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Report

Estrogen receptor analysis in primary breast tumors by ligand-binding assay, immunocytochemical assay, and northern blot: a comparison

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Key words: akaike information criterion (AIC), breast cancer, DCC, estrogen receptor, immunocytochemical assay (ICA), ligand-binding assay, mRNA, northern-blot

Summary

Estrogen receptor (ER) status is an important parameter in breast cancer management. In this study, ER protein contents established by two conventional techniques were confronted to ER mRNA level, to analyze whether the latter may be introduced in routine assay. Eighty-seven breast tumor samples were examined. ER amounts were determined by ligand-binding assay (LBA) and by computer-assisted immunocytochemical assay (ICA), ER mRNA was analysed and quantified by northern blot. Seventy-seven percent of tumor samples examined were positive for ER mRNA and they all expressed the 6.7-kb receptor signal. No trace of small-sized ER mRNA variants was detected in any sample. Following akaike information criterion (AIC) discriminant analysis, a simple linear correlation was found between ER mRNA levels and ER amounts provided by LBA. This was not observed when either mRNA or LBA values were compared to ICA values. These latter were found to rapidly reach a plateau at increasing mRNA or LBA values. In conclusion, our data points to the linear correlation between ER amounts determined in breast tumors at both protein and mRNA levels by quantitative methods; they also indicate that the semi-quantitative computer-associated ICA may complement rather than replace these quantitative methods.

Introduction

Estrogen receptor (ER) has gained clinical importance in the treatment of breast carcinomas. ER status is used as a prognostic factor and a predictor of patient's response to therapy by endocrine treatments. The presence of ER is associated with increased survival and longer disease-free intervals for the patients. ER-positive tumors have a 50–60% overall response rate to the anti-estrogen tamoxifen [1]. Moreover, several techniques have been developed to analyse qualitatively and/or quantitatively the expression of ER by breast tumors.

For more than 20 years, ER content has been determined by ligand-binding assay (LBA), also called dextran-coated charcoal assay. By this technique, 50–80% of breast tumors are found to be ER-positive, depending on the cut-off value for positivity [2, 3]. Some problems are, however, associated with LBA. It requires relatively large amounts of fresh-frozen tissue and is insensitive and non-specific in accounting to differences in the cellular composition of samples, such as those with a low tumor cell content or contaminating benign cells that might be ER-positive.

The development of highly specific monoclonal antibodies to ER has allowed the evaluation of receptor status by immunocytochemical assay (ICA), which further permits the cellular localization of ER. Moreover, quantification of immunostaining data provided by ICA may be assisted by computer through the use of the CAS 200 image analysis system [4]. Compared with LBA, ICA is easier to perform, cheaper, safer, applicable to a wider variety of samples (eg. cytology preparations, frozen tissue sections, fixed archival tissue sections, etc), and more sensitive and specific in the identification of rare ER-positive tumor cells or contaminating ER-positive benign epithelium under direct microscopic visualization.

By applying non-computer-assisted ICA and LBA to the same tumor samples, several laboratories have reported a high (80–90%) concordance between the two assays regarding their evaluation of positivity [1, 5]. The relationships between data obtained by computer-assisted ICA and LBA have been less investigated.

A different way used to access ER in tumor samples has been through the measurement of its mRNA, first by northern- and dot-blot, more recently by reverse transcription-polymerase chain reaction (RT-PCR). The main advantage of this latter technique is that it may be applied to very small samples from which RNA can be easily extracted. Moreover, a number of samples may be processed at the same time. However, one major criticism addressed to all mRNA-measuring techniques is that the amount of a specific mRNA in a tumor might not reflect accurately the amount of the corresponding protein. Regarding ER, this problem is thought to be complicated by the presence in breast tumors of several mRNA variants, whose translation into protein remains, however, uncertain to date (see for instance Dowsett et al.) [6].

In this paper, we assessed the ER mRNA level in breast tumors, using the northern-blot technique. We also searched for a correlation between the amounts of ER determined at both protein, by LBA and ICA, and mRNA levels, by northern-blot, in tumor samples. Thus, we compared two quantitative (LBA and northern-blot) and one semi-quantitative (ICA) technique(s) and confirmed their complementarities.

Table 1. Patients and tumors clinicopathological characteristics

	$n = 87$
Age (years)	
Median	54
Range	29–94
Tumor stage	
T1 (≤ 2 cm)	9
T2 (> 2 and < 5 cm)	53
T3 (> 5 cm)	11
T4 (extension to skin/chest wall)	3
Unknown	11
Lymph node involvement	
Positive	46
Negative	24
Unknown	17
Histology	
Ductal	71
Lobular	9
Ductal + lobular	6
Other (mucinous)	1
Histological grade*	
G1	11
G2	30
G3	42
Unknown	4

*G1: well-differentiated tumor.
G2: moderately-differentiated tumor.
G3: poorly-differentiated tumor.

Materials and methods

Patient population

Tumor specimens from 87 patients with primary breast cancer were included in this study. They were accessioned from 1998 to 1999 from the Anatomical Pathology Laboratory at Jules Bordet Institute. Patients and tumors clinicopathological characteristics are summarized in Table 1. Three fragments of each tumor were independently analysed by LBA, ICA, and northern-blot, respectively.

Materials

Earle's based minimal essential medium (MEM), fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin were obtained from Life Technologies (Ghent, Belgium), plastic culture materials were from Falcon (Ghent, Belgium). [^{3}H]Estradiol (87,0 Ci/mmol), [^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham (Little Chalfont, United Kingdom). The ER-alpha cDNA clone λOR3 was kindly provided by Prof. P. Chambon, Strasbourg, France.

MCF-7 and MDA-MB-231 breast cancer cell lines (BCC) were from American Tissue-type Culture Collection (ATCC). They were routinely cultured and passaged in MEM supplemented with 10% heat-inactivated FCS and L-glutamine, penicillin, and streptomycin at the usual concentrations, and maintained at 37°C in an humidified atmosphere of 95% air-5%CO_2.

RNA isolation and northern-blot analysis

Total RNA was extracted from frozen tumor samples powdered in liquid nitrogen and from monolayers of

BCC with RNAZol (Life Technologies, Ghent, Belgium), according to the manufacturer's instructions. Fifteen to 25 μg were separated on a 0.9% agarose gel in 2.2% formaldehyde, 0.02 M 3-(N-morpholino) propane sulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA before transfer onto a nylon membrane (Hybond-N, Amersham, UK). Prehybridization, hybridization with the ^{32}P-labeled DNA probes and autoradiography were performed as previously described [7, 8]. The ER-alpha cDNA and 28S rRNA oligonucleotide probes were prepared as described [9, 10]. Signals were quantified by densitometry, using a Hoefer GS300 densitometer (Hoefer Instruments, San Francisco, CA) and the GS370 Densitometry Analysis System 3.0 for Macintosh. Each signal was measured three times and the mean value was used, which was normalized to the 28S rRNA. All membranes were exposed in autoradiography for various periods of time to ensure that only signals obtained in the linear range of sensitivity were quantified. To rehybridize filters, former probes were first removed by incubating the membranes in pure water at 85°C. Due to their heterogeneous nature, the tumor samples provided highly variable amounts of total RNA. We estimated that at least 50 mg of tissue were necessary to ensure the obtention of 25 μg total RNA.

Ligand-binding assay (LBA)

Homogenization of tissues samples was carried out with a whole glass homogenizer in phosphate buffer (10 mM K_2HPO_4/KH_2PO_4, 1 mM monothioglycerol, 1.5 mM EDTA, 10% glycerol, pH 7.5). Cytosolic ER was assessed on 1 h $100000 \times g$ supernatant fraction of ultracentrifugation by multipoint Dextran-Coated Charcoal (DCC) assay according to European Organization for Research and Treatment of Cancer (EORTC) recommendations [11] using $[^3H]E_2$ as labelling ligand. Receptor concentrations assessed by Scatchard plot analysis were expressed in fmol/mg total protein, the latter being measured using the Bio-Rad reagent (Bio-Rad, Richmond, CA). Cytosols of which protein content was less than 1 mg/ml were excluded (possibility of false negative cases). Binding data were analyzed according to Scatchard with Ligand 4.5 program (P.J. Munson, NIH, Bethesda, MD20892). The positive threshold fixed by the laboratory was of 10 fmol/mg of proteins. Due to their heterogeneous nature, the tumor samples provided highly variable amounts of cytosolic protein. We estimated that at least 150 mg of tissue were necessary to ensure a successful LBA assay.

Immunocytochemical assay (ICA)

Anti-ER (clone 6F11 [12]) antibody was used. Paraffin-embedded sections (4 μm) were obtained from a representative block of each tumor. The slides were dewaxed, rehydrated and immunoreactivity was enhanced by antigen retrieval treatment for all the antibodies. This treatment consisted of heating the slides in a microwave oven (Panasonic NN-5252B) in a 10 μM sodium citrate buffer (pH 6) for 3×10 min at 600 W power followed by a 20 min cooling at room temperature. The antigen antibody reaction was revealed by the streptavidin-biotin complex and diaminobenzidine was used as chromogen. Quantification of immunostaining (obtention of the QIC score) was done using CAS 200. The QIC score incorporates both the intensity and distribution of staining according to the formula: Percent stained nuclear area × staining intensity/10 [4]. The score was expressed in arbitrary units (AU). The positive threshold fixed by the laboratory was of 18 AU.

Statistics

The akaike information criteria (AIC) [13] was used to discriminate between simple and piecewise (with two populations) linear relationship distribution models, the model with minimum AIC being favored. AIC was calculated according to the formula: $\mathrm{AIC} = n \times \ln(\text{residual sum-of-squares}) + 2 \times p$, where n is the number of observations (points) and p the number of parameters in the model.

To analyse the agreement on ER status obtained by northern-blot, LBA, and ICA, several statistics were used. Concordance was defined as the percentage of tumors that were found positive or negative by both compared methods. Discordance (= 100 − concordance) was the percentage of tumors for which discordant ER status was obtained. Sensitivity was the percentage of cases positive by one method compared with the total number of positive cases by the other method. Specificity was the percentage of cases negative by one method compared with the total number of negative cases by the other method. χ^2-test was used to establish the statistical significance of the relationship between methods. The strength of agreement was measured by the κ-statistics.

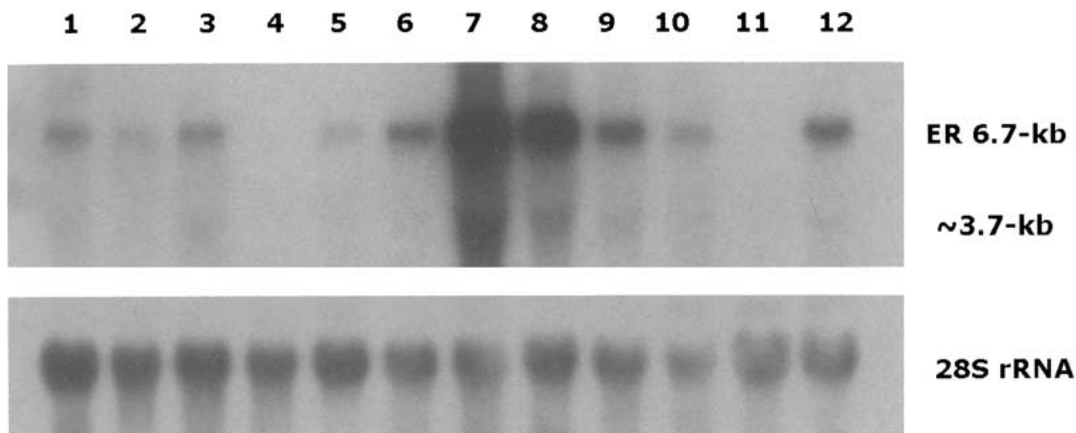

Figure 1. Northern-blot analysis of ER transcripts in breast tumors. 15 μg total RNA extracted from 10 different tumors (lanes 1–10) and from MDA-MB-231 (negative control, lane 11) and MCF-7 (positive control, lane 12) breast cancer cell (BCC) lines were separated by electrophoresis, transferred to nylon membrane and successfully hybridized to a ^{32}P-labeled full-length ER-alpha cDNA and a 28S rRNA oligonucleotide probe. For ER, the 6.7-kb signal is shown.

Results

ER mRNA analysis

Total RNA was successfully extracted from 84 of the 87 tumors examined. In the three remaining samples, RNA was partly degraded and could not be further analysed. Despite prolonged exposure of northern-blot membranes to autoradiography, we were unable to detect any ER mRNA signal in 19/84 (23%) tumors.

Figure 1 shows a typical autoradiogram obtained after successive hybridization of total RNA from 10 unselected tumor samples with DNA probes specific to ER-alpha mRNA and 28S rRNA. MDA-MB-231 (lane 12) and MCF-7 (lane 11) BCC were used as ER-negative and -positive control, respectively.

All ER mRNA-expressing tumors, as well as MCF-7 BCC, contained a 6.7-kb mRNA species. In all these samples, we also detected a ∼3.7-kb band. Such signals were never found in ER mRNA-negative tumors nor in MDA-MB-231 BCC. No other signal was found.

Comparison of data provided by northern-blot (ER mRNA) and LBA (ER)

Fifty of 87 tumor cytosols contained more than 1 mg of total protein, thus providing suitable ER values in LBA. Of these 53 samples, 28 (53%) were negative for ER (LBA value < 10 fmol/mg protein). Because RNA was degraded in one case, 52 tumor samples were used to compare LBA and mRNA data. Prior to comparison, ER mRNA amounts were normalized to the ER amount expressed by MCF-7 BCC (arbitrary level = 1), using the same preparation of MCF-7 RNA for the whole study.

All tumor samples that were ER-positive by LBA also expressed ER mRNA. In contrast, 33% of the samples found ER-negative by LBA contained detectable levels of ER mRNA. Figure 2 depicts the repartition of points obtained by plotting LBA against northern-blot values. Their distribution was described at best by a simple linear relationship model. The following parameters were obtained for the regression curve: y-intercept = 0.48; slope = 25.46; $r = 0.81$.

Comparison of data provided by northern-blot (ER mRNA) and ICA (ER)

On 69 tumor samples for which a QIC score had been obtained, 25 (36%) were ER-negative (QIC scores < 18). All samples were used to compare ICA and mRNA values. All the samples that were ER-positive by ICA also expressed ER mRNA. In contrast, 36% of the samples found ER-negative by ICA contained detectable levels of ER mRNA.

ICA values were plotted against northern-blot values (Figure 3). As the distribution of points suggested that it could not be accurately described by a simple linear model, the AIC was used. This criterion allows the comparison of distribution models with different parameters and different least sum of squares [13]. According to it, the distribution of points appeared to be best fitted by a two-populations piecewise linear

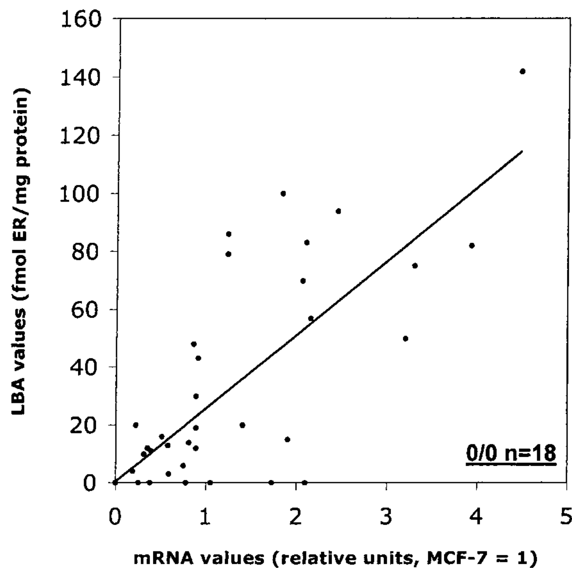

Figure 2. Distribution of ER values obtained by LBA versus ER mRNA values obtained by northern-blot analysis. LBA values are expressed in fmol ER/mg total protein in tumor samples. mRNA values are expressed in relative units (ER mRNA level in tumor samples / ER mRNA level in MCF-7 BCC). According to Akaike information criterion, the distribution of points was best described at best by a one-population model. Linear regression parameters: slope = 25.46; intercept = 0.48, $r = 0.81$. Number of points for which both LBA and mRNA values were equal to 0 : 18.

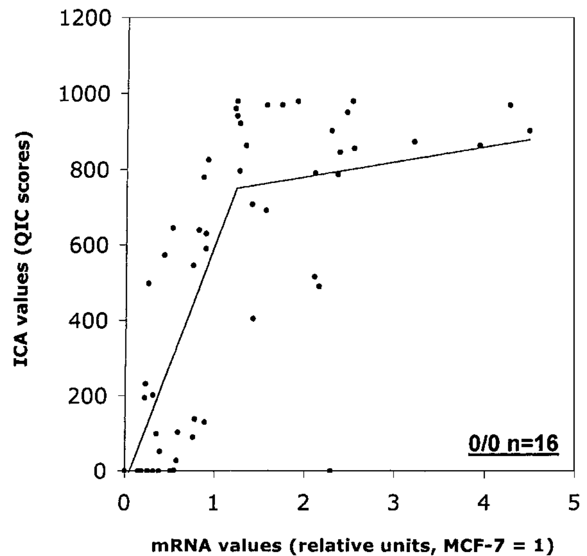

Figure 3. Distribution of ER values obtained by ICA versus ER mRNA obtained by northern-blot analysis. ICA values are expressed as QIC scores. mRNA values are expressed in relative units (ER mRNA level in tumor samples / ER mRNA level in MCF-7 BCC). According to Akaike information criterion, the distribution of values was best described by a two-populations model. For mRNA amounts higher than 1.23, the linear regression gave a slope not significantly different of 0 (95% confidence interval: [−32.5–112]). Hence, QIC scores were no longer discriminatory for mRNA variations above 1.23. Number of points for which both ICA and mRNA values were equal to 0 : 16.

model (AIC = 1032) rather than by a one-population (AIC = 1065) model. For the population of points with a mRNA value lower than or equal to 1.23, a regression line was obtained, with the following parameters: y-intercept = −34.88; slope = 638.41. For values higher than 1.23, the slope of the regression line was not significantly ($P < 0.05$) different of 0.

Comparison of data provided by LBA and ICA

Forty-four tumor samples were used to compare LBA and ICA values. 37 (84%) of them were either ER-positive or -negative by both LBA and ICA. Of the seven remaining samples, six were positive by ICA and negative by LBA.

ICA values were plotted against LBA values (Figure 4). The distribution of points suggested that it could not be accurately described by a simple linear model. According to AIC, the distribution of points appeared to be best fitted by a two-populations piecewise linear model (AIC = 639) rather than by a one-population linear (AIC = 647) model. For the population of points with a mRNA value lower than or equal to 43,01, a regression line was obtained, with the following parameters: y-intercept = 62.13; slope = 16.77. For values higher than 43.01, the slope of the regression line was not significantly ($P < 0.05$) different of 0.

Qualitative relationships between ER status by northern-blot (mRNA), LBA, ICA

The agreement on ER status obtained by northern-blot, LBA, and ICA, was investigated. A cut-off value for mRNA data was first calculated. By using the linear regression curve describing the relationship between mRNA and LBA data, 10 fmol ER/mg protein (the cut-off value for LBA) was found to correspond to a mRNA value of 0.37.

Figure 5 summarizes the concordance/discordance, sensitivity, and specificity data (as defined in Materials and methods) obtained for the three ER evaluation methods compared one to another. χ^2-test revealed that the relationship between methods was statistically significant in all cases. κ-statistics indicated that the strength of agreement was good (κ-values > 0.60).

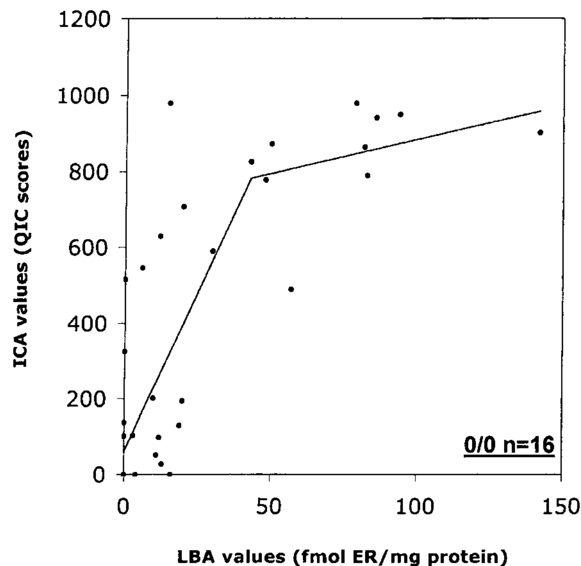

Figure 4. Distribution of ER values obtained by ICA versus ER values obtained by LBA. ICA values are expressed as QIC scores. LBA values are expressed in fmol ER/mg total protein in tumor samples. According to Akaike information criterion, the distribution of values was best described by a two-populations model. For LBA values higher than 43.01 fmol/mg protein, the linear regression gave a slope not significantly different of 0 (95% confidence interval: [−29.6–147.8]). Hence QIC scores were no longer discriminatory for LBA variations above 43.01 fmol/mg. Number of points for which both ICA and LBA values were equal to 0 : 16.

Discussion

To the best of our knowledge, this study is the first to compare three techniques evaluating ER amount at both protein and mRNA levels in breast tumors.

No major difference was found in the mRNA transcript sizes within our samples. Besides the 6.7-kb band whose presence has been repeatedly reported in tumors and breast cancer cell lines, we observed a smaller ∼3.7-kb band already described by others as early as in 1987 [14]. Contrasting with these authors, however, we failed to find that this smaller band was more abundant in most human breast tumors compared to BCC. A close relationship to ER is, however, highly suggested by its absence in ER-negative (by LBA and ICA) tumors, as well as in MDA-MB-231 BCC. Degradation of ER mRNA seems unlikely, in view of the fact that the band is not diffuse (no smear). Moreover this band does not seem to appear when only poly(A)+ mRNA is used [15, 16]. We thus suggest that it could result, at least in part, from the disturbing presence of a very high amount of 28S ribosomial RNA located in its close vicinity. In any case, we did not use this ∼3.7-kb band for the quantification of ER mRNA.

ER mRNA was found in 77% (65/84) of the tumors. This percentage is in good agreement with data from the literature, reporting a ER-positivity in about 50–85% of breast carcinomas [17]. It is, however, higher than those provided by LBA and ICA, according to which tumor samples were positive in 47% and 64% of cases, respectively. This could be explained by the fact that both LBA and ICA use non-null cutoff values to discriminate between ER positivity and negativity. In contrast, at the messenger level, only samples for which no trace of ER mRNA was found, even after prolonged (1 week) exposure of the radioactive northern-blot membranes in autoradiography, were counted as negative for the receptor. This points out to the necessity of defining appropriate, but also clinically-relevant, cut-off values for studies evaluating the ER through the expression of its messenger. On the other hand, the prevalence of ER positivity by LBA (47%) and ICA (64%) was unusually low. Indeed, several published studies have reported a prevalence of at least 70% in primary tumors. Although the tumors examined here were primary, Table 1 shows that a high amount of them were of grade 3, had a size superior to 2 cm, and were characterized by a lymph node positivity. Such features are generally associated to a low ER amount. The reduced number of small (≤2 cm) tumors may be explained by the fact that it was difficult to cut them into three pieces large enough to allow the realization of the three ER analyses (mRNA, LBA, ICA).

Besides the 6.7-kb and the ∼3.7-kb bands, northern-blot analysis did not reveal any other signal. A number of ER splicing variants, lacking one or more exons, have been identified in breast cancer specimens and breast cancer cell lines [6]. However, their presence has always been revealed by PCR-based amplification and, to the best of our knowledge, never by northern-blot applied to total tumor RNA. Note that the faint ∼3.7-kb band has never been associated to any receptor variant. The apparent absence of variants could be due to their multiplicity of size and to the fact that their cumulative contribution to the total breast carcinoma ER mRNA seems not to be higher than 25% [18]. In any case, the linear correlation that we observed between the level of 6.7-kb ER mRNA and the receptor amount given by LBA highly suggests that the measurement of this sole receptor mRNA, even by RT-PCR, might provide a valuable estimation of the E_2-binding form(s) of ER. The significance

mRNA vs LBA

		mRNA -	mRNA +	Total
LBA	-	20	8	28
	+	2	22	24
	Total	22	30	52

Concordance = 81%
Discordance = 19%
Sensitivity = 92%
Specificity = 71%
χ2-test: P < 0.001
κ–statistics = 0.71

mRNA vs ICA

		mRNA -	mRNA +	Total
ICA	-	21	4	25
	+	5	39	44
	Total	26	43	69

Concordance = 87%
Discordance = 13%
Sensitivity = 89%
Specificity = 84%
χ2-test: P < 0.001
κ–statistics = 0.72

LBA vs ICA

		LBA -	LBA +	Total
ICA	-	17	6	23
	+	1	20	21
	Total	18	26	44

Concordance = 84%
Discordance = 16%
Sensitivity = 95%
Specificity = 74%
χ2-test: P < 0.001
κ–statistics = 0.68

Figure 5. Qualitative relationships between ER evaluations by northern-blot (mRNA), LBA, and ICA. The respective cut-off values for ER positivity were 0.37 (obtained from Figure 2), 10 fmol ER/mg cytosolic protein, and 18 AU (arbitrary units). For the definition of statistics, see Materials and methods.

of ER variants in cancer, notably their contribution to the cancer response to estrogens and antiestrogens remains unclear.

ER mRNA is increasingly used to access the amount of the receptor itself. In particular, RT-PCR is a rapid method allowing the simultaneous quantification of multiple mRNAs from a very small tumor sample. Moreover, the recourse to mRNA analysis should increase in the next years through the introduction of DNA microarrays ('biochips'). However, besides potential problems related to the amplification process, RT-PCR (and, more widely, all RNA-based techniques) has been questioned about the existence of a simple relationship between ER mRNA and receptor amounts in tumors. We found that the 6.7-kb ER mRNA amounts in breast tumor samples could be linearly correlated to the receptor levels measured by LBA. We suggest that these LBA values could be used to infer an accurate cut-off value for studies involving mRNA measurements, including RT-PCR. By evaluating ER in a few tumor samples both at mRNA and protein levels, a linear regression curve could be obtained. From the LBA cut-off value, this curve would allow the calculation of an equivalent at the mRNA level. This latter value could then be used to study a wider series of tumor samples for which LBA analysis would be unnecessary.

Northern-blot, LBA, and ICA exhibited few divergences in establishing the positivity or negativity of tumor samples (qualitative data, see Figure 5). However, as compared to LBA and northern-blot, ICA tended to overestimate the ER level in tumor samples (quantitative data). The 'plateau effect' that we observed was already visible in precedent works [14, 19, 20], but was not explicitly discussed by these authors. Several hypotheses may be advanced to explain the divergence.

Thus, one could propose that this is attributable to a degree of subjectivity presiding to the choice of fields for ICA analysis. Current recommendations include the measurement of cells only from areas in which some positive staining is observed. This method would appropriately exclude populations of non-tumor (endothelial, stromal, blood,...) cells biologically negative for ER. As a consequence, however, truly biologically negative tumor cell populations could also be ignored and the ER level of the entire carcinoma overestimated. While this hypothesis underlines the character essentially non-quantitative of ICA, it does not explain why the overestimation due to ICA was apparently not observed at relatively low ER concentrations.

Another possibility is that discrepancies between ICA and LBA values could result from a tumor necrosis event degrading a fraction of ER in the samples. As a consequence, this fraction would become undetectable biochemically, ICA values being less affected due to field selection. However, tumor necrosis is believed also to degrade, in a similar way, both the ER mRNA and the 28S rRNA that we used as internal control for northern-blot analysis, leading to no change in the relative level (ER mRNA/28S rRNA) of ER messenger. Hence, no discrepancy should be observed between mRNA and ICA values, which contrast with our data.

ICA is based on the ER 6F11 antibody [12] recognizing the A/B region in the *N*-terminal part of the ER. On the other hand, in LBA, the ligand binds to

the *C*-terminal region of the receptor. One could suggest, according to Maaroufi et al. [21] that a fraction of ER is uncompletely proteolysed in tumor samples, leading to the disappearance of the *C*-terminal region of the receptor, thus no longer detected by LBA, while the *N*-terminal region, measured by ICA, remains intact. However, such a specific disappearance of the *C*-terminal region of ER due to limited proteolysis should not exist at the mRNA level. Our data showed a same clear discrepancy between mRNA and ICA, suggesting the invalidity of the proteolysis-based explanation. Supporting this view, a discrepancy between LBA and ICA, characterized by an overestimation of high ER values by ICA, was previously reported by Parl et al. [20] which, however, used the H222 antibody, raised against the *C*-terminal region of ER.

Finally, we asked whether overestimation of ER amounts by ICA could result from unadequate settings of the detection device (CAS 200). However, despite modifications brought to these settings, notably a reduction of device sensitivity, we were unable to prevent the apparition of the 'plateau effect' (data not shown).

This plateau cannot recommend to use computer-assisted method to quantify ER in breast cancer, although ICA, as described in the literature, is a good method to quantify the ER status in breast cancer [1, 5].

The decreasing size of the tumor samples obtained at surgery should progressively restrict the use of LBA and other biochemical techniques such as enzyme-immunoassay. Our data suggest that mRNA-based techniques (i.e., RT-PCR) could constitute a valuable substitute in determining ER amounts. Future studies in our laboratory should examine ER (as determined by ICA) and ER mRNA (as determined by RT-PCR and/or DNA microarray) expression on large series of tumors (including small samples $< 2\,\mathrm{cm}$) and try to correlate their amounts to clinical outcome (disease-free survival, overall survival).

In short, we have shown that ER amounts, measured at both the protein (by LBA) and the 6.7-kb mRNA levels, may be linearly correlated. This suggests that ER studies based on mRNA measurement may accurately reflect measures of the receptor itself. We have also confirmed that ICA, although displaying specific advantages, is essentially a qualitative technique. Biochemical methods for evaluating ER amounts in breast tumor samples will progressively fail to be feasible, due to the decreasing size of these samples. Our data suggest that besides ICA, methods based on ER mRNA estimation may be reliable and should gain importance in the future.

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