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Estrogen Receptor Messenger RNA Splice Variants Are Not Involved in Antiestrogen Resistance in Sublines of MCF-7 Human Breast Cancer Cells

Mogens W. Madsen, Birgit E. Reiter, Søren S. Larsen, Per Briand, and Anne E. Lykkesfeldt

Department of Tumor Endocrinology, Division for Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark

Abstract

Development of resistance to tamoxifen is a serious problem in treatment of breast cancer patients. Although the mechanisms for development of resistance are unclear, an altered expression of alternatively spliced estrogen receptor (ER) mRNA has been suggested to be involved. We have looked for differential expression of ER splice variants lacking exon 2 (ERΔE2), exon 3 (ERΔE3), exon 4 (ERΔE4), exon 5 (ERΔE5), exon 7 (ERΔE7), and exons 4 and 7 (ERΔE4, 7) in the human breast cancer cell line MCF-7 and 10 ER-positive MCF-7 sublines resistant to the antiestrogens tamoxifen, ICI 164,384 or ICI 182,780. No major differences in the expression were demonstrated between MCF-7 cells and resistant cells, indicating that ER splice variants are not involved in antiestrogen resistance in this model system. Furthermore, despite a high mRNA level of some of the ER splice variants, no corresponding proteins could be detected using Western blot analysis.

Introduction

Breast cancer patients with an ER-³ positive primary tumor often benefit from treatment with endocrine therapy. The most common first-line treatment is the nonsteroidal antiestrogen tamoxifen. Unfortunately, almost all patients with advanced disease will eventually develop resistance toward the treatment. New more efficient pure antiestrogens, ICI 164,384 and ICI 182,780, have been developed, and ICI 182,780 has been evaluated in clinical treatment and found to be effective in treatment of breast cancer patients with advanced disease relapsing on tamoxifen treatment (1). Although the response to the ICI 182,780 compound appears to be unusually long (1), resistant cell lines to both the ICI 164,384 and ICI 182,780 compound have been established in tissue culture (2). The mechanisms for development of antiestrogen resistance are poorly understood. Since the antiestrogens act through the ER, alterations in ER expression, structure, or function may be involved in antiestrogen resistance (3). Loss of ER expression is observed in tamoxifen-resistant breast tumors, although most of the resistant tumors remain ER positive (4). Unlike other members of the nuclear receptor gene superfamily, a significant proportion of the ER mRNA in human breast tumors is expressed as splice variants (5). It has been questioned whether altered expression of the ER splice variants could be involved in antiestrogen resistance. The human ER gene is composed of eight exons and splice variants deleted for exons 2, 3, 4, 5, 7, and 4 + 7 are found both in normal and malignant breast epithelial cells (5—9). The first ER splice variant described was the ER

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: ER, estrogen receptor; ERΔE2, ER lacking exon 2; ERΔE3, ER lacking exon 3; ERΔE4, ER lacking exon 4; ERΔE5, ER lacking exon 5; ERΔE7, ER lacking exon 7; ERΔE4,7, ER lacking exon 4 and 7; RT, reverse transcription; nt, nucleotide.

Materials and Methods

Cell Culture. The MCF-7 cell line was originally obtained from The Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA). The establishment of the antiestrogen-resistant cell lines: MCF-7/164R-1, MCF-7/164R-5, MCF-7/164R-7, MCF-7/182R-1, MCF-7/182R-6, and MCF-7/182R-7 has been described earlier (2). The MCF-7/TAMR-4, MCF-7/TAMR-7, and MCF-7/TAMR-8 cells were generated by long-term treatment of MCF-7 cells with 10—8 M tamoxifen (Zeneca Pharmaceuticals, Macclesfield, United Kingdom) using a procedure described previously (12). The MCF-7/182R-6T cell line originated from a 9 X 9 X 9-mm tumor of MCF-7/182R-6 cells grown for 12 weeks in a nude mouse supplemented with estrogen. Tissue handling and establishment of the explant culture was performed as described previously with the modification that 1% FCS was included in the growth medium and the culture flasks were not coated with collagen (13). The ER-negative human breast epithelial cell line HMT-3522 (13) was used for expression of the ERΔE5 variant protein. Transient transfection was performed according to the calcium phosphate transfection protocol (Promega Corporation, Madison, WI).

All MCF-7 cell lines were propagated in growth medium consisting of DME/F12 medium (1:1) supplemented with 1% FCS, 2.5 mM Glutamax (Life Technologies, Roskilde, Denmark), and 6 ng/ml insulin (Novo-Nordisk, Copenhagen, Denmark). Growth medium was renewed every second or third day, and the cultures were passaged every week by trypsinization and seeded with 8 ¥ 10³ cells/cm². The antiestrogen-resistant cell lines were propagated and passaged in the presence of the respective antiestrogen in the following concentrations: 10—6 M tamoxifen, 10—7 M ICI 164,384 (Zeneca Pharmaceuticals), and 10—7 M ICI 182,780 (Zeneca Pharmaceuticals). The HMT-3522 cell line was propagated in chemically defined growth medium as described earlier (13).

ER Determination. Near confluent cultures were harvested and cytosol was prepared after hypotonic treatment and homogenization in tightly fitting glass-glass Potter-Elvehjem homogenizers as described previously (14). The content of free ER was determined using a ligand-binding assay (2).

RNA Preparation and Northern Blot Analysis. Total RNA was isolated from the cell lines by lysing the cells in 4 M guanidine thiocyanate followed by centrifugation over a CsCl cushion as already described (9). Poly(A)⁺ RNA

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was isolated using Dynabeads Oligo(dT)25 (Dynal, Oslo, Norway) according to the manufacturer’s instructions. Two μg of poly(A)+ RNA were denatured by glyoxal/DMSO, electrophoresed on a 1.0% agarose gel, and analyzed using Northern blotting. The Northern blot was hybridized with a 32P-labeled ER cDNA probe containing the 2.1-kb EcoRI cDNA fragment of pOR8 (15). The Northern blot was rehybridized with a 1.2-kb PstI glyceraldehyde-3-phosphate dehydrogenase cDNA probe (9) as a control for differences in poly(A)+ RNA loading. The Northern blot analysis was repeated twice on independent RNA preparations, and the results were reproducible.

**Western Blot Analysis**. Cells for Western blot analysis were grown for 15 days without the respective antiestrogen to avoid reminiscence effects. Cultures were harvested at about 60–70% confluency, cell lysis, SDS-PAGE, electrophoresing to nitrocellulose membrane, and immunostaining were performed as described previously (9) using a primary mouse monoclonal antibody against human ER (DAOK-ER 1D5; DAKO A/S, Glostrup, Denmark) and alkaline phosphatase-conjugated secondary antibody (DAKO D314). The enhanced chemiluminescence Western blotting protocol (Amersham, Buckinghamshire, England) has been used for detection of the ERΔE5 protein with the 1D5 primary antibody and a horseradish peroxidase-conjugated secondary antibody (DAKO P0260).

**RNase Protection Assay**. RNase protection assay was performed as described before (9). Briefly, 50 μg of total RNA were solution hybridized with a [α-32P]CTP-labeled antisense RNA probe (5 X 10^6 cpm) which was synthesized in vitro by transcription from a linearized plasmid using either T3, T7, or Sp6 promoter. A sample with 50 μg of tRNA served as a control for background hybridization. The unhybridized RNA fragments were removed by RNase T1 (1.8 μg/ml) and RNase A (36 mg/ml). RNAs were inactivated by the addition of proteinase K (300 μg/ml) and SDS (0.5%/w/v), followed by extraction with phenol and chloroform, and unincorporated nts were removed by repeated ethanol precipitation. RNA samples were denatured by boiling in a formamide loading buffer, electrophoresed on a 4–6% denaturing polyacrylamide gel, and visualized by autoradiography. Protected fragments representing the variant ER mRNA or the wt ER mRNA were quantified by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA), measuring the amount of radioactivity incorporated into the fragments. The data were corrected for differences in the content of the used 32P-labeled nt, and the molar ratio between variant ER mRNA and wt ER mRNA was calculated. For each investigated splice variant, the RNase protection assay was performed on two independent RNA preparations per cell line.

**Antisense Probes Used for RNase Protection Assay**. The antisense probes used in this study were generated from MCF-7 or MCF-7/TAMR cell lines by RT-PCR using primers located in the flanking exons of the exon of interest (9). The following cloned ER cDNAs were used for generation of antisense probes: ERAE2: nts 447–975, deleted for exon 2 (nts 685–875); ERAE3: nts 707–1323, deleted for exon 3 (nts 876–992); ERAE4: nts 891–1413, deleted for exon 4 (nts 993–1328); ERAE5: nts 1175–1536, deleted for exon 5 (nts 1329–1467); ERAE7: nts 1499–2051, deleted for exon 7 (nts 1602–1785); ERAE4,7: nts 966–2091, deleted for exon 4 (nts 993–1328) and for exon 7 (nts 1602–1785). The nt positions are according to Green et al. (15).

**Construction of ERAE5 Expression Vector**. A fragment of the ER cDNA containing the exon 5 deletion was RT-PCR amplified from MCF-7 cells and cloned into the pCR-II vector (Invitrogen, San Diego, CA). The cloned cDNA was found free of mutations using dyeoxy sequencing. The ERAE5 expression vector was constructed from the eukaryotic expression vector, pSGS-HEGO, containing the wt ER cDNA (16), by replacing the wt exon 5 and flanking sequences with the cloned exon 5-deleted cDNA fragment using a flanking HindIII and a BglII site.

**Results**

All of the antiestrogen-resistant breast cancer cell lines (MCF-7/TAMR4, MCF-7/TAMR5, MCF-7/TAMR6, MCF-7/TAMR8, MCF-7/TAMR14, MCF-7/TAMR4,5, MCF-7/TAMR5, MCF-7/TAMR6, MCF-7/TAMR8, MCF-7/TAMR7, MCF-7/TAMR18-7, MCF-7/TAMR18-6, MCF-7/TAMR7) expressed ER as determined by the dextran-coated charcoal ligand-binding assay on cytosol preparations from cells grown in medium with 1% FCS. Generally, the levels of ER were lower than in the parental MCF-7 cells (616 fmol/mg cytosol protein) and varied between 67 and 398 fmol/mg cytosol protein (Table 1). Northern blot analysis and Western blot analysis on total cell lysates revealed some discrepancies between mRNA steady-state level and expressed protein suggesting an impaired relationship between mRNA and protein levels (Fig. 1, A and B). For all of the cell lines, the Western blot analyses disclosed only one distinct band located like wt ER at Mr 65,000 (Fig. 1B). To verify that the 1D5 mouse monoclonal antibody also recognizes variant ER protein, we have transfected an ER-negative human breast epithelial cell line HMT-3522 (13) with an ERAE5 expression vector, and Fig. 1C shows that the 1D5 antibody recognizes a protein with the expected molecular weight for the ERΔE5 protein of approximately 41,000. This protein is not present in the untransfected HMT-3522 cells. Since none of the resistant cell lines had lost the ER, we looked for differential expression of ER mRNA splice variants between the parental MCF-7 cell line and the resistant cells. An altered expression of specific ER splice variants could indicate an involvement in antiestrogen resistance. By using fragments of cloned ER cDNAs encoding different ER mRNA splice variants, six different RNase protection assays were set up to determine the ERAE2, ERAE3, ERAE4, ERAE5, ERAE7, and ERAE4,7 mRNA expression (Fig. 2, Lanes A-F). The ratio between variant ER and wt ER mRNA expression in the different cell lines was determined by PhosphorImaging analysis of the RNase protection assays (Fig. 3). The relative expression of ERAE3, ERAE4, and ERAE5 mRNAs was higher (0.24–1.11) than the expression of ERAE2, ERAE7, and ERAE4,7 (0.016–0.071). No significant differences in the relative expression of ERAE2, ERAE3, ERAE4, and ERAE7 could be observed between MCF-7 cells and resistant cell lines. However, the ERAE5 wt ER ratio was higher in MCF-7 cells compared to the resistant cells, and within the resistant cell lines the ERAE5:wt ER ratio was slightly higher in the tamoxifen-resistant cell lines than in the ICI 164,384- and ICI 182,780-resistant cell lines. Regarding the ERAE4,7 mRNA expression, the variant ER:wt ER ratio in parental MCF-7 cells was almost equal to that of the ICI-resistant cells, whereas a higher level was seen in the tamoxifen-resistant cell lines. Overall, no major differences in the variant ER:wt ER ratio between the parental MCF-7 cell line and the resistant cell lines were found.

**Discussion**

It has been suggested that alterations in the ER structure and function could be involved in development of antiestrogen resistance in breast cancer (3). By using a model system of 10 antiestrogen-resistant cell lines derived from the ER-positive human breast cancer cell line MCF-7, we have investigated the role of alternative splicing of ER mRNA in antiestrogen resistance. All of the resistant cell lines expressed ER as determined by Northern blotting, Western blotting,
The ER gene appears to be particularly prone to generate mRNA splice variants (5), and a differential expression of specific ER splice variants between the parental cells and the antiestrogen-resistant cell lines would strongly indicate an involvement in antiestrogen resistance. However, the RNase protection assay of the ERL\(\Delta E2\), ERL\(\Delta E3\), ERL\(\Delta E4\), ERL\(\Delta E5\), ERL\(\Delta E7\), and ERL\(\Delta E4,7\) revealed no major differences between the resistant cell lines and MCF-7 cells. The first ER splice variant identified was ERL\(\Delta E5\) which was found in an ER-negative, progesterone receptor-positive breast tumor by Fuqua et al. (8). The ERL\(\Delta E5\) mRNA-encoded protein is truncated in the beginning of the hormone-binding domain and exhibits a constitutive transactivation of reporter genes in yeast and of endogenous genes in ERL\(\Delta E5\)-transfected MCF-7 cells (8, 10). Moreover, MCF-7 cells stably transfected with ERL\(\Delta E5\) cDNA and expressing the variant \(M\), 40,000 protein at levels equivalent to or slightly less than wild-type ER were tamoxifen resistant but not resistant to the pure antiestrogen ICI 164,384 (10). We find that the ERL\(\Delta E5\) variant mRNA is expressed at levels equal to the wt ER mRNA in both the antiestrogen-sensitive parental MCF-7 cell line and the antiestrogen-resistant cell lines. This high expression level of the ERL\(\Delta E5\) mRNA variant in the MCF-7 cells that are sensitive to treatment with tamoxifen, ICI 164,384, or ICI 182,780 indicates that an endogenously expressed corresponding variant protein is not able to exhibit a significant estrogen/antiestrogen-independent transcriptional activity. A similar high expression level of the ERL\(\Delta E5\) mRNA has been reported in MCF-7 cells and ZR-75-1 cells from another laboratory (17), again demonstrating that significant levels of ERL\(\Delta E5\) mRNA expression are present in antiestrogen-sensitive human breast cancer cell lines in tissue culture. Furthermore, our results with no difference in the ERL\(\Delta E5\)-wt ER ratio in sensitive and resistant cell lines suggest that the ERL\(\Delta E5\) variant mRNA cannot explain tamoxifen nor ICI 164,384 or ICI 182,780 resistance in our model system. This is in accordance with the results published by Daffada et al. (18), who, in a series of tamoxifen-resistant human

and ligand-binding assay. Loss of ER expression is therefore not the underlying cause of the antiestrogen-resistant phenotype in these cell lines and this is in accordance with the clinical observation that the majority of patients with acquired tamoxifen resistance maintain ER expression (4). However, reduced expression of ER is often observed in breast tumors from patients with acquired tamoxifen resistance (4), and all of our cell lines, which have developed acquired antiestrogen resistance in cell culture, have a reduced ER protein level according to the ligand-binding assay. In some of our resistant cell lines, a high level of ER mRNA expression was not reflected at the protein level and vice versa. These differences could, for example, be due to expression of ER splice variants without hormone binding.

Fig. 1. Expression of ER in MCF-7 cells and in the antiestrogen-resistant sublines. A, Northern blot, 2 \(\mu\)g of poly(A)\(^+\) RNA from each cell line were analyzed with Northern hybridization with a specific probe against ER. The Northern blot was rehybridized with a rat glyceraldehyde-3-phosphate dehydrogenase probe as control for differences in poly(A)\(^+\) RNA loading. B, Western blot (alkaline phosphatase staining), total cell extracts (containing about 40 \(\mu\)g of protein) from each cell line were separated by SDS-PAGE, electroblotted, and probed with a monoclonal mouse anti-human ER antibody, 1D5. C, poly(A)\(^+\) RNA loading. B, Western blot (alkaline phosphatase staining), total cell extracts a rat glyceraldehyde-3-phosphate dehydrogenase probe as control for differences in hybridization with a specific probe against ER. The Northern blot was rehybridized with Northern blot, 2 \(\mu\)g of poly(A)\(^+\) RNA from each cell line were analyzed with Northern blot, 2 \(\mu\)g of poly(A)\(^+\) RNA from each cell line were separated by SDS-PAGE, electroblotted, and probed with a monoclonal mouse anti-human ER antibody, 1D5. C, poly(A)\(^+\) RNA loading.

Fig. 2. RNase protection assay of ER mRNA splice variant expression in MCF-7 cells. In each assay, 50 \(\mu\)g of RNA from the MCF-7 cell line were analyzed by RNase protection assay using \(^{32}\)P-labeled antisense probes containing ER RNA deleted for either exon 2 (\(\Delta E2\)), exon 3 (\(\Delta E3\)), exon 4 (\(\Delta E4\)), exon 5 (\(\Delta E5\)), exon 7 (\(\Delta E7\)) or exons 4 + 7 (\(\Delta E4,7\)). The marked bands represent the protected fragments from either exon deleted or wt RNA used for quantification by PhosphorImaging. Lane A, \(\Delta E2\): 338 nt, wt: 100 nt; Lane B, \(\Delta E3\): 500 nt, wt: 331 nt; Lane C, \(\Delta E4\): 187 nt, wt: 102 nt; Lane D, \(\Delta E5\): 223 nt, wt: 154 nt; Lane E, \(\Delta E7\): 369 nt, wt: 266 nt; Lane F, \(\Delta E4,7\): 606 nt, wt: 273 nt. The major unmarked band (238 nt) in Lane A represents the other protected wt fragment generated from the antisense probe, and the unmarked bands in Lane F represent the ERL\(\Delta E7\) mRNA (579 nt) and another protected wt fragment (306 nt) generated from the same antisense probe.
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breast tumors versus a control group, did not find any differences between the ER@E5:wt ER mRNA ratio using RT-PCR.

Regarding the dominant negative ER variants (ER@E2, ER@E3, and ER@E7), no significant differences in the variant ER:wt ER ratio were observed between the antiestrogen-resistant cells and MCF-7 cells. Therefore, expression of these variants does not appear to be important for selection of our antiestrogen-resistant cell lines. This is in contrast to the suggestion that the expression of the ER@E7 variant may render a tumor unresponsive to antiestrogens (11). The low ER@E7:wt ER ratio (mean, 0.023) determined in this study also excludes that ER@E7 is involved in antiestrogen resistance of these cell lines. However, the relative ER@E4,7 mRNA expression fluctuated, with a tendency of higher levels in the tamoxifen-resistant cell lines. Since the biological activity of the ER@E4,7-encoded protein still is unknown (9), the implication of this observation is unclear.

No protein bands corresponding to any of the ER variant proteins were observed with Western blotting, although the 1D5 monoclonal antibody recognizes an epitope mapped in the N-terminal part (A/B domain) of the ER which is present in all of the variant ER proteins. The 1D5 antibody recognizes an epitope mapped in the N-terminal part (A/B domain) of the ER which is present in all of the variant ER proteins. Therefore, the observed lack of expression of significant levels of variant ER protein suggests that variant ER mRNAs are not translated or that the corresponding proteins may have a decreased stability compared to wt ER. The latter hypothesis is now being investigated in our laboratory. Proteins translated from ER mRNA splice variants will have a tertiary structure different from that of wt ER. It has been shown that the turnover rate of the ER protein depends dramatically on the tertiary structure of the protein since estradiol-bound ERs are more stable than free ERs, and ICI 164,384-bound ERs are very unstable (19). Despite the fact that many groups have identified ER mRNA splice variants in breast cancer cells, expression of a naturally occurring corresponding protein has only been demonstrated in the human breast cancer cell line BT-20 (20). Unfortunately, this study included no further investigation of the biological function of the endogenously expressed variant protein. Therefore, it is still unproven whether the endogenously expressed ER variant splice variants have any biological significance or represent by-products made by the splicing machinery. In some subsets of breast tumors which apparently are ER negative, but still express the estrogen-inducible progesterone receptor and pS2 genes, an overexpression of the ER@E5 mRNA relative to wt ER mRNA was demonstrated (18), suggesting the presence of a biologically active ER@E5 protein. ER splice variants have also been found in human brain and liver, and a tissue-specific alternative splicing was observed in rats which expressed a significantly higher level of the ER@E4 mRNA in hypothalamus than in uterus (7, 21, 22), indicating a physiological function of this ER splice variant. However, the finding that the ER@E4 protein obtained by in vitro translation was unable to bind to a synthetic estrogen-responsive element or to bind estradiol (7) suggests that this protein is nonfunctional. Furthermore, a recent publication describes that although the ER@E4 mRNA variant was expressed at levels close to wt ER mRNA in MCF-7 cells, no variant protein could be detected by immunoprecipitation supporting the hypothesis that variant mRNAs are not translated (23). The differential expression of the mRNA splice variant may therefore be without biological significance.

This study shows that differential expression of ER mRNA splice variants is not involved in resistance toward the antiestrogens: tamoxifen, ICI 164,384, and ICI 182,780 using a model system of 10 ER-positive antiestrogen-resistant breast cancer cell lines established from the human breast cancer cell line MCF-7. The investigation also demonstrates that the expression levels of the corresponding variant proteins are extremely low, implying that, if expressed, the variant ER proteins may have a high turnover rate.

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