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Quantitation of estradiol receptors alpha and beta and progesterone receptors in human breast tumors by real-time reverse transcription-polymerase chain reaction Correlation with protein assays

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Abstract

Hormone receptor content is the most useful parameter for predicting hormone response therapy in breast cancer. The high frequency of primary and secondary resistance to treatment makes it necessary to find out other parameters in order to improve the prediction of response to treatment. The newly described estrogen receptor β (ERβ) is a potential candidate. Using real-time quantitative RT-PCR, we evaluated estrogen receptor α (ERα), ERβ, and progesterone receptors (PR) in comparison with ERα and PR protein content measured with the enzyme immunoassay (EIA), in a retrospective series of 98 breast tumors. We obtained a highly significant correlation between mRNA and EIA assays for ERα and PR ($r = 0.79$ and $r = 0.71$, respectively; $P < 0.001$). We confirmed the low level of ERβ mRNA transcripts in comparison to ERα in breast tumors. We did not find any statistically significant correlation between the absolute ERβ mRNA level and ERα or PR mRNA level, and ERα or PR-EIA. We found a significant correlation between ERα mRNA and PR mRNA expressions. We did not find any correlation between ERβ mRNA and clinical features of the patients (age, menopausal status, tumor size, and nodal status), nor with the histological type of the tumor. In conclusion, the accuracy of the present quantitative RT-PCR assay makes it suitable for a routine clinical use. In addition, the present results suggest that, ERβ mRNA expression is independent of the classical parameters.

Keywords: Breast cancer; Quantitative RT-PCR; Hormone receptors; ERα and β; PR

1. Introduction

Endocrine manipulations are the most effective and least toxic of the systemic therapies currently available for the management of hormone-dependent breast cancer. The estradiol receptor (ER) assay was proposed first by Jensen *et al.* [1], to determine which patients would respond to endocrine therapy. It was then established that patients with ER-rich tumors respond to endocrine therapy, whereas patients with ER-negative tumors are unlikely to respond [2]. Ligand binding assay, then EIA, that represented the gold standard, and more recently immunohistochemistry were used for the routine determination of ER and PR in breast cancer tumors [3]. Whereas response rates of up to 70% are achieved with endocrine therapy in patients with tumor expressing ER and PR, most responsive tumors will eventually acquire resistance. This demonstrates that the determination of ER and PR is necessary but probably not sufficient to determine hormone responsiveness. The mechanisms driving resistance in tumors that express ER and/or PR remains unclear but

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Abbreviations: ERα, estrogen receptor α; ERβ, estrogen receptor β; PR, progesterone receptor; PCR, polymerase chain reaction; mRNA, messenger RNA; ROC, receiver operating characteristic.

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may be multifactorial. The recent discovery of a new ER (ERβ) with a different trans-activating transcription pathway from that of the classical ER (now ERα), has led to question its potential role in hormonal resistance. Hypotheses including alterations in the ratio of the ERα/ERβ and other changes in estrogen-driven transcription function have been advocated. However, few studies have been conducted so far in breast cancer, due to the absence of validated analytical methods. Molecular biotechnology offers a great potential for developing multiparametric analysis of messenger RNA expression of clinically relevant biological parameters in estrogen-dependent breast tumors. Real-time polymerase chain reaction (PCR) technology using fluorescent quenching detection probes (Taqman™) is quantitative, sensitive, specific, and robust [4]. This study was designed to validate the use of real-time RT-PCR for the measurement of hormone receptors (ERα, ERβ, and PR) using a retrospective series of breast tumors that have been previously evaluated for their content in ERα and PR proteins by EIA.

2. Material and methods

2.1. Human breast cancer cell lines

Well-characterized hormone-dependent and hormone-independent breast cancer cell lines were used to assess basal levels of target genes as reference for calibration of the assay and for quality control: MCF7 and T47D, established from hormone-dependent human breast cancers and MDA-MB 231 established from a hormone-independent human breast cancer [5]. Cells were maintained in 75-cm^2 flasks in DMEM (Gibco BRL) supplemented with 10% fetal calf serum (Dutscher), and glutamine 2 mM (Sigma) at 37° in a 5% CO_2 air humidified incubator. Dilutions of RNA of relevant cell lines were used to assess the PCR efficiency and were used as calibrator in each experiment.

2.2. Human breast tumor samples

Ninety-eight primary untreated breast tumors from patients undergoing surgery at Curie Institute from 1996 to 1998 were retrospectively analyzed. Tumor sampling was performed extemporaneously by a pathologist. Samples were frozen stored in liquid nitrogen within 20 min after the excision. Half of each tumor sample was stored for ERα and PR protein content. The residual fragment was stored in liquid nitrogen in RNAse-free conditions until use. In 2001, we retrospectively analyzed this residual tissue for ERα, ERβ, and PR mRNA content. Adjacent fragments of frozen material were fixed in AFA and stained with hematoxylin and eosin in order to determine the proportion of tumor cells in each sample. The clinical staging and histological diagnosis were routinely determined in each case.

2.3. ER and PR protein assays

Tissue homogenization was routinely performed on consecutive samples, within a week after sampling. Briefly, breast tissue was homogenized in TK buffer (Tris 10 mM, glycol 10%, Na-molybdate 10 mM, dithiothreitol 0.5 mM, and KCl 0.4 M) and centrifuged at 105,000 *g* for 1 hr. ERα and PR were assayed in the supernatant (cytosol fraction) according to the recommendations of the EORTC receptor Study Group (1980). ER and PR were assayed using an EIA (ER-EIA, PR-EIA, Abbott laboratories), as described previously [6]. The EIA kit for ER can detect ERα but not ERβ [7]. ERα and PR levels were expressed relative to tissue DNA status (fmol/mg DNA). The hormone receptor content (+ or −) was defined by the cut-off value for ERα and PR positivity that has been previously determined as 250 fmol/mg DNA.

2.4. ERα and β and PR mRNA assays by real-time RT-PCR

2.4.1. RNA extractions

Total RNA was extracted from 2×10^6 cells or from crushed tumor samples by RNA plus® kit (Bioprobe) following the manufacturer instructions. The RNA samples were stored in 50 μL RNAse-free distilled water at −80°. The quality of RNA samples was determined by electrophoresis through a 2% agarose gel, and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under UV light.

2.4.2. cDNA synthesis

RNA was reverse transcribed in a final volume of 20 μL containing 1× reverse transcriptase buffer (1.25 mM each dNTP, 6.7 mM $MgCl_2$, 2.5 U RNase inhibitor (Amersham), and 5 μM/L random Hexamer (Boehringer-Mannheim), 10 U Murine Moloney Leukemia virus reverse Transcriptase (Life Technologies Inc.), and 1 μg total RNA aliquots were incubated at 42° for 32 min, reverse transcriptase was then inactivated at 72° for 2 min, followed by cooling at 5° for 5 min.

2.4.3. Real-time PCR amplification

ERα, ERβ, and PR transcripts were quantified using real-time quantitative RT-PCR assays. Transcripts of β2 microglobulin and RPLPO (36B4) were also quantified as endogenous RNA of reference genes to normalize each sample [8].

Primers and probes were chosen with the assistance of the computer program Primer Express (Applied Biosystems). Then nucleotidic sequence were blasted against dbEST and nr to confirm the total gene specificity of the nucleotide sequences chosen as primers at probes. Each primer couple was positioned in different exons of the gene in order to avoid amplification of contaminating genomic DNA. The nucleotide sequences are shown in Table 1.

Table 1
Primers and probe sequences for RT-PCR amplification of ERα, ERβ, and PR mRNA

mRNA	Localization	Product size (nt)	Primers	Probe
ERα	Exons 5/6	155	agcacccagtgaagctact tgaggcacacaaactcct	tggctacatcatcggttccgca
ERβ	Exons 6/7	143	aagaatatctctgtgtcaaggccatg ggcaatcacccaaaccaaag	ttgctgaacgccgtgaccgatg
PR	Exons 4/5	122	gaaccagatgtgatctatgcagga cgaaaacctggcaatgatttagac	acctgacacctccagttctttgctgacaag
β2 microglobulin	Exons 2/4	121	gatgagtatgcctgccgtgt aattcatccaatccaaatgag	aaccacgtgactttgtcacagcccaa

Primers and probe sequence for RPLPO have been previously described by Bieche *et al.* [8].

PCR reactions were performed using an ABI Prism 7700 Sequence Detection System and Core Reagent Kit (Applied Biosystems). Real-time detection was performed using oligonucleotide probes containing a fluorescent dye at its 5′-end and a quencher at its 3′-end, (PE Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95° for 10 min, then 40 cycles at 95° for 15 s, and an annealing temperature depending upon the parameter: 57° for ERα, 65° for ERβ and RPLPO, and 60° for PgR and β2 microglobulin. PCR products were then migrated on 2% agarose gel in order to confirm the presence of a single band with the expected size.

To prevent carry over of contaminating DNA, the reaction was carried out in the presence of dUTP. Two non-template controls were included in every amplification run. Accurate quantification was achieved through the generation of calibration curves by serial dilutions of human cell line for each parameter.

Quantitative values were obtained from the threshold cycle (Ct) number at which the increase in fluorescent signal associated with an exponential increase of PCR products can to be detected (i.e. 10-fold the standard deviation of background signal, using Applied Biosystems analysis software).

ΔCt values of the sample are determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. The relative gene expression level was also normalized relative to a positive calibrator, consisting of one of the samples of the calibration curve of the assay. Results were expressed as "N target" determined as follows [8]:

$$\text{N target} = 2^{(\Delta\text{Ct sample} - \Delta\text{Ct calibrator})}.$$

2.5. Statistical analysis

The log-transformed values were used in all subsequent calculations and figures to more closely approximate a normal distribution. Pearson correlation coefficients (r) and associated probabilities (P) were calculated to determine the relation between the different measurements of hormone receptors. We used Student's t-test to compare the mRNA levels of qualitative variables. We used receiver operating characteristic (ROC) analysis on the mRNA levels.

The best cut-off level of positivity/negativity was determined by testing every possible cut off and choosing the one with the maximal value of chi-square test, by taking in account the level of the reference gene. The association of gene levels with tumor variables and patient age was assessed using multiple linear regression. A two-sided P-value of 0.05 or less was considered as statistical significance.

3. Results

The clinical and pathological characteristics of the tumors are shown in Table 2. Most patients (77%) were postmenopausal. Sixty-six percent of the tumors were infiltrating ductal carcinoma. The ERα and PR protein content were initially measured by EIA: 74 tumors were ERα-positive (76%) and among which 60 had detectable or high level of PR, whereas 14 had no detectable or low level of PR. Twenty-four tumors had no detectable ER level and among them, four had a detectable level of PR and 20 had no detectable or low level of PR by EIA. The respective median values were 1280 (3–4060) fmol ERα/mg DNA and 824 (50–10268) fmol PR/mg DNA for premenopausal patients and 1640 (0–9580) fmol ERα/mg DNA and 1110 (0–26199) fmol PR/mg DNA for postmenopausal patients.

3.1. PCR optimization

ERα, ERβ, and PR primers and probes were localized in exons coding for the steroid binding or E domain (exons 4–7) of the receptor protein (Table 1). The respective size of the PCR products was 155, 143, and 122 nt and the specificity of PCR products was initially ascertained by the presence of a single band of the expected size after migration of PCR products on agarose gels. For quantification of cDNA by real-time fluorescence PCR, a calibration curve was generated by analyzing serial dilutions of human breast cancer cell lines and a pool of human breast tumors for each hormone receptor and reference genes. The amplification curves of the calibrator and the calibration curve generated by the Sequence Detector Computer software (Applied Biosystems) are shown in Fig. 1 for ERβ. Similar results were obtained for ERα and PR. The PCR efficacy was 98, 99, and 92%, respectively, for ERα, ERβ, and PR. Each measurement was performed in duplicate. The intra-assay CV was <2% and the inter-assay CV was <5% as calculated by the standard error. The linearity for

Table 2
Clinical and pathological characteristics of the samples

	Number (%)
Patients and clinical tumor characteristics	
Age	
≥50 years	75 (77)
<50 years	23 (23)
Tumor size	
T1	34 (35)
T2	45 (46)
T3/T4	15 (15)
Nodal status	
N0	69 (70)
N1	27 (28)
Unknown	2 (2)
Histological characteristics	
Macroscopic tumor size	
<30 mm	62 (63)
≥30 mm	33 (34)
Unknown	3 (3)
Nodal status	
N^-	44 (45)
N^+	49 (50)
No axillary dissection	5 (5)
Histological type	
Infiltrating ductal	65 (66)
Infiltrating lobular	20 (20)
Others	13 (13)
Grade (SBR)	
1	19 (19)
2	43 (44)
3	31 (32)
Unknown	5 (5)
Hormone receptor status	
ER-positive	74 (76)
ER-negative	24 (24)
PR-positive	71 (72)
PR-negative	27 (28)

each hormone receptor was observed for >5 log dilutions, including the observed samples values.

3.2. Determination of threshold and correlation of analysis of hormone receptor by RT-PCR vs. EIA

3.2.1. ERα

A cut off of 250 fmol/mg DNA is the validated cut-off value used for the "presence" of ER protein in our laboratory [6]. We performed the method of research of threshold as described in Section 2 to determine the optimal cut-off level of positivity/negativity for each parameter taking into account the level of the reference gene. We confirm these results using ROC curves analysis [9]. For ERα, the optimal cut off expressed as N target was 19 for β2 microglobulin as reference gene (Fig. 2) and one for RPLPO as reference gene. The sensitivity of the ERα assay was 0.79 and 0.86 and the specificity was 0.93 and 0.91, respectively, for β2 microglobulin and RPLPO as reference genes. In these conditions, with these cut-off values, 80 and 81% of breast tumors were positive for ERα by RT-PCR, respectively, with β2 microglobulin and RPLPO as reference genes, and 76% were positive for ERα by EIA. The concordance status between RNA and protein levels is shown in Fig. 3A. There is a significant correlation between the two methods ($r = -0.79$, $P < 0.001$ and $r = -0.81$, $P < 0.001$, respectively, with β2 microglobulin and RPLPO as reference genes). As anticipated, there is a good correlation between ERα mRNA and PR mRNA ($P < 0.001$).

3.2.2. PR

The same cut off of 250 fmol/mg DNA was the cut-off value used for the presence of PR protein [6]. Using the method of research of threshold we also determined the optimal cut-off level of positivity/negativity expressed as arbitrary units (N target) for PR (Fig. 2). It was 1378 for β2 microglobulin as reference gene and 64 for RPLPO as reference gene. The sensitivity of PR assay was respectively 0.69 and 0.87 and the specificity was respectively 0.92 and 0.83 for β2 microglobulin and RPLPO as reference genes. With these cut-off values, 73 and 71% of samples were positive for PR by RT-PCR, respectively, for β2 microglobulin and RPLPO as reference genes. Seventy-two percent of samples were positive by EIA. The concordance status between RNA level and protein level is shown in Fig. 3C for β2 microglobulin. A similar correlation is observed with RPLPO as reference gene (data not shown). There is a significant correlation between the two methods using β2 microglobulin and RPLPO as reference genes ($r = -0.71$, $P < 0.001$ and $r = -0.76$, $P < 0.001$, respectively).

We found a statistically significant correlation between ERα (either protein and mRNA) and PR (either protein and mRNA) ($P < 0.001$) and between ERα and PR with histological grade ($P = 0.03$ and $P = 0.002$, respectively). No correlation was observed with other clinical or pathological characteristics in this limited series of large tumors.

3.2.3. ERβ

Results were expressed as a continuous variable. No statistically significant correlation was found with either ERα (protein and RNA content) or PR (protein and RNA content) (Fig. 3B). No correlation was found between ERβ transcripts and clinical or pathological characteristics (age, tumor size, nodal status, histological subtype, grade, histological size, and nodal status). ERβ protein level was not evaluated on the initial sample, so no comparison could be performed.

The mean ERα, ERβ, and PR mRNA levels were 88, 14, 586 and 9, 1, 39 with β2 microglobulin and RPLPO as reference genes, respectively. The mean ERβ mRNA values was lower than ERα mRNA.

The median values of ERα, ERβ, and PR mRNA expressing tumors according to ERα and PR-EIA are shown in

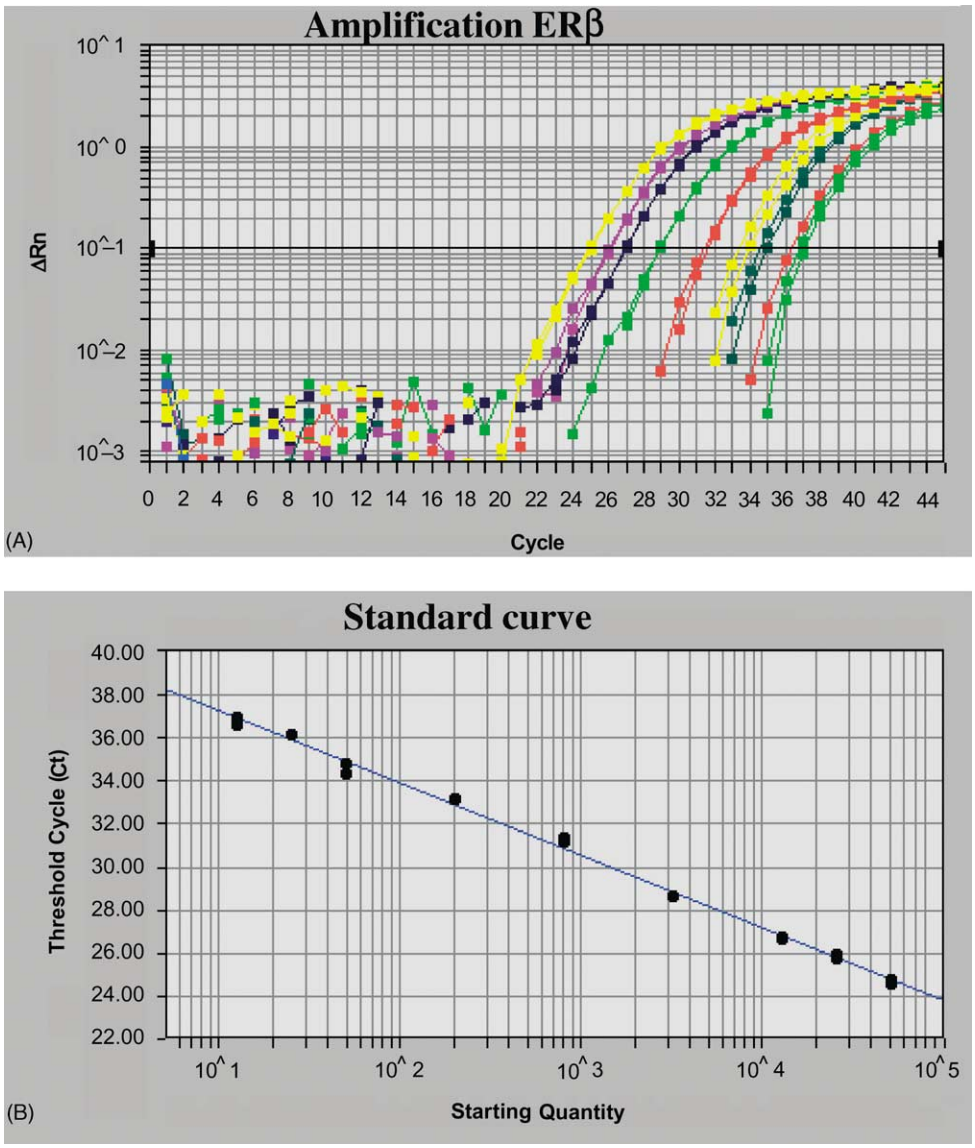

Fig. 1. ERβ standard curve by real-time RT-PCR. (A) Amplification plot. Serial dilutions of cDNA (1–10^5-fold) from MDA-MB 231 carcinoma cell line were used as standards and were subjected to real-time PCR. Ct is plotted vs. change in normalized reporter signal (Rn), ΔRn. For each reaction tube, the fluorescence signal of the reporter dye Fam for ERα, ERβ, and Vic for β2 microglobulin and RPLPO was divided by the fluorescence signal of the passive reference dye TAMRA to obtain a ratio defined as the Rn. ΔRn represents the Rn minus the baseline signal established in the first 15 PCR cycles. Each point sample was analyzed in duplicate and a mean value was considered. (B) Standard curve plotting log initial copy number vs. Ct.

Table 3. In ER-positive group, the level of ERβ mRNA is always lower than ERα.

3.3. Interpretation of discrepancies

3.3.1. ERα

Although the overall concordance in expression levels of protein and RNA is good (92%), eight tumors displayed a discordant ERα status using β2 microglobulin as reference genes (Fig. 3A) and seven tumors displayed a discordant ERα status using RPLPO as reference gene. One case was borderline ER-positive by EIA (370 fmol/mg DNA) and also borderline ERα-positive by RT-PCR using RPLPO as reference gene, whereas it expressed very few ERα transcripts (78 expressed with β2 microglobulin as reference gene). This sample was PR-positive by EIA (750 fmol/mg DNA), whereas it expressed very few PR transcripts (2876 expressed with β2 microglobulin as reference gene).

Seven tumors (7%) did not express ERα by EIA, whereas they expressed ERα transcripts either using β2 microglobulin (Fig. 3A) or RPLPO as reference genes. In these latter cases, the values of ERα transcript levels were not borderline values (3–30) and the value of ER protein was unambiguously ‘‘negative’’ (53–170 fmol/mg DNA). Interestingly, all samples are PR-negative by EIA, whereas four of them express PR transcripts.

3.3.2. PR

The quantification of PR proteins and transcripts in this series of 98 breast tumors shows 17/98 discordant cases

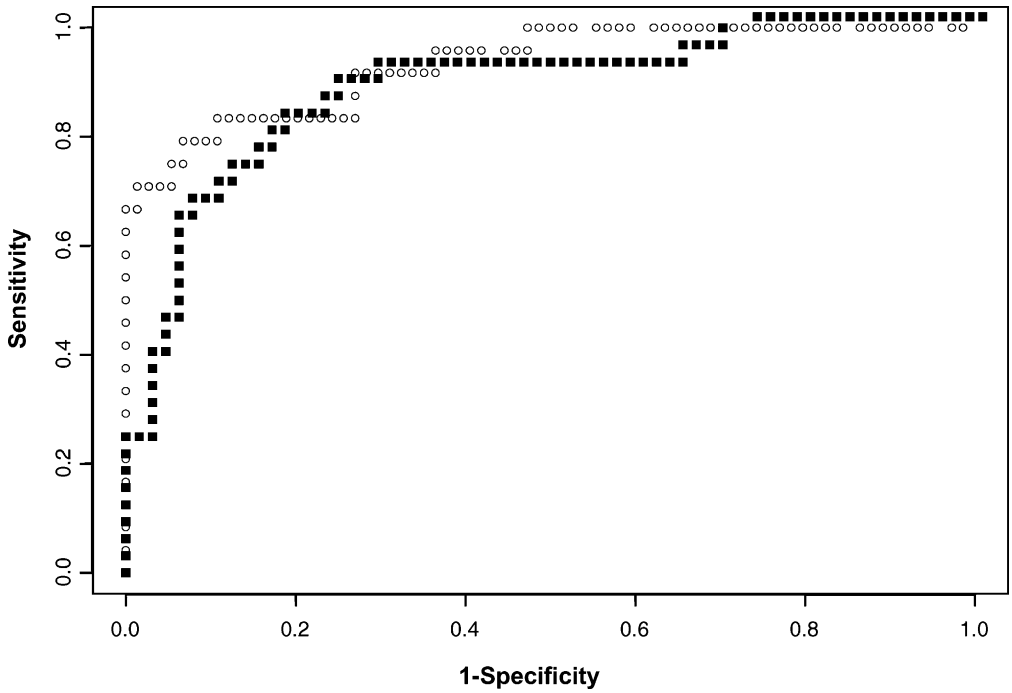

Fig. 2. ERα and PR ROC curves. ROC curves for ERα-RT-PCR vs. EIA (○) and PR-RT-PCR vs. EIA (■).

(83% concordance) using β2 microglobulin as reference genes (Fig. 3C) and 15/98 discordant cases (85% concordance using RPLPO as reference gene). Eight tumors were borderline positive (three) or clearly positive (five) for PR-EIA, whereas they had low levels of PR transcripts (1595–30,573 expressed with β2 microglobulin as reference gene, among them, one sample was concordant with EIA using RPLPO as reference gene). Among these eight samples, five were ERα-positive by EIA and by RT-PCR, two were ER-negative by EIA and RT-PCR, and one was ERα-negative by EIA and ERα-positive by RT-PCR.

Nine tumors were PR-EIA negative, whereas they had borderline positive levels of PR transcripts (1024–1278 expressed with β2 microglobulin as reference gene for five samples) or positive levels of transcripts (144–749 expressed with β2 microglobulin as reference gene for four other samples). Among the five borderline PR discordant samples using β2 microglobulin as reference gene, one sample was concordant using RPLPO as reference gene. Among the eight PR RT-PCR negative tumors, five gave ''false positive'' results in ERα RT-PCR and are described earlier. Two were ERα-positive by EIA and RT-PCR and one was ERα-negative by EIA and RT-PCR whatever may be the reference gene used.

4. Discussion

In this study, we validated a RT-PCR method for the quantification of hormone receptors. The method is based on real-time analysis of PCR amplification and Taqman™ technology that has several advantages over other RT-PCR-based quantitative and biochemical methods. The real-time PCR method does not require post-PCR sample handling, thereby avoiding problems related to carry over. It possesses a wide dynamic range. The method is quantitative and robust, the intra-assay and inter-assay coefficients of variation are low (<2 and <10%, respectively). A standard

Table 3
Distribution of ERα and β and PR-EIA and PR mRNA levels according to hormone receptor status

Hormonal status	Number of patients	Hormone receptor (EIA, median (range))		Hormone receptor (RT-PCR, median (range))		
		ERα (fmol/mg DNA)	PR (fmol/mg DNA)	ERα (N target)	ERβ (N target)	PR (N target)
ER-negative/PR-negative	20	65 (0–190)	64 (0–200)	127 (3.5–2320)	3.7 (0.7–65)	3113 (144–91405)
ER-negative/PR-positive	4	148 (3–360)	447 (210–824)	103 (5.5–516)	12 (1–67)	2044 (508–8364)
ER-positive/PR-negative	14	2434 (410–5900)	148 (30–300)	5.3 (0.5–34)	6.5 (0.3–244)	940 (111–30573)
ER-positive/PR-positive	60	2402 (280–9580)	3994 (326–26199)	2.7 (0.25–56)	5.8 (0.5–67)	340 (4.5–9877)
All	98	1401 (0–9580)	969 (0–26199)	5.48 (0.25–2320)	5.62 (0.3–244)	586 (4.5–91405)

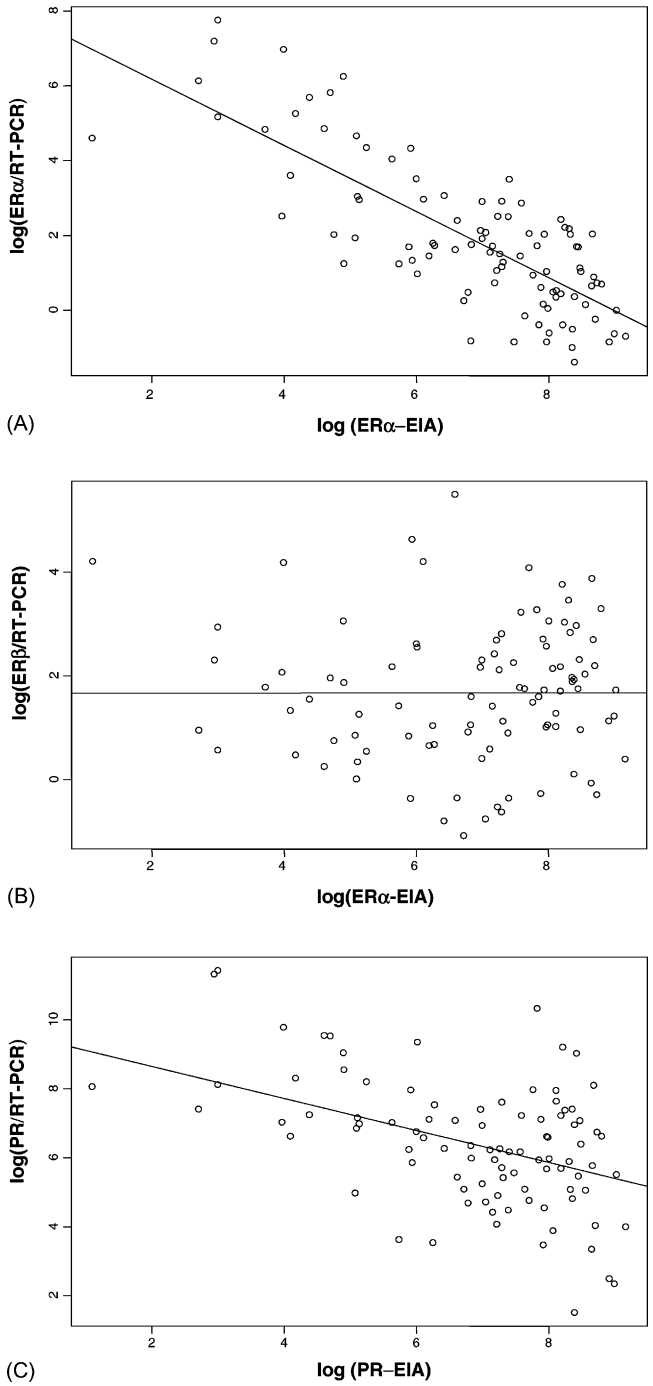

Fig. 3. Regression analysis between hormone receptor mRNA and protein (EIA) levels in breast tumors (β2 microglobulin as reference gene). (A) ERα mRNA vs. ERα-EIA, (B) ERβ mRNA vs. ERα-EIA, and (C) PR mRNA vs. PR-EIA.

curve of a calibrator (a human breast cancer cell line) is analyzed in each experiment in the same conditions as that for unknown samples. The inter-assay variation of the quantification of the calibrator was <5%. This method is, therefore, adapted to routine evaluation of hormone receptors on clinical samples.

We evaluated this method in a retrospective series of 98 consecutive breast tumors. The high and statistically significant correlation between ERα and PR-EIA and ERα-negative and PR mRNA assays, validate the present RT-PCR assay. At the level of individual samples, concordant results between EIA and RT-PCR were slightly better using RPLPO as reference gene for three samples, either for ERα and PR, leading us to prefer it in a routine clinical use.

It is well established that ER-EIA can detect ERα but not ERβ protein [7]. We analyzed ER and PR on two adjacent samples, and we observed a highly significant correlation between protein expression and transcripts for ERα and PR (83 and 86%, respectively, and $P < 0.001$). The analysis of discrepant samples showed that for ERα, two samples were considered as "false negative samples" by RT-PCR. However, these samples had very low cellularity (low DNA content), which made the EIA results overestimated. The "false mRNA positive samples" were highly positive and the discrepancy with protein assay may either be due to the heterogeneity of the samples, since EIA and RT-PCR analysis were performed on two adjacent samples of the same tumor, or to the higher sensitivity of the RT-PCR assay as previously found by other using semi-quantitative or quantitative RT-PCR [10,11]. The same conclusions could be derived for PR. This molecular method represents an alternative for the biochemical assessment of hormone receptors and allows a more complete analysis of hormone dependency profile of the tumor. In addition, this method requires very little tumor material and can be used either on residual tumor material, or on fine needle aspirations or core biopsies. One microgram total RNA (approximately 10^5 tumor epithelial cells) allows one to analyze 10 different parameters on a unique analyte and in a cost- and manpower-effective way.

Other parameters, adapted to analyze hormone responsiveness, can thus be derived from the same analyte in an attempt to increase the predictive value of response to endocrine treatment.

ERβ is a new ER that has a structure and function similar to ERα, i.e. hormone-dependent transcription regulation [7,12]. Estrogens bind to ERβ with high affinity and this is followed by an activation of transcription of target genes containing hormone responsive element in an estrogen-dependent manner [7]. However, the functions of ERα and ERβ are not identical because differential activation of ERE-regulated reporter genes and differential activation of activator protein-1-regulated reporter genes by these two receptors have been reported using anti-estrogens [13]. Heterodimerization of both receptors has been demonstrated and suggests a putative cross-signaling of the two signaling pathways [14]. ERβ is expressed in several human tissues including the breast [12,15]. ERβ mRNA expression is regulated by estradiol and antagonized by anti-estrogens [13,16].

In the present series of samples, the level of transcripts of ERβ was always lower than the level of ERα, confirming other previous publications [17–19]. We did not find any correlation between the absolute value of ERβ expression

and ERα-EIA in our series of 98 breast tumors, nor with ERα-EIA and PR-EIA or RNA levels. These results are similar to those of Cullen *et al*. [20] and Dotzlaw *et al*. [19] on a smaller series of patients, but different with those of Iwao *et al*. [17], Bieche *et al*. [18], Brouillet *et al*. [11], and Knowlden *et al*. [21] who observed either an inverse correlation between ERβ expression and ERα protein content or an inverse correlation between ERβ/ERα mRNA and ERα protein content. All these analyses were performed by semi-quantitative RT-PCR, with the exception of the study of Iwao *et al*. [17] and Bieche *et al*. [18] who analyzed ERα and ERβ mRNA expression in 116 and 131 breast carcinoma using real-time RT-PCR. They are the unique groups, in addition to us, using real-time for ERβ. They observed an inverse correlation between ERβ mRNA and ERβ and PR-EIA. In Iwao's study the distribution of ERβ/ERα values has a wide range with a large overlapping between of ERβ distribution in ERα "positive" and ERα "negative" groups of tumors, as observed in our series (data not shown). However, the statistical analysis was significant ($P < 0.02$). We have also evaluated ERβ/ERα ratio in our series and found an inverse correlation with ERα and PR-EIA (data not shown), but this was linked to the observed correlation of ERβ mRNA with ERα and PR-EIA. In Bieche's study, they divided their tumors in tertile with low, intermediate, and high ERβ values. They also found a statistically significant negative correlation of ERβ mRNA with ERα and Cyclin D1 mRNA. The primers and probes used in our study are located in exons 6/7 of the ERβ gene closed to those of Iwao *et al*. that are located in exons 5/7, and this assay detected the same ERβ variants. Those of Bieche *et al*. were located in domain A/B (exons 1/2). The size of Iwao's PCR products was larger and we could expect a possible increase in PCR efficiency with Bieche's smaller PCR products and ours. But the design of the PCR could not explain the differences observed in these studies as it could not explain the differences observed in other study using semi-quantitative RT-PCR. However, the populations studied were not the same, since we had mainly T2 tumors and 20 lobular carcinoma, and not strictly ductal carcinoma as observed in Iwao's series. A major difference was also observed regarding the endocrine status of the patients, since in the series of Iwao, 59% of patients were premenopausal, in the series of Bieche, 34% patients were premenopausal, in contrast to 23% in our series. Even if no data were available on the regulation of ERβ in different endocrine and therapeutic status, we could not eliminate a major difference between the populations analyzed, since we were not in the same part of the distribution of ERα having a high proportion of high values of REα-EIA, in contrast to the Iwao's study, comprising more ERα-negative tumors. However, the major discrepancies in all studies and the great overlapping in the distribution of ERβ in ERα "positive" and ERα "negative" tumors strongly suggested that ERα and ERβ were independent biological parameters. In addition, we did not find any correlation of ERβ with other clinical or pathological features as also shown by others [12,20,21]. Recently, Jensen *et al*. [23] demonstrated, using a polyclonal antibody, that ERβ is expressed in stromal and epithelial breast cells whereas ERα is only expressed in epithelial cells. In addition, breast tumors expressing high level of ERβ were also associated with high level of proliferation markers (Ki67 and Cyclin A). They also showed that recurrent disease expressed higher level of ERβ than primary tumors. This is not observed for ERα [23]. Other studies analyzed the expression of ERβ using different polyclonal antibodies, either in normal mammary gland or in benign and malignant disease [24,25]. Using polyclonal antibodies and mRNA *in situ* hybridization, Jarvinen *et al*. [24] found high levels of ERβ in normal and benign breast disease, but in their series of 92 primary breast cancers, they found a statistically significant association between ERβ level determined as >20% nuclear staining and low grade and low S phase fraction. Using different polyclonal antibody, Roger *et al*. [25] also found an inverse correlation between ERβ and Ki67 expression in ductal carcinoma *in situ*. The low level of expression of ERβ, as compared to ERα and PR, should probably increase the difficulties of standardization of immunohistochemical methods for determining ERβ status.

The role of ERβ in breast cancer was not clearly elucidated. Its implication in the resistance of breast tumors to anti-estrogens has been advocated but not demonstrated [19,22,24]. Its evaluation in prospective adjuvant or/and neoadjuvant protocol of hormonotherapy may answer this question. No validated standardized method exists yet to quantify routinely ERβ protein, even if polyclonal antibodies are available for a research use [23] and the present quantitative RT-PCR method is clearly adapted for prospective studies.

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