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Standardization of Steroid Receptor Assays in Human Breast Cancer—II. Samples with Low Receptor Content

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Abstract—*Four different lyophilized cytosols with low receptor content were assayed for estrogen and progesterone receptors by 13 laboratories in 7 countries. Each sample was analyzed on three different working days. Three laboratories reported unsatisfactory ER results, whereas the other 10 participants were in good agreement. Employing a cut-off limit of 10 fmol/mg cytosol protein, 95% agreement of ER-positivity was achieved for the two samples with the lowest receptor contents (18 and 21 fmol/mg cytosol protein). For estrogen receptor analyses the inter-laboratory coefficients of variation ranged between 15 and 18%. Both the intra- and inter-laboratory variations and the rate of concurrence on receptor status were less satisfactory for the progesterone than for the estrogen receptor analyses. The variation in protein results among the participating 13 laboratories is decreased to 10%. It is concluded that multi-center clinical trials based upon the estrogen receptor status assigned by individual laboratories are feasible.*

INTRODUCTION

THE EORTC Breast Cancer Cooperative Group decided in 1978 that identification of the estrogen receptor (ER) status of the patients' tumor would be a prerequisite for some future clinical trials. It was recognized, however, that before clinical trials based upon ER status could be initiated, each participating institution should agree on the classification of the receptor status in the tumor. Characterization of tumor samples as either receptor-positive or -negative depends on the lower limit of sensitivity of the assay employed, as well as on the definition of the cut-off limit between positive and negative samples. During the previous trials conducted by the EORTC Receptor Group very little attention was paid to the analyses of samples with low receptor content. Therefore a trial was designed to study the concurrence of classification among participating institutions for samples with ER values of 50 fmol/mg cytosol protein and less.

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MATERIALS AND METHODS

Preparation of the lyophilized reference samples

Cytosol from calf uterine tissue was prepared and lyophilized as previously described [1, 2]. In order to prepare calf uterine cytosol with a low steroid hormone receptor content while maintain-

ing a constant protein concentration, calf serum was diluted with phosphate buffer to the same protein concentration as the undiluted cytosol. Cytosol was then diluted 10-, 15-, 20- and 25-fold with the diluted calf serum to yield samples A, B, C and D respectively. The ratio of receptor values of these four samples is 2.5 (sample A):1.67 (sample B):1.25 (sample C):1 (sample D). The mean ER and progesterone receptor (PgR) contents of sample A were 57 and 64 fmol/mg cytosol, as determined in Nijmegen.

Study protocol

Each participant received three vials of the four different samples. Samples were mailed at ambient temperature. The stability of the samples under these conditions was confirmed by returning a set of samples for analysis to the laboratory in Nijmegen via one of the participants in Italy (data not shown). The 12 vials were analyzed in 3 consecutive receptor assays with one vial of each sample in each assay in different sequences that were unknown to the participants.

Methods

A description of the preparation of cytosol from the lyophilized material as well as the details of the DCC receptor assay conditions are published in the companion article [3]. However, it may be noted that during this trial the participants employed their own batches of radioactive and non-radioactive ligands, Coomassie brilliant blue (Bio-Rad Laboratories) and Kabi human serum albumin. The concentrations of radioactive ligands used ranged between 0.5 and 5.0×10^{-9} M.

RESULTS

Estradiol receptor

The results of 11 of the 13 participating laboratories are summarized in Table 1. The reasons for the two exclusions are given in the footnote of the table. All laboratories, with the exception of lab. C, performed a multipoint Scatchard analysis. Laboratory C performed single-dose saturating analysis using 5.0 nM [^{3}H]-estradiol and obtained results that are within the range of the values reported by the others. The magnitude of intra-laboratory variation differed greatly, but despite this appreciable difference in inter-assay reproducibility, the median values of the samples in each lab. agreed reasonably well (Table 2). The inter-laboratory coefficients of variation for the median values ranged between

Table 1. Estradiol receptor values (fmol/mg protein) reported by individual laboratories

	Sample A		Sample B		Sample C		Sample D	
Lab.	Range	Median value	Range	Median value	Range	Median value	Range	Median value
A	47–82	77	28–65	31	16–34	18	15–147	50
C	43–51	49	29–32	30	20–21	21	14–19	17
D	55–59	57	31–33	33	19–25	22	15–17	16
E	41–62	54	21–42	42	20–25	24	6–23	23
F	54–61	56	37–41	38	26–27	26	13–20	17
G	44–60	50	26–38	26	14–17	16	14–18	16
H	47–62	51	34–37	35	nd*–27	18	nd–17	17
I	42–50	44	28–35	32	20–23	21	20–21	21
J	52–60	55	19–25	23	16–24	18	13–24	15
L	45–64	49	31–40	34	23–26	24	15–24	15
M	47–63	55	35–49	41	20–34	23	22–32	23

*nd = no specific binding detectable (i.e. receptor-negative).

Laboratory B performed only one assay. Due to adsorption problems, no results could be calculated. Laboratory K obtained many unsatisfactory Scatchard plots, which did not allow calculation of the concentration of specific receptor binding sites. These anomalous curves were probably caused by an overestimation of non-specific binding.

Table 2. Summary of median estradiol receptor values (fmol/mg protein)

Sample	No. of labs	Range of median values	Mean	S.D.	V.C. (%)
A	11	44–77	54	8.5	15.6
B	11	23–42	33	5.8	17.5
C	11	16–26	21	3.2	15.1
D	10*	15–23	18	3.2	17.4

*Excluding lab. A.

15.1 and 17.5%. Linear regression analysis of values reported by each lab. in relation to the dilution factor revealed significant correlations ($r = 0.97 - 0.999$) in all but one lab. (lab. A). The addition of calf serum to the calf uterine cytosol had a detrimental influence on the measurement of estradiol receptor, which is illustrated by the fact that the regression line of the mean values ($y = 62.5\,x - 8.5$, $r = 0.9967$) does not pass through the origin.

The definitions employed by the individual laboratories for discrimination between receptor-positive and receptor-negative samples differ; cut-off levels between positive and negative samples vary from 5 to 20 fmol/mg cytosol protein. Common cut-off values of 5, 10, 15 and 20 fmol/mg cytosol protein were applied to the receptor results of all participants, and these results are shown in Table 3. Only one of the 66 assays performed by all 11 laboratories on samples A and B would be classified as ER-negative even if the highest limit of 20 fmol/mg cytosol protein is employed as a cut-off limit. However, 30 and 61% of samples C and D respectively would be classified as ER-negative using this level of discrimination. Use of a limit of 15 fmol reduces the latter values to 6 and 18% respectively, while a cut-off limit of 10 fmol results in only 3 and 6% disagreement in these two samples. The agreement of ER-positivity for all four samples is 97.7% (129/132 assays) when a limit of 10 fmol/mg cytosol protein is implemented as a discriminant.

Progesterone receptor

The ranges and median values of the PgR analyses from 10 laboratories are given in Table 4. Laboratories B, C and L are omitted from the table for reasons given in the footnote. Laboratory L performed the receptor analyses 3–4 months after the samples had been mailed. Their results are included in the report of ER results but omitted from the PgR results since it has been observed that progesterone receptor binding may decline gradually even in lyophilized preparations, whereas estradiol receptors appeared to be stable in all lyophilized uterine preparations studied so far in Nijmegen (manuscript in preparation). Nevertheless, lab. L's results remained within the range reported by the other participants. A wide range of values with appreciable inter-assay variation was reported for all four samples (see

Table 3. Classification of receptor status

	Estradiol receptor					Progesterone receptor				
	Mean value	% assays classified ER-negative*				Mean value	% assays classified PgR-negative*			
Sample	(fmol/mg protein)	<5	<10	<15	<20	(fmol/mg protein)	<5	<10	<15	<20
A	54	0	0	0	0	75	0	0	0	0
B	33	0	0	0	3	43	0	0	3	3
C	21	3	3	6	30	25	7	17	17	40
D	18	3	6	18	61	24	7	10	23	47

*Threshold value for negatively expressed as fmol/mg protein.

Table 4. Progesterone receptor values (fmol/mg protein) reported by individual laboratories

	Sample A		Sample B		Sample C		Sample D	
Lab.	Range	Median value	Range	Median value	Range	Median value	Range	Median value
A	82–200	88	22–54	38	7–61	36	35–241	71
D	58–61	60	32–38	33	16–18	17	12–13	13
E	99–115	109	49–60	59	29–46	43	nd*–44	34
F	58–74	60	29–40	37	20–24	22	17–19	17
G	83–121	89	49–53	50	25–28	28	15–26	25
H	67–104	85	42–48	44	nd–39	15	15–42	20
I	61–69	66	37–51	42	16–26	19	16–22	21
J	36–66	40	11–32	24	9–16	9	9–18	12
K	55–98	71	51–112	51	nd–67	35	nd–43	42
M	72–101	81	42–61	55	23–36	25	27–43	28

*nd = no specific binding detectable (i.e. receptor-negative).

Laboratory B performed only a single determination obtaining the following values: sample A, 54 and 56; sample B, 26 and 27; and sample D, 12 and 27 fmol/mg protein. Laboratory C used [^{3}H]-R 5020 instead of the agreed ligand [^{3}H]-Org 2058; moreover, single-dose assays were performed. Laboratory L did not follow the agreed time protocol. The reported values varied between 46 (sample A) and 17 fmol/mg protein (sample D).

also Table 5). The inter-laboratory coefficients of variation ranged from 25 to 43%.

Highly significant correlation coefficients ($r>0.98$) were found between PgR values and dilution factors for all but two labs (A and K). All the labs observing a statistically significant correlation also observed a relative decrease of PgR values with increasing concentrations of calf serum in the samples. The line of linear regression analysis of the mean values of all participants given in Table 5 is described by: $y = 90.1\ x - 16.2$, $r = 0.9910$.

Four of the 60 (6.7%) assays performed on the nominally positive samples C and D demonstrated no specific high affinity binding at all and were, therefore, reported as PgR-negative. The percentage of disagreement in relation to the four arbitrary cut-off levels are presented in Table 3. Analogous to the situation with classification of ER status, while there is good agreement using each of the four cut-off limits for the two samples with highest receptor content (A and B), difficulties arise in classification of the samples with lowest receptor content. A cut-off limit of 10 fmol/mg cytosol protein results in agreement of receptor-positivity in all four samples for 93% (112/120) of the assays.

Protein measurement

For each participating laboratory the protein values of the four samples were very similar, which is in accord with the fact that the calf uterine cytosol and diluted calf serum contained approximately equal protein concentrations. Therefore a single mean value was calculated from all twelve results from the individual laboratories (Table 6). The reproducibility of the protein analyses differed considerably from one laboratory to another, with intra-laboratory variation coefficients ranging from 1.5 to 15.5%. The mean values of all participants were between 4.97 and 6.91 mg/ml cytosol, with an inter-laboratory coefficient of variation of 10.3%.

Table 6. Summary of protein results (mg/ml cytosol)

Lab.	Range	Mean	S.D.
A	4.6–6.0	5.36	0.48
B	5.2–5.7	5.34	0.16
C	6.1–7.3	6.67	0.38
D	5.3–5.6	5.44	0.08
E	5.2–6.0	5.46	0.26
F	5.9–6.8	6.32	0.26
G	5.6–6.3	5.85	0.23
H	4.7–5.2	4.97	0.18
I	6.7–7.2	6.91	0.17
J	4.8–6.2	5.47	0.39
K	4.3–7.0	5.73	0.89
L	4.5–5.4	5.10	0.24
M	5.6–6.2	5.82	0.24

DISCUSSION

Characterization of the steroid hormone receptor status of tumors is one of the most important factors in selecting patients with advanced breast cancer for endocrine therapy. When multicenter trials are to be conducted it is imperative that receptor analyses are comparable in the participating institutions and that classification of a given sample as negative or positive is the same. Ten of the 13 laboratories participating in this study reported ER results that were in good agreement. Employing a cut-off limit of 10 fmol/mg cytosol protein, 94.5% agreement of ER-positivity was achieved by 10 of the 13 laboratories for the two samples with the lowest receptor content (C and D). In comparison, 58–77% agreement was observed in an ECOG study [4] and a study by Witliff *et al.* [5] for samples with approximately the same ER content as those studied here. It should be noted, however, that both of these two cited studies utilized frozen tissue powders as the reference material rather than lyophilized cytosols, as has been the case in the present study. A greater degree of heterogeneity as well as a greater lability is conceivable for tissue powders contrasted to lyophilized cytosols.

The present trial demonstrates the feasibility of designing multicenter trials based upon the estrogen receptor status of the tumor tissue for treatment of breast cancer patients. However, both the reproducibility and the rate of agreement on receptor status of the progesterone receptor analyses are unsatisfactory in the present trial.

Table 5. Summary of median progesterone receptor value (fmol/mg protein)

Sample	No. of labs	Range of median values	Mean	S.D.	C.V. (%)
A	10	40–109	75	20	26.1
B	10	24–59	43	11	24.8
C	10	9–43	25	11	42.7
D	9*	12–42	24	10	41.9

*Excluding lab. A.

Similar results have been reported by the Southeastern Cancer Study Group (SECSG) quality assurance program. Using pulverized frozen tissue powders, not even 65% agreement was achieved by the participating laboratories [6]. It must therefore be concluded that the present state of the art of PgR analysis is inadequate for multicenter trials at the present moment.

While interassay reproducibility of ER assays still needs improvement in some of the laboratories, the reduction in inter-laboratory variation seen in the previous trial [3] is confirmed here. The coefficients of variation of the median values reported by each lab. are between 15 and 18%, and they compare favorably to results reported by other inter-laboratory quality control programs [4–9].

Inter-laboratory variation in protein analysis is lower in the present trial (c.v. = 10%) than in the previous trial (c.v. = 14–17%). Reproducibility of the assay must still, however, be improved in a few laboratories. Nevertheless, the variation in quantitation of protein is reduced to such an extent that analysis of tissue preparations is now possible with little interference resulting from protein determinations in each laboratory.

Dilution of the calf uterine cytosol with calf serum resulted in underestimation of ER and PgR values that increased proportional to the content of serum in the sample. The interference was most pronounced in PgR analysis, and it precluded detection of the PgR with the ligand R5020 (results from 6 labs, not included in the present report). Reasons for these effects of addition of calf serum to the cytosols are unclear, but they may either be due to the addition of endogenous steroids in the serum or to the presence of excessive amounts of proteins that bind to the radioactive ligands with low affinity.

The significance of individual procedures of tissue homogenization for receptor analysis will be evaluated in the forthcoming EORTC Receptor Group trial. To achieve this, minced tissue frozen on solid CO_2 will be distributed to participants simultaneously with lyophilized tissue cytosol prepared from the same minced tissue.

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