Validation of real-time RT-PCR for analysis of human breast cancer cell lines resistant or sensitive to treatment with antiestrogens

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Abstract

Using a quantitative real-time RT-PCR technique we have compared the expression of a number of genes in two different human breast cancer model systems for development of acquired resistance to antiestrogens. The model system developed at the Danish Cancer Society comprises the cell lines MCF-7, MCF-7/TAMR-1, MCF-7/182R-6 and MCF-7/182R-7, and the model system developed at the Lombardi Cancer Research Center consists of the cell lines MCF-7/LCC1, MCF-7/LCC2 and MCF-7/LCC9. The findings on the well-known parameters estrogen receptor (ER)α, progesterone receptor (PR) and epidermal growth factor receptor (EGFR) are in good agreement with previous reports, thus documenting the usefulness of the real-time RT-PCR technique for multiparametric RNA analysis. The gene expression levels in the two model systems were found to be quite similar in relation to ERα, AIB1 (amplified in breast cancer-1), breast cancer antiestrogen resistance gene 1 (BCAR1) and ErbB-2 mRNA expression, whereas significant differences were observed on the expression of ERβ, multidrug resistance gene 1 (MDR1), PR and EGFR. Furthermore, the presented data suggest that ERβ, AIB1, BCAR1, CYP19 and MDR1 are unlikely to be causally involved in development of antiestrogen resistance in these breast cancer cell lines.

Endocrine-Related Cancer (2003) 10 409–418

Introduction

Endocrine therapy is the most effective and least toxic of the systemic therapies currently available for the management of hormone-dependent breast cancer. The estrogen receptor (ER) assay, first described by Jensen et al. (1971), has long been used to predict which patients could be expected to respond to endocrine therapy. It is primarily the ER-positive tumors that respond to endocrine therapy with antiestrogens, aromatase inhibitors and progestins, whereas patients with ER-negative tumors rarely respond (McGuire et al. 1975). Response rates of up to 70% have been achieved with anti-estrogen therapy in patients with ER- and progesterone receptor (PR)-expressing tumors (Ravdin et al. 1992). Unfortunately, after a period, the responsive tumors often acquire resistance to the treatment (Osborne 1998). However, the mechanisms involved in development of resistance in tumors that express ER and/or PR are so far unclear but seem to be multifactorial (Clarke et al. 2001). The fact that about 30% of the ER- and PR-positive tumors display innate endocrine resistance demonstrates that receptor determination alone is insufficient to determine hormone responsiveness in all cases.

The ligand binding assay (LBA) and the enzyme immunoassay for detection of hormone receptors have long represented the ‘gold standard’ for measurement of steroid hormone receptors in breast cancer cell lines and in breast cancer tissues. However, recently immunohistochemistry has been introduced in routine determination of ER and PR proteins in breast tumors (Zafrani et al. 2000). With the new advances in molecular biology, and in methods for multiparametric RNA analysis, real-time RT-PCR is becoming implemented in varies laboratories around the world. The real-time RT-PCR technology, using fluorescent quenching detection probes (Taqman), is quantitative, sensitive, specific and robust (de Cremoux et al. 2002), which makes it an attractive method for fast and reliable multiparametric RNA analysis.
The discovery of a new ER isoform (ERβ) (Kuiper et al. 1996, Mosselman et al. 1996), with different transactivating function compared with ERα (Paech et al. 1997, Liu et al. 2002), has led to different hypotheses regarding ERβ’s potential role both in progression of breast cancer and in the development of antiestrogen resistance. It has been suggested that the ERα/ERβ ratio represents a dynamic balance between growth-promoting and growth-inhibiting signals within the cells (Gustafsson & Warner 2000). Involvement of ERβ in development of antiestrogen resistance was first suggested by Speirs & Kerin (2000), who found high ERβ mRNA expression to be directly correlated with tamoxifen (TAM) resistance.

In this study, we first validated the use of real-time RT-PCR for the measurement of hormone receptors (ERα, ERβ and PR) using a series of breast cancer cell lines which previously have been evaluated for their content of ERα and PR protein by LBA (Lykkesfeldt et al. 1994, Madsen et al. 1997). Secondly, we determined the expression of a number of potential ‘candidate genes’ which could be expected to play a role in development of antiestrogen resistance. For this purpose a large collection of cell lines resistant to different antiestrogens was screened for the mRNA expression of the steroid receptors ERα, ERβ and PR, the co-activator AIB1 (amplified in breast cancer-1), the CYP19 gene (encoding the aromatase enzyme), the breast cancer antiestrogen resistance gene 1 (BCAR1), the multidrug resistance gene 1 (MDR1), the epidermal growth factor receptor (EGFR/ErbB-1), and ErbB-2, which in different ways could be expected to play a role in development of antiestrogen resistance.

Materials and methods

Human breast cancer cell lines and culture conditions

The MCF-7 cell line was originally obtained from The Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA, USA). In these experiments the MCF-7/S0.5 subline of the original MCF-7 cell line, which has been adapted to grow at low serum concentration, has been used (Briand & Lykkesfeldt 1984). The antiestrogen-resistant cell lines MCF-7/TAMα-1, MCF-7/164β-5, MCF-7/186β-6 and MCF-7/182β-7 have been established from MCF-7/S0.5 by long-term selection with 10^-6 M TAM (Sigma-Aldrich), 10^-7 M ICI 164,384 (AstraZeneca) and 10^-7 M ICI 182,780 (subsequently referred to simply as ICI) (AstraZeneca) respectively (Lykkesfeldt et al. 1994, 1995). These cell lines are referred to as cell lines from DCS (Danish Cancer Society). These MCF-7 sublines are maintained in phenol red-free DMEM/F12 (1:1) medium supplemented with 1% fetal calf serum (Gibco BRL), 2 mM glutamine and 6 ng/ml insulin. Resistant cell lines are maintained in the presence of the respective antiestrogen (stem cells) or withdrawn from the antiestrogen 1 week before onset of experiments (control cells). Estradiol (E2) was obtained from Collaborative Research, Waltham, MA, USA.

Another set of MCF-7 sublines is the LCC sublines established at the Lombardi Cancer Research Center. The MCF-7/LCC1 is a hormone-independent and hormone-responsive MCF-7 variant (Clarke et al. 1989, Brunner et al. 1993, Thompson et al. 1993). MCF-7/LCC2 and MCF-7/LCC9 cells were established by a stepwise in vitro selection of MCF-7/LCC1 cells against 4-OH-TAM and ICI respectively (Brunner et al. 1993, 1997). All LCC cell lines were maintained in phenol red-free DMEM/F12 (1:1) medium supplemented with 5% charcoal-stripped fetal calf serum, 2 mM glutamine and 6 ng/ml insulin.

RNA extraction

Total RNA was either extracted from 2 x 10^6 cells by using the RNA plus kit (Bioprobe, Montreuil, France), or extracted from cell cultures which were about 70% confluent using the Trizol Reagent (Invitrogen). Purification was carried out according to the manufacturer’s instructions. The quality of the RNA was similarly high with the two extraction methods used at Institut Curie and at DCS respectively. The purified RNA samples were stored in RNase-free distilled water at –80°C. The quality of RNA samples was ensured by electrophoresis in an agarose gel followed by ethidium bromide staining, where the 18S and 28S RNA bands could be visualized under UV light. Quantification of RNA was performed in duplicate by spectrophotometry at 260 nm.

cDNA synthesis

One microgram of total RNA was reverse-transcribed in a final volume of 20 μl containing 1 × reverse transcriptase buffer (1.25 mM each dNTP, 6.7 mM MgCl2, 2.5 U RNase inhibitor (Amersham) 5 μM/1 random hexamer (Boehringer-Mannheim) and 10 U murine Moloney leukemia virus reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA). The reaction mix was incubated at 42°C for 30 min, before the reverse transcriptase was inactivated by a 2 min incubation. cDNA was stored at –80°C.

Real-time PCR amplification

All target transcripts were detected using quantitative real-time RT-PCR (Taqman) assays. RPLPO (also known as 36B4) was the endogenous control used for normalization of the data (Bie`che et al. 2001a).

Primers and probes were chosen with the assistance of the computer program Primer Express (Applied Biosystems, Foster City, CA, USA). Then nucleotidic sequence was ‘Blasted’ against the dbEST and nr databases to confirm the
For each sample, the lysis software, was used for the calculation of the ‘N-target’. Ground signal (recommended by Applied Biosystems products is 10-fold above the standard deviation of the background signal associated with an exponential increase of PCR products. Controls were necessary for the calculations described below. For each target gene, the efficiency (E) of the real-time PCR reaction was calculated from the given slope of the standard curve using the Taqman software. E was calculated as 

\[
E = 10^{(-\frac{1}{\text{slope}})}
\]

from the exponential part of the standard curve according to the equation 

\[
\Delta C_{\text{t}} = \Delta C_{\text{t sample}} - \Delta C_{\text{t calibrator}}.
\]

The threshold cycle (Ct) number, at which the fluorescent signal associated with an exponential increase of PCR products is 10-fold above the standard deviation of the background signal (recommended by Applied Biosystems analysis software), was used for the calculation of the ‘N-target’. For each sample, the ΔCt values were determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene (RPLPO). The relative gene expression level was also normalized relative to a positive calibrator, consisting of one of the samples from the calibration curve. The relative gene expression level of the calibrator (ΔCt calibrator) was also determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. Results were expressed as ‘N-target’ and determined as follows (Bie`che et al. 2001a):

\[
\text{N-target} = 2^{\Delta C_{\text{t sample}} - \Delta C_{\text{t calibrator}}}
\]

Table 1 Primmers and probes sequences for RT-PCR amplification. Primers and probe sequence for ERα, ERβ and PR have been previously described (de Cremoux et al. 2002). Primers and probe sequence for EGFR/ErbB-1 have been previously described (Pawlowski et al. 2001). Annealing temperature was 65°C for BCAR1, AIB1, and CYP19, and 60°C for ErbB-2 and MDR1.

<table>
<thead>
<tr>
<th>Localization</th>
<th>PCR product (bp)</th>
<th>Primers</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAR1</td>
<td>—</td>
<td>188</td>
<td>5’-gactgccagatggcagta-3’</td>
</tr>
<tr>
<td>AIB1</td>
<td>Exons 14/15</td>
<td>145</td>
<td>5’-taagccaggtgtaaactgct-3’</td>
</tr>
<tr>
<td>CYP19</td>
<td>Exons 4/5</td>
<td>73</td>
<td>5’-tcttgccctgcgctctgt-3’</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>Exons 3/4</td>
<td>205</td>
<td>5’-cttgatcaagacccctcctt-3’</td>
</tr>
<tr>
<td>MDR1</td>
<td>Exons 21/22</td>
<td>89</td>
<td>5’-aaagaccaacagcttcaggttacgtgc-3’</td>
</tr>
</tbody>
</table>

Results

PCR optimization and validation

The specificity of the RT-PCR products was documented using high-resolution gel electrophoresis and resulted in a single product with the desired length (ERα: 145 bp; ERβ: 143 bp; PR: 122 bp; CYP19: 188 bp; MDR1: 89 bp; EGFR: 205 bp, ErbB-2: 249 bp). We have already demonstrated high accuracy of this quantitative real-time RT-PCR technique for ERα and PR (de Cremoux et al. 2002).

For each target gene, the efficiency (E) of the real-time PCR reaction was calculated from the given slope of the standard curve using the Taqman software. E was calculated from the exponential part of the standard curve according to the equation 

\[
E = 10^{(-\frac{1}{\text{slope}})}
\]

The investigated transcripts showed high real-time PCR efficiency rates (ERα: 98%; ERβ: 99%; PR: 92%; CYP19: 95%; AIB1: 94%; BCAR1: 97%; MDR1: 99%; EGFR: 98%; ErbB-2: 99%) in the dynamic range of the experiments. Samples of between 0.4 and 25 ng cDNA exhibited high linearity as shown in Fig. 1b.

To confirm the accuracy and reproducibility of the method, the intra-assay precision was determined in two repeats within one Taqman run and the inter-assay variation was investigated in three different Taqman runs showing less than 2% intra-assay variation and less than 5% inter-assay variation as calculated by the standard error.
Real-time RT-PCR analysis of human breast cancer cell lines

Figure 1 Real-time RT-PCR PR mRNA standard curve. The T47D carcinoma cell line was used as a standard. (a) Cycle threshold was plotted vs change in normalized reporter signal ($\Delta R_n$). For each reaction tube, the fluorescence signal of the reporter dye 6-carboxyfluorescein for PR and Vic for RPLPO was divided by the fluorescence signal of the passive reference dye 6-carboxy-tetramethyl-rhodamine to obtain a ratio defined as the normalized reporter signal ($R_n$). $\Delta R_n$ represents the normalized reporter signal ($R_n$) minus the baseline signal established in the first 15 PCR cycles. Each sample was analyzed in duplicate and a mean value was considered. (b) Standard curve plotting log initial copy number vs Ct.

Basal expression and E2, TAM and ICI-regulated RNA expression of ER$\alpha$, ER$\beta$, PR, AIB1, CYP19, BCAR1, MDR1, EGFR and ErbB-2 in MCF-7 cells

The expression levels of mRNA for the hormone receptors, co-activator, BCAR1, CYP19, and growth factor receptors in MCF-7 cells are shown in Table 2. ER$\alpha$, PR, AIB1, BCAR1 and ErbB-2 were expressed at significant amounts, whereas the levels of ER$\beta$, CYP19, MDR1 and EGFR were low or undetected. E2 treatment for 48 h induced a slight decrease (non-significant) in ER$\alpha$ mRNA expression and a significant increase in PR mRNA expression (3.3-fold). No other regulation was observed in the presence of E2 under these experimental conditions. Treatment for 48 h with TAM had no effect on the expression of the measured parameters with one exception, MDR1, which was increased by 2.4-fold. In contrast, treatment for 48 h with ICI induced numerous changes in the level of the different transcripts. A slight increase in ER$\alpha$ level was observed (non-significant), a significant increase in AIB1 (2.4-fold), ErbB-2 (3-fold) and MDR1 (2.3-fold) mRNA expression was detected, whereas a significant decrease was observed for ER$\beta$ (2.1-fold), PR (7.2-fold) and CYP19 (3.6-fold) mRNA expression, as summarized in Table 2.
Basal expression of ERα, ERβ, PR, AIB1, CYP19, BCAR1, MDR1, EGFR and ErbB-2 mRNA in parental MCF-7 and antiestrogen-resistant cell lines from DCS and LCC

The expression of the investigated parameters was compared between antiestrogen-resistant cell lines and parental MCF-7 cells from DCS (Table 3). The level of expression of ERα was decreased by 6-, 3.5-, 6- and 6-fold in MCF-7/TAMR-1, MCF-7/164R-5, MCF-7/182R-6 and MCF-7/182R-7 respectively. The level of ERβ was equally low in all antiestrogen-resistant cell lines and comparable with the level in parental MCF-7 cells. The PR mRNA was readily detected in MCF-7 cells but this E2-inducible transcript was reduced to extremely low levels in all resistant cell lines (decreased by 500- to 3000-fold). In the case of AIB1, BCAR1, CYP19 and ErbB-2 these transcripts remained mainly unchanged in the antiestrogen-resistant cell lines when compared with the parental MCF-7 cell line, whereas the expression of EGFR mRNA was clearly increased in all DCS antiestrogen-resistant cell lines by 9-, 6-, 33- and 6-fold in MCF-7/TAMR-1, MCF-7/164R-5, MCF-7/182R-6 and MCF-7/182R-7 respectively. Also MDR1 was induced in the resistant DCS cell lines by 9-, 6-, 33- and 2.3-fold respectively.

In the LCC cell lines, we analyzed the same set of parameters (data shown in Table 4). The MCF-7/LCC1 cell line is a hormone-independent but hormone-responsive MCF-7 subline (Brünn et al. 1993). This cell line can be considered the parental cell line for the TAM-resistant MCF-7/LCC2 and the ICI- and TAM-resistant MCF-7/LCC9 cell lines (Brünn et al. 1993b, 1997). The basal expression level of the investigated mRNAs in MCF-7/LCC1 cells differs from the MCF-7 cells (DCS) with respect to a number of parameters. MCF-7/LCC1 contains about 2-fold more ERα transcripts than MCF-7 (DCS) whereas the expression of ERβ, PR, AIB1 and MDR1 remains unchanged. BCAR1, EGFR and ErbB-2 are expressed at higher levels in MCF-7/LCC1 compared with MCF-7 (2.4-, 50- and 2.3-fold respectively). The expression level of CYP19, which is low in MCF-7 cells, is decreased further to a level close to the detection level in the LCC cell lines.

Comparison of MCF-7/LCC1 cells with the corresponding antiestrogen-resistant sublines (MCF-7/LCC2 and MCF-7/LCC9) revealed decreased ERα mRNA levels (2.5- and 19-fold respectively) and increased ERβ levels (2.6- and 2.5-fold) in both resistant cell lines. We also observed a decrease in PR mRNA expression in MCF-7/LCC2 (25-fold) and an increase in PR mRNA expression in the MCF-7/

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**Table 2** Basal expression levels and estrogen and antiestrogen regulation in MCF-7 cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MCF-7/S0.5 Control</th>
<th>E2</th>
<th>TAM</th>
<th>ICI 182,780</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>ERβ</td>
<td>+</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>PR</td>
<td>++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>AIB1</td>
<td>+++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>BCAR1</td>
<td>++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>CYP19</td>
<td>Low level</td>
<td>NC</td>
<td>NC</td>
<td>Decrease (2.2-fold)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Low level</td>
<td>NC</td>
<td>NC</td>
<td>Increase (2-fold)</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>++</td>
<td>NC</td>
<td>NC</td>
<td>Increase (2-fold)</td>
</tr>
<tr>
<td>MDR1</td>
<td>+</td>
<td>NC</td>
<td>NC</td>
<td>Increase (5-fold)</td>
</tr>
</tbody>
</table>

NC, no change.

Low level means that the Ct is just above of the limit of sensitivity of detection.

---

**Table 3** Basal expression levels in cell lines from DCS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MCF-7/S0.5 control</th>
<th>MCF-7/TAMR-1</th>
<th>MCF-7/164R-5</th>
<th>MCF-7/182R-6</th>
<th>MCF-7/182R-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+++</td>
<td>Decrease (6-fold)</td>
<td>Decrease (3.5-fold)</td>
<td>Decrease (6-fold)</td>
<td>Decrease (6-fold)</td>
</tr>
<tr>
<td>ERβ</td>
<td>+</td>
<td>Decrease (800-fold)</td>
<td>Decrease (3400-fold)</td>
<td>Decrease (2300-fold)</td>
<td>Decrease (570-fold)</td>
</tr>
<tr>
<td>PR</td>
<td>++</td>
<td>Decrease (6-fold)</td>
<td>Decrease (6-fold)</td>
<td>Decrease (6-fold)</td>
<td>Decrease (6-fold)</td>
</tr>
<tr>
<td>AIB1</td>
<td>+++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>BCAR1</td>
<td>++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>CYP19</td>
<td>Low level</td>
<td>Increase (2.2-fold)</td>
<td>Increase (9-fold)</td>
<td>Increase (33-fold)</td>
<td>Increase (6-fold)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Low level</td>
<td>Increase (6-fold)</td>
<td>Increase (6-fold)</td>
<td>Increase (33-fold)</td>
<td>Increase (6-fold)</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>MDR1</td>
<td>+</td>
<td>Increase (5-fold)</td>
<td>Increase (4.4-fold)</td>
<td>Increase (3.2-fold)</td>
<td>Increase (2.3-fold)</td>
</tr>
</tbody>
</table>

NC, no change.

Low level means that the Ct is just above of the limit of sensitivity of detection.
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Table 4 Basal expression levels in MCF-7 control and in cell lines from LCC. The protein levels in MCF-7/LCC1 cells are compared with the MCF-7 control, whereas the levels in MCF-7/LCC2 and MCF-7/LCC9 are compared with their parental MCF-7/S0.5 cell line

<table>
<thead>
<tr>
<th>Gene</th>
<th>MCF-7/S0.5 control</th>
<th>MCF-7/LCC1</th>
<th>MCF-7/LCC2</th>
<th>MCF-7/LCC9</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+++</td>
<td>Increase (2-fold)</td>
<td>Decrease (2.5-fold)</td>
<td>Decrease (19-fold)</td>
</tr>
<tr>
<td>ERβ</td>
<td>+</td>
<td>NC</td>
<td>Increase (2.6-fold)</td>
<td>Increase (2.5-fold)</td>
</tr>
<tr>
<td>PR</td>
<td>++</td>
<td>NC</td>
<td>Decrease (25-fold)</td>
<td>Increase (2.3-fold)</td>
</tr>
<tr>
<td>AIB1</td>
<td>+++</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>BCAR1</td>
<td>++</td>
<td>Increase (2.4-fold)</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>CYP19</td>
<td>Low level</td>
<td>Decrease (4.2-fold)</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>EGFR</td>
<td>Low level</td>
<td>Increase (50-fold)</td>
<td>NC</td>
<td>Decrease (16-fold)</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>++</td>
<td>Increase (2.3-fold)</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>MDR1</td>
<td>+</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC, No change.
Low level means that the Ct is just above of the limit of sensitivity of detection.

LCC9 cell line (2.3-fold). In addition, we observed a decrease in EGFR mRNA expression in LCC9 cell line (16-fold). No change was observed for all other parameters in the resistant cell lines.

Comparison between gene expression in parental MCF7 from DCS treated with E2, TAM or pure antiestrogens (Table 2) and resistant DCS cell lines (Table 3) revealed different profiles of CYP19 and EGFR mRNA expression. Cyp19 gene expression is decreased in parental MCF7 cells under ICI treatment, whereas it is increased in TAM- and ICI-resistant cell lines. EGFR mRNA expression is not modified by treatment of the parental MCF7 cell line, whereas it is clearly increased in resistant DCS cell lines. This led us to conclude that these alterations in mRNA expression may be due to the resistance profile rather than exposure to treatment. In contrast, the increase in MDR1 mRNA gene expression may just reflect that antiestrogens up-regulate MDR1 expression, as shown for MCF-7/S0.5 cells.

Discussion

For mRNA detection, we used a recently developed quantitative RT-PCR method (Taqman) based on real-time analysis of PCR amplification. This assay is sensitive and allows accurate quantification of mRNA expression as previously demonstrated for ERα and PR transcripts (Iwao et al. 2000, Bioche et al. 2001b, de Cremoux et al. 2002). The real-time PCR method had several advantages over other RT-PCR-based quantitative and biochemical methods. It does not require post-PCR sample handling, thereby avoiding problems related to carry-over. Furthermore, it has a wide dynamic range, and the intra- and inter-assay coefficients of variation are very low (<5%). Each target gene is normalized with an endogenous reference gene (RPLO) to compensate for sample-to-sample variation of the mRNA amount. Real-time RT-PCR-specific errors in the quantification of mRNA transcripts are usually related to possible variation of starting material. However, this is not a significant problem in this study, where we have used mRNA from cell lines in which the quality and the quantity of starting material were excellent and sufficient.

To compensate for any inter-PCR variation between the runs, calibration of the expression of each target gene was carried out. A standard curve for a calibrator (a human breast cancer cell line expressing detectable level of the studied parameter) was analyzed in each experiment under the same conditions as used for unknown samples. Also the PCR efficiency was calculated from the calibration curve in each experiment and for each transcript the possible variation from one run to the next was taken into account.

The real-time RT-PCR analyses of MCF-7 cells (Table 2) revealed a significant level of ERα mRNA, whereas the ERβ mRNA level was found to be very low. Previously, we have found high levels of ERα mRNA in MCF-7 cells by Northern analysis (Larsen et al. 1997, Jensen et al. 1999), and the ERα protein level has been measured as 400–600 fmol/mg cytosol protein in MCF-7 cells (Lykkesfeldt et al. 1994, Madsen et al. 1997). By Western analysis a specific protein band has been detected at 65 kDa with ERα-recognizing antibodies (Madsen et al. 1997). We have not been able to detect the ERβ protein in a total MCF-7 cell lysate (30 µg protein) by Western analysis with an ERβ-specific antibody. However, we were able to detect recombinant ERβ protein down to a level of 2 ng in the same experiment (data not shown). For comparison the same MCF-7 lysate contained about 20 ng ERα protein. The real-time RT-PCR data on ERα and ERβ obtained by Taqman analysis correspond very nicely with our previously published data and recent experiments with Western analyses using recombinant ERα and ERβ proteins. The estrogen and antiestrogen regulation of ERα mRNA expression in MCF-7 cells (DCS) did not reveal significant changes (cut-off value equals 2-fold) in relation to the control culture in an individual set of experiments (Table 2).

However, the three independent experiments that were carried out showed a 1.5-fold decrease of ERα mRNA in E2-treated cultures and a 1.5-fold increase in ERα mRNA in...
all three experiments with ICI-treated cultures. These observations are in concert with our previously published data, using Northern analyses (Jensen et al. 1999), and support the usefulness and reproducibility of the real-time RT-PCR analysis.

A significant level of PR mRNA was found in control cultures of MCF-7 cells. The observed E2-induced increase and ICI-induced decrease in PR mRNA level are in agreement with previously published Northern analyses from our laboratory (Larsen et al. 1997, Jensen et al. 1999) and in concert with publications from other laboratories (Horwitz & McGuire 1979, May et al. 1989, Kraus et al. 1994).

MCF-7 cells were found to express significant amounts of the ERα co-activator AIB1 and the BCAR1 mRNA. The AIB1 level was found to be increased by ICI treatment, whereas no change in BCAR1 and MDR1 mRNA expression was observed upon hormonal treatment. The CYP19 gene expression was close to the detection level in both the control cultures and E2- and TAM-treated cultures. This is in agreement with the fact that we previously have been unable to detect CYP19 by Northern analyses, probably due to the low expression level in our cell lines. In spite of that, we consistently found evidence that ICI treatment down-regulated the CYP19 gene expression further in MCF-7 cells (DCS). The data presented here suggest that ICI, apart from interacting directly with the aromatase enzyme (CYP19 protein) and thereby decreasing the aromatase activity as reported by Long & Tilghman (1998), also acts as a suppressor at the transcriptional level in our model system. These data represent the first report on the expression of AIB1, BCAR1 and CYP19 mRNA in hormone-treated cultures of MCF-7 cells. The role of CYP19 gene expression in endocrine resistance may be advocated, since it has previously been demonstrated that even low levels of aromatase protein and mRNA may be responsible for the high level of synthesis of estrogens in situ found in breast cancer tissue in postmenopausal women (Miller et al. 1997), but our data cannot prove that hypothesis.

The observation of low EGFR mRNA levels and a significant level of ErbB-2 mRNA is in concordance with data obtained by semi-quantitative RT-PCR analysis and Northern analysis (Larsen et al. 1999).

In conclusion, the real-time RT-PCR analyses have revealed new information about our cell culture model system. We have confirmed previous findings and the analysis has proven its usefulness as a sensitive, fast and quantitative multiparametric technique for measuring selected transcripts in human breast cancer cell lines.

A large panel of antiestrogen-resistant breast cancer cell lines, derived from two different laboratories and selected by different methods, have also been analyzed with respect to expression of the above-mentioned parameters (Tables 3 and 4). The cell lines from DCS have been selected by long-term treatment with a high concentration of the respective antiestrogen prior to clonal selection of the resistant cells (Lykkefeldt & Briand 1986, Lykkefeldt et al. 1994, 1995). The LCC cell lines have been selected for the ability to adapt to a stepwise increase in antiestrogen concentration (Brünnér et al. 1993b, 1997).

The ERα mRNA expression levels were decreased in all resistant cell lines compared with their respective parental cell lines (MCF-7 (DCS) or MCF-7/LCC1 (LCC)). This appears to be a general feature for both human breast tumors and breast cancer cell lines with acquired antiestrogen resistance (Brünnér et al. 1993a, 1997, Johnston et al. 1995, Madsen et al. 1997). In the resistant cell lines from DCS, the ERβ level was similar to the level in the parental MCF-7 cell line, whereas an increase in ERβ mRNA expression level was observed in MCF-7/LCC2 and MCF-7/LCC9 when compared with the MCF-7/LCC1 cells. When calculating the ERα/ERβ ratio, based on the N-target value (data not shown), we found it to be about 1000 in MCF-7 and the lowest ratio in the resistant DCS cell line was about 60. In the LCC cell lines the ERα/ERβ ratio was found to be about 1000 and 200 in MCF-7/LCC1 and MCF-7/LCC2 respectively. Even in the MCF-7/LCC9 cell line, which has the lowest ERα level and highest ERβ level, the ERα/ERβ mRNA ratio is about 30, still demonstrating a rather large excess of ERα. Whether the small increase in ERβ expression is involved in antiestrogen resistance as suggested by Speirs & Kerin (2000) is very difficult to anticipate, although Liu et al. (2002) have demonstrated a clear dominance of ERβ over ERα in transactivating activity in transfection assays with E2-treated cells at a 10-fold excess of ERα.

All antiestrogen-resistant cell lines, except the MCF-7/LCC9 cell line, have a significantly reduced expression of PR mRNA, probably reflecting the reduced ERα expression level in these cell lines. The MCF-7/LCC9 cell line displays high PR expression and this expression has been found to be estrogen-independent (Brünnér et al. 1997), indicating that the PR gene could be amplified in this particular cell line. Whether the corresponding PR protein is functional in this cell line is questionable, since treatment of the MCF-7/LCC9 cell line with progesterone or the synthetic progestin, meigace, had no effect on cell proliferation (data not shown).

Overexpression of the BCAR1 gene in breast cancer cell lines confers antiestrogen resistance (Brinkman et al. 2000), and it has been reported that high expression levels of BCAR1 in primary breast tumors is associated with a greater risk of intrinsic resistance to TAM therapy (van der Flier et al. 2000b), whereas tumors from patients with acquired TAM resistance did not appear to express increased BCAR1 level (van de Flier et al. 2000a). In this study with a large panel of cell lines with acquired antiestrogen resistance, we could confirm the clinical data demonstrating that antiestrogen resistance is not associated with increased BCAR1 expression.

It is interesting to note the increased EGFR expression level in the antiestrogen-resistant cell lines from DCS.
This probably reflects the inverse relationship between EGFR and ERβ expression that has been demonstrated in several studies on both cell lines and clinical material (Sainsbury et al. 1985, Davidson et al. 1987, Klijn et al. 1994, Larsen et al. 1999) and found to be associated with progression towards estrogen-independence and lack of response to endocrine therapy (Harris et al. 1989, Nicholson et al. 1994). In the estrogen-independent LCC cell system, however, the basal expression level of EGFR in MCF-7/LCC1 is dramatically increased compared with MCF-7 (DCS) but without a concomitant decrease in ERα mRNA level. The EGFR remains high during selection of MCF-7/LCC2 cells, whereas a significant decrease in EGFR expression is observed in the estrogen- and antiestrogen-resistant cell line MCF-7/LCC9. However, compared with MCF-7 cells from DCS, the MCF-7/LCC9 cells have a 3-fold increase in EGFR expression. Thus, all cell lines from LCC have increased EGFR expression levels compared with MCF-7 from DCS, but no inverse relationship between ERβ and EGFR expression is observed in this model system. Whether this represents the multifactorial nature of antiestrogen resistance mechanisms, or the difference in the selection procedures, is not clear.

Analyses of the MDR1 gene revealed that this transcript was expressed at a very low level and was probably not involved in development of antiestrogen resistance in the LCC cell lines. Theoretically, MDR1 may be involved in development of antiestrogen resistance in the DCS cell lines, as the expression level was found to be increased in some of them. Regarding the MCF-7/182R6 cells, which display no increase in MDR expression, we have previously demonstrated that resistance to ICI is not due to reduced intracellular antiestrogen concentration, thus excluding an MDR-mediated increased efflux of the drug (Brockdorff et al. 2000). In summary, these data do not indicate a general involvement of the MDR gene in antiestrogen resistance.

The general conclusions from this study are listed below. First, the data presented here support already published data, thus confirming the real-time RT-PCR technique to be a very reliable and convenient method for detection of a large number of genes even in a small amount of sample material. Secondly, we have for the first time compared the expression of a broad range of transcripts in two different model systems of antiestrogen-resistant human breast cancer. The LCC and the DCS model system displayed both similar and different gene expression characteristics. The similarities are decreased ERβ and no change in expression of AIB1, BCAR1 and ErbB-2 in the resistant cell lines compared with their respective parental cell lines. The observed differences between the model systems are increased MDR1 expression in some of the resistant DCS cell lines, increased EGFR expression in MCF-7/LCC1, slightly increased expression of ERβ in MCF-7/LCC2 and MCF-7/LCC9 and increased expression of PR in MCF-7/LCC9. Overall, the expression profiles for the most commonly measured parameters (ER, PR and EGFR) in the DCS cell lines showed complete correlation with clinical data. The LCC cell lines have the advantage of being able to grow in nude mice without estrogen supplementation, rendering these cell lines useful both as an in vitro and an in vivo model. Lastly, the presented data demonstrate that changes in ERβ, AIB1, BCAR1, CYP19 and MDR1 gene expression are not likely to be involved in acquired antiestrogen resistance in the analyzed model systems. Further studies of gene expression in antiestrogen-sensitive and -resistant cell lines will be important for elucidating the mechanisms involved in development of acquired resistance to antiestrogen treatment, and for this purpose the real-time RT-PCR technique will be very useful.

Acknowledgements

We thank P Mandé for her help in typing the manuscript and C Barbaroux, I Heiberg and B E Reiter for excellent technical assistance. This work supported by grants from the Curie Institute (PIC (Programme Incitatif et Coopératif), the Union Internationale Contre le Cancer (ICRETT) fellowship), and from the Danish Cancer Society.

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Endocrine-Related Cancer (2003) 10 409–418
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