The favourable prognostic value of oestrogen receptor β immunohistochemical expression in breast cancer


Aims: Oestrogen receptor β (ERβ) is present in breast tumours, although its prognostic and pathophysiological roles remain to be established.

Methods: Standard immunohistochemistry with a specific monoclonal antibody was performed on paraffin wax embedded sections; 10% of strongly immunostained carcinoma cells was used as the cutoff point to classify tumours as ERβ positive. Statistical correlations were sought with clinicopathological variables (including hormone receptor status) and disease free (DFS) and overall survival (OS) in a well documented series of 181 invasive breast carcinomas. Cell proliferation was assessed immunohistochemically by topoisomerase IIa (TopoIIa) index; p53 protein accumulation and c-erbB-2 oncoprotein expression were also taken into account.

Results: ERβ immunoreactivity was detected in most specimens (71.2%); it was positively linked to ERα immunoreactivity and increased TopoIIa index, and inversely to c-erbB-2 overexpression. There were no correlations with p53 immunostaining or other clinicopathological parameters. A significant favourable impact of ERβ immunoreactivity emerged with regard to DFS and OS in both univariate and multivariate analysis; ERβ immunoreactivity retained its favourable significance with regard to DFS in the subgroups of stage I and II patients when they were examined separately. Progesterone receptor expression also had an independent favourable influence on survival, albeit with less significance. In contrast, survival was not significantly influenced by ERα status.

Conclusions: Because of the positive association between ERβ immunoreactivity and TopoIIa expression, the presence of ERβ in breast cancer cells could be considered an indication of increased proliferation. Nevertheless, ERβ immunoreactivity emerges as a valuable, independent indicator of favourable prognosis.

Estrogen is associated with the induction and growth of breast cancer. Hormone receptor status is among the factors shown to have prognostic impact and to be useful in the clinical management of patients with breast cancer. In fact, oestrogen receptor (ER) status, as assessed by immunohistochemistry, is superior to the ligand binding assay for predicting response to adjuvant endocrine treatment in breast cancer. Oestrogen mediates most of its functions through two specific intracellular receptors, ERα and ERβ, which are hormone dependent transcription regulators.

Evidence for the presence of ERβ in the normal human breast and in breast cancer has been provided from reverse transcription polymerase chain reaction studies and from in situ hybridisation of mRNA; full length ERβ protein has been identified in human breast tumours by western blotting. Human ERβ has a structure highly homologous to the previously known human ER (ERα) within its DNA and ligand binding domain, but the two receptors are encoded on different chromosomes. ERα and ERβ can form biologically functional receptor heterodimers in the tissues in which they are coexpressed. However, ERα and ERβ have distinct cellular distributions, regulate separate sets of genes, and can oppose each other’s actions on some genes. The two receptors are thought to play different roles in breast cancer, partly by mediating the transcription of various genes via different types of DNA enhancer. It has been suggested that ERβ inhibits proliferation and invasion of breast cancer cells, and its status is arguably a significant predictor of response to adjuvant hormonal treatment with tamoxifen.

The function of ERβ in breast pathobiology remains unclear, partly because most studies have focused on its mRNA rather than the protein. Large studies with multivariate analysis are needed to confirm the independent predictive value of ERβ.

“Oestrogen receptor α and β have distinct cellular distributions, regulate separate sets of genes, and can oppose each other’s actions on some genes”

Our present study was undertaken to investigate the relation between ERβ immunohistochemical expression and various clinicopathological variables, including disease free and overall patient survival in a large series of invasive breast carcinomas.

METHODS

Patient sample

Our study comprised 181 women with M0 invasive breast cancer, either ductal invasive of no special type (n = 149) or lobular invasive (n = 32). They had undergone segmental resection or mastectomy and had received no preoperative chemotherapy or endocrine treatment. Information was recorded concerning the patient’s age at surgery (mean, 61

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; TIMP-1, tissue inhibitor of metalloproteinases 1; TopoIIa, topoisomerase IIa
years; SEM, 1; range, 36–88), menopausal status, tumour size, the pathological diagnosis according to standard criteria, stage of disease (according to UICC criteria), and ERα and progesterone receptor (PR) positivity, as determined immunohistochemically (table 1). Additional data concerning the expression of several immunohistochemical markers (c-erbB-2 oncoprotein overexpression (cutoff point, 10%; antibody clone, CB 11; Biogenex, San Ramon, California, USA), p53 protein accumulation, anti-apoptotic protein Bcl-2, topoisomerase IIα immunostatus, p53 protein accumulation, anti-apoptotic protein Bcl-2, topoisomerase IIα, and tissue inhibitor of metalloproteinases 1 (TIMP-1) expression (cutoff point, 10%; antibody clone, 147–6D11; Biogenex, San Ramon, California, USA), p53 protein accumulation, anti-apoptotic protein Bcl-2, topoisomerase IIα, and tissue inhibitor of metalloproteinases 1 (TIMP-1) expression, a and ERα immunostatus

### Immunohistochemistry

For ERα immunostaining, paraffin wax embedded sections (4 μm) were dewaxed in xylene and rehydrated through graduated ethanol to water. The samples were stained in four runs after strict standardisation of the staining procedure. Endogenous peroxidase was blocked by incubation for 30 minutes with a solution of 1% hydrogen peroxide, and antigen retrieval was performed by autoclaving sections in 0.01M citrate buffer, pH 6.0, for 20 minutes at 800 W. The Serotec ERα mouse monoclonal antibody (clone PPG5/10; Raleigh, North Carolina, USA) was applied at a dilution of 1/25. As an immunogen, a synthetic peptide derived from the C-terminus of the human ERα isoform was used; this type of antibody is specific for ERα protein and does not crossreact with ERβ protein.29 Biotinylated secondary antibody and avidin–biotin kits were obtained from Vector Laboratories (Burlingame, California, USA). For substitute negative controls, the primary antibody was replaced with phosphate buffered saline alone. Immunostaining of the normal ductal epithelial cells adjacent to cancer tissues was used as a positive internal control for ERβ. Sections of tonsillar lymphoid tissue served as negative controls.

### Assessment of tissue staining

The slides were examined by two observers who were blinded to the clinicopathological features of the patients. The percentage of positively stained cells was an average after counting the intensely stained and the total number of cancer cells from at least 10 high power fields; when the evaluations differed, final agreement was reached by consensus using a two headed microscope. In parallel with Mann et al.,21 ERβ positivity was defined as nuclear staining in more than 10% of the cancer cells; this cutoff point was associated with better survival in both Mann’s study and ours. After having taken into account the mean percentage of ERβ positive cells in the immunopositive cases, we divided them as follows: cases with “low” positivity (percentages of immunoreactive cells between 10% and 40%) and cases with high positivity (percentages > 40%). When assessing ERα and both relapse free and overall survival, 10% was used as the cutoff point to discriminate between ERα negative and ERα positive cases.

### Statistical analysis

Chi square tests were used to compare the distribution of ERβ expression with all categorical variables of interest. The association of TopoIIα with ERβ expression was examined by one way analysis of variance after logarithmic transformation.
of the data. Univariate analyses were performed using the log rank test to establish the value of each variable as a separate predictor of overall survival/disease free survival. The results are illustrated by Kaplan–Meier curves. Cox’s proportional hazard model was used to identify those variables that were independent predictors of overall survival and disease free survival. Significance was set at 5%.

RESULTS
ERβ immunostaining
One hundred and twenty nine of 181 (71.2%) tumours were defined as ERβ positive; in these receptor positive specimens, the abundance of cells containing ERβ varied from 12% to 82% (median, 35%). In the ERα/ERβ phenotype (n = 94), ERβ was always expressed in greater amounts than ERα. In general, strong ERα nuclear immunoreactivity was seen both in cancer cells and tumour adjacent, morphologically normal, ductal epithelium (figs 1 and 2). The in situ component of the tumour, when noticeable, was characterised by immunostaining similar to that of the invasive compartment. ERβ (but not ERα) was also focally present in the nuclei of cells other than epithelial cells, in both “normal” and tumoral structures, particularly in fibroblasts.

ERβ relations to clinicopathological variables
ERβ immunostaining did not differ with regard to patients’ menopausal status ($\chi^2 = 0.949; p = 0.622$), tumour size ($\chi^2 = 2.136; p = 0.711$), lymph node status ($\chi^2 = 2.298; p = 0.317$), stage ($\chi^2 = 4.761; p = 0.313$), histological type ($\chi^2 = 3.530; p = 0.171$), histological grade of differentiation ($\chi^2 = 1.836; p = 0.766$), nuclear grade of malignancy ($\chi^2 = 4.564; p = 0.335$), PR status ($\chi^2 = 0.699; p = 0.705$), p53 protein accumulation ($\chi^2 = 3.779; p = 0.151$), bcl-2 immunoreactivity ($\chi^2 = 1.157; p = 0.561$), or TIMP-1 protein expression in stromal cells ($\chi^2 = 2.245; p = 0.326$). ERβ immunostaining was positively related to ERα immunostaining ($\chi^2 = 16.107; p = 0.001$; fig 3A), in addition to TopoIIα quantitative expression (TopoIIα index) ($F = 3.255; p = 0.045$; fig 3B) and TIMP-1 protein overexpression in cancer cells ($\chi^2 = 13.287; p = 0.001$; fig 3C). ERβ immunopositivity was inversely associated with overexpression of the c-erbB-2 oncoprotein ($\chi^2 = 6.513; p = 0.039$; fig 3D).

ERβ and survival
Patients were followed every three months postoperatively (mean follow up period, 76 months; SD, 26). During the follow up period, 42 patients died of their disease and 57 patients developed recurrence. Systemic recurrence was noticed in 47 patients; in nine of these last patients concomitant locoregional recurrences were observed. Only local recurrence was noticed in 10 patients. As far as relapse free survival is concerned, in univariate statistical analysis, ERβ immunopositivity appeared to exert a favourable influence both on its own (log rank, $p = 0.0002$; fig 4C) and combined with ERα immunopositivity (log rank, $p = 0.0008$; fig 4D). In Cox’s regression analysis, ERβ immunoreactivity emerged as a strong favourable prognostic indicator, along with PR positivity, although PR positivity had a lesser influence; disease free survival was also significantly influenced by lymph node status, TopoIIα index, p53 accumulation, and stage. ERα immunolabelling did not affect relapse time (table 2). With regard to overall survival, ERβ immunopositivity was again an important favourable prognostic indicator, both on its own (log rank, $p = 0.0002$; fig 4A) and in combination with ERα immunoreactivity (log rank, $p = 0.0039$; fig 4B). When each stage was separately evaluated, log rank tests showed that ERβ immunopositivity had a significant influence on relapse free survival of stage I and II patients ($p = 0.0168$ and $p = 0.0045$, respectively) and in overall survival of stage II and III patients ($p = 0.0089$ and $p = 0.0307$, respectively). In multivariate analysis, stage was the most powerful prognostic indicator; ERβ expression retained its significant favourable influence, being the second most powerful prognostic factor. TopoIIα index and PR status also emerged as important independent prognostic indicators, whereas ERα status by itself was again not found to influence overall survival (table 3). Interestingly, with regard to the combined ERβ/ERα immunophenotype, both disease free and overall survival were significantly longer in patients with ERβ/ERα double immunopositive tumours; the second most powerful immunophenotype was ERβ/ERα, ERβ+/ERα− was next and, finally, patients with tumours simultaneously negative for ERβ and ERα had the worst prognosis (fig 4B, D).

DISCUSSION
In accordance with previous immunohistochemical studies, ERβ was expressed in a considerable proportion of carcinomas, in addition to non-malignant breast ducts. The observation that both ERs are expressed in morphologically normal ductal epithelium implies that ERβ probably has a function in the normal mammary gland. Despite previously reported correlations in smaller series between ERβ immunoreactivity and premenopausal/perimenopausal status, histological low grade, lack of axillary node metastasis, and PR status, we found no associations between ERβ immunostaining and these clinicopathological factors. These
earlier studies used a polyclonal and a different monoclonal antibody to the one used in our study. In our series, using the PPG5/10 monoclonal antibody, we found a

Figure 3  Statistical associations of (A) oestrogen receptor (ER) immunopositivity and ERα expression in cancer cells (ERα negative, black bars; ERα positive, shaded bars); (B) ERβ immunopositivity and topoisomerase IIa index in cancer cells; (C) ERβ immunopositivity and tissue inhibitor of metalloproteinases 1 (TIMP-1) expression in cancer cells (<30% cells positive, black bars; >30% cells positive, shaded bars); and (D) c-erbB-2 expression in cancer cells (c-erbB-2 negative, black bars; c-erbB-2 positive, shaded bars).

Figure 4  The prognostic impact of oestrogen receptor (ER) immunopositivity on disease free and overall survival (all patients; log rank tests).

earlier studies used a polyclonal and a different monoclonal antibody to the one used in our study. In our series, using the PPG5/10 monoclonal antibody, we found a
significant association between positive ERβ status and positive ERα status, in addition to lack of c-erbB-2 oncoprotein overexpression, reinforcing the findings of other investigators. In contrast to ERα status, ERβ status appears to be independent of PR status; in other words, unlike ERα, ERβ does not appear to induce PR. Transcription of the PR gene is enhanced and maintained by oestrogens; thus, a positive PR status has long been regarded as a marker of a functional ER pathway. According to our findings, ERβ does not seem to be an important factor defining the expression of PR in breast cancer; this may indicate indirectly that ERβ has a smaller role in defining the responsiveness to hormonal treatment in breast cancer. In those tissues that exclusively express either ERα or ERβ, signalling would be via the specific receptor, whereas in those tissues expressing both subtypes, signalling would be mediated by ERα/ERβ heterodimers. It could be hypothesised that one function of ERβ is to antagonise the effects of ERα in epithelial cells. Because one of the main functions of ERα in the epithelium is the induction of PR, it is possible that an ERα/ERβ heterodimer can inhibit this action.

The molecular study of Bieche et al. found no definite associations between activation and cell proliferation. In our present immunohistochemical study, ERβ immunoreactivity was positively associated with a reliable marker of actively proliferating cells in breast cancer, TopolIα. This molecule is a cycle related enzyme that functions in the segregation of newly replicated chromosome pairs in chromosome condensations and in altering DNA superhelicity. TopoIIα is thought to provide an exact measure of the growth fraction and its increased expression has been positively associated in breast cancer with more commonly used proliferation markers, such as Ki-67. The view that ERβ is important in cell proliferation in breast cancer has been discussed in a previous study, where the expression of ERβ was associated with a higher content of Ki-67 and cyclin A; in that study, in specimens where ERβ was much more abundant than ERα, there were many Ki-67 positive cells, 98% of which expressed ERβ. In contrast, ERα expressing cells in breast cancer do not express proliferation markers, so that it is possible that ERβ and not ERα is related to proliferation in breast cancer. The proliferative action of oestradiol on the epithelium is indirect—oestrogen is thought to stimulate the secretion of growth factors from breast stroma and these factors stimulate epithelial cells to proliferate. Interestingly, ERβ mRNA positive cancers are more frequently epidermal growth factor receptor protein positive than their negative counterparts. Two distinct types of responses to oestrogen in the breast have been described, namely: (a) an indirect action of oestradiol in the mammary epithelium by inducing ER containing stromal cells to produce growth factors that stimulate epithelial cells to divide, and (b) a direct effect on ERα containing cells that occurs at low oestrogen concentrations and results in the induction of PR. As far as the breast carcinomas in our present study are concerned, the detection of ERβ in tumour stromal elements and the lack of correlation between ERβ and PR status lead us to suggest that ERβ is involved in the first type of oestrogen response. In any case, our finding that ERβ positive carcinomas have a proliferative advantage potentially illuminates the role of ERβ selective antagonists as a useful addition to the therapeutic regimen with a novel target—namely proliferating cells expressing ERβ—which, probably, are not targets of tamoxifen; it is accepted that the beneficial effect of tamoxifen in breast cancer is caused by its effect on ERα positive cells, very few of which are proliferating.

Table 2: Cox's regression analysis of the prognostic value of the evaluated parameters with regard to disease free survival

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B</th>
<th>SE</th>
<th>df</th>
<th>p Value</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERβ status</td>
<td>-1.333</td>
<td>0.403</td>
<td>1</td>
<td>0.001</td>
<td>0.264</td>
<td>0.120 to 0.580</td>
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<tr>
<td>Lymph node status</td>
<td>0.851</td>
<td>0.310</td>
<td>1</td>
<td>0.006</td>
<td>2.342</td>
<td>1.274 to 4.302</td>
</tr>
<tr>
<td>Stage</td>
<td>1.154</td>
<td>0.446</td>
<td>1</td>
<td>0.010</td>
<td>3.172</td>
<td>1.324 to 7.597</td>
</tr>
<tr>
<td>TopoII index</td>
<td>0.039</td>
<td>0.018</td>
<td>1</td>
<td>0.032</td>
<td>1.039</td>
<td>1.003 to 1.077</td>
</tr>
<tr>
<td>p53 accumulation</td>
<td>0.945</td>
<td>0.446</td>
<td>1</td>
<td>0.034</td>
<td>2.573</td>
<td>1.074 to 6.164</td>
</tr>
<tr>
<td>PR status</td>
<td>-0.817</td>
<td>0.407</td>
<td>1</td>
<td>0.045</td>
<td>0.442</td>
<td>0.199 to 0.981</td>
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<tr>
<td>Tumour size</td>
<td>0.722</td>
<td></td>
<td></td>
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<tr>
<td>Histological grade</td>
<td>0.622</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nuclear grade</td>
<td>0.622</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ERα status</td>
<td>0.966</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c-erbB-2 overexpression</td>
<td>0.470</td>
<td></td>
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</tbody>
</table>

CI, confidence interval; df, degrees of freedom; ER, oestrogen receptor; PR, progesterone receptor; TopoIIα, topoisomerase IIα.

Table 3: Cox's regression analysis of the prognostic value of the assessed variables with regard to overall survival

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B</th>
<th>SE</th>
<th>df</th>
<th>p Value</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>1.331</td>
<td>0.394</td>
<td>1</td>
<td>0.001</td>
<td>3.783</td>
<td>1.747 to 8.193</td>
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<tr>
<td>ERβ status</td>
<td>-0.957</td>
<td>0.376</td>
<td>1</td>
<td>0.011</td>
<td>0.384</td>
<td>0.184 to 0.802</td>
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<tr>
<td>TopoIIα index</td>
<td>0.038</td>
<td>0.018</td>
<td>1</td>
<td>0.030</td>
<td>1.039</td>
<td>1.004 to 1.075</td>
</tr>
<tr>
<td>PR status</td>
<td>-0.956</td>
<td>0.467</td>
<td>1</td>
<td>0.041</td>
<td>0.384</td>
<td>0.154 to 0.960</td>
</tr>
<tr>
<td>Tumour size</td>
<td>0.999</td>
<td></td>
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<tr>
<td>Lymph node status</td>
<td>0.096</td>
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<tr>
<td>Histological grade</td>
<td>0.427</td>
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<td>Nuclear grade</td>
<td>0.411</td>
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<tr>
<td>ERα status</td>
<td>0.833</td>
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<tr>
<td>c-erbB-2 overexpression</td>
<td>0.066</td>
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</tbody>
</table>

CI, confidence interval; df, degrees of freedom; ER, oestrogen receptor; PR, progesterone receptor; TopoIIα, topoisomerase IIα.
According to recent reports, ERβ immunopositivity had a significantly favourable impact on disease free survival and overall survival in patients with breast cancer in both univariate and multivariate analysis. This was despite the fact that there was a positive association between ERβ immunoreactivity and topoisomerase IIα expression, indicating that ERβ is associated with increased proliferation.

Despite these correlations, ERβ immunopositivity emerged as an independent indicator of favourable prognosis with regard to both disease free and overall survival. It is of particular interest that ERβ immunostatus appears to have clinical significance with regard to the relapse free survival of stage I patients. In contrast to the proposed prognostic impact of ERβ, ERα is now considered a weak prognostic factor. According to recent reports, ERα positive tumours may have a more indolent course during the first years after primary breast cancer treatment; however, longer term disease free and overall survival are not significantly affected by ERα status in the primary tumour in terms of the natural aggressiveness of the tumour.34 In our present series, multivariate statistical analysis revealed no significant influence of ERα status on survival, whereas patients with ERα positive tumours had an improved long-term prognosis. To the best of our knowledge, this is the first time that the independent value of ERβ immunoreactivity on prognosis in breast cancer has been reported. Nevertheless, the finding that ERβ immunopositivity emerged as an independent indicator of favourable prognosis, despite the fact that ERβ positivity was associated with a proliferative advantage, requires verification in other series, which should examine whether this favourable prognostic influence results purely or partly from the fact that all patients received tamoxifen.

In conclusion, we found that although the ERβ protein is associated with increased cell proliferation it could be useful as an indicator of good prognosis in breast cancer.

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