Evaluation of ERα, PR and ERß isoforms in neoadjuvant treated breast cancer

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Abstract. The actual predictive value of oestrogen receptor (ER) ß for treatment decisions in breast cancer is still unclear. Retrospective studies using preoperative systemic therapy (PST) revealed that chemotherapy but also endocrine therapy can lead to alterations in expression levels of ERα and progesterone receptor (PR). The main purpose of this study was to compare ERß expression levels before and after neoadjuvant chemotherapy or endocrine therapy and to explore a possible predictive value of ERß. Matching 'baseline' biopsies and post-therapy surgical specimens of 69 breast cancer patients treated with neoadjuvant anthracycline- or taxane-based chemotherapy or with aromatase inhibitors were analyzed for expression levels of ERα, PR, total ERß (ERßt), ERß1, ERß2 and the proliferation-related antigen Ki-67 using immunohistochemistry. A marked expression of ERßt significantly correlates with low proliferation rates after PST (p=0.0013) and with response to it. Further most tumours decreased ERß1 expression with PST. A marked ERß2 expression was observed predominantly in responders and significantly decreased during chemotherapy (p=0.047). Results on ERα and PR corroborate findings of previous studies. Our data demonstrate that changes of ERß expression occur during PST and that total ERß expression and ERß2 may have a predictive value for PST.

Introduction

The oestrogen receptor ß (ERß) and its isoforms are promising new potential biological markers in breast cancer. It is likely that ERß can be used as a predictive marker for response to endocrine therapy or as a therapeutic target in the future. Therapy decisions in breast cancer are to a great extent guided by hormone receptor expressions. So far only estrogen receptor α (ERα) and progesterone receptor (PR) are routinely evaluated in breast cancer patients (1). For several years now research is focusing on the potential prognostic and predictive role of ERß and its variant isoforms in breast cancer. Most of these isoforms are splice variants or exon deletion isoforms (2,3). The so far best characterized isoforms are ERß1, the wild-type form, and ERß2 (also known as ERßcx) which is a splice variant of ERß1 (4,5; reviewed in refs. 6 and 7). Data on the role of ERß are often conflicting due to the circumstance that the different ERß isoforms most likely have different biological functions or clinical values (3,5-10). However, there seems to be a consensus on a role of ERß as a tumour suppressor (reviewed in refs. 11 and 12). Another challenge in the process of establishing ERß and its isoforms as new biological markers in routine diagnostics remains the development of a standardized and quality controlled scoring system for immunohistochemical detection of ERß and its isoforms (1,13,14).

Neoadjuvant or preoperative systemic therapy (PST) provides an excellent model for researchers to evaluate the expression of biological markers such as hormone receptors before and after therapy in order to gain a deeper understanding of tumour biology. Several research groups observed significant changes in expression levels of tumour parameters like ERα, PR or Her-2 during PST (15-23). Therefore, it is strongly recommended that hormone receptor expression and other biological markers should be re-evaluated after PST in order to make sure that post-surgery treatment is tailored adequately. ERα and PR are established predictive markers to select patients for an endocrine therapy in breast cancer. However, some research groups also found that ERα and PR are likely to have a predictive value for chemotherapy as well, most of them stating a correlation of ERα and PR negativity with chemotherapy response (18,24-27). Rody and colleagues observed in a neoadjuvant study that Her-2 seems to be highly predictive for chemotherapy outcome (24). Concerning ERß Miller and colleagues focused on ERß expression with

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Table I. Treatment applied.

PST	Regimen	Dose (mg/m^2)
Anthracycline-based	A/E+C	60/90+600
Taxane-based	A/E+T	50/90+75
	E+C+T	90+500+75
Aromatase inhibitor	Letrozole	2.5 mg
	Exemestane	25 mg

A, adriamycin; C, cyclophosphamide; E, epirubicin; T, docetaxel.

Table II. Classification of hormone receptor expression.

IRS-score	Classification I	Classification II
0	0 = negative	0 = negative
1-3	1 = weakly positive	1 = weakly positive
4-7	2 = intermediately positive	1 = weakly positive
8-12	3 = strongly positive	2 = strongly positive

neoadjuvant tamoxifen therapy and observed a decrease of ERß1 (28). Possible effects of aromatase inhibitors or chemotherapy on ERß or a possible predictive value of ERß for cytotoxic drugs remain to be investigated. Advancing our knowledge about interactions of different cytoreductive drugs with ERß will lead to a better understanding about the relevance of ERß and its isoforms for therapy decisions in breast cancer.

Based on this background the purpose of this study was to assess possible effects of different PST regimens on ERα and PR but also on ERß1, ERß2 and total ERß (ERßt) (including all isoforms) expression levels. We also focused on the question whether ERßt, ERß1 or ERß2 may have any predictive relevance for these therapies. Hormone receptor proteins were detected semi-quantitatively using immunohistochemistry. We compared expression levels before and after anthracycline-based or taxane-based neoadjuvant chemotherapy or neoadjuvant endocrine therapy with the aromatase inhibitors letrozole and exemestane.

Patients and methods

Tissue samples from 69 breast cancer patients with non-metastatic invasive primary breast carcinoma (cT1-4, Mo) have been included into the study. They have undergone PST at the Department of Gynaecology and Obstetrics, University Hospital, Tuebingen, Germany, from January 1999 until January 2003. All patients provided diagnostic core biopsy of the breast tumour to confirm invasive cancer before starting treatment. All specimens were obtained after written informed consent and collected using a protocol approved by the local ethics committee (AZ 266/98).

Chemotherapy schedules and surgery. Patients received 4-6 cycles of either an anthracycline (n=17) or taxane (n=30) based therapy administered at 21-day intervals or neoadjuvant endocrine treatment with letrozole or exemestane (n=22) daily for 6 months (Table I). Surgery was performed ~1 month after the final cycle of chemotherapy. Patients who had no remaining invasive cancer in the breast and who were lymph node negative were considered to have a pathologically complete response (CR).

Assessment of response. Response to PST was evaluated pathologically by classifying the regressive changes using a semi-quantitative scoring system from 0 to 4 [0, no effect; 1, resorption and tumour sclerosis; 2, minimal residual invasive tumour (<0.5 cm); 3, residual non-invasive tumour only; 4, no tumour detectable] according to the tumour regression grading described by Sinn *et al* (29). A consultant pathologist (U.V.) blinded to clinical outcome reviewed all paired biopsy and surgical specimens. Labelled sections were investigated in a blinded fashion by M.W. and U.V. who did not know the kind of treatment used. All sections were digitally documented and labelling was semi-quantitatively scored. Labelling for Ki-67 was scored in a different way: only the percentage of positive tumour cells was scored. If >10% of the cells were labelled the score was positive.

Labelling of hormone receptors was scored according to the 'immune reactive score' (IRS) established by Remmele and Stegner (30). This score calculates the percentage of positive nuclei (0, 0%; 1, <10%; 2, 10-50%; 3, 50-80% and 4, >80% of positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong staining). The IRS is calculated by multiplying both values providing scores between 0 and 12.

Tumour samples were classified according to their receptor expression in two ways (Table II): classification I was used to investigate co-expressions, proliferation and changes in receptor expression; classification II was applied to investigate receptor expression and response to therapy.

Immunohistochemistry. The immunohistochemical (IHC) analysis was performed on tissue microarrays (TMA) produced from cut core biopsies and surgical resection specimens. Tissue samples have been fixed in 4.5% buffered formalin (pH 7.0) and embedded in paraffin. IHC was performed on TMA sections (4 μm) mounted onto Superfrost glass slides. In total four TMAs have been produced with 150 cores each. For IHC CytoChem-Plus HRP kit, Broad Spectrum (Zytomed, Berlin, Germany) was used. Briefly, before incubation with primary antibody unspecific binding was blocked with Blocking Solution-SuperBlock for 5 min. After washing once primary antibodies were incubated in appropriate dilutions for optimized incubation times (Table III). Primary antibodies were diluted in Antibody Diluent (Dako, Hamburg, Germany) and applied according to the manufacturer's instruction. DAB (3,3'-diaminobenzidine) was used as chromogen. Finally, the slides were counterstained with Mayer's haematoxylin for 10 sec and mounted for examination.

For each antibody a positive tissue sample was used as positive control. For negative control the same section was incubated without the primary antibody. Reactions were performed in a humified chamber. Counter staining was done

Table III. Antibodies and dilutions.

Antibody	Species	Dilution	Supplier	Refs.
ERßt	Mouse monoclonal (14C8; specific for as 1-153 of human ERß)	1:1000	GeneTex, Inc., San Antonio, TX, USA	(13,46)
ERß1	Mouse monoclonal (PPG5/10, specific for c-terminal peptide of ERß1)	1:1000	Serotec, Oxford, UK	(47,48)
ERß2	Mouse monoclonal (57/3, specific for c-terminal peptide of ERß2)	1:500	Acris, Hidden-hausen, Germany	(49,50)
ERα	Rabbit monoclonal (SP1, specific for c-terminal peptide of ERα)	1:200	DCS, Hamburg, Germany	(51)
PR	Rabbit monoclonal (SP2, specific for as 412-526 of human PR)	1:200	DCS	(52-54)
Ki-67	Mouse monoclonal (Mib-1)	1:200	DakoCytomation, Hamburg, Germany	(55-57)
CK18	Mouse monoclonal (DC 10)	1:2000	Dako	(58)

with Papanicolaous solution 1a (Harris' Hematoxylin) for 30 sec.

Statistical analysis. To find correlations between two parameters χ^2 test was performed. P-values <0.05 were regarded as significant. For analysis of receptor co expression multivariate correlation was performed and the correlation coefficient was calculated according to Spearman, since classes were not equally distributed.

Results

Clinical characteristics and response to treatment. Sixty-nine breast cancer patients were investigated in our study. Clinical data are presented in Table IV. After PST response to treatment (partial remission, complete remission) was reached in 49.3% of the cases. 50.7% were non-responders. Positive lymph nodes were seen in 65.2% of the patients. The predominant histological tumour type was invasive ductal carcinoma (63.8%) followed by invasive lobular carcinoma in 20.3% of the cases. The majority of the patients was post-menopausal.

ERα. ERα was detectable only in nuclei of epithelial cells (Fig. 1). Most of the tumours (79.7%) were ERα positive. A correlation of ERα negativity and proliferation could be observed (χ^2=4.2; p=0.04). After PST this correlation was more pronounced and significant (χ^2=17.5; p<0.0001). During PST ERα expression decreased in 27.5% of the tumours and stayed unchanged in 68.1%. Furthermore, ERα expression was observed to decrease more often in responders to PST (40.7%) than in non-responders (10%).

PR. Like ERα PR was detectable only in nuclei of epithelial cells. Most of the tumours were classified as PR positive. We observed a distinct decrease of PR during PST in all 3 therapy groups (66.7%) but most prominent in the endocrine therapy group (77.3%). Such a decrease could be made out more often in pre-menopausal than in post-menopausal women (data not shown).

Table IV. Basic patient characteristics after primary systemic therapy.

Total	N (%)
	69 (100)
Menopausal status	
Pre	12 (17.4)
Peri	8 (11.6)
Post	49 (71.0)
Tumour size	
ypT1	17 (42.6)
ypT2	33 (47.8)
ypT3	12 (17.4)
ypT4	7 (10.1)
Nodal status	
yN negative	24 (34.8)
yN positive	45 (65.2)
Grading	
I	3 (4.3)
II	47 (68.1)
III	19 (27.5)
Histology	
Ductal	44 (63.8)
Lobular	14 (20.3)
Ductulo-lobular	8 (11.6)
Others	3 (4.3)
Primary systemic therapy	
Anthracycline-based	17 (24.6)
Taxane-based	30 (43.5)
Aromatase inhibitors	22 (31.9)
Therapy response	
Responders	34 (49.3)
Non-responders	35 (50.7)

ypT, post-chemotherapy pathologic T classification; yN, post-chemotherapy pathologic N classification.

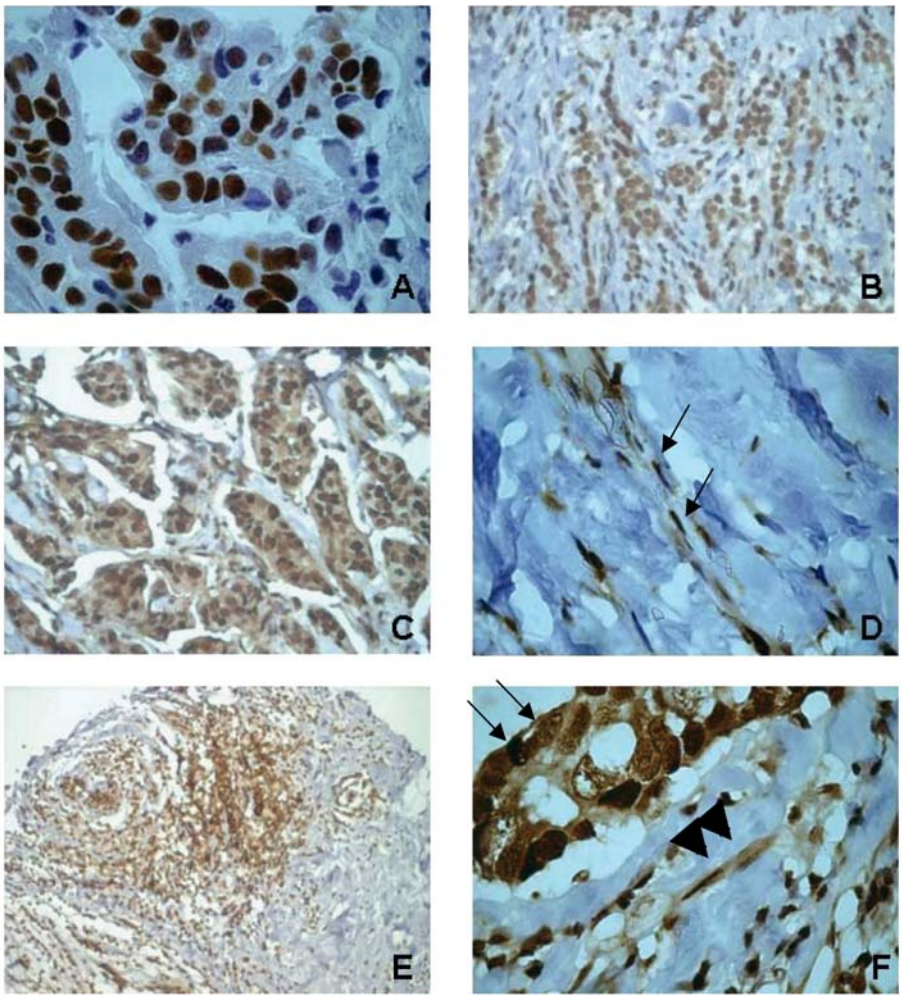

Figure 1. Immunohistochemistry for hormone receptors. Depicted are typical staining obtained for (A) ERα expression is detected only in nuclei of epithelial cells; (B) ERß isoforms, nuclear expression; (C) ERß isoforms, nuclear and weak cytoplasmic staining; (D) ERß isoforms in fibroblasts (arrows); (E) ERß isoforms in inflammatory cells; (F) ERß isoforms in breast cancer cells (arrows) and fibroblasts (arrowheads). Magnifications: (A, D and F), x40; (B and C), x20; (E), x10.

Total ERβ expression (ERβt, including all isoforms). ERßt could be detected in nuclei as well as in the cytoplasm of epithelial cells, fibroblasts and inflammatory cells (Fig. 1). The majority of the tumours (73.9%) were strongly positive for ERßt. Tumours with a strong expression of ERßt were significantly more often classified as non-proliferating after chemotherapy than tumours which were only weakly positive for ERßt (χ^2=10.4; p=0.0013). Furthermore a strong ERßt expression was tendentially more often observed in responders (81.4%) to chemotherapy than in non-responders (50%).

ERβ1. Like ERßt, ERß1 could be detected in nuclei as well as in the cytoplasm of epithelial cells, fibroblasts and inflammatory cells (Fig. 1). Most of the tumour specimens (62.3%) were classified as strongly positive for ERß1. We observed a decrease of ERß1 during chemotherapy in 55.3% of cases.

ERβ2. Like ERß1 and ERßt, ERß2 was detectable in nuclei and in the cytoplasm of mammary epithelial cells, fibroblasts and inflammatory cells (Fig. 1). The majority of the tumours (47.8%) were strongly positive for ERß2.

Responders to chemotherapy often showed a stronger ERß2 expression than non-responders (Table V). This could be observed predominantly in the taxane-group. We further observed that responders to chemotherapy decreased ERß2 significantly more often than non-responders (χ^2=6.1; p=0.047) particularly in the taxane-group (Table V).

Hormone receptor co expressions. ERα and PR were significantly co-expressed in our tumour specimens (r=0.58; p<0.0001), but neither ERα nor PR expression correlated with expression levels of ERßt, ERß1 or ERß2 (data not shown). Since ERßt includes all ERß isoforms we found a significant co expression of ERßt with ERß1 (r=0.4; p=0.0008) and also with ERß2 (r=0.38; p=0.0013) as expected. There was also a significant correlation of ERß1 and ERß2 (r=0.35; p=0.0031) detectable.

Discussion

ERβ isoforms vary and correlate with chemotherapy response. Although the number of samples investigated is small this neoadjuvant immunohistochemical study provides evidence that oestrogen receptor ß expression can change during PST and that especially a change in ERß2 expression significantly correlates with chemotherapy response. Our data on ERα and PR mainly substantiate findings of other research groups.

Table V. Oestrogen receptor ß2.

A. ERß2 expression and therapy response.[a]

	ERß2					
	Anthracycline + Taxane			Taxane		
	Negative (%)	Weak (%)	Strong (%)	Negative (%)	Weak (%)	Strong (%)
Responders	0	13 (48.1)	14 (51.9)	0	9 (60)	6 (40)
Non-responders	0	14 (70)	6 (30)	0	12 (80)	3 (20)

B. Change in immunohistochemical score with PST.

	ERß2						
	Anthracycline + Taxane			Taxane			
	Decrease (%)	No change (%)	Increase (%)	Decrease (%)	No change (%)	Increase (%)	P-value
Responders	13 (48.1)	9 (33.3)	5 (18.5)	7 (46.7)	5 (33.3)	3 (20)	χ^2=6.1; P=0.047
Non-responders	3 (15)	12 (60)	5 (25)	1 (6.7)	9 (60)	5 (33.3)	

[a]No significant differences between responding and non-responding tumours.

Variations of oestrogen receptor α and progesterone receptor during neoadjuvant therapy. A decrease of ERα expression in responders to PST has already been described (18). Our data confirm these findings and support the hypothesis that chemotherapy can induce significant variations in ERα expression. We further observed a down-regulation of PR expression levels in all therapy groups. A decrease of PR during neoadjuvant therapy with aromatase inhibitors but also during an anthracycline-based chemotherapy is also known from the literature (15,16,21-23,31). Therefore, a re-evaluation of ERα and PR status after PST is strongly recommended in order to optimize individual therapy decisions based on the actual receptor expression. In the endocrine therapy group we found a decrease of PR predominantly in premenopausal women. This is most likely due to the fact that endocrine therapy induces menopause resulting in a down-regulation of PR.

Specific detection of ERß isoforms. In the process of establishing ERß as a prognostic or predictive parameter, in order to guide individual decision making for the treatment of breast cancer, it is necessary to develop a standardized scoring system for a specific detection of the different isoforms (1,5,13,14). In this study we established an immunohistochemical staining approach and scoring system which allows detecting expression levels of ERß1 and ERß2 specifically. The detection of ERß and its isoforms not only in nuclei of mammary epithelial cells but also in the nuclei and cytoplasm of endothelial cells, fibroblasts and inflammatory cells is already known from the literature (6,32,33). However, we evaluated and compared ERß expression only in mammary epithelial cells in order to gain as valid data as possible on ERß in carcinoma cells.

ERßt might act as a predictive marker for chemotherapy. There is a solidifying consensus that ERß acts as a tumour suppressor in breast cancer and has a protective role against the development of a malignancy (reviewed in ref. 11). Moreover, Lazennec and colleagues stress an antiproliferative effect of ERß on breast cancer cells (34). Our data demonstrate that a strong expression of ERßt significantly correlates with low proliferation rates after chemotherapy. This corroborates the assumption of an antiproliferative effect of ERßt. We further observed that responders to chemotherapy tend to be more often strongly positive for ERßt than non-responders. These findings suggest that ERßt might act as a positive predictive parameter for chemotherapy. Since the phenomenon was most pronounced in the anthracycline group it might be speculated that ERßt has a predictive value especially for an anthracycline-based chemotherapy.

ERß1 decreases during neoadjuvant chemotherapy. Our data demonstrate that ERß1 expression decreases in the tumour cells during primary systemic chemotherapy. Since the different chemotherapy groups show a similar decrease in ERß1 we assume that the down-regulation might not be due to specific effects or signal transductions of the drugs on the tumour cells. A possible explanation for the unspecific decrease of ERß1 might be alterations in the tumour extracellular matrix (ECM). However, this hypothesis needs to be confirmed. A lot of studies revealed that changes in the composition of the ECM or in cell-matrix-interaction processes frequently occur in breast cancer which also leads to alterations in expression levels of tumour relevant proteins such as hormone receptors (35-38). We recently observed a decrease of ERß1 in MDA-MB-231 breast cancer cells by a laminin-rich basement membrane matrix (39).

ERß2 might have a predictive value for chemotherapy. Expression of ERß2 is suggested to exert an antiproliferative or tumour suppressive effect (40,41). Furthermore, we find that responder to chemotherapy more often show a strong expression of ERß2 compared to non-responder, which highly suggests that ERß2 might have a predictive value for chemotherapy, especially for a taxane-based chemotherapy since the effect could be observed most prominent in the taxane-group of our study. Taxanes have many biological effects which appear to be related to its ability to promote an assembly of microtubules stabilizing them against depolymerising agents (19,42). However, taxanes are also known to exert several effects which are mediated by other mechanisms (19,43-45). It might be speculated that tumour cells strongly positive for ERß2 are more sensitive to taxane-mediated effects. Based on this hypothesis ERß2-positive tumour cells are primarily annihilated by chemotherapy. Remaining tumour tissue after chemotherapy therefore consists of cells with weak ERß2 expression levels, or cells mainly ERß2 negative. This might explain why responders to taxanes in our study show significantly more often a marked decrease of ERß2 expression (or a shift from ERß2 positivity to negativity) after therapy than non-responders. Taken together, we conclude from our data that ERß2 may have a predictive value for a taxane-based chemotherapy, however, the underlying molecular biological reason remains to be investigated.

In conclusion, this study reveals that PST influences ERß expression in breast cancer and that tumour proliferation and chemotherapy response are correlated with ERß expression. ERßt and ERß2 seem to have a predictive value for chemotherapy.

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