UC 577,1; 61 ISSN 0354-3447

Jugoslov. Med. Biohem. 21: 15–19, 2002 *Originalni naučni rad*
Original paper

SINGLE-POINT *VERSUS* FIVE-POINT BIOCHEMICAL METHOD FOR DETERMINATION OF ESTROGEN AND PROGESTERONE RECEPTORS

Dragica Nikolić-Vukosavljević, Milan Markićević, Slađana Todorović

Institute of Oncology and Radiology of Serbia, Belgrade, Yugoslavia

Summary: Receptors for estrogen and progesterone are accepted by international consensus as biomarkers of breast carcinoma responsiveness to endocrine therapy. Numerous current studies are aimed at consideration of importance of »the new generation« of estrogen-regulated biomarkers in treatment of breast cancer patients. Simultaneous knowledge of all these biomarkers may help in medical decision making. However, the amount of tumor material available from breast carcinoma can make impossible determination of estrogen-regulated biomarkes together with estrogen and progesterone receptors. To assess whether we could replace our current five-point ligand binding assay for measurement of estrogen and progesterone receptors with a single-point ligand binding assay, we compared simultaneous measurements in same samples of breast carcinomas by both methods. A linear regression analysis shows that single-point assay can be confidently used instead of five-point assay. In addition, there were no variations over time in estrogen and progesterone receptors phenotypes, as well as in estrogen and progesterone receptors contents determined by single-point assay. Accordingly, the results clearly demonstrate the validity of intralaboratory quality control and give a possibility for the establishment of interlaboratory quality control of single-point ligand binding assay.

Key words: estrogen and progesterone receptors, single-point ligand binding assay, breast cancer.

Introduction

A variety of patient (age and menopausal status) and tumor characteristics (lymph node status, histological size, type and grade) can provide prognostic information useful in the management of patients with breast cancer (1). In addition to the above »classical« prognostic factors, it has been generally accepted today that determinations of receptors for estrogen (ER) and progesterone (PR) in breast cancer tissue are an important task in the management of breast cancer (2). The importance of the knowledge of ER and PR status in breast cancer has especially arisen with the introduction of chemotherapy in oncological practice as a more aggressive therapy. Thus, some means are needed to distinguish the patients with hormone-dependent, as well as hormone-sensitive carcinomas, who are favorable candidates for endocrine therapy, from majority of patients, whose tumors are unresponsive to hormonal therapy and who should directly receive chemotherapy. Although very useful, the knowledge of ER and PR status in combination with patient and tumor characteristics does not always provide sufficient information for accurate prediction of response/survival for any individual patient. For this reason, several new biomarkers have been proposed. These biomarkers are related directly or indirectly to the differentiation, proliferation and apoptosis within growth rate of tumor, as well as to invasive and metastatic potential of cancer cells. Among the most thoroughly studied of so called »new-generation« biomarkers are estrogen-regulated proteins: pS2 as a biomarker of endocrine growth control (3), receptor for epidermal growth factor as a biomarker of autocrine/paracrine growth control (4) and cath-D as a biomarker of malignant cell invasion and metastasis (5).

It is important to point out that limitations in the amount of tumor material available from breast carcinoma can preclude determination of the estrogen-regulated proteins together with ER and PR. Our aim in this study was to assess the possibility of ER and

Address for correspondence

Dragica Nikolić-Vukosavljević
Institute of Oncology and Radiology of Serbia
Department of Experimental Oncology
Pasterova 14
11 000 Belgrade, Yugoslavia
fax: 381 11 685 300
e-mail: tut@infosky.net

PR determination by a single-point instead of five-point biochemical method and, therefore, to enable determination of the other molecular biomarkers from the same breast carcinoma cytosolic fraction.

Material and methods

Tumors

Excised tumors were obtained from breast cancer patients treated by ablative breast surgery at the Institute of Oncology and Radiology of Serbia. None of the patients have received preoperative radiotherapy, chemotherapy or endocrine therapy. At the time of surgery, tumor specimens were divided into two representative parts by the pathologists. One part of the specimen was proceeded for histology and the other was immediately placed into liquid nitrogen for biochemical receptor assays. ER and PR contents were obtained by the single-point and five-point biochemical method using 49 and 36 breast carcinoma samples, respectively. Quality control of the single-point biochemical assay was performed using 500 consecutive breast carcinoma samples.

Standard biochemical assay: five-point assay

Storage of tumors specimens, homogenization of the tissue, cytosol preparation, incubation of cytosol with radiolabeled steroid hormone and separation of receptor-bound steroid hormone from excess of free steroid hormone, were performed exactly according to procedures recommended by the EORTC (6 12). The saturation kinetics of ER and PR breast carcinoma was analyzed using five concentrations of each ^{3}H-estradiol (2, 4, 8, 16 and 32 nmol/L) and ^{3}H-ORG 2058 (4, 8, 16, 32 and 64 nmol/L).

Binding observed in the presence of excess of unlabeled steroid hormone is related to nonreceptor or nonspecific (low affinity, high capacity) binding of the ligand. Specific binding was estimated as the difference between total and nonspecific binding (*Figure 1A)*. The saturation kinetics data from *Figure 1A.* were used (13) for determination of the dissociation constant (Kd) and the number of specific binding sites (Bmax) of the ER and PR *(Figure 1B)*. Usually, the number of specific binding sites is expressed in fmol of labeled steroid hormone bound *per* mg cytosol protein. Cytosol protein concentration was determined by the method of Lowry at al. (14).

Single-point assay

To verify precise quantification of the number of specific binding sites of the ER and PR , it is necessary to use the highest, saturable hormone concentration from five-point assay. The number of specific binding sites of ER and PR is estimated as the difference between total and nonspecific binding. Standardization of single-point assay was performed in accordance with recommendations of EORTC (15).

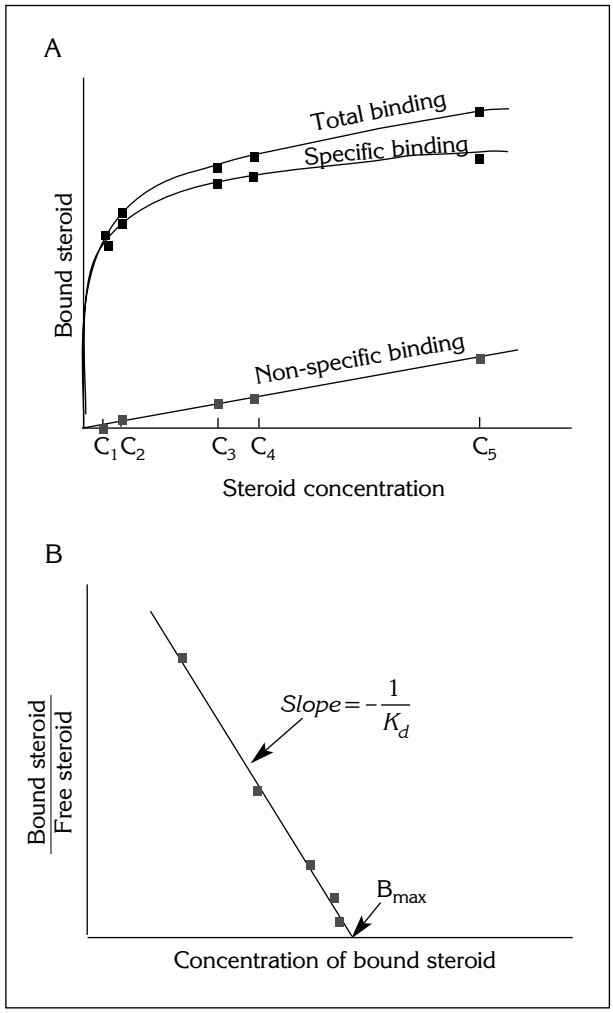

Figure 1. The saturation kinetics analysis of ER and PR in breast carcinoma. (A) Specific binding was estimated as the difference between total and nonspecific binding. (B) The saturation kinetics data from (A) were plotted according to the method of Scatchard, where Kd represents dissociation constant of estrogen receptor- and progesterone receptor-hormone complex and Bmax the maximum number of receptor sites.

Statistical methods

A linear regression analysis was used to compare five-point and single-point assays for ER and PR content determination.

Non-parametric statistic methods were used to analyze the obtained results within single-point biochemical assay. Chi-square test was performed to examine distribution of phenotypes of steroid receptor status among the whole group and each subgroup, or

among subgroups. Mann-Whitney U-test was performed to examine the distribution of characteristic quantitative receptor contents or the distribution of all receptor individual contents.

Results

Comparison of five-point and single-point assays

Results of comparison of five-point and single-point assays for estrogen receptor (A) and progesterone receptor (B) contents are shown in *Figure 2*.

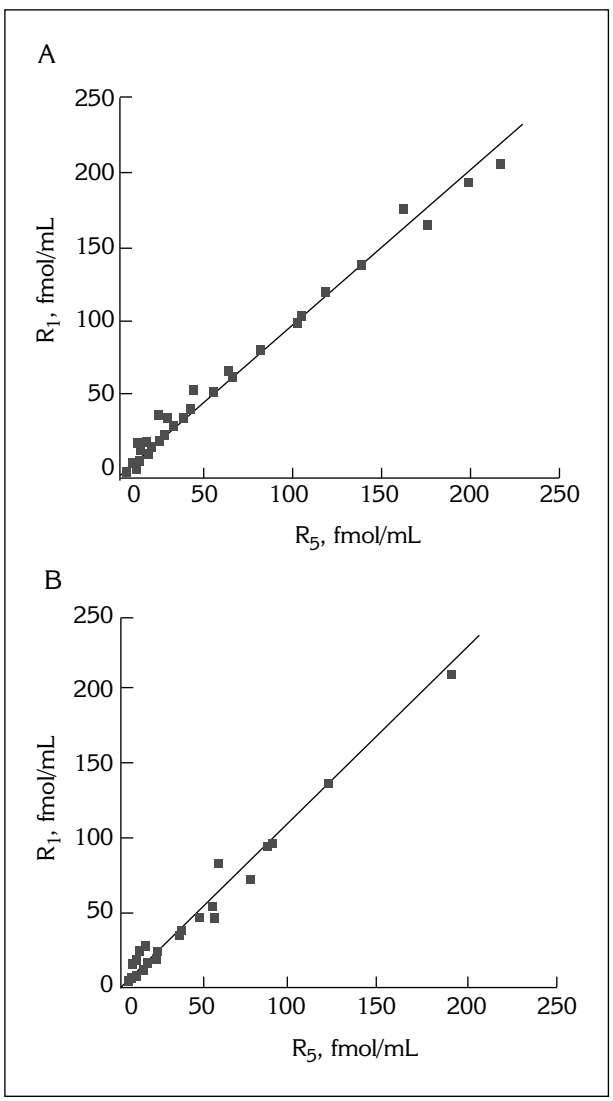

Figure 2. Measurement of estrogen receptors (A) and progesterone receptors (B) by five-point assay (R_5) and single-point assay (R_1). Linear regression analysis between receptor contents obtained by five-point assay and single-point assay in the total population yielded: (A) $y = 1.009x$ ($r = 0.996$, $P < 0.05$), (B) $y = 0.974x$ ($r = 0.99$, $P < 0.05$).

There were 49 and 36 samples analyzed for estrogen and progesterone receptor contents, respectively. Measurements were done on the same breast carcinoma cytosols. Our results demonstrated that single-point assay can be correlated with five-point assay. The correlation between measurements of estrogen receptor and progesterone receptor contents obtained by five-point assay and single-point assay in the total population was very high ($r = 0.996$ and $r = 0.990$, respectively).

Intralaboratory standardization of single-point assay

Intralaboratory standardization was performed in accordance with recommendation of EORTC (9).

Qualitative manner of standardization related to examination of distributions of ER and PR phenotypes within the whole group and each subgroup, as well as within subgroups is shown in *Figure 3*. Homogeneity was striking in distribution of each phenotype among the whole group and each subgroup, or among subgroups.

Quantitative manner of standardization related to examination of the distribution of the 5th, 25th, 50th, 75th and 95th percentile values of estrogen receptor or progesterone receptor contents within the whole group and each subgroup, as well as within subgroups, is shown in *Figure 4*. Homogeneity was striking in distribution of represented ER and PR contents among the whole group and each subgroup, or among subgroups. These findings were confirmed by the analyses of all individual quantitative values of ER and PR (data not shown).

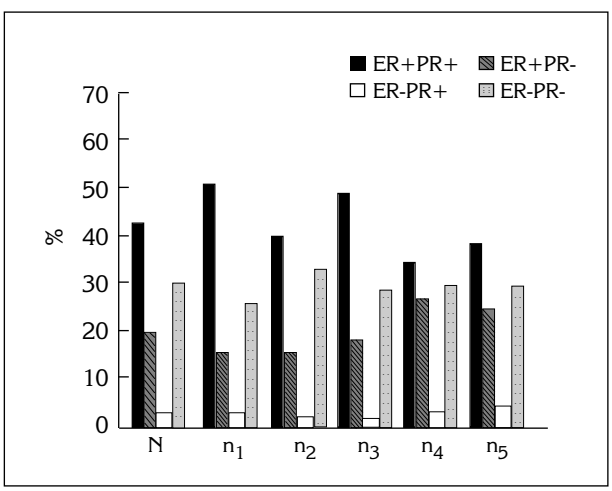

Figure 3. Distribution of ER and PR phenotypes, obtained by single-point biochemical assay, within the whole group of 500 carcinomas (N) and five consecutive subgroups of 100 carcinomas (n_1 n_5).

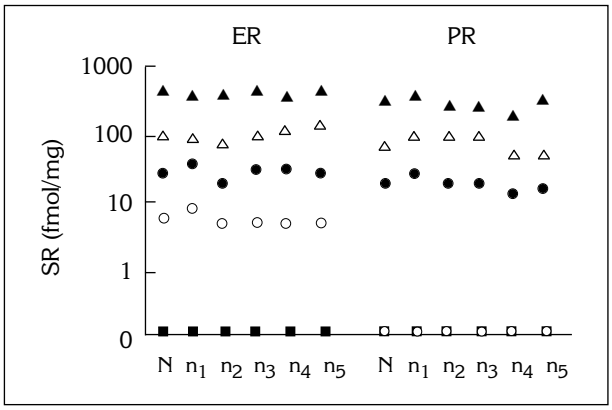

Figure 4. Distribution of the 5 (■), 25 (○), 50 (●), 75 (△) and 95 (▲) percentile values of ER and PR contents, obtained by single-point biochemical assay, within the whole group of 500 carcinomas (N) and five consecutive subgroups of 100 carcinomas (n_1 n_5).

Discussion

The aim of this study was to investigate potential usefulness of single-point biochemical method for ER and PR determination in breast carcinomas. Our reported method-comparison study generally shows that single-point assay is equivalent to five-point assay in relation to the content of both ER and PR. Consequently, we could use single-point assay instead of five-point assay for ER and PR determination, thus making possible determination of the other molecular biomarkers from the same breast carcinoma cytosol fraction.

Our further analysis in this study was aimed at examining of validity of intralaboratory quality control of single-point biochemical assay. We analyzed distributions over time of ER and PR status in qualitative and quantitative manner. No variations have been observed over time in ER and PR phenotypes, as well as in ER and PR quantitative values.

It is important to point out that consideration of interlaboratory variations of ER and PR phenotypes may not be suitable due to two reasons, so crucial for the biological nature of breast carcinoma, which are often ignored (16). Firstly, the ER and PR quantitative contents have a wide range of values. Secondly, the threshold quantitative value used to define receptor positivity, which varies from 3 to 20 fmol/mg, belongs to the level with the highest frequencies in distribution of the ER and PR levels. Therefore, the distribution of ER and PR quantitative values gives a more realistic view of the interlaboratory comparability.

Studies of estrogen-regulated proteins may offer an insight into the molecular pathogenesis of estrogen-dependent carcinomas and they may help in medical decision making, as well. There is a sufficient number of small pilot studies for most of the estrogen regulated biomarkers of »new generation« (17 19) and there is a wealth of information on the more established "classical" biomarkers. It is time for extensive prospective studies in which the majority of the old and new biomarkers would be assessed together. Such studies are necessary to fully evaluate and validate significance of each biomarker and to determine the combinations of biomarkers which can identify accurately subgroups of patients with different courses of disease. In this way, both under and over treatment can be avoided.

Acknowledgments

This work was supported by the Ministry for Science and Technology of Serbia, contract No 13M13. The authors truly thank Ms Anđelka Lukač, Milica Matas and Jelica Perović for excellent technical assistance.

ODREĐIVANJE RECEPTORA ZA ESTROGEN I PROGESTERON BIOHEMIJSKOM METODOM JEDNA TAČKA VS. PET TAČAKA

Dragica Nikolić-Vukosavljević, Milan Markićević, Slađana Todorović

Institut za onkologiju i radiologiju Srbije, Beograd, Jugoslavija

Kratak sadržaj: Receptori za estrogen i progesteron su međunarodnim konsenzusom prihvaćeni kao biomarkeri odgovora karcinoma dojke na endokrinu terapiju. Danas postoje brojne studije usmerene ka sagledavanju važnosti »nove generacije« estrogenom regulisanih biomarkera u lečenju pacijenata sa rakom dojke. Istovremeno poznavanje svih ovih biomarkera može pomoći pri donošenju odluke o terapiji. Međutim, količina tumorskog materijala, koja je raspoloživa od karcinoma dojke, može da onemogući određivanje estrogenom regulisanih biomarkera zajedno sa receptorima za estrogen i progesteron. Da bi procenili da li možemo biohemijski metod za određivanje receptora za estrogen i progesteron sa pet tačaka da zamenimo metodom sa jednom tačkom, uporedili smo istovremena merenja, istih uzoraka karcinoma dojke, dobijena obema metodama. Linearna regresiona analiza ukazuje da se biohemijski metod sa jednom tačkom može pouzdano upotrebiti umesto metode sa pet tačaka. Uz to, utvrđeno je da nije bilo promena kako u fenotipovima receptora za estrogen i progesteron tako ni u sadržaju receptora za estrogen i progesteron koji su određeni pomoću metode sa jednom tačkom. Shodno tome, rezultati jasno pokazuju pouzdanost intralaboratorijske kontrole kvaliteta i daju mogućnost da se uvede interlaboratorijska kontrola kvaliteta biohemijske metode sa jednom tačkom.

Ključne reči: receptori za estrogen i progesteron, biohemijski metod sa jednom tačkom, kancer dojke.

References

1. Sunderland MC, McGuire WL. Prognostic indicators in invasive breast cancer. Surg Clin North Am 1990; 2: 989 1004.
2. McGuire WL. Hormone receptors: Their role in predicting prognosis and response to endocrine therapy. Semin Oncol 1978; 5: 428 433.
3. May FE, Westley BR. Trefoil proteins: Their role in normal and malignant cells. J Pathol 1997; 183: 4 7.
4. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocrinol Rev 1995; 16: 559 589.
5. Rochefort H. Biological and clinical significance of cathepsin D in breast cancer. Acta Oncol 1992; 31: 125 130.
6. Breast Cancer Cooperative Group. Standards for the assessment of hormone receptors in human breast cancer. Eur J Cancer 1973; 9: 379 381.
7. Breast Cancer Cooperative Group. Revision of the standards for the assessment of hormone receptors in human breast cancer. Eur J Cancer 1980; 16: 1513 1515.
8. Koenders A, Thorpe SM on behalf of the EORTC Receptor Group. Standardization of steroid receptor assays in human breast cancer I. Reproducibility of oestradiol and progesterone receptor assays. Eur J Cancer Clin Oncol 1983; 19: 1221 1229.
9. Thorpe SM on behalf of the EORTC Receptor Group. Standardization of steroid receptor assays in human breast cancer II. Samples with low receptor content. Eur J Cancer Clin Oncol 1983; 19: 1467 1472.
10. Koenders A, Thorpe SM on behalf of the EORTC Receptor Group. Standardization of steroid receptor assays in human breast cancer III. Selection of reference material for intra and inter laboratory quality control. Eur J Cancer Clin Oncol 1986; 22: 939 944.
11. Koenders A, Thorpe SM on behalf of the EORTC Receptor Group. Standardization of steroid receptor assays in human breast cancer IV. Long-term within- and between laboratory variation of estrogen and progesterone receptor assays. Eur J Cancer Clin Oncol 1986; 22: 945 952.
12. Blankenstein MA, Benarad TJ. Multilaboratory assessment of tissue prognostic factors in breast cancer: the EORTC Receptor Study Group experience. Can J Oncol 1992; 3 (Suppl.): 58 64.
13. Scatchard G. The attraction of proteins for small molecules and ions. Ann NY Acad Sci 1949; 51: 660 672.
14. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193: 265 275.
15. Romain S, Laine Birdon C, Martin PM, Magdelanat H on behalf of the EORTC Receptor Study Group. Steroid receptor distribution in 47 892 breast cancers. A collaborative study of 7 European Laboratories. Eur J Cancer 1995; 31A: 411 417.
16. Thorpe SM, Rose C. Oestrogen and progesterone determination in breast cancer. Technology, biology and clinical significance. Acta Oncol 1988; 27: 1 19.
17. Luqmani Y, Temmim L, Memon A, Parkar A, Ali M, Motawy M *et al.* Immunoradiometric measurements of pS2 in breast cancer: Correlation with steroid receptors and plasminogen activators. Acta Oncol 1999; 38: 805 814.
18. Rochefort H. The prognostic value of cathepsin D in breast cancer. A long road to clinic. Eur J Cancer 1996; 32A: 7 8.
19. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocrine Rev 1995; 16: 559 589.

Received: October 8, 2001

Accepted: December 4, 2001