has a heterozygous frequency of ~1/600, leads to substitution of glutamine for arginine in the variant gene product (6). Individuals carrying the mutant allele in the heterozygous state are affected by a disorder termed "familial defective apo B-100" (FDB), characterized by delayed clearance of LDL from plasma, hypercholesterolemia, and high concentrations of apo B in plasma (6). This mutation supposedly alters the structural conformation of the protein in the vicinity of the receptor-binding domain, thus preventing the binding of LDL to the LDL receptor (7). Because high concentrations of plasma cholesterol are associated with an increased risk for development of coronary heart disease (CHD) (8), FDB may represent an additional example of a genetic defect that predisposes carriers to this disease.

To date, FDB subjects have been identified by use of either a monoclonal antibody that binds with higher affinity to the abnormal LDL (9) or radiolabeled allele-specific oligonucleotides that differentiate between the normal and mutant apo B allele by their alternative ability to form, under stringent conditions, stable homoduplexes with the allelic-gene segments that had been amplified by the polymerase chain reaction (PCR) (5).

We describe here a method that further simplifies detection of the point mutation on codon 3500 of the apo B gene. This method is based on the selective creation of an artificial Map I restriction site in the wild-type allele, but not in the mutant allele, by the use of sequence-modifying PCR primers. This principle was described previously in studies aimed at the detection of point

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4 Nonstandard abbreviations: apo, apolipoprotein; LDL, low-density lipoprotein; CHD, coronary heart disease; PCR, polymerase chain reaction; FDB, familial defective apo B-100; and RFLP, restriction fragment length polymorphism.

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