Correlation of mRNA for oestrogen receptor beta splice variants ER\(\beta_1\), ER\(\beta_2\)/ER\(\beta_{cx}\) and ER\(\beta_5\) with outcome in endocrine-treated breast cancer

M P A Davies, P A O’Neill, H Innes, D R Sibson, W Prime\(^1\), C Holcombe\(^2\) and C S Foster\(^3\)

Clatterbridge Cancer Research Trust, J K Douglas Laboratories, Clatterbridge Hospital, Bebington, Wirral CH63 4JY, UK
\(^1\)Cancer Tissue Bank Research Centre, University of Liverpool, Liverpool L69 3GA, UK
\(^2\)Breast Services, Linda McCartney Centre, Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP, UK
\(^3\)Department of Cellular and Molecular Pathology, University of Liverpool, Liverpool L69 3GA, UK

(Requests for offprints should be addressed to M P A Davies; Email: Mike.Davies@ccrt.nhs.uk)

Abstract

This study has been performed to test the hypothesis that different oestrogen receptor beta (ER\(\beta\)) splice variants may be important determinants of clinical parameters, including outcome, in post-menopausal women with breast cancer receiving adjuvant endocrine treatment but no chemotherapy. Splice variants ER\(\beta_1\), ER\(\beta_2\) and ER\(\beta_5\) have been analysed by semi-quantitative RT-PCR in a cohort of 105 patients with primary breast cancer. Clinical correlates included age, grade, size, nodal status, ER\(\alpha\), progesterone receptor, Ki67, relapse-free survival (RFS) and overall survival (OS). Seventy per cent of cases were ER\(\beta_1\) positive, 69% ER\(\beta_2\) positive and 70% ER\(\beta_5\) positive. Within the cohort, 47% were positive for all three variants while 10% were negative for all three. ER\(\beta_1\) exhibited no discernible relationship with disease outcome. ER\(\beta_2\) and ER\(\beta_5\) expression was significantly associated with better RFS (\(P<0.005\)), and ER\(\beta_2\) with better OS (\(P=0.0002\)). In multivariate analysis, ER\(\beta_2\) (\(P=0.006\)), nodal status and the level of Ki67 expression were independent predictors for RFS while ER\(\beta_2\) (\(P=0.0008\)) and Ki67 status were independent predictors for OS. In the ER\(\alpha\)-positive cases, or in the subset of those receiving adjuvant tamoxifen, ER\(\beta_2\) was significantly associated with good RFS (\(P<0.0005\)) and was the only independent marker of OS. We conclude that precise identification of splice variants of ER\(\beta\) are more important assessors than is ER\(\beta_1\) alone of the biological status of individual breast cancers, and hence in predicting their response to endocrine therapy.

Journal of Molecular Endocrinology (2004) 33, 773–782

Introduction

Oestrogen receptor alpha (ER\(\alpha\)), as a measure of steroid hormone receptor status, is a currently accepted prognostic marker used to predict the response of an individual breast cancer to hormone therapy (Pertschuk & Axiotis 1999). However, it is known that up to 40% of breast tumours with positive ER\(\alpha\) status do not respond to endocrine therapy (Locker 1998). Reasons for this lack of response are poorly understood. However, the relative expression of ER\(\beta\) may be an important modulator of oestrogenic hormone manipulation. A variety of studies have employed RT-PCR or in situ hybridisation to analyse the mammary expression of ER\(\beta\) mRNA (Enmark et al. 1997, Vladusic et al. 1998, Dotzlaw et al. 1999, Leygue et al. 1999, Speirs et al. 1999a, Iwao et al. 2000a,b, Kurebayashi et al. 2000, Bieche et al. 2001, Cullen et al. 2001, de Cremoux et al. 2002, Omoto et al. 2002, Tong et al. 2002). However, these studies are conflicting, reflecting either differences in case selection or limitations of the detection techniques. Nevertheless, it has been suggested that the level of ER\(\beta\) expression has prognostic significance in breast cancer (Speirs et al. 1999a, Jarvinen et al. 2000, Knowlden et al. 2000, Speirs & Kerin 2000, Mann et al. 2001, Fuqua et al. 2003). ER\(\beta\) has been
associated with markers of low biological aggressiveness such as negative axillary nodal status (Jarvinen et al. 2000), low morphological grade (Jarvinen et al. 2000, Speirs & Kerin 2000) and improved survival (Mann et al. 2001, Omoto et al. 2001). The ratio of ERα:ERβ has been shown to predict progression to neoplasia and malignancy in the breast (Shaaban et al. 2003a) and the ratio ERα:ERβ mRNA changes during mammary carcinogenesis (Leygue et al. 1998) together with relative amounts of different ERβ variants (Leygue et al. 1999, Omoto et al. 2002).

ERβ1, considered to be the full-length or wild-type ERβ, is able to form heterodimers with ERα (Cowley et al. 1997) and in this may contribute to differences in ER-dependent gene expression. Some ERβ variants resulting from differential splicing are expressed in a cell-type and tissue-type specific manner (Saunders 1998), are expressed as proteins (Saunders et al. 2002) and can interact with both ERβ1 and ERα (Moore et al. 1998). Hence these splice variants may be considered as functionally distinct isoforms of ERβ. For example, ERβ2 (also known as ERβcx) is a variant that utilises an alternative exon 8 (Moore et al. 1998), a change that results in relatively poor binding to oestradiol and a dominant negative effect on ERα function (Ogawa et al. 1998). The putative ERβ5 protein sequence diverges from ERβ1 at the same point as ERβ2 (amino acid 469) by alternative splicing (Moore et al. 1998). As in ERβ2 the C-terminal of this ERβ5 protein is truncated, resulting in loss of the AF-2 core domain and differences in ligand binding (Peng et al. 2003). Such effects may modulate the action of therapies directed against ERα, such as the endocrine treatments used in breast cancer. Expression of ERβ variants has been shown to vary between different tumours and cell lines (Leygue et al. 1999, Iwao et al. 2000a, b, Omoto et al. 2002, Tong et al. 2002). However, expression of individual splice variants is not known with respect to particular clinical parameters, including treatment or outcome, principally because many mRNA studies utilised RT-PCR primers designed against sequences common to all splice variants (Leygue et al. 1998, Dotzlaw et al. 1999, Speirs et al. 1999a, b, Iwao et al. 2000a, b, Knowlden et al. 2000, Kurebayashi et al. 2000, Bieche et al. 2001, Cullen et al. 2001, de Cremoux et al. 2002) and variant-specific antibodies have not been widely available. Levels of mRNA for ERβ2 and ERβ5 are recognised to be higher than those for the ERβ1 variant (Leygue et al. 1999, Iwao et al. 2000a, b), hence many of the non-specific RT-PCR studies to date may reflect expression of these variants. It is therefore important to determine the individual relationships between ERβ variants and clinical and pathological factors, so as to understand their relative contributions to breast disease. To this end, we have analysed a retrospective cohort of primary breast cancers, representing a series of cases receiving endocrine therapy but not chemotherapy. Typically such clinically defined cohorts are heterogeneous, allowing associations with other pathological factors to be considered, but requiring that multivariate statistical analysis be applied when considering outcome data.

The literature contains detailed consensual reference to ERβ splice variants ERβ1, ERβ2/ERβcx and ERβ5, including the agreed primer sequences for PCR. Although not exhaustive, analysis of these three splice variants is sufficient to identify those splice variants commonly expressed by normal breast and breast carcinomas. Therefore, the purpose of the present study was to test the hypothesis that ERβ splice variant expression in breast cancer is related to patient outcome data when patients receive only endocrine therapy in the absence of chemotherapy.

Materials and methods

Subjects

Patients undergoing treatment, at the Royal Liverpool University Hospital, for invasive breast cancer during the period 1993–1999 were identified by the Cancer Tissue Bank Research Centre (CTBRC). Tissues were collected by CTBRC with full consent and appropriate ethical committee approval and RNA was prepared by standard methods. Clinical follow-up data were recorded by retrospective case-note review, but were unavailable for some variables in a limited number of cases. All cases were subjected to histopathological review according to the guidelines of the UK NHSBSP (National Coordinating Group for Breast Screening Pathology 1997), by three investigators (P A O’N, C S F and Prof. J P Sloane). Histopathological sections of tissues adjacent to that used for...
RNA preparation were assessed and cases comprising at least 50% tumour cells were included (90% of such cases contained 75% or more tumour cells). Cases were only included if the quality of the prepared cDNA was considered adequate (see below). Consequent upon these caveats, the study population (Table 1) comprised 105 post-menopausal women treated with surgery, either with or without radiation treatment and no chemotherapy. The median age was 68 years (range 48–88). Most cases were invasive ductal carcinomas of no special type. Other invasive carcinoma types included lobular (n = 10), mucinous (n = 4), medullary (n = 1), papillary (n = 1), tubular (n = 1), metaplastic (n = 1) and mixed mucinous/papillary (n = 1).

All patients received adjuvant hormone therapy but no chemotherapy. For 89 of the cases, endocrine therapy comprised only adjuvant tamoxifen; adjuvant treatment for seven further patients was received as part of the Arimidexor Tamoxifen Alone or in Combination (ATAC) trial and could not be determined. Only nine patients received primary tamoxifen therapy prior to surgery, six of these patients then received adjuvant tamoxifen and three received adjuvant anastrozole. Hence surgical specimens for all but these nine cases were collected prior to commencement of endocrine therapy. Since routine histopathology did not include steroid receptor analysis until 1996, some cases were subsequently found to be ER\textsuperscript{α} negative.

Ethical approval for this study was obtained from all relevant bodies.

**Immunostaining**

Immunostaining data for ER\textsuperscript{α}, progesterone receptor (PgR) and Ki\textsuperscript{67} were made available by the CTBRC. Briefly, a mouse anti-human ER\textsuperscript{α} monoclonal antibody (Clone 1D5; Dako Ltd, Ely, UK) and a polyclonal rabbit antihuman PgR antibody (Novocastra, Newcastle upon Tyne, UK) were used. Ki\textsuperscript{67} was detected using a monoclonal antibody (Clone 8H11; Dako Ltd).

**Table 1** Histological, clinical and molecular characteristics of 105 breast cancer cases receiving adjuvant endocrine treatment but no chemotherapy with outcome data: RFS and OS. Log-rank P values are from Kaplan–Meier analysis, hazard ratios (with 95% confidence intervals (CIs)) and multivariate P values are from Cox’s regression analysis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group</th>
<th>n</th>
<th>Log-rank P value</th>
<th>Hazard ratio (95% CI)</th>
<th>Multivariate P value</th>
<th>Poor outcome associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td>Invasive ductal</td>
<td>86</td>
<td>0·035 OS</td>
<td>0·35 (0·13–0·97) OS</td>
<td>NS</td>
<td>Invasive ductal</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>19</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td>Wide local excision</td>
<td>63</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mastectomy</td>
<td>42</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Radiotherapy</strong></td>
<td>No</td>
<td>55</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>49</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td>I</td>
<td>18</td>
<td>0·005 RFS</td>
<td>2·0 (1·3–3·0) RFS</td>
<td>NS</td>
<td>Higher grade</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>42</td>
<td>0·018 OS</td>
<td>1·8 (1·2–2·8) OS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>45</td>
<td>—</td>
<td>—</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>Up to 2 cm</td>
<td>38</td>
<td>0·038 OS</td>
<td>1·9 (1·0–3·6) OS</td>
<td>NS</td>
<td>Size &gt; 2 cm</td>
</tr>
<tr>
<td></td>
<td>2–5 cm</td>
<td>60</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5 cm and up</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td>Negative</td>
<td>47</td>
<td>0·0002 RFS</td>
<td>3·4 (1·7–6·8) RFS</td>
<td>0·003 RFS</td>
<td>Node positive</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>42</td>
<td>0·011 OS</td>
<td>2·3 (1·2–4·5) OS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td><strong>ER\textsuperscript{α}</strong></td>
<td>Negative</td>
<td>37</td>
<td>0·0002 RFS</td>
<td>0·35 (0·20–0·63) RFS</td>
<td>NS</td>
<td>ER\textsuperscript{α} negative</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>65</td>
<td>0·008 OS</td>
<td>0·46 (0·26–0·83) OS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td><strong>PgR status</strong></td>
<td>Negative</td>
<td>49</td>
<td>0·022 RFS</td>
<td>0·49 (0·26–0·91) RFS</td>
<td>NS</td>
<td>PgR negative</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>42</td>
<td>0·043 OS</td>
<td>0·53 (0·28–0·99) OS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td><strong>Ki\textsuperscript{67} status</strong></td>
<td>Low</td>
<td>44</td>
<td>0·0002 RFS</td>
<td>3·3 (1·7–6·2) RFS</td>
<td>0·0004 RFS</td>
<td>High Ki\textsuperscript{67}</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>45</td>
<td>0·002 OS</td>
<td>2·7 (1·4–5·3) OS</td>
<td>0·003 OS</td>
<td>—</td>
</tr>
<tr>
<td><strong>ER\textsuperscript{β1} RT-PCR</strong></td>
<td>Negative</td>
<td>32</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>73</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>ER\textsuperscript{β2} RT-PCR</strong></td>
<td>Negative</td>
<td>33</td>
<td>&lt;0·0001 RFS</td>
<td>0·31 (0·17–0·56) RFS</td>
<td>0·006 RFS</td>
<td>ER\textsuperscript{β2} negative</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>72</td>
<td>0·0002 OS</td>
<td>0·35 (0·20–0·62) OS</td>
<td>0·0008 OS</td>
<td>—</td>
</tr>
<tr>
<td><strong>ER\textsuperscript{β5} RT-PCR</strong></td>
<td>Negative</td>
<td>31</td>
<td>0·005 RFS</td>
<td>0·45 (0·25–0·79) RFS NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>74</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Cambridge, UK) was used for the immunohistochemical detection of ERα. PgR status was assessed using a mouse monoclonal anti-PgR antibody (Clone 1A6; Novacastra, Newcastle upon Tyne, UK) and Ki67 status was assessed using polyclonal rabbit anti-human Ki67 antibody (Ki67p; Novacastra). Formalin-fixed and paraffin wax-embedded sections were immunostained by methods identical to those previously described (Shaaban et al. 2002, 2003b). To maximise consistency of scoring, only nuclei having moderate or strong staining were regarded as positive, independently of cytoplasmic staining. The percentage of nuclear-stained epithelial cells was calculated as a proportion of the total number of epithelial cells present. A 10% cut-off was the conventional criterion to define ERα staining, as previously described (Shaaban et al. 2002) and PgR staining. Staining for Ki67 was confirmed as high if >20% cells stained (using a median cut-off from a larger cohort). Immunostaining data were unavailable for a limited number of cases.

RT-PCR analysis

Total RNA was provided by the CTBRC. Following a DNAaseI digestion step (Invitrogen), reverse transcription (RT) was performed in duplicate on 0.5 µg RNA according to the manufacturer’s instructions. RT reactions incorporated Superscript II Reverse Transcriptase (Invitrogen), 0.5 µg Oligo (dT)17 and 0.5 µl Prime Recombinant Ribonuclease Inhibitor (Eppendorf, Cambridge, UK). Parallel reactions were performed in which the RT enzyme was omitted and these acted as controls for genomic DNA contamination.

PCR reactions (20 µl) were performed in duplicate in 96-well plates, each using 2 µl of a 1/20 dilution of cDNA (equivalent to cDNA from approximately 2.5 ng total RNA). PCR reactions included 0.2 mM dNTPs, 0.5 U HotstarTaq DNA polymerase (Qiagen) and 1xPCR Buffer (containing 1.5 mM MgCl2; Qiagen). Primer and MgCl2 concentrations varied as per Table 2. The oligonucleotide primers (Table 2) for ERβ1 and ERβ2/5 RT-PCR were taken from Moore et al. (1998). β-Actin and hypoxanthine ribosyltransferase (HPRT) were used as control genes for determination of RNA integrity and RT efficiency and positive controls (testis and MCF7 cells) were

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>PCR</th>
<th>Cycling conditions</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERβ1</td>
<td></td>
<td>36 cycles of 94°C – 30 s, 60°C – 60 s</td>
<td>477</td>
</tr>
<tr>
<td>ERβ2/5</td>
<td></td>
<td>40 cycles of 94°C – 30 s, 64°C – 30 s, 72°C – 40 s</td>
<td>291</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td>35 cycles of 94°C – 15 s, 58°C – 15 s, 72°C – 30 s</td>
<td>370</td>
</tr>
<tr>
<td>HPRT</td>
<td></td>
<td>36 cycles of 94°C – 30 s, 60°C – 60 s</td>
<td>367</td>
</tr>
</tbody>
</table>

Table 2 Primer sequences, conditions and product sizes for RT-PCR
included. Each PCR reaction was limited in cycle number and/or primer concentration, so as to avoid the plateau phase of the reaction, but otherwise cycle number was maximised to give maximum sensitivity for each gene product. PCR was performed on Perkin Elmer 9600 thermal cyclers and all reactions (Table 2) included a 94°C 13 min activation and a 72°C 3 min final extension.

PCR products were separated by electrophoresis on gels containing 2·5% Seakem Agarose (Flowgen, Ashby de la Zouch, Leics, UK) and 40 mM Tris-acetate, 1 mM EDTA, pH 7·6 buffer. Molecular mass markers (PhiX174/HaeIII; Abgene, Epson, Surrey, UK) were included on each gel and DNA was visualised by inclusion of 0·5 µg/ml ethidium bromide, scanning with a Molecular Dynamics FluorimagertSI and analysis with ImageQuant v. 4·1 (Amersham Biosciences). Control genes were scored as weak or strong positive and individual RT reactions excluded from ERα/afii9826 assessment if either was negative, or if both were weak. Cases were considered positive for ERβ RT-PCR if any band was scored as positive by two independent investigators.

Identity of PCR products was confirmed by direct sequencing using DYEnamicET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Biosciences) and analysed on a MegaBACE1000 (Amersham Biosciences). Alternatively PCR products were cloned using TOPO-TA cloning (Invitrogen) prior to sequence analysis.

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### Statistical analysis

All statistical analyses were performed using the SPSS package (Windows, v. 11). To compare immunostaining percentage values in different groups, data were analysed by the non-parametric, two-sided Mann–Whitney test. Association between categorical data was assessed by the Chi-squared test, with contingency coefficients (Cont. Coeff.) calculated to assess the strength of different associations. Survival curves were generated using the Kaplan–Meier method for censored data and compared using the log-rank test. Cox’s regression models were used to determine hazard ratios and for multivariate survival analysis.

### Results

#### RT-PCR

The identities of representative RT-PCR products for each gene were confirmed by sequence analysis. No evidence of artefactual PCR products due to genomic DNA contamination was seen. The use of control genes β-actin and HPRT identified 105 cases in which cDNA was considered of appropriate quantity and integrity for further analysis. RT-PCR analysis of these 105 cases categorised 70% of cases ERβ1 positive, 69% ERβ2 positive and 70% ERβ5 positive (Table 1) and these percentages were similar in the cohort of 56 ERα-positive, adjuvant tamoxifen-only cases. A wide variety of expression...
patterns was seen (Fig. 1). Nearly half of the cases (47%) expressed all three splice variants and almost three-quarters expressed at least two variants (72%). Strong relationships were identified between expression of all three mRNAs. However, based on Cont. Coeff. values ERβ2 and ERβ5 were more closely associated (Cont. Coeff. 0·45, \( P=2 \times 10^{-7} \)) than either variant was to ERβ1 (ERβ2 Cont. Coeff. 0·24, \( P=0·01 \); ERβ5 Cont. Coeff. 0·22, \( P=0·02 \)).

**Relationship of ERβ RT-PCR with clinical and immunohistological parameters**

The relationships between each of the ERβ RT-PCR scores (ERβ1, ERβ2, ERβ5) and the clinical and immunohistological parameters, presented in Table 1 were examined by the Chi-squared test. RT-PCR data for ERβ showed no association with any treatment groups, histology or tumour size. There was no association of any ERβ RT-PCR with ERα, PgR or Ki67 staining and this was confirmed using a Mann–Whitney test for differences in staining strength (per cent positive cells) between RT-PCR-positive and -negative groups.

Some association was seen between ERβ2 and tumour grade; ERβ2 was positive in 89% of grade I, 52% of grade II and 76% of grade III tumours and RT-PCR-positive scores were significantly associated with grade I (Chi-squared, \( P=0·007 \)) or grade III (Chi-squared, \( P=0·024 \)) tumours as opposed to grade II tumours.

**Relationship of ERβ RT-PCR with disease outcome**

To examine the possible effect of ERβ status in relation to endocrine treatment, outcome data have been restricted to those 105 women receiving endocrine treatment either with or without neoadjuvant tamoxifen treatment, but with no primary or adjuvant chemotherapy (Table 1). The median time to relapse for this cohort was 75·7 months (inter-quartile range 61·2–90·3) and the median time to death was 93·8 months (inter-quartile range 68·4–119·2). Subgroup analysis within this cohort was also carried out on the 65 ERα-positive cases and the 56 ERα-negative cases receiving adjuvant tamoxifen only (Table 3).

In the 105 case cohort, high grade, positive-nodal status, larger size, ERα-negative status, PgR-negative status and high Ki67 status were all associated with worse prognosis (Table 1). In addition, histopathological features other than invasive ductal carcinoma, were associated with a moderately better overall survival (OS) (Table 1). The choice of surgery, radiotherapy or endocrine treatment was of no significance for patient outcome. ERβ2 was associated with significantly better relapse-free survival (RFS) (Fig. 2) and OS...
Table 1), while ERβ5 was also associated with better RFS. ERβ1 was not significantly associated with any measure of survival. For ERβ2-negative patients, the median time to relapse was 38.1 months (inter-quartile range 6.6–69.5) and the median time to death was 51.2 months (inter-

quartile range 32.6–69.8). In contrast, median time to either measure of outcome was not reached for ERβ2-positive cases. In multivariate analysis, nodal status (P=0.003), Ki67 (P=0.0004) and ERβ2 (P=0.006) were independent markers of RFS and only Ki67 (P=0.003) and ERβ2 (P=0.0008) were independent markers of OS.

With respect to the ERα-positive cases, irrespective of treatment (n=65), the only significant markers for RFS are nodal status (log-rank P=0.011), Ki67 (P=0.039), ERβ2 (P<0.0001) and ERβ5 (P=0.002). Of these, only nodal status (P=0.011) and Ki67 (P=0.028) are independently significant for RFS in multivariate analysis. For OS, grade (P=0.033), ERβ2 (P=0.0001) and ERβ5 (P=0.042) are significant by log-rank, but only ERβ2 is independent in multivariate analysis (P=0.0002). The same markers were significant even in the smallest sub-group analysed (the 56 ERα-positive, tamoxifen-only cases). As shown in Table 3, ERβ variants, grade, nodal status and Ki67 were all significantly related to RFS or OS, with ERβ2 being the best predictor of OS.

Discussion

The study reported herein strongly supports the initial hypothesis that different ERβ splice variants are important determinants, and hence predictors of clinical parameters including outcome, in post-menopausal women with breast cancer treated only by adjuvant hormonal manipulation. The study also confirms that individual primary breast cancers are heterogeneous with respect to the spectrum of ERβ splice variants expressed. Thus, a single breast cancer might simultaneously express different isoforms of the receptor that are potentially agonist, antagonist or unresponsive with respect to interaction with a potential inhibitor (e.g. tamoxifen). Of the three splice variants examined, expression of ERβ2 was clearly beneficial with respect to RFS (Fig. 2B). The advantage conferred by ERβ5 was less pronounced (Fig. 2C). These two variants are likely to contribute to the association of ERβ with better outcome in RT-PCR studies using primers that amplify total ERβ. Conversely, expression of ERβ1 mRNA apparently confers no statistically significant advantage, with respect to RFS or OS.

Figure 2 Kaplan–Meier plots of RFS for subgroups defined by RT-PCR status for ERβ1 (A), ERβ2 (B) and ERβ5 (C). Dotted lines are negative cases and unbroken lines positive cases, crosses represent censored data, P values are given for log-rank tests.
In common with most previous observations, the current data reveal few associations between ERβ mRNAs and either clinical or histological features. This observation alone emphasises the complexity of the multiplicity of factors contributing to clinical behaviour and to histopathological appearances. Both grade I and III groups did have significantly higher proportions of RT-PCR-positive cases for ERβ2 when compared with grade II cases. Although not reported in other RT-PCR studies, an association of ERβ protein and high grade was identified by Myoshi et al. (2001) using an antibody that is likely to be pan-variant. Others using similarly non-specific antisera (Skliris et al. 2001) have reported associations with low-grade tumours.

Unlike ERα, which is bimodally distributed with respect to expression in breast cancers, others have reported a diverse range of ERβ mRNA levels using quantitative RT-PCR (Bieche et al. 2001) making assignment of meaningful cut-off values difficult. Use of semi-quantitative RT-PCR in the manner described here imposes an arbitrary cut-off dependent on the sensitivity of the RT-PCR reaction and the detection technique. The assignment of a meaningful and valid cut-off is complex and would rely on more quantitative approaches. Despite this caveat, the use of our cut-off has distinguished statistically significant groups of cases based upon ERβ2 or ERβ5 status. Use of a higher cut-off, e.g. by reassigning the ten weakest ERβ2 RT-PCR-positive cases as negative leads to a diminished log-rank score, but a significant relationship with outcome is maintained (P<0.005). It is possible that the lack of significant association between ERβ1 and disease outcome is due, at least in part, to the technical limitations of this approach. However, the relative number of cases positive for ERβ1, ERβ2 and ERβ5 RT-PCR product is similar, suggesting that the distribution of ERβ1 is distinct. Despite almost half of the cases expressing all three variants, expression of ERβ1 was found to be more diverse while ERβ2 and ERβ5 were more closely related.

In previous studies the relationship between ERβ and disease outcome in breast cancer patients has not been comprehensively evaluated, primarily since most early studies concentrated on the role of ERβ in the wider context of breast cancer pathology. Two studies that included outcome data reported no relationship with ERβ RT-PCR (Kurebayashi et al. 2000, Bieche et al. 2001). Both studies employed non-specific RT-PCR, which has now been demonstrated to be an inferior marker to variant-specific RT-PCR. Furthermore, case selection was different such that premenopausal patients were included with no account being taken of treatment. Speirs et al. (1999a) reported increased ERβ mRNA expression in tamoxifen-resistant breast cancer patients. Again non-specific RT-PCR was used, follow-up was limited to time receiving tamoxifen and the association was based on a small number of cases (n=17).

While demonstration that particular ERβ splice variant mRNAs are related to disease outcome is potentially important as a molecular marker, more direct involvement in the disease process would rely on protein expression. Some relationship between ERβ expression and outcome has been reported using immunohistochemistry (Mann et al. 2001, Omoto et al. 2001, 2002, Saji et al. 2002). Nevertheless, it is difficult to compare the current RT-PCR data with immunohistological appearances; although splice variants are expressed as protein, the quantitative relationship between mRNA and protein for ERβ is not clear. Omoto et al. (2001, 2002) found a weak association between ERβ1 protein and better outcome, but no significant results for other variants, albeit with smaller numbers of cases from non-selective cohorts. Saji et al. (2002) found that a greater proportion of ERβ2-negative cases responded to primary tamoxifen treatment, implying an association of ERβ2 with worse response. This observation is in contrast to our current findings, but the number of cases studied was very small (18 ERα-positive core biopsies), and it is unclear how response in this neoadjuvant setting relates to adjuvant treatment and longer-term outcome. Mann et al. (2001) studied the immunohistochemical expression of ERβ in patients treated with adjuvant tamoxifen and showed ERβ-positive patients to have a better survival when compared with ERβ-negative patients. Although the contribution of individual ERβ variants is unclear since the employed antibody was of broad specificity, if ERβ2 expression contributes to the ERβ expression measured, these findings support the current data. Recently further supportive information has emerged from a study of ERβ2 using variant-specific Western blot analysis (Palmieri et al. 2004a). Here the presence of ERβ2 was associated significantly with longer survival and with a better endocrine-treatment response. Our results indicate a stronger association...
between ERβ2 and outcome and reasons for this include differences in the patient groups studied and the method of detection for ERβ2 expression. Palmieri et al. (2004b) do not report the treatment received by the majority of patients in their study, cases included locally advanced and metastatic disease and endocrine-treatment response was evaluated in only 23 patients receiving neoadjuvant or palliative therapy. That significant associations between ERβ2 and outcome or response were found is testament to the potential importance of this marker, as the authors failed to find any association between outcome and ERα status (Palmieri et al. 2004a). Hence our results more directly address the importance of ERβ2 in the important clinical setting of adjuvant endocrine treatment, while those of Palmieri et al. suggest that ERβ2 protein expression as well as ERβ2 mRNA is related to a better outcome.

Both our findings and those of Palmieri et al. (2004a) make use of heterogeneous breast samples and it is possible that non-tumour cells contribute to the expression seen. This is potentially of some importance as it seems that, unlike ERα, ERβ and some of its splice variants can be expressed in stromal cells of human breast (Palmieri et al. 2004b). However, using Western blots and immunostaining on the same tumour samples (Palmieri et al. 2004a) these techniques result in >80% agreement and immunostaining is predominantly epithelial. Such results would suggest that in most cases results from heterogeneous samples reflect tumour cell expression of ERβ2, but that in situ techniques should be applied to address this issue in more detail.

Since it has been proposed that ERβ2 can act as a dominant negative regulator of ERα (Ogawa et al. 1998), the association of ERβ2 expression with better disease outcome is not unexpected. We suggest that, in breast cancer, inhibition of ERα function associated with high levels of ERβ2 acts alongside tamoxifen to promote a better outcome. In summary, this study has provided new evidence to support the hypothesis that ERβ splice variant expression is an important determinant of breast cancer phenotype, including response to hormonal manipulation in post-menopausal women with ERα-positive tumours. Further studies are now required to relate expression of individual ERβ isoforms to particular patterns of tumour behaviour, including response to different ER antagonists.

Acknowledgements

We wish to thank Clatterbridge Cancer Research Trust for funding this project; the Cancer Tissue Bank Research Centre for providing RNA samples and patient information; all medical professionals who contribute towards tissue bank donations and all patients who kindly donated their tissue for research use. In addition, we would like to thank Amanda Torevell and Mathew Bode (a Nuffield Science Bursary student) for technical assistance. The authors are unaware of any conflict of interest that would prejudice the impartiality of this study. The late Professor John P Sloane (University of Liverpool) was instrumental in the initiation of this work, his involvement is respectfully acknowledged.

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Received 21 July 2004
Accepted 12 August 2004

Made available online as an Accepted Preprint 24 August 2004