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DETERMINATION OF ESTROGEN RECEPTORS IN PARAFFIN-EMBEDDED TISSUE

Techniques and the value in breast cancer treatment

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Estrogen receptor (ER) analysis in breast cancer has been used in three clinical situations: to select patients with advanced breast cancer for hormonal therapy, as a prognostic parameter, and for selection of women with early breast cancer to adjuvant hormonal treatment. ER has traditionally been measured using labelled hormone in binding assays—often in dextran-coated charcoal assays (DCC). Monoclonal antibodies to ER has permitted development of a solid phase enzyme immunoassay (ER-EIA) used for quantitative determination of ER in tissue homogenates, and have also been used for determination of ER using an immunohistochemical assay in frozen sections (ER-ICA) or in formalin-fixed, paraffin-embedded tissue (ER-PAR). A large number of studies has compared ER-EIA with ER-DCC assays. There is a good linear correlation between the two types of assay but ER-EIA measure more ER and classify a larger fraction of tumors ER-positive than conventional ER assays. Lack of clinical data makes the significance of this uncertain. Numerous studies have reported on the correlation between ER-ICA and ER-DCC or ER-EIA. There is a good correlation among the assays on classification of ER status with a median 86% concordance, but a somewhat poorer correlation between semiquantified ER of immunohistochemical assays and ER determined by the quantitative methods (median coefficient of correlation 0.67). There is a large variation in the cut-off level for definition of ER-positive in immunohistochemical assays emphasizing the need for quality control studies. The major problem involved in ER analysis in paraffin-embedded tissue is a considerable loss of immunoreactivity compared to sections from frozen tissue. This can partly be overcome by modifications of the immunohistochemical technique using enzyme pretreatment and other amplification systems, but the sensitivity of ER-PAR remains lower than ER-ICA despite these modifications, and the ER status is less reliably determined in tumors with low ER contents (<100 fmol). The prognostic value of ER-PAR was evaluated with a multivariate analysis. The endpoint was disease-free interval in systemically untreated patients with early breast cancer, and the variables used were: ER-DCC, ER-PAR, age, tumor size, tumor grade, and nodal status. A total of 133 patients from the Danish Breast Cancer Cooperative Group's (DBCG) 77c protocols had a complete set of variables. The analysis showed that only nodal status, ER-DCC, and tumor grade were significant and independent prognostic variables. An overview of larger multivariate studies on mainly node-negative patients failed to show independent prognostic significance of ER-DCC. The predictive value of

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ER-PAR in adjuvant hormonal treatment of women with early breast cancer was examined in 349 patients from the DBCG 77c protocols randomized to radiotherapy or radiotherapy + tamoxifen. A previously published analysis at 42 months' follow-up had shown only women with > = 100 fmol ER/mg protein to benefit from tamoxifen. ER-PAR was determined in these patients and data reanalyzed. This showed tamoxifen to be without benefit in any of the receptor-defined subgroups at 86 months and that there was no difference between ER-PAR and ER-DCC. It is difficult on the basis of publicized studies, to interpret the relationship between ER and the benefit of adjuvant hormonal treatment, and most large studies fail to disclose receptor-defined subgroups who do not benefit from hormonal treatment. Stratification of patients using receptor status should thus be planned with caution. ER-PAR was analyzed in 137 previously untreated patients with advanced breast cancer. Fifty percent of the ER-PAR positive patients responded to endocrine treatment compared to 10% of the ER-PAR negative. This is comparable to results from ER analyses in fresh tissue. Studies on response to hormonal treatment in breast cancer and immunohistochemical analyses are generally very small and difficult to compare. ER-PAR was used to compare ER status of the primary tumor and its metastases. ER status was concordant in the primary and 84 of 92 regional lymph node metastases and in 44 of 51 distant metastases. The discordance is readily explained by methodological limitations. The observations cannot explain the lack of response in 40–50% of patients with ER positive primary tumors.

In 1896, Beatson was the first to describe regression of metastases in advanced breast cancer, achieved by removal of the ovaries in young women (1). More recently it has been shown that additive or ablative hormonal treatment yields a response in roughly 1/3 of women with advanced mammary carcinoma (2). However, the physiological and molecular basis on which this response rests remained unknown until the synthesis of radiolabeled hormones was achieved in the late 1950s. It was then observed that hormones could be taken up by a target tissue against a concentration gradient (3) and that the uptake of labeled estradiol in breast cancer metastases corresponded with the response to adrenalectomy (4). This fostered the concept of estrogen receptors (ER) as mediators of steroid hormone action and provided a rationale for understanding the effect of hormonal treatment in breast cancer (5, 6). Determination of ER was first made possible by the use of radiolabeled hormones in ligand binding assays (6), and the development of monoclonal antibodies against human ER has later paved the way for quantitative enzyme immunoassays and immunohistochemical ER assays in frozen tissue (7).

The ER status has shown its clinical usefulness in three areas. First, it has served as a criterion for selection of advanced breast cancer patients for endocrine therapy: response to hormonal manipulation is achieved in some 40–75% of patients with positive tumors but only in 5–15% of patients with ER-negative tumors (8–11). Second, a certain prognostic value of the ER status has been demonstrated (12–14). Third, the ER status may be a parameter that can guide the selection of primary breast cancer patients for adjuvant hormonal treatment (15–18).

It has been suggested (19) that progesterone receptors indicate the presence of functional ER and they may therefore add to the value of ER determinations (11). However, in this overview it has been decided to focus exclusively on ER analysis because of the relative scarcity of clinical data on PgR analysis using monoclonal antibodies.

Aims. To develop a method for ER determination in formalin-fixed paraffin-embedded tissue.

To compare this method with other methods of ER determination.

To study the value of ER analysis in paraffin-embedded breast cancer tissue in three clinical situations: as a prognostic tool in early breast cancer and as a criterion according to which patients with early breast cancer can be selected for adjuvant endocrine therapy and patients with advanced breast cancer for endocrine therapy.

Background. Estrogen receptors (ER) are nuclear proteins (20–22), present in at least two different forms, physically characterized by their sedimentation constant. The 8S form, which is ER unoccupied with hormone, is loosely bound and extracted into the homogenization buffer used in ligand-binding ER assays (21–24). The 4S form is extracted in high-salt media and represents hormone-bound ER (25). Unoccupied ER has been found to consist of a dimer of two steroid binding subunits of M_r 65 000 each bound to a 90 000 M_r heat shock protein, while the ligand-bound form is a dimer of the hormone-binding subunit (26). Biochemical characterization has shown that steroid receptors are composed of three functional domains: a steroid binding site, a DNA binding site, and a site responsible for transcriptional activation of

selected genes (27, 28). ER is furthermore characterized by the kinetics of the hormone-receptor binding, which must be specific, i.e. ER binds estradiol with a higher affinity than other classes of steroid hormones (29), of high affinity, i.e. the equilibrium dissociation constant K_d is in the order of 1×10^{-10} (30), and saturable as there is a limited amount of receptor molecules in each cell, roughly 20 000 in an ER-rich breast cancer cell (31). The more physiological marker of a receptor: a tissue or cell response to hormonal stimulation is rarely determined.

Hybridoma techniques have permitted the development of monoclonal antibodies directed against ER and have allowed receptor determination based on monoclonal antibody recognition in tissue extracts and in tissue sections. Greene et al. purified ER from a human ER-positive breast cancer cell line, MCF-7, and used it for immunization of male Lewis rats (32). Splenic cells from these animals were fused with a mouse myeloma cell line and the antibody-producing hybridomas were expanded as ascites tumors in athymic mice (7, 32). The ER antibodies from these experiments were designated with D-numbers, and in a parallel, collaborative study at the Abbott Laboratories a corresponding H-series was developed (33).

Monoclonal antibodies. Three antibodies, all IgG2a immunoglobulins, have been used: H222, H226, and D547. H222 recognizes a site near the ligand-binding domain of the receptor (34) and recognizes ER in reproductive tissue from all tested mammals (including rabbits) and in the hen oviduct (22, 34, 35). H226 binds near the DNA-binding domain of the receptor (34) and covers the same spectrum of species as H222 (22, 34, 35). D547 binds in the central part of the receptor (34) and has a lower affinity to human ER than H222 and H226 (36). D547 recognizes only mammalian ER and among these rat and calf ER with decreased affinity (37). The specificity of these three antibodies for ER has been extensively documented by sucrose gradient and immunoblot analyses (32, 33, 38). The H222 antibody is widely used in immunohistochemical ER-assays manufactured by the Abbott Laboratories and has been used in studies (42, 52, 62, 67, 68, 101). The D547 antibody in conjunction with H222 has been used in the commercially available enzyme immunoassay (ER-EIA) employed in study (42). The H226 antibody has been used for ER immunohistochemistry in frozen sections in study (53).

Methods for determination of estrogen receptors

Dextran-coated charcoal assays

This ligand-binding ER assay (ER-DCC) has previously been reviewed by Poulsen (39) and Thorpe (40). In brief: the assay is performed by incubating extracts of tumor tissue with a radiolabeled estrogen, removing the unbound hormone with dextran-coated charcoal, and estimating the ER content from a binding plot. The assay is technically difficult to use and critical points encompass tissue sampling and storage, the homogenization procedure, composition of extraction buffers, the method for removal of excess ligand, compensation for non-specific binding, and the interpretation of binding plots. The method is furthermore dependent on a high cytosol protein concentration which demands relatively large amounts of tissue (0.5–1 g). Histopathologic control must ensure that the sample contains malignant tissue, and the use of homogenate inherently hides potential tumor heterogeneity. The assay is, however, widely used and almost all clinical information has so far come from DCC assays. In the present studies ER-DCC assays were performed according to EORTC recommendations (41).

Enzyme-immunoassays

The technology employed in this assay was first suggested by Greene & Jensen (7). The principle is that a primary monoclonal antibody, D547, is bound to a polystyrene bead and incubated with the cytosol. After washing a second monoclonal antibody (H222) labeled with peroxidase enzyme is added and labels the bound receptor. The beads are then incubated with hydrogen peroxide and o-phenylenediamide, which yields a colored reaction product measurable in a spectrophotometer. The extinction of cytosols is compared with a run-to-run standard curve obtained from dilutions of a standard cytosol extracted from a MCF-7 cell line. Thus the results are referred back to the manufacturer's (Abbott Laboratories) own DCC assay used for calibration of standards. The method is technically easy to perform and ER can be reliably measured in cytosols with protein concentrations as low as 0.5 mg/ml (42–44). Many studies have compared ER-EIA with steroid binding assays as summarized in Table 1. A significant correlation is found between two methods but the slope of the regression line is usually >1 indicating that the ER-EIA assay determines more ER than ligand-binding assays.

A slope significantly larger than 1 was also found in our own comparative study (42) and in the EORTC Receptor Study Group quality control studies in which the ER-EIA in general determines more ER than DCC assays (45). The trend has persisted in the 1989 EORTC quality control study (Benraad and Geurts, personal communication). There could be several explanations why the ER-EIA measures more receptor than the ligand-binding assays. First, ligand-binding assays do not normally measure ER already occupied by endogenous estrogen (46). Second, ER-EIA may measure degraded ER which has lost its steroid binding ability but retained its epitopes for antigenic recognition. This theory is, however, refuted by experiments showing that the ER-EIA does not detect inactivated receptor (47). Third, it is possible that the

Table 1

Estrogen receptor analyses in breast cancer. Comparison of steroid-binding and enzyme-immunoassays. Correlation coefficient, slope and intercept given for the linear correlation of ER-DCC vs ER-EIA with ER-EIA = slope × ER-DCC + intercept

Reference	Tumors	Correlation coefficient	Slope	Intercept	Binding assay
(42)	57	0.92	1.3	+12 fmol	Multi-point DCC
(43)	19	0.97	0.8	+16 fmol	Multi-point DCC
(44)	80	0.96	1.0	+21 fmol	Multi-point DCC
(44)	153	0.98	1.3	+5 fmol	Single-point DCC
(49)	82	0.71	1.9	−6 fmol	Multi-point DCC
(50)	115	0.90	1.3	NA	Multi-point DCC
(51)	25	0.94	NA	NA	Multi-point DCC
(113)	75	NA	1.9	+3 fmol	Isoelectric focusing
(113)	74	NA	0.8	+0 fmol	Single-point DCC
(113)	87	NA	1.0	+3 fmol	Multi-point DCC
(113)	75	NA	1.0	+1 fmol	Multi-point DCC
(113)	74	NA	1.3	+3 fmol	Multi-point DCC
(113)	16	NA	1.2	+5 fmol	Multi-point DCC
(113)	72	NA	1.0	+1 fmol	Multi-point DCC
(113)	84	NA	1.1	+2 fmol	Multi-point DCC
(114)	63	0.92	1.8	+8 fmol	Single-point DCC
(114)	65	0.97	1.5	+6 fmol	Multi-point DCC
(114)	59	0.97	1.1	+10 fmol	Single-point DCC
(115)	76	0.95	0.9	NA	Multi-point DCC
(116)	70	0.85	1.8	NA	Multi-point DCC
(117)	24	0.88	2.0	NA	Multi-point DCC
(118)	61[1]	0.86	0.9	+1 fmol	Single-point DCC
(119)	75	0.75	1.3	NA	Isoelectric focusing
(120)	23	0.77	NA	NA	Multi-point DCC
(121)	100[1]	0.85	6.0	NA	Isoelectric focusing
(122)	108	0.95	1.5	+33 fmol	Multi-point DCC

1: Fine-needle biopsies
NA = Not available, DCC: Dextran-coated charcoal

amounts of ER determined by the two methods vary because differences between the ER-DCC assays used in the individual laboratories and the ER-DCC assay used for standardization of the ER-EIA by the manufacturer (Abbott Laboratories) are actually greater than should be expected.

The ER-EIA assays seemed originally to have smaller interlaboratory variations than the ER-DCC assays (48), but as more and more laboratories shift from the ER-DCC to the ER-EIA, EORTC quality control studies have shown that there is a broadening of the coefficients of variation between these laboratories approximating the 40% known from ER-DCC assays (Benraad and Geurts, personal communication). The overall result is that using a given cut-off level for a definition of what ER positive means (normally 10 fmol/mg protein), more and more tumors are being classified ER-positive by the ER-EIA assay, especially in the low range (42, 43, 45, 49, 50). The implication is that another cut-off level should possibly be used for obtaining a given relationship between ER-status and clinical parameters, which is supported by the only clinical study comparing ER-EIA and ER-DCC (51). The true virtue of the assay in patient management is so far undetermined and future comparisons of receptor-response relationships among different patient groups will be virtually impossible if the use of fundamentally different techniques adds to the confidence problems already caused by interlaboratory variations. The only possible solution will be quality assurance studies and the production of massive clinical documentation.

Immunohistochemical assays using monoclonal ER antibodies in frozen sections

Almost all published studies have used the same immunohistochemical method, ER-ICA, developed and manufactured by Abbott Laboratories. In brief: cryostat sections are lightly fixed in formalin and treated with methanol and acetone. The sections are incubated with monoclonal ER antibody and the ER anti-ER binding is amplified with a secondary goat anti-rat antibody followed by a peroxidase-antiperoxidase system and visualized with di-amino-benzidine. The same technique is used with minor modifications for ER staining in fine-needle aspirates.

Table 2

Comparison of immunohistochemical ER assays in frozen sections or fine-needle biopsies with ER assays in tissue homogenates

Ref.	Tumors	Tissue	Score system	Cut-off[1] level	ER[2] pos.	Assay	Cut-off level fmol	ER status agreement	Correl. coeff.	Senstivity advantage
(36)	48	FS	IMA	>0 AU	NA	DCC	NA	NA	0.72	NA
(49)	40	FS	EYE	>1 AU	50%	EIA	20	68%	NA	EIA
(54)	127	FN	IMA	>0 AU	76%	NA	NA	NA	NA	NA
(55)	35	FN	IMA	>5%	69%	DCC	10	91%	0.77	DCC
(56)	115	FS	IMA	>0?	70%	DCC	10	88%	NA	DCC
(57)	100	FS	IMA	>18 AU	75%	DCC	10	99%	0.86	No diff
(58)	600	FS	EYE	>10%	45%	DCC	10	77%	0.56	DCC
(59)	117	FS	EYE	>0%	58%	SG	300	86%	0.76	FS
(60)	114	FS	EYE	>10%	57%	DCC	11	86%	0.50	DCC
(61)	28	FS	EYE	>0%	50%	DCC	5	86%	NA	FS
(70)	114	FS	EYE	>0%	77%	DCC	10	88%	0.83	DCC
(71)	257	FS	EYE	>75 AU	54%	DCC	10	69%	0.63	NA
(72)	163	FS	EYE	>2%	68%	EIA	10	89%	NA	EIA
(88)	97	FS	EYE	>100 AU	53%	DCC	NA	92%	NA	DCC
(89)	56	FS	EYE	>0.5 AU	NA	DCC	15	NA	NA	FS
(104)	78	FN	EYE	>0%	80%	DCC	15	89%	0.48	FN
(105)	56	FN	EYE	>0%	82%	DCC	5	93%	0.73	DCC
(105)	41	FN	EYE	>0%	73%	DCC	5	NA	0.65	DCC
(106)	69	FS	EYE	>5 AU	83%	DCC	10	93%	NA	FS
(106)	31	FN	EYE	>5 AU	77%	DCC	10	90%	0.55	No diff
(107)	63	FS	EYE	>10%	64%	DCC	10	86%	NA	DCC
(107)	63	FN	EYE	>10%	59%	DCC	10	78%	0.45	DCC
(108)	35	FN	EYE	>10%	80%	DCC	10	94%	0.49	FN
(109)	33	FN	EYE	>75 AU	58%	DCC	10	85%	0.74	DCC
(122)	108	FS	EYE	>70 AU	NA	EIA	10	NA	0.72	NA
(122)	225	FS	EYE	>70 AU	59%	DCC	10	77%	0.67	DCC
(123)	359	FS	EYE	>0%	70%	DCC	10	NA	NA	FS
(124)	34	FS	EYE	>0%	74%	DCC	5	94%	0.87	DCC
(125)	118	FS	EYE	>0%	78%	DCC	10	91%	0.60	FS
(126)	36	FS	EYE	>0%	75%	DCC	3	94%	NA	FS
(127)	32	FS	EYE	>0%	66%	DCC	5	91%	NA	FS
(128)	212	FS	EYE	>100 AU	69%	DCC	11	78%	NA	DCC
(129)	82	FS	EYE	>10%	70%	DCC	10	85%	NA	DCC
(129)	82	FS	EYE	>10%	70%	EIA	10	71%	NA	NA
(130)	48	FS	EYE	>0%	71%	DCC	10	81%	0.76	No diff
(131)	185	FS	EYE	>100 AU	71%	DCC	10	79%	NA	FS
(132)	62	FS	EYE	>75 AU	NA	DCC	NA	NA	0.79	NA
(132)	262	FS	EYE	>75 AU	NA	DCC	NA	NA	0.64	NA
(133)	78	FS	EYE	>10%	78%	DCC	10	86%	0.73	FS
(134)	116	FS	EYE	>30%	53%	DCC	10	80%	0.48	DCC
(135)	20	FS	EYE	>10%	65%	DCC	10	85%	0.85	FS
(136)	123	FS	EYE	NA	64%	DCC	10	89%	0.75	FS
(137)	46	FS	EYE	>10%	77%	IF	10	85%	NA	FS
(138)	15	FS	EYE	>0%	81%	DCC	NA	92%	NA	FS
(139)	50	FS	EYE	>25%	42%	DCC	5	65%	NA	No diff
(140)	50	FS	EYE	>35 AU	60%	DCC	10	82%	0.65	No diff
(141)	159	FS	EYE	>100 AU	69%	DCC	10	80%	0.52	DCC
(142)	94	FS	EYE	>0%	70%	DCC	5	94%	0.44	FS
(143)	100	FS	EYE	>0%	71%	DCC	10	91%	NA	DCC
(144)	37	FS	EYE	>0%	71%	DCC	20	90%	0.60	NA
(145)	91	FS	EYE	>1 AU	80%	DCC	10	93%	NA	FS
(145)	71	FS	EYE	>1 AU	69%	DCC	10	75%	NA	DCC
(145)	55	FS	EYE	>1 AU	78%	DCC	10	91%	NA	DCC
(146)	62	FN	EYE	>0%	71%	DCC	10	92%	NA	DCC
(147)	179	FS	EYE	>100 AU	69%	DCC	10	82%	0.59	FS
Median					64%			83%		
Range					42%–83%			65%–99%		

1: Cut-off level for definition of ER positive. 2: Percent ER positive defined from immunohistochemical analysis. FS: Frozen sections. FN: Fine-needle biopsies. EYE: Scored by eye. IMA: Scored by image analysis. AU: Arbitrary units. DCC: Dextran-coated charcoal. SG: Sucrose gradient. IF: Isoelectric focusing. EIA: Enzyme immunoassay. NA: Not available. Diff. = difference.

The specificity of the staining is normally ensured by parallel incubation of the section, replacing the anti-ER with a non-immune serum and in addition by staining a known ER-positive control slide. This control consists of a smear from a strongly ER-positive MCF-7 cell-line. The results from comparative studies on ER-ICA and assays in tissue homogenate are summarized in Table 2. The studies are not easily compared. First, the overall percentage of ER-positive tumors ranges from 42–83%. Second, the definition of a receptor positive tumor varies from 1 positive cell to 30% positive cells in the immunohistochemical assays and from 5–20 fmol ER/mg cytosol protein in the ER-DCC or ER-EIA assays. Third, a variety of scoring systems have been applied, including video-image analysis systems. However, with a median agreement of 86% the immunohistochemical and the biochemical assays show a fairly good overall concordance. This is probably the best result that can be obtained considering the inherently different nature of the assays. Using the given cut-off levels, the sensitivity of the immunohistochemical assays in terms of their ability to detect ER seems to match that of the biochemical assays (Table 2). Our own study comparing ER-DCC, ER-ICA and ER-PAR showed the ER-DCC assay to be slightly more sensitive that the ER-ICA (52).

A great deal of effort has been put into semiquantifying the staining features to allow comparison with biochemical analyses, either by use of a microscopic grid or more recently by advanced imaging systems (53–58). Significant quantitative correlations can be obtained as shown in Table 2 and the use of imaging systems seems to reduce the discordance between observers (36). Nevertheless, the correlation coefficients are far from impressive and efforts would probably be rewarded better if attempts were made to define a relevant cut-off level based on clinical data.

Quality control studies on immunohistochemical assays have not yet been undertaken.

Immunohistochemical assays using monoclonal ER antibodies in paraffin sections

A major problem in ER analysis in paraffin-embedded tissue (ER-PAR) is the loss of immunoreactive ER during tissue processing and fixation. Several studies have accordingly reported results in formalin-fixed material to be inconsistent (59–61). We found many fixatives unsuitable for ER immunohistochemistry, i.e. Carnoy's Zenker's Bouin's Lilly's and Helly's fixatives and ethanol, while other commonly used fixatives like 3.6% formaldehyde, 2.5% glutaraldehyde or Zamboni's fixative made ER determination possible in spite of a considerable loss of immunoreactivity compared to frozen sections (62). The decreased immunoreactivity necessitated modifications of the immunoperoxidase technique. We obtained a significant increase in staining intensity after digestion with trypsin (62) and thus confirmed the ability of proteolytic enzymes to unmask antigenic determinants in fixed tissue (63, 64). In addition, we found it necessary to change the amplification system from peroxidase-antiperoxidase to the more sensitive avidin-biotin-peroxidase-complex system (65). Similar modifications have been necessary in other studies on ER analysis in paraffin-embedded tissue, for an overview see Table 3. We established controls for each specimen by replacing the primary antibody with normal rat IgG in the same dilution. In addition, we processed a known medium-stained ER-positive and a known ER-negative specimen in parallel with the unknown sections. The strongly ER-positive MCF-7 control delivered with Abbott's ER-ICA kit seems less satisfactory for two reasons. First, minor run-to-run variations in staining intensities are disclosed if the control is always maximally stained. Second, the ER antibody used is raised from MCF-7 ER and then used for staining MCF-7 cells which makes this control less acceptable.

Table 3

Estrogen receptor analyses in paraffin-embedded tissue. Comparison of methods

Reference	Fixative	Pretreatment	Amplification system	Chromogen
(42, 62) (52, 67)	10% formalin	trypsin	ABC	carbazole
(61)	cold 10% formalin	none	ABC	DAB
(86)	Bouin's	none	ABC	DAB
(127)	10% formalin	DNase-I	PAP	DAB
(148)	10% formalin	pronase	Alk. Phosph.	BCIP/NBT
(149)	10% formalin	trypsin/DNase	PAP	DAB/$CoCl_2$
(150)	picric acid/formalin acetic acid	none	ABC	DAB
(151)	Bouin's	none	PAP	DAB
(152)	10% formalin	trypsin	Peroxidase*	DAB

ABC: Avidin-biotin-peroxidase. PAP: Peroxidase-antiperoxidase. DAB: Diamino-benzidine. BCIP: 5-bromo-4-chloro-3-indoyl phosphate. NBT: Nitroblue tetrazolium. *: Peroxidase conjugated secondary antibody.

We scored the staining features semiquantitatively to establish a correlation with the biochemical ER content. The intensity of nuclear staining was subjectively scored 0, 1, 2, 3, and the percentage of ER-positive epithelial cells was estimated using a microscopic grid. This method was also used to estimate the percentage of the specimen occupied by malignant epithelium ('cellularity'). The resulting histochemical 'ER score', derived by multiplying staining intensity, percentage of stained epithelial cells and cellularity, correlated closely with the ER-DCC results (42, 62). We also found that a simpler score system combining staining intensity and the number of positive cells each graded 0–3 correlated equally well with ER-DCC (62, 66). We used the simple score system to disclose intra- and interobserver differences in the evaluation of slides. ER scores were highly reproducible as evidence by a correlation coefficient between 1st and 2nd scores by the same observer of 0.97 and an interobserver correlation between two observers of 0.93 (62). The main purpose of making a score system was that we wanted to obtain a semiquantitative correlation with the quantitative ER assays. We found satisfactory correlations with the ER-DCC assays when fixation and tissue processing was controlled in our own laboratory (42, 62, 66), while routine processed tissue blocks from other laboratories produced a less impressive overall agreement between ER statuses (52, 67) and poorer semiquantitative correlations (52). The ER status was in particular less reliably determined in tumors with low ER contents (< 100 fmol) (52, 67). This observation together with lack of correlation between response and semiquantified ER content in a clinical study (68) made us give up semiquantitation of ER in paraffin blocks processed outside our own laboratory, and we consequently defined a tumor ER-positive if we could recognize any degree of specific staining exceeding that of the control section.

Results obtained from studies in paraffin sections are compared with the results achieved in fresh tissue in Table 4. Paraffin assays are generally less sensitive (i.e. less able to detect ER) than fresh tissue assays in spite of the modifications adopted in the immunohistochemical techniques, and ER-positiveness is often defined at a lower cut-off level in paraffin sections than in frozen sections.

The clinical value of estrogen receptor determination in breast cancer

Background

The value of ER determination from a prognostic point of view, i.e. its ability to forecast disease outcome, can only be established by considering disease-free survival of early breast cancer patients who have received no prior systemic treatment. The term prognostic value is thus used to forecast the outcome in patients with early breast cancer. The only relevant end-point to use in attempts to

Table 4

Estrogen receptor analyses in breast cancer. Comparison of immunohistochemical ER assays in paraffin sections and ER assays in tissue homogenates or in frozen sections

Reference	Tumors	Score system	Cut-off level	ER pos	Assay	Cut-off level	ER status agreement	Correl. coeff.	Sensitivity advantage
(42)	56	EYE	>0%	68%	DCC	10 fmol	96%	0.88	DCC
(42)	55	EYE	>0%	67%	EIA	10 fmol	95%	0.60	EIA
(52)	116	EYE	>0%	59%	DCC	10 fmol	75%	0.62	DCC
(52)	109	EYE	>0%	60%	FS	>0%	82%	0.61	FS
(61)	24	EYE	>0%	50%	FS	>0%	88%	NA	FS
(61)	109	EYE	>0%	39%	DCC	5 fmol	82%	NA	DCC
(62)	111	EYE	>0%	63%	DCC	10 fmol	91%	0.81	DCC
(67)	309	EYE	>0%	75%	DCC	10 fmol	80%	NA	DCC
(86)	44	EYE	>0%	68%	DCC	10 fmol	73%	NA	DCC
(127)	32	EYE	>0%	59%	FS	>0%	94%	NA	FS
(127)	70	EYE	>0%	59%	DCC	5 fmol	87%	NA	DCC
(148)	27	EYE	>0%	41%	DCC	5 fmol	93%	NA	PAR
(149)	12	EYE	>0%	83%	FS	>0%	100%	NA	No difference
(149)	12	EYE	>0%	83%	DCC	NA	92%	NA	PAR
(150)	115	EYE	NA	NA	DCC	10 fmol	NA	NA	NA
(151)	68	EYE	>0%	40%	DCC	3 fmol	65%	NA	DCC
Median				59%			88%		
Range				39%–82%			65%–100%		

AU: Arbitrary units. DCC: Dextran-coated charcoal, EYE: Scored by eye. FS: Frozen sections. NA: Not available. PAR: Paraffin assay.

evaluate ER as a prognosticator is the disease-free survival of systemically untreated patients. Because the disease-free survival is the sum of the natural course of the disease and the effect of systemic treatment, we do not here consider patients in adjuvant endocrine therapy or adjuvant chemotherapy because such therapy may partly work through cytotoxic castration (69), and the effect may in both cases be a function of receptor status. Survival is not a relevant endpoint because it is a sum of the natural course of the disease and hormonal therapy of recurrent disease, a treatment received at some time by nearly all patients with recurrent breast cancer.

The value of ER determinations can also be assessed in terms of its ability to predict the benefit of adjuvant therapy. In this situation response cannot be measured and the endpoint is prolongation of disease-free survival or survival in receptor-defined subgroups.

Finally, ER determination may provide a tool for prediction of response to endocrine therapy of advanced disease and may thus assist the selection of categories of patients who are expected either to respond or not to respond to endocrine therapy.

Patients

The prognostic value of ER analysis in paraffin-embedded tissue was analyzed in two patient groups. The patients in study (52) had previously been selected for a comparison between ER-DCC and ER-ICA assays in a study published by Desombre et al. (70). This data was supplemented with data obtained in paraffin-embedded tissue in order to directly compare the ER-DCC, the ER-ICA and the ER-PAR assays. The 130 biopsies available were all from postmenopausal high-risk patients with early breast cancer. A total of 108 patients had been entered in the Danish Breast Cancer Cooperative Group (DBCG) protocols: 84 in DBCG-77 and 24 in DBCG-82. Patients in the DBCG-77 protocols had received adjuvant radiotherapy and were then randomized to either observation or adjuvant tamoxifen 10 mg × 3 for 48 weeks. Patients in the DBCG-82 protocols were randomized to either 1) radiotherapy and tamoxifen (the same schedule as in the DBCG-77 protocols), 2) tamoxifen, or 3) tamoxifen, cyclophosphamide, methotrexate, and 5-fluorouracil. Only 40 of the patients in study (52) had received no systemic treatment, which is too low a figure to permit adequate statistical evaluation of the prognostic value of ER. The prognostic value of ER-PAR was accordingly re-analyzed in study (67), in which 176 patients were systemically untreated as described in the following.

The ability of ER-PAR to predict the benefit of adjuvant tamoxifen treatment was analyzed in study (67) in a subset of 349 patients from the DBCG 77c protocols, in whom the ER content had been measured at the time of operation. As described above these postmenopausal high-risk patients had been randomized to either radiotherapy (176 patients) or radiotherapy + tamoxifen (173 patients). A previous study with a median follow-up of 42 months has shown that a primary tumor content of more than 100 fmol ER/mg cytosol protein is required if patients should be expected to benefit from tamoxifen therapy (15). This data was supplemented with data obtained in paraffin-embedded tissue in these 349 biopsies to examine the value of ER-PAR compared to that of a conventional binding assay.

The ability of ER-PAR to predict the response to hormonal treatment in advanced breast cancer was analyzed in study (68). The records of all patients treated for advanced or recurrent breast cancer at one institution during a 10-year period were reviewed, while observing the following inclusion criteria: 1) recurrent or advanced breast cancer with evaluable disease parameters, 2) no previous systemic treatment, 3) evaluable disease treated with hormones only, 4) no previous hormone receptor analysis. These criteria were met by 145 patients, but histologic material could not be obtained in 8, leaving 137 for analysis. This was less than 10% of the total number of patients treated for recurrence. Nevertheless, this selected group corresponded with the one seen in clinical practice, where receptor analysis was required to assist the choice of therapy of recurrent disease in those patients whose ER status had not been measured at the time of the primary operation.

ER and prognosis in patients with early breast cancer

The relationship between prognosis and ER-EIA has only been described in a single study, which unfortunately included patients who received adjuvant systemic therapy. However, this study did provide one important piece of information: a 30 fmol ER/mg cytosol protein level in the ER-EIA assay was clinically equivalent to a 18 fmol ER/mg cytosol protein level in the ER-DCC assay (51).

The literature on prognosis and immunohistochemical ER analysis is sparse and the data, which is discrepant, has exclusively been obtained from analysis of frozen sections: Kinsel et al. found that neither ER-ICA positive nor ER-DCC positive patients had a disease-free survival advantage (71). Pertschuk et al. (58) found a significant disease-free survival advantage of ER-ICA positive patients, but some or all patients in the two studies seemed to receive adjuvant therapy. Walker et al. found a significant disease-free survival advantage of ER-ICA positive patients, none of whom received systemic adjuvant therapy, but they also found a close correlation between the ER status and other prognostic factors, and the statistical analysis used was univariate (72). In study (52) we found that neither of the applied immunohistochemical ER-assays (ER-ICA ($p = 0.25$), ER-PAR ($p = 0.51$)) could statistically significantly discriminate a high-risk group from a

low risk-group among systemically untreated patients, but the study lacked statistical power.

The difficulty of precisely determining the role of the ER status as a prognosticator lies partly in the fact that it is correlated with other prognostic parameters and its individual prognostic value may best be determined by use of a multivariate analysis. Few such studies have been published on ER and disease-free survival in systemically untreated patients and they have all dealt with biochemical ER analyses. A review of a number of large studies in which multivariate analysis was used is shown in Table 5. The majority of these studies have focused on low-risk groups and only few have analyzed systemically untreated patients. None of the studies selected ER as an independent prognostic parameter.

A comparison of the relative prognostic value of the ER-PAR and the ER-DCC was performed in a multivariate analysis of the systemically untreated patients described in (67). A Cox's proportional hazards model was used (73) (for covariates and scores, see Table 6). A complete set of covariates was present in 133 patients who did not receive systemic adjuvant therapy and they form the basis for the analysis. The regression variables with statistics, shown in Table 7, confirm the strong prognostic influence of lymph node status and tumor size. In addition we found the ER-DCC but not the ER-PAR assay to be a significant prognosticator. In the final regression model, also shown in Table 6, the significant contributors only included lymph node status, tumor differentiation and ER-DCC, which in this study was a significant and independent prognostic variable. Closely correlated factors can displace each other during the analysis in the Cox's model and we consequently tested the model with ER-DCC and ER-PAR, respectively as the only covariate. The test showed that ER-DCC remains an independent variable,

Table 6

Scoring of covariates in a proportional hazards model analysis of prognostic factors in 133 postmenopausal patients with high-risk breast cancer

Covariate	Scores		
Age	Actual age		
Tumor size	1: > 5 cm. 0:	< = 5 cm.	
Nodal status*	Actual fraction		
Tumor grade#	2: Grade III	1: Grade II	0: Grade I
ER-DCC	1: < 10 fmol	0: > = 10 fmol	
ER-PAR	1: Negative	0: Positive	

*: Nodal status given as the number of positive nodes divided by the number of nodes excised. #: Bloom and Richardson (158).

Table 5

Multivariate studies on the prognostic significance of ER in primary breast cancer

Reference	No. of pts.	Stage	Premenop.[1]	Adjuvant therapy given		ER pos.	Variables tested	Independent variables found
				Radiotherapy	Systemic			
(14)	414	I, II, III	NA	NA	NA	63%	No. pos ln., Tumor size, ER, PgR, age, TLI	No. pos. ln., Tumor size, TLI
(75)	807	N-	36%	0	0	75%	Menopausal status, age, tumor size, nuclear pleomorphy, ER, PgR, no. of lymph nodes found	Nuclear pleomorphy
(153)	215	N-	66%	0	0	77%	Tumor size, ER, TLI	TLI
(154)	345	N-	NA	NA	NA	71%	Ploidy status, tumor size, ER, PgR, Age	Ploidy status
(155)	250	N-	28%	35%	28%	69%	Age, tumor size ER, PgR, ploidy S-phase-fraction	S-phase-fraction
(156)	506	NA	26%	NA	NA	72%	No. pos ln., tumor size, ER, PgR, age, menopausal status	No. pos ln., PgR, tumor size, age, menopausal status
(157)	1 157	N-	NA	33%[2]	0	74%	Nuclear grade, ER, histologic grade, PgR	Nuclear grade

ER: Estrogen receptor. PgR: Progesterone receptor. TLI: Tumor labeling index. NA: Not available. N-: Node-negative patients. 1: Percentage premenopausal patients. 2: Radiotherapy to breast after tumorectomy.

Table 7

Regression variables with statistics

Covariate	χ^2	p-value	Relative risk (95% CL)
Complete model			
Age	0.10	0.76	
Tumor size	6.97	0.008	
Nodal status*	24.88	0.000	
Tumor grade#	4.38	0.036	
ER-DCC	7.90	0.005	
ER-PAR	0.55	0.46	
Final			
Nodal status*	24.88	0.000	5.6 (3.9–7.9)
ER-DCC	8.76	0.003	2.1 (1.6–2.7)
Tumor grade#	3.78	0.05	1.6 (1.2–2.0)

*: Nodal status given as the number of positive nodes divided by the number of nodes excised. #: Bloom and Richardson (158)

while ER-PAR stays insignificant (data not shown). ER-PAR is probably inferior because a cut-off level of 5 fmol seems close to optimal for prognostication (74, 75).

In conclusion, the ER-DCC assay, not the ER-PAR assay, is useful as a prognostic parameter in the high-risk postmenopausal patient group analyzed, but the prognostic value of estrogen receptor analyses is in general low because they fail to achieve significance in most studies using multivariate analysis.

Prediction of benefit of adjuvant hormonal treatment in early breast cancer

Meta-analyses show a small but significant benefit of adjuvant endocrine therapy in postmenopausal women with early high-risk breast cancer (76, 77). The value of ER status in predicting the benefit from adjuvant endocrine treatment is controversial. Thus, adjuvant tamoxifen treatment has been reported in some studies (16–18) to benefit only patients with ER-positive tumors, whereas the NATO trial found that the ER status had no such importance (78). However, the Scottish trial demonstrated that all women benefitted, but mostly those with the highest ER content (79). The NSABP study selected only ER-positive patients for adjuvant tamoxifen but found the benefit to be independent of the actual ER level (80).

The DBCG 77c study found an overall effect of tamoxifen (81), but in the subset with ER-DCC analyses an effect was only seen in patients with high ER levels. We reanalysed the DBCG data in 1990 as described above. The results showed that the benefit of tamoxifen had diminished below statistical significance in all receptor-defined subgroups at a median follow-up of 86 months and that the advantage approached significance only for patients with higher receptor concentrations. Results from the paraffin analyses showed that the effect of tamoxifen treatment, although non-significant, was of the same magnitude in ER-PAR positive and ER-DCC positive patients with high ER contents. This implies that ER-PAR analyses might be valuable in the retrospective analysis of adjuvant trials. The fading of the treatment effect with time in the DBCG study speaks in favor of prolonging treatment beyond one year as also suggested by the NATO trial where the treatment advantage fell off after discontinuation of tamoxifen (78). Cell kinetic studies have shown that the main effect of tamoxifen is to reversibly cause a slow cell cycle transity (82, 83). This suggests that adjuvant tamoxifen treatment should be continued until recurrence or death, as in the treatment of advanced disease.

Some of the problems of interpreting the relationship between ER and the effect of adjuvant hormonal treatment are undoubtedly secondary to the fact that only a fraction of the patients in the relatively large studies mentioned above had their ER measured (DBCG: 20%, Scottish: 43%, NATO: 41%). In addition there must be differences in the quality of receptor analyses among the laboratories as can be seen from the comparison in Table 8, where substantial variation among receptor classifications are seen in these 3 trials despite relatively comparable patient populations. Further definition of the role of ER in predicting the benefit from adjuvant endocrine therapy is obviously required and can best be obtained from controlled clinical trials in which ER is measured with high-quality ER assays. A possible shortcut could be retrospective analysis of tumors from one of the larger randomized trials using a paraffin-based ER assay as shown in our own study (67). Pending the collection of such data stratification based upon receptor status should be cautious, considering the risk of withholding ER-negative patients a potential benefit from an adjuvant endocrine treatment with modest toxicity.

Table 8

Comparison of receptor data in adjuvant tamoxifen trials

Trial	Patients total No.	Menopause		ER data % of tot.	Distribution of ER-levels (fmol)					
		pre	post		0–4	0–9	5–19	10–99	20–99	>=100
NATO[1]	1 211	12%	88%	41%	NA	41%	NA	31%	NA	28%
Scottish	1 312	18%	82%	43%	29%	NA	12%	NA	29%	30%
DBCG	1 700	0%	100%	20%	NA	21%	NA	29%	NA	50%

1: Receptor data taken from (40)

Prediction of response to hormonal treatment in advanced breast cancer

About one third of unselected patients with advanced breast cancer will respond to endocrine therapy (84, 85). The effect of hormonal therapy seems to be mediated through hormone receptors and ER analyses can be used for predicting the probability of response. Biochemical assays generally show response rates of 50–60% among patients with ER-positive tumors and 5–10% when the tumor is ER-negative (8–11, 39). Clinical data on response and ER-EIA analyses is not available and although several studies using immunohistochemical ER analyses have been published, the majority have used fresh tissue i.e. frozen sections or fine-needle aspirates. The results of these studies, which generally include very few patients, are summarized in Table 9. An overall 60% of ER-positive patients responded to endocrine therapy in contrast to 10% of ER-negative patients. They are very similar to those obtained from ER-DCC techniques. The studies utilizing paraffin-embedded tissue seem to have the lowest predictive power with 49% (68) and 55% (86) responses among patients classified as ER-positive, but this does not seem to be secondary to a decreased sensitivity of the assays as the overall frequency of ER-positive tumors is similar to that of studies in frozen tissue. These results should be interpreted with caution because they are virtually incomparable. Thus the frequency of overall response differs two-fold between different studies, which must reflect differences among the study populations and/or the criteria of response. Table 9 also shows that the criteria for classification of ER-status are different, resulting in large differences in the frequency of ER-positive tumors among the studies.

Increasing response rates with increasing ER content has been reported in most studies based on biochemical ER determinations (8–10, 87) and in three studies based on immunohistochemical ER analysis in frozen tissue (36, 60, 88). In our own study (68) and in a study utilizing immunohistochemical ER analysis in frozen tissue (89), no such relationship could be established. We consider this point to be of minor clinical importance because the true power of the assay lies in its ability to select those patients who would not respond to endocrine therapy. The overall impression is that immunohistochemical ER analyses can be useful predictors of response to endocrine therapy in patients with advanced breast cancer, notwithstanding the differences in the material used for analyses or even the differences among the criteria for interpretation of ER status.

The clinical relevance of estrogen receptor heterogeneity

The treatment of metastatic disease based on the receptor status of the primary tumor rather than on that of the metastasis rests on the assumption of an identical ER status and is often necessitated by the inaccessibility of tissue from the metastatic site. The question why a large fraction of patients with ER-positive tumors do not respond to endocrine therapy whereas some patients with ER-negative tumors do respond could, among several reasons, be intratumoral clonal differences in ER status caused by tumor heterogeneity with a subsequent propensity of ER-negative clones to metastasize. This theory is based on the known genetic instability of cancer cells (90) and is supported by several studies showing a difference in ER status between multiple specimens from individual breast tumors (91–94), between simultaneous samples from primary and metastatic lesions (95–97) and between sequential biopsies (97–100). The studies cited have all used biochemically

Table 9

Immunohistochemical ER analysis and response to endocrine treatment.

Ref.	Tumors	Tissue	Response rate %	ER positive definition	ER positive %	Response ER pos. %	Response ER neg. %
(36)	49	frozen	33	>0 AU	67	48	0
(58)	47	frozen	38	>10% pos	64	57	6
(60)	43	frozen	33	>10% pos	47	60	9
(68)	137	paraffin	37	>0% pos	70	49	7
(86)	28	paraffin	43	>0 AU	71	55	13
(88)	20	frozen	35	>1 AU	55	86	11
(89)	56	frozen	39	>0 AU	70	75	4
(105)	14	fine-needle	43	>0% pos	79	55	0
(132)	22	frozen	64	>75 AU	64	93	11
(134)	29	frozen	69	>10% pos	86	64	100
(159)	52	fine-needle	52	>0% pos	NA	63	0
(160)	12	frozen	33	>7 AU	58	57	0
Median			39		67	59	8
Range			33–69		47–86	49–93	0–100

determined ER status, and the differences reported could be explained by limitations of the applied methods for ER determination as discussed in (101).

The theory seems to be supported by immunohistochemical ER assays which invariably report cell-to-cell differences of ER expression in tissue sections from breast cancers. We have addressed this question by performing immunohistochemical ER analysis in paraffin-embedded tissue from breast tumors and their regional and distant metastases. The ER status of the primary tumor was found identical with that of a regional lymph node metastasis in 84 of 92 (91%) cases and with that of a distant metastasis in 44 of 51 (86%) cases. It is accordingly concluded that the disparaties can result from methodological limitations (101). A similar immunohistochemical study in paraffin-embedded tissue by Kamby et al. showed a similar discordance between the ER status of the primary and the regional metastases but differences were larger if distant metastases were analyzed (102). The frequency of ER-positive tumors was only 40% and the ER analysis used in the study probably suffers from a decreased sensitivity of the paraffin assay as emphasized in our own studies on paraffin-embedded tissue (62). A small immunohistochemical study in frozen tissue showed a 100% concordance in ER status of the primary and regional lymph node metastases (103), but cell-to-cell differences in ER staining were still observed.

The cell-to-cell ER heterogeneity observed in immunohistochemical studies may possibly be based on phenotypic rather than clonal or genotypic differences. First, good qualitative and quantitative correlations have been established between biochemical ER assays in homogenates of tumor tissue and immunohistochemical ER assays applied on a few cells obtained from fine needle biopsies (55, 104–109). Second, the ER expression in MCF-7 breast cancer cells has been shown to vary with the nutritional condition (110) and the cell cycle phase (110, 111). Third, ER-positive colonies may arise from ER-negative clusters of breast cancer cells (111). Fourth, structurally and functionally normal ER has been found in two antiestrogen resistant cell lines (112).

In conclusion, the cited studies offer only modest evidence in support of any major difference in ER status between the primary tumor and the metastatic sites, and they cannot explain the lack of response in 40–50% of patients with ER-positive primary tumors.

Conclusions

It has been established that immunoenzyme and immunohistochemical assays for estrogen receptor determination in fresh tissue correlate closely with conventional steroid binding assays. This implies that their value in the management of patients with breast cancer is of the same magnitude as that of the steroid binding assays, although the direct clinical evidence is so far very limited. With the present knowledge, a change from binding assays to immunoenzyme and immunohistochemical assays for estrogen receptors can only be recommended if clinical correlations can be established and national and international quality control studies of the same kind as for laboratories performing binding assays are implemented. Immunohistochemical analysis for estrogen receptors in paraffin-embedded tissue suffers from a loss of immunoreactive ER due to tissue processing and fixation, but modifications of the immunohistochemical techniques can partly compensate for the decreased sensitivity.

The value of ER as a prognosticator in patients with early breast cancer is in general low, and significance is not achieved in most studies using multivariate analysis. ER-PAR cannot be used as a prognosticator, but ER-DCC is an independent but weak prognostic factor in selected subgroups.

ER analyses in patients with early breast cancer receiving adjuvant hormonal treatment. Meta-analyses show a significant effect of adjuvant tamoxifen in postmenopausal patients with early breast cancer. The use of ER status as a criterion for treatment allocation is attractive as a treatment benefit cannot be ruled out initially. The use of ER analyses for treatment stratification should be done with caution because at least two large-scale adjuvant trials have shown that also ER-negative patients benefit from adjuvant tamoxifen treatment. The value of ER-PAR is undetermined, but it may be of the same magnitude as ER-DCC. Further definition of the role of ER is required, and can best be obtained from controlled clinical trials in which ER is measured with high-quality ER assays. A possible shortcut may consist in a retrospective analysis of tumors from one of the larger randomized trials using a paraffin-based ER assay.

Immunohistochemical ER analyses are useful predictors of response to endocrine therapy in patients with advanced breast cancer. The predictive value seems to be of the same magnitude as that of ER-DCC assays despite differences in the material used for analysis and the differences among the criteria for interpretation of the ER-status. The value of ER-PAR appears to be equivalent to that of assays in fresh or frozen tissue.

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