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Enzyme Immunoassay of Estrogen and Progesterone Receptors in Drill Biopsy Specimens from Breast Cancer

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Abstract—*We have applied enzyme immunoassay (EIA) to the detection of estrogen and progesterone receptors (ER and PR, respectively) in small samples obtained by drill biopsy of primary breast cancers. Thirty patients with breast cancer underwent drill biopsy of the tumors just before mastectomy. Both the drill biopsy and surgical specimens were assayed for ER and PR in the cytosol and nuclear fractions by EIA. ER and PR in the cytosol fraction (ERc and PRc, respectively) of the drill biopsy specimens (DBS) correlated very well with those of the surgical specimens (SS): ERc (DBS) = 1.02 × ERc (SS) + 3.85 fmol/mg protein (r = 0.958) and PRc (DBS) = 1.05 × PRc (SS) + 3.87 fmol/mg protein (r = 0.958). ER and PR in the nuclear fraction (ERn and PRn, respectively) of the drill biopsy specimens also correlated very well with those of the surgical specimens: ERn (DBS) = 1.02 × ERn (SS) + 59.18 fmol/mg DNA (r = 0.932) and PRn (DBS) = 0.98 × PRn (SS) + 54.28 fmol/mg DNA (r = 0.898). These results demonstrate that EIA for ER and PR of the drill biopsy specimens is a very useful method for the estimation of the receptor status of breast cancers.*

INTRODUCTION

It is well known that the presence of estrogen receptors (ER) and progesterone receptors (PR) in breast cancer is not only predictive of the response to endocrine therapy but is also a useful independent prognostic factor. Nowadays, it seems essential to know the ER and PR status for planning a suitable treatment strategy for breast cancer.

Tritiated-ligand binding assays, such as the dextran-coated charcoal (DCC) method, have been the main methodology for the determination of ER and PR in breast cancer for 20 years. However, recent success in preparing monoclonal antibodies against ER and PR has enabled immunological detection of these receptors [enzyme immunoassay (EIA)]. The EIA kits for ER (ER-EIA) and PR (PR-EIA) produced by Abbott Laboratories (Chicago, IL) have recently been demonstrated to give an excellent correlation with the conventional DCC method and their validity has been established [1–3]. EIA has several advantages over the DCC method, particularly the elimination of radioisotope from the assay procedure and small sample volumes required for the assay. Goussard *et al.* reported that the level of protein concentration needed in the enzyme immunoassay is as low as 0.2 mg/ml while the DCC method in routine use can be performed accurately only on cytosol with a protein concentration of more than 1 mg/ml [2]. This advantage of EIA is of great importance in the assay of ER and PR in small samples.

Another advantage of EIA lies in its ability to detect the receptors whether or not they are occupied with the corresponding endogenous hormones whereas the DCC method can detect only the unoccupied receptors. Moreover, assay results of ER-EIA and PR-EIA have been demonstrated not to be influenced by the high ionic conditions (0.6 M KCl) required for the extraction of ER and PR from the nuclear pellet [3, 4]. Therefore, EIA can be considered to be suitable for ER and PR assay in the nuclear fraction.

In this report, we have applied EIA for the detection of ER and PR both in the cytosol and nuclear fractions of small samples obtained by drill biopsy of primary breast cancers. Correlation of the receptor values was also studied between drill biopsy specimens and surgical specimens.

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MATERIALS AND METHODS

Patients and drill biopsy

Thirty consecutive patients (15 premenopausals and 15 postmenopausals) with primary breast cancer measuring more than 2 cm in diameter were entered in this study. Drill biopsy of the breast tumor was performed with an electric drill biopsy apparatus (Nipro, Tokyo, Japan) in the operation room after general anesthesia was induced. A rotating bore (1.8 mm in inner diameter) was percutaneously introduced into the central portion of the tumor. After insertion, the bore was kept rotating in the tumor for several seconds and then slowly withdrawn. Cylindrical specimens were easily obtained at the first attempt in 27 patients. When the specimens were missed, drill biopsy was repeated in three patients until the specimens could be obtained. Finally, we could obtain the drill biopsy specimens from each of the 30 patients entered in this study.

After the drill biopsy, a modified radical mastectomy was performed on every patient. Immediately after the removal of the breast, the tumor was cut with a surgical knife and correct sampling of the tumor tissue by drill biopsy was assured by confirming a needle track in the tumor. Surgical specimens were obtained from the tumor tissue adjacent to the needle track. The residual tumor was processed for histological examination and a diagnosis of infiltrating ductal carcinoma was obtained in every patient.

Both drill biopsy and surgical specimens were snap frozen and kept at −80°C until the assay which was carried out within 2–4 weeks after the operation.

Preparation of cytosols and nuclear extracts

Every procedure was carried out at 0–4°C unless otherwise specified. Surgical specimens were homogenized in 5 volumes of TEDMG buffer [10 mM Tris, 1.5 mM EDTA, 0.5 mM dithiothrcitol, 10 mM sodium molybdate, 10% (v/v) glycerol] using a Polytron P-10 by three 10 s runs with 30 s cooling between each run. After removal of an aliquot of the homogenate for DNA assay, the homogenate was centrifuged at 800 ***g*** for 10 min. The supernatant was removed and the pellet was resuspended in 5 volumes of TEDMG buffer. After centrifugation at 800 ***g*** for 10 min, the supernatant was removed, combined with the former supernatant and centrifuged at 105,000 ***g*** for 1 h. The resultant supernatant was obtained as a cytosol without a superficial lipid layer.

The washed pellet was extracted in 5 volumes of TEDMGK buffer (TEDMG plus 0.6 M KCl) for 1 h. The crude extract was centrifuged at 105,000 ***g*** for 1 h and the resultant supernatant was obtained as nuclear extract.

Drill biopsy specimens were homogenized in 1 ml of TEDMG buffer using a Teflon–glass homogenizer by three 10 s runs with 30 s cooling between each run. The homogenate was centrifuged at 800 ***g*** for 10 min. The supernatant was removed and centrifuged at 105,000 ***g*** for 1 h and the resultant supernatant was obtained as cytosols without superficial lipid layer. The pellet was extracted in 1 ml of TEDMGK buffer for 1 h. The crude extract was centrifuged at 105,000 ***g*** for 1 h and the resultant supernatant was obtained as nuclear extract.

Enzyme immunoassay for estrogen and progesterone receptors

Enzyme immunoassay kits for estrogen receptor (ER-EIA) were purchased from Abbott Laboratories (Chicago, IL). All the procedures for ER-EIA were performed according to the manufacturers' instructions. ER-EIA is a solid phase enzyme immunoassay based on the 'sandwich' principle. In brief, samples (100 μl) and standards were incubated with beads coated with anti-ER monoclonal antibody (rat) for 18 h at 4°C and unbound material was removed by aspiration and washing of the beads with distilled water. A second anti-ER monoclonal antibody (rat) conjugated with horseradish peroxidase was incubated with ER–bead complex at 37°C for 1 h. After aspiration of excess conjugate and washing of the beads with distilled water, the beads were incubated in enzyme substrate (hydrogen peroxide and *o*-phenylenediamine) for 30 min at room temperature. The reaction was stopped by the addition of 1 N sulfuric acid. The intensity of color developed was recorded at 492 nm using a Quantum II. A standard curve was obtained by plotting the absorbance of the standards versus their ER concentrations, and ER concentrations of samples were determined from this standard curve.

Enzyme immunoassay kits for progesterone receptor (PR-EIA) were generous gifts from Abbott Laboratories. All the assay procedures were performed according to the manufacturer's instructions. The principle on which the PR-EIA is based is identical to that of ER-EIA and the assay procedure for PR-EIA is quite similar to that of ER-EIA except that the second incubation is performed at 4°C.

Cytosols and nuclear extracts from surgical specimens were diluted two-fold with homogenization buffer and extraction buffer, respectively and assayed for ER and PR, while those from drill biopsy were assayed in non-diluted forms. Every sample was assayed in duplicate.

Intra-assay coefficients of variation (%CV) of ER-EIA and PR-EIA were 5.6 and 4.1%, respectively and inter-assay coefficients of variation of ER-

EIA and PR-EIA were 8.7 and 7.9%, respectively [3].

Miscellaneous

Protein and DNA were assayed according to the methods of Lowry *et al.* [5] and Burton [6], respectively.

RESULTS

Drill biopsy specimens

The mean net weight and DNA content of 30 drill biopsy specimens were 28.9 ± 2.3 (S.E.) mg and 88.3 ± 7.5 (S.E.) μg, respectively. The mean protein concentration of cytosols prepared from these specimens was 1.03 ± 0.07 (S.E.) mg/ml ranging from 0.52 to 2.20 mg/ml and fell into an appropriate assay range.

The mean protein concentration of cytosols prepared from surgical specimens was 3.21 ± 0.15 (S.E.) mg/ml ranging from 1.88 to 4.60 mg/ml.

Correlation of ER and PR assay results between drill biopsy specimens and surgical specimens

ER and PR values in the cytosol fraction (ERc and PRc, respectively) were compared between drill biopsy specimens and surgical specimens and are shown in Fig. 1. An excellent correlation was obtained with correlation coefficients of 0.958 ($Y = 1.02X + 3.85$) for ERc and 0.958 ($Y = 1.05X + 3.87$) for PRc. The slopes of the regression curves for ERc and PRc were close to 1. The concordance rate of ERc and PRc results between drill biopsy specimens and surgical specimens is shown in Table 1, where ERc and PRc values were assessed qualitatively with a cut-off value of 10 fmol/mg protein. The concordance rate was as high as 90.0% for ERc and 93.3% for PRc. Discordance of ERc and PRc assay results was found in three and two patients, respectively (Table 2).

Table 1. Concordance of ERc and PRc assay results between drill biopsy specimens and surgical specimens

Drill biopsy specimens	Surgical specimens			
	ERc*		PRc†	
	Positive	Negative	Positive	Negative
Positive‡	16	2	15	1
Negative	1	11	1	13

*Estrogen receptor in the cytosol fraction.
†Progesterone receptor in the cytosol fraction.
‡Positive when ≥10 fmol/mg protein.

ER and PR values in the nuclear fraction (ERn and PRn, respectively) were compared between drill biopsy specimens and surgical specimens (Fig. 2). An excellent correlation was obtained with correlation coefficients of 0.932 ($Y = 1.02X + 59.18$) for ERn and 0.898 ($Y = 0.98X + 54.28$) for PRn. The slopes of the regression curves for ERn and PRn were close to 1.

Influence of surgical procedure (mastectomy) on ER and PR contents of tumors

A routine receptor assay is performed on surgical specimens obtained after mastectomy. Tumor tissue is considered to be exposed to significant changes

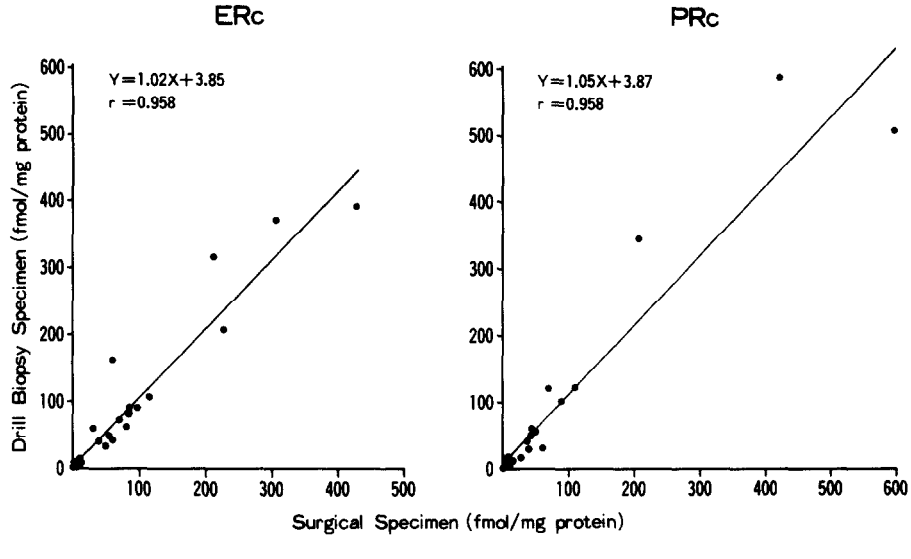

Fig. 1. Correlation between drill biopsy specimens and surgical specimens in terms of ERc (left panel) and PRc (right panel) in the cytosol fraction. Regression curves were (Y = 1.02X + 3.85 (r = 0.958) *for ERc curves* Y = 1.05X + 3.87 (r = 0.958) *for PRc.*

Table 2. Discordance of ERc and PRc assay results between drill biopsy specimens and surgical specimens

Case*	ERc		PRc	
	Drill biopsy specimen	Surgical specimen	Drill biopsy specimen	Surgical specimen
1	12†(+)‡	4 (−)	49 (+)	46 (+)
2	11 (+)	3 (−)	0 (−)	0 (−)
3	5 (−)	11 (+)	2 (−)	2 (−)
4	4 (−)	5 (−)	9 (−)	11 (+)
5	101 (+)	115 (+)	10 (+)	4 (−)

*Cases 1–3: discordance of ERc assay results. Cases 4,5: discordance of PRc assay results.
†fmol/mg protein.
‡(+): ERc or PRc positive when ≥10 fmol/mg protein. (−): ERc or PRc negative when <10 fmol/mg protein.

in temperature and blood flow for extended periods of time during mastectomy before the biopsy for receptor determination is excised and frozen. Therefore, there is a considerable possibility that receptor assay results obtained from surgical specimens are different from the true receptor levels of the tumors. On the other hand, drill biopsy specimens can be obtained with a minimal perturbance of the physiology of the tumor tissue and assay results obtained from drill biopsy specimens can be considered to be closer to the true receptor levels of the tumor. For the purpose of addressing the problem that damage to the tumor tissue incurred during mastectomy may influence the receptor contents, we compared the assay results of ER and PR between drill biopsy specimens and surgical specimens in terms of total receptor contents (cytosol plus nuclear fraction) and their distribution.

Tumors, of which drill biopsy specimens were positive for ERc ($n = 18$) or PRc ($n = 16$), were analyzed. The total ER concentration was compared between the surgical and drill biopsy specimens (Fig. 3). The Rank sum test revealed that the total ER concentration of the surgical specimens was significantly lower than that of the drill biopsy specimens ($P < 0.01$). The median percentage concentration of the total ER in the surgical specimens relative to the drill biopsy specimens was 87% ranging from 53 to 118%. The total PR concentration was compared between the surgical and drill biopsy specimens (Fig. 4). The Rank sum test revealed that the total PR concentration of the surgical specimens was significantly lower than that of the drill biopsy specimens ($P < 0.01$). The median percentage concentration of the total PR in the surgical specimens relative to the drill biopsy specimens was 80% ranging from 27 to 131%.

The distribution of ER and PR in the cytosol and nuclear fractions was also compared between the surgical and drill biopsy specimens. However, no significant difference was found in the ER and PR distribution (Rank sum test).

DISCUSSION

Our results demonstrate that ER and PR assay results from drill biopsy specimens, whether in cytosol or nuclear fraction, correlate very well with those of surgical specimens and that the regression curves have slopes close to 1. The concordance rate of ER and PR assay results were as high as 90.0 and 93.3%, respectively. Therefore, EIA for ER and PR both in the cytosol and nuclear fractions of drill biopsy specimens can be considered to be a

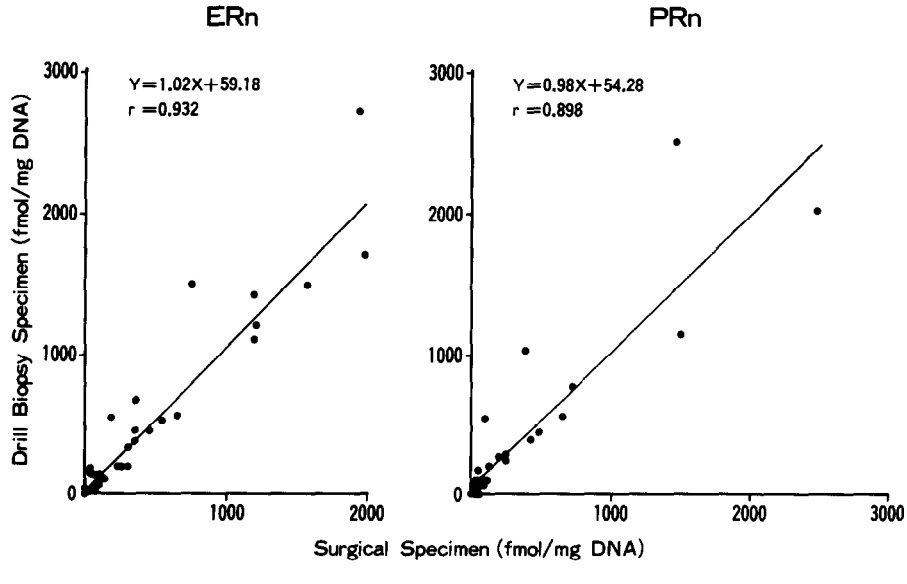

Fig. 2. Correlation between drill biopsy specimens and surgical specimens in terms of ERn (left panel) and PRn (right panel) in the nuclear fraction. Regression curves were Y = 1.02X + 59.18 (r = 0.932) *for ERn and* Y = 0.981X + 54.28 (r = 0.898) *for PRn.*

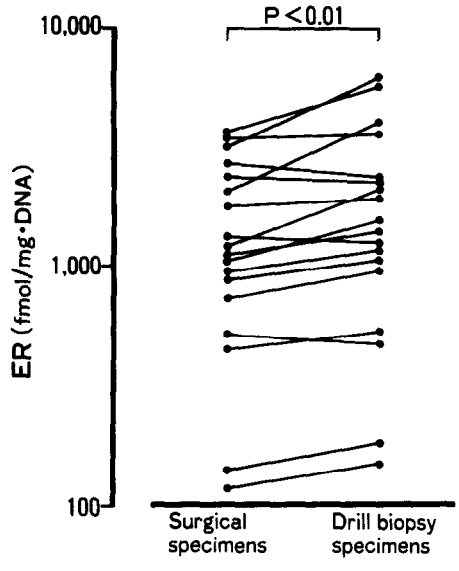

Fig. 3. Comparison of the total ER concentration between the surgical and drill biopsy specimens. Each line represents an individual patient and connects the ER values of the surgical and drill biopsy specimens.

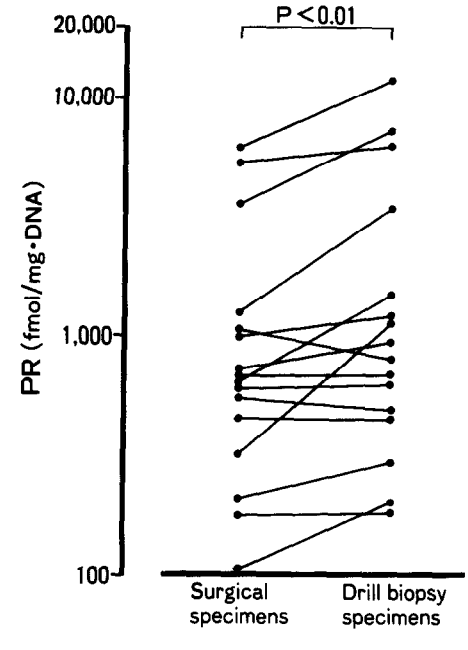

Fig. 4. Comparison of the total PR concentration between the surgical and drill biopsy specimens. Each line represents an individual patient and connects the PR values of the surgical and drill biopsy specimens.

very accurate and useful method for the estimation of the receptor status of primary breast tumors.

There have been several studies on tritiated-ligand binding assay for ERc and/or PRc of small samples obtained from drill biopsy and fine needle aspiration [7–9]. However, it is not so easy to perform conventional tritiated-ligand binding assay on cytosols of low protein concentrations with such an accuracy and reproducibility as was shown in the present study. For the detection of ERn and PRn, exchange assay has been usually employed. However, this assay is not suitable for ERn and PRn assay of small samples since it requires a relatively large sample volume and it is sensitive to procedural details. On the other hand, EIA is applicable to both ERn and PRn detection in drill biopsy specimens since it requires small sample volumes and detects both occupied and unoccupied forms of receptors. At present, EIA seems to be best suited for the assay of ERn and PRn of small samples.

Intratumoral variation of ER and PR has been demonstrated to range from 17 to 32% [10–12]. The high concordance rate obtained in our series i.e. 90.0% for ER and 93.3% for PR, is not consistent with these reports. We obtained surgical specimens from the tumor tissue adjacent to the needle track of the drill biopsy in order to minimize the heterogeneity due to different sampling sites. This may explain the high concordance rate in our series.

Though there is a considerable possibility that tissue damage caused by the surgical procedure (mastectomy) influences the receptor content of the tumors, this problem has rarely been studied. Our results suggest that the surgical procedure decreases the ER and PR contents of the tumors without affecting their distribution, i.e. the total ER and PR values obtained before the operation (drill biopsy specimens) were significantly higher than those obtained after the operation (surgical specimens) while ER and PR distribution in cytosol and nuclear fractions were almost constant. This result has an important clinical implication in terms of the interpretation of ER and PR assay results since receptor assay based on the surgical specimens might give a false negative result in some cases. The limited number of tumors analyzed in this study prevents us from drawing a firm conclusion on this problem; however, it should be studied in more detail because of its clinical importance.

Nowadays, several drugs are used for endocrine treatment of breast cancer but their mechanisms of action are not fully understood. It seems very useful to obtain sequential samples for receptor assay from the same tumor during endocrine treatment in order to elucidate the mechanism of action of these drugs. Drill biopsy is suitable for this purpose since ER and PR of drill biopsy specimens can be assayed accurately by EIA and this procedure does not affect the prognosis of the patients. Moreover, the advantage of EIA in detecting the receptors in the nuclear fraction of small samples would contribute a lot to investigating the change of the receptor dynamics provoked by these drugs. A study on elucidating the action mechanism of tamoxifen and medroxyprogesterone acetate, which are in routine use for endocrine treatment, is currently being undertaken by comparing the receptor status of drill biopsy specimens sequentially obtained from the primary breast cancer.

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