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Activated ERK1/2 and phosphorylated oestrogen receptor α are associated with improved breast cancer survival in women treated with tamoxifen ☆

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ABSTRACT

The aim of this study was to investigate the expression of activated (phosphorylated) ERK1/2, oestrogen receptor α phosphorylated at S118 (ERα^{S118}), and HER2 in primary breast cancer, and to make correlations with the outcome of tamoxifen therapy. We performed immunohistochemical analysis to determine the expression of HER2, ERα^{S118}, and activated ERK1/2 in tumours obtained from 279 women with primary breast cancer. HER2 status was also estimated by fluorescence in situ hybridisation. We identified 108 women with ERα-positive tumours who had received adjuvant tamoxifen. Activated ERK1/2 (pERK1/2) and ERα^{S118} were found to be associated with each other and with other factors correlated with good prognosis. HER2 was inversely associated with pERK1/2. Positive staining for pERK1/2 (particularly intense staining) indicated better relapse-free survival ($P = 0.05$) and a trend towards better breast cancer-corrected survival in women treated with tamoxifen. To conclude, this study shows that activated ERK1/2 and ERα^{S118} are associated with improved survival. The poorer outcome in HER2-positive women who receive adjuvant tamoxifen cannot be explained by the crosstalk between HER2 and ERα^{S118} via activated ERK1/2 alone.

1. Introduction

Endocrine therapy has been widely used for almost 30 years to treat breast cancer in both adjuvant and metastatic disease. In a survey conducted by the Early Breast Cancer Trialists' Collaborative Group,[1] it was found that adjuvant tamoxifen reduced the risk of recurrence by 41% and lowered the rate of mortality by 34%. In metastatic breast cancer patients positive for oestrogen receptor α (ERα) and/or progesterone receptor (PgR), it has been estimated that tamoxifen results in an objective response rate of 24%, whereas the clinical benefit rate is 50%, with a median time to progression of 6.4 months.[2] Even patients who express the ERα can show *de novo* resistance to tamoxifen, and the majority of those who initially respond develop resistance to the drug after a few years of therapy.[3]

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It has been proposed that HER2 (also known as c-erbB-2 and HER2/neu) can partly explain the problems associated with tamoxifen resistance. It is a matter of debate whether HER2 is a factor that can predict the outcome of endocrine therapy. Indeed, some reports even indicate that women who have HER2-positive tumours and receive tamoxifen therapy will do worse than those without such treatment,[4–6] although the results are conflicting.[7,8] The human mitogen-activated protein kinases (MAPKs) are signal transducers located downstream of HER2, and it has been suggested that resistance to tamoxifen may involve crosstalk between growth factor signalling and the oestrogen receptor α (ERα) via the MAPK called extracellular signal-regulated kinase (ERK1/2).[9]

The ERα receptor contains two transcription activation motifs, AF-1 and AF-2, the former of which can be phosphorylated (activated) in a ligand-independent manner at the serine residue 118 by ERK1/2.[10] Pre-clinical studies have confirmed that overexpression of HER2 in breast cancer cell lines is associated with phosphorylated ERK1/2 (pERK1/2) and resistance to tamoxifen, but there are few reports concerning the significance of pERK1/2 in human breast cancer in vivo.[11] Therefore, we hypothesised that the outcome of tamoxifen therapy might be affected by HER2 signalling that occurs through activation of the ERK1/2 signalling cascade, leading to phosphorylation of ERα.

The present study had two objectives: first, to investigate breast cancer cells and connective tissue surrounding tumours with regard to expression of pERK1/2, ERα phosphorylated at S118 ($ER\alpha^{S118}$), and HER2; second, to ascertain whether these factors are of prognostic value after adjuvant tamoxifen therapy in ERα-positive breast cancer patients, especially in relation to relapse-free survival (RFS), breast cancer-corrected survival (BCCS), and overall survival (OAS).

2. Patients and methods

2.1. Patients and tumours

The study was approved by the Ethics Committee of Karolinska Hospital. Five hundred and twenty-four patients with primary invasive breast cancer who had undergone surgery at Karolinska Hospital from 1 January 1 1994 to 31 December 1996 were identified in the Regional Cancer Registry. Formalin-fixed paraffin-embedded tissue blocks were available for 279 of the patients. According to medical records, 51 of the 524 patients initially identified did not meet the inclusion criteria given above (primary invasive breast cancer without distant metastases; operated without previous neoadjuvant therapy), and another 194 tumours were excluded due to lack of remaining invasive cancer or because they could not be located. Information on patients and tumour characteristics was collected from the mentioned registry, and supplementary data were obtained from the medical records of the included patients. Considering biological characteristics, the patients that were excluded and those who were included were similar, except that more of the cancers in the latter group were lymph-node positive (33% vs. 24%) and ERα positive (85% vs. 71%). After correction for multiple comparisons, only the percentage of ERα-positive tumours still differed between the two groups. The histological slides of the primary tumours were re-examined and re-classified according to the Elston–Ellis grading system by a pathologist (H.N.) without any knowledge of the patients. Receptor contents had previously been estimated in tumour cytosolic extracts by enzyme immunoassays (ER-EIA, Abbott Laboratories, Abbott Park, IL, USA), and, based on the results, the tumours were characterised as ERα or PgR positive at values $\geqslant 0.05$ fmol μg^{-1} DNA. The characteristics of the patients ($n = 279$) and their primary tumours are summarised in Table 1.

2.2. Therapy

All but 13 patients included in the study had primary surgery consisting of modified radical mastectomy or sector resection and axillary dissection ($n = 266$). Nodal exploration was not performed on the other 13 women. Patients with lymph node-positive disease received loco-regional radiotherapy, and those who underwent sector resection (no lymph node metastases) were given local radiotherapy to the remaining breast parenchyma. Systemic adjuvant endocrine therapy consisted of one of the following: tamoxifen for two or five years; tamoxifen and/or goserelin; comparison of sequential and alternating tamoxifen-medroxyprogesteron and tamoxifen, as part of a prospective randomised study. The adjuvant chemotherapy chosen most often was cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), which were given intravenously on days 1 and 8, q 4 weeks for six courses. High-risk patients were asked to enter the randomised SBG 9401 study.[12] We identified 108 women with ERα-positive tumours who had received tamoxifen but no chemotherapy as adjuvant treatment. Thirteen of these 108 women had received tamoxifen for less than two years, 52 patients received tamoxifen for 2 years while the remaining 43 women received tamoxifen for 5 years.

2.3. Immunohistochemical (IHC) analyses

The following primary antibodies were used: CB11 (anti-HER2/neu), diluted 1:300 (NovoCastra Laboratories Ltd., Newcastle, UK); phospho-p44/42 MAPK (Thr202/Thr204) (E10) monoclonal antibody (ERK1/ERK2), diluted 1:400, and phospho-estrogen receptor α (Ser118) 16J4 monoclonal antibody (oestrogen receptor α phosphorylated at serine 118), diluted 1:400 (Cell Signalling Technology, Inc., Beverly, MA). Examples of positive staining for HER2, $ER\alpha^{S118}$, and pERK1/2 are shown in Fig. 1.

Sections (4 μm) of paraffin-embedded primary tumours were mounted on glass slides and then deparaffinised in xylene and rehydrated in graded concentrations of ethanol to TBS. Heat-induced epitope retrieval was performed to analyse HER2 and pERK1/2. In short, the prepared tissue sections were covered with citric acid buffer (pH 6) and heated in a microwave oven at 800 W until the buffer started to boil. This microwave processing was continued for 20 min at 240 W, after which the sections were allowed to cool at room temperature for further 20 min. For analysis of $ER\alpha^{S118}$, heat-induced epitope retrieval was done using TRIS/EDTA buffer (pH 9). In this case, the tissue sections were also heated at 800 W in the microwave until the buffer started to boil, but the processing was subsequently continued for 5 min at 400 W and finally

Table 1 – Characteristics of the patients (n = 279) and their primary tumours

	No. of patients	(%)	Missing data	
			No. of Patients	(%)
Age (years)				
<50	77	28		
⩾50	202	72		
Tumour size (mm)				
⩽20	196	70	1	
21–50	71	25		
>51	11	4		
Lymph node metastases				
0	179	64	13	(5)
<4	65	23		
⩾4	22	8		
Hormone receptor status				
ER and PgR positive	184	66	33	(12)
ER and PgR negative	27	10		
ER positive and PgR negative	25	9		
ER negative and PgR positive	10	4		
Histological grading (Elston–Ellis)				
1	61	22	33	(12)
2	117	42		
3	68	24		
Menopausal state				
Premenopausal	92	32	9	(3)
Postmenopausal	178	64		
Relapse				
Yes	60	22		
Loco-regional	18	6		
Distant	42	15		
No	219	78		
Death				
Due to breast cancer	48	17		
Not due to breast cancer	27	9		
Alive	204	73		
Follow-up, years (median)	8.7, range 0.3–10.4			
HER2-amplified tumours	24	9		
Tumours with pERK1/2 staining	87	31		
Tumours and surrounding tissue with pERK1/2 staining	49	18		
Tumours with ERα^{S118} staining	151	54		
Tumours with both ER and ERα^{S118} staining	121	43	33	(12)

5 min at 250 W, after which the sections were allowed to cool at room temperature for 20 min. Automatic immunostaining was performed in a DAKO Tech Mate instrument (DAKO, Glostrup, Denmark). Staining of HER2 and pERK1/2 was done using the recommended DAKO ChemMate Detection Kit (Peroxidase/DAB Rabbit/Mouse), and staining of ERα^{S118} was achieved with a DAKO ChemMate EnVision Detection Kit (Peroxidase/DAB, Rabbit/Mouse). The slides were counterstained with haematoxylin and dehydrated through graded concentrations of ethanol to xylene before mounting.

2.4. *Evaluation of the immunohistochemical (IHC) analyses*

2.4.1. *HER2*

All slides were evaluated by one pathologist (G.E.), and tumours with no staining or only cytoplasmic staining were classified as negative for HER2. The evaluation was done as recommended by DAKO: partial or incomplete, weak to moderate, and moderate to strong membranous staining in more than 10% of the tumour cells were scored as 1+, 2+, and 3+, respectively. As controls in each experiment, we had sections from a multiple cell line block made of four human breast cancer cell lines with known intensity scores of 0, 1+, 2+, and 3+.

2.4.2. *pERK1/2*

The immunostained slides were scored by one pathologist (G.E.) according to a slightly modified version of the classification system described by Harvey and colleagues.[13] First, the proportion of tumour cells with positive nuclear staining was estimated as <10%, 10–25%, 50%, or 75–100% (classified 0, 1, 2, and 3, respectively). Next, the average intensity of the nuclear staining was scored as none (0), weak (1), moderate (2), or strong (3). In heterogeneous tumours, hot spots were examined to approximate the number of cells that were stained. A total score was obtained by combining the proportion and intensity scores to give a scale of 0–6. Values

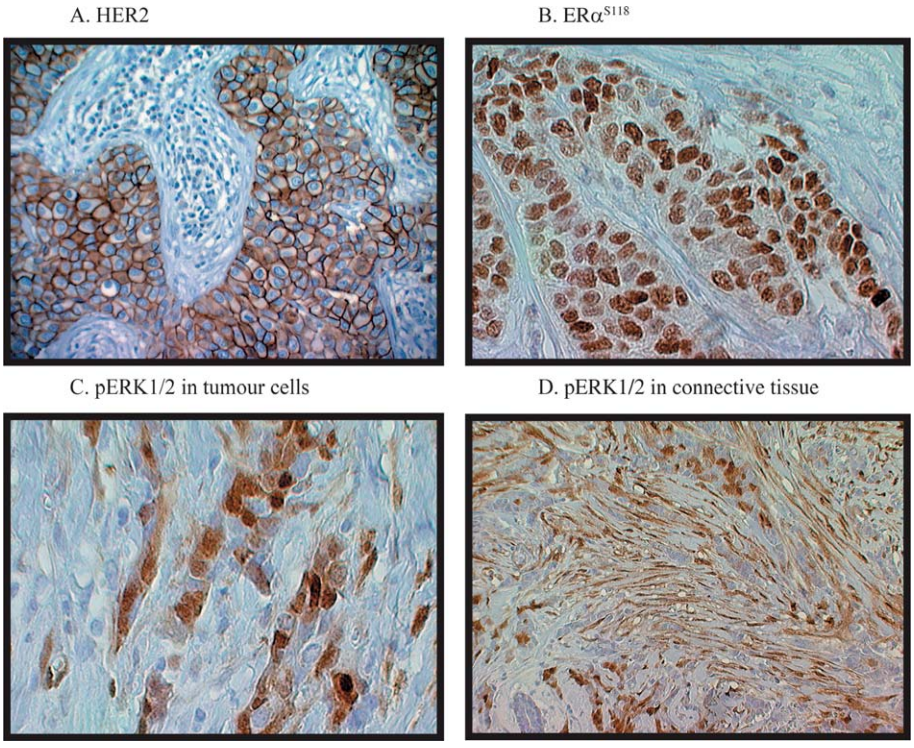

Fig. 1 – Positive immunohistochemical staining for expression of the proteins (A) HER2, (B) ERα^{S118}, and (C) pERK1/2 in tumours and (D) surrounding connective tissue.

⩽1 and >1 were dichotomised into negative versus positive, respectively. In the 108 women treated with tamoxifen, we compared tumours of higher vs. lower scale values for pERK1/2 staining (i.e., 5–6 vs. 1–4, respectively). This was done to address the assumption that strong and weak ERK1/2 phospho-signals might have different impacts on cell signalling.[14] Tissue sections of a tonsil with and without the primary antibody were used as negative and positive controls in every run.

We also applied the above-mentioned intensity score (0, none; 1, weak; 2, moderate; 3, strong) to evaluate staining of activated ERK1/2 in slides with connective tissue surrounding the tumours. Values ⩽1 and >1 were dichotomised as being, respectively, negative and positive for pERK1/2.

2.4.3. *ERα^{S118}*

The ERα^{S118} antibody gives a nuclear staining pattern in ERα-positive cells in which the ERα is phosphorylated at serine 118. Breast cancer tissue samples that were known to show ERα^{S118} staining with the primary antibody were used as positive and negative controls (i.e., with and without the primary antibody) in every run. The immunostained slides were scored in the same manner as pERK1/2 and by the same pathologist (G.E.).

2.4.4. *Fluorescence in situ hybridisation of HER2*

All tumours with scores of 2+ or 3+ in the immunohistochemical analysis were also evaluated by fluorescence in situ hybridisation (FISH) using a PathVysion HER2 DNA Probe Kit (Vysis Inc., Downers Grove, IL, USA). The FISH procedure has been described in detail in a recent publication.[15] Briefly, sections of formalin-fixed paraffin-embedded tissue specimens were mounted on slides and evaluated for *HER2* gene copy number using a Leica DMLB microscope equipped with appropriate filters as recommended by the manufacturer. DNA was denatured and allowed to hybridise with two fluorescent signals (one each for HER2 and chromosome 17). The cell nuclei were stained with DAPI. The three fluorescent signals were observed sequentially in the same field of view. The ratio of *HER2* to chromosome 17 is expected to be 2.0 for normal or unamplified breast tissue specimens. The scoring conditions used were those recommended by Vysis Inc., and a ratio greater than 2.0 was considered amplified. The pathologist (H.N.) did not know the immunohistochemistry (IHC) score for HER2.

3. Statistical methods

We used χ^2 analysis to compare all patients (i.e., all 524 that were initially identified in the cancer registry, both those who were and were not subsequently included in the study) and the tamoxifen-treated women with regard to descriptive statistics. Two-tailed Pearson and Spearman's correlations was used to analyse relationships between HER2 (determined by FISH), pERK1/2, ERα^{S118}, tumour size (<21 vs. ⩾21 mm), lymph node status (0–3 vs. ⩾4 node metastases), age (⩽50 vs. >50 years old at diagnosis), ERα, PgR and histologic grading (Elston–Ellis).

3.1. *Survival analyses*

The Kaplan–Meier method was used to estimate survival in terms of relapse-free survival (RFS), breast cancer-corrected

survival (BCCS), and overall survival (OAS). RFS was calculated from date of diagnosis to date of first metastasis (local or distant). Contralateral and/or new primary breast cancer was not considered as a relapse. OAS was calculated from date of diagnosis to date of death due to any cause, as compared to BCCS, which indicated death from breast cancer only. For the multivariate analyses, we used the Cox proportional hazard model constructed with forward stepwise selection and the level of significance to enter or stay in the model set at $P \leqslant 0.05$. We analysed the entire group of patients included in our study ($n = 279$) and also a subgroup ($n = 108$) that had ERα-positive tumours and received only tamoxifen as adjuvant treatment (i.e., no chemotherapy).

4. Results

4.1. Analysis of HER2, pERK1/2, and ERα^{S118} (n = 279)

Expression data of HER2, pERK1/2 and ERα^{S118} are presented in Table 1. We detected moderate (2+) or strong (3+) overexpression of HER2 in 31 of the 279 tumours (11%), and further analysis of these 31 tumours by FISH verified that 24 (9%) were truly amplified (Fig. 2). There was a significant correlation between HER2 amplification and high Elston score ($P < 0.01$) and larger tumour size ($P = 0.05$). We found an inverse relationship between HER2 amplification and positive pERK1/2 staining in tumours cells ($P = 0.05$). pERK1/2 in the cancer cells was significantly associated with the levels of pERK1/2 expressed in the surrounding connective tissues ($P < 0.01$), low Elston score ($P < 0.01$) and smaller tumour size ($P = 0.01$). In addition we found a significant correlation between pERK1/2 and ERα^{S118} ($P < 0.01$). Moreover, we observed significant associations between levels of expression of ERα^{S118} and the hormone receptors ERα ($P = 0.01$) and PgR (0.01), low Elston score ($P = 0.05$) and smaller tumour size ($P = 0.05$).

4.2. Analyses of survival in all patients (n = 279)

Results of univariate analysis of RFS and BCCS are shown in Table 2. HER2, pERK1/2 and ERα^{S118} were not significant prognostic factors according to the multivariate analysis with reference to RFS and BCCS. Only histologic grade according to Elston–Ellis (RFS and BCCS) and lymph node status (BCCS) were statistically significant in multivariate survival analyses.

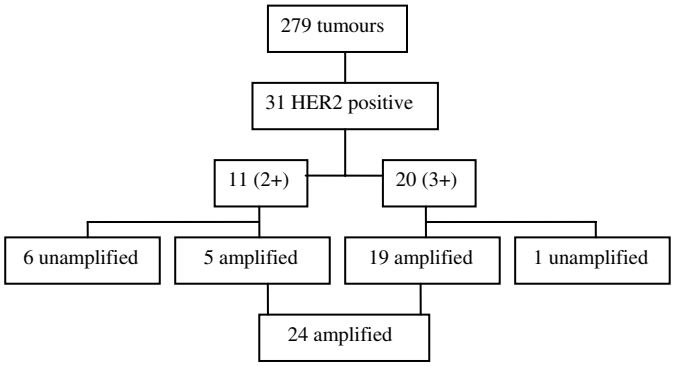

Fig. 2 – Flowchart of immunohistochemical and fluorescence in situ hybridisation analyses of HER2 in all patients (n = 279).

Table 2 – Univariate analysis (Kaplan–Meier log-rank) of relapse-free survival (RFS) and breast cancer-corrected survival (BCCS) in all 279 patients

Factor	Univariate RFS		Univariate BCCS	
	Patients surviving (%)	P	Patients surviving (%)	P
pERK1/2 positive vs. negative	90 vs. 73	0.004	92 vs. 79	0.013
pERK1/2 weak vs. intense staining	–		–	
pERK1/2 in surrounding tissue positive vs. negative	90 vs. 76	0.032	–	
ERα^{S118} positive vs. negative	–		–	
HER2 amplified vs. unamplified	–		–	
ER positive vs. negative	80 vs. 68	0.050	–	
PgR positive vs. negative	82 vs. 64	0.002	85 vs. 75	0.037
Histological grade[a] 1 vs. 2 vs. 3	**95 vs. 79 vs. 60**	**<0.0001**	**98 vs. 84 vs. 66**	**<0.0001**
Tumour size ⩽20 mm vs. >20 mm	–		–	
0–3 vs. ⩾4 lymph node metastases	80 vs. 56	0.012	**84 vs. 62**	**0.008**
Age ⩽5 0 vs. >50 years	–		–	

–, Not significant.
Values which do not remain significant in multivariate analyses in bold type.
a Histological grade according to Elston–Ellis. Estimates at median follow-up of 104 months (8.7 years).

4.3. *Analysis of HER2, pERK1/2, and ERα^{S118} in the tamoxifen treated women (n = 108)*

In these 108 women, we found 10 with HER2 amplified tumours (9%) and HER2 determined by FISH was significantly associated with high Elston score ($P = 0.01$) and inversely associated with the PgR ($P = 0.01$). In addition we found pERK1/2 to be correlated with pERK1/2 in the surrounding connective tissue ($P = 0.01$), ERα^{S118} ($P = 0.01$) and low Elston score ($P = 0.01$).

4.4. *Analyses of survival in the tamoxifen-treated patients (all ERα positive; n = 108)*

Patients with HER2 amplification had significantly worse RFS ($P = 0.001$; Fig. 3), BCCS ($P = 0.0002$; Fig. 3), and OAS ($P = 0.006$; not shown). Women with intensely pERK1/2-stained tumours had significantly better RFS ($P = 0.05$) and a trend towards more favourable BCCS ($P = 0.07$) compared to those with tumours that were weakly to moderately stained for that protein (Fig. 3). These trends were also seen for pERK1/2-positive tumours when the results were dichotomised into negative and positive staining (RFS $P = 0.16$ and BCCS $P = 0.09$, not shown). When performing the two different pERK1/2 analyses, we took into consideration the differences in cellular responses to high and low expression of pERK1/2 that had been reported by Rul and colleagues.[14] Univariate and multivariate estimates of RFS and BCCS are given in Tables 3 and 4, respectively. In multivariate analyses, only HER2 established by FISH was of prognostic significance for both RFS ($P = 0.007$) and BCCS ($P = 0.007$).

5. Discussion

We used immunohistochemical and FISH techniques in our study and demonstrate the expression of pERK1/2, ERα phosphorylated on the serine residue 118 and HER2 in human breast cancer. To date, our investigation represents the largest group of breast cancer patients to be studied concerning the expression and possible prognostic value of the mentioned crosstalk factors in relation to tamoxifen therapy.

We detected staining of activated ERK1/2 in 31% of the tumours, which can be compared with the wide range of 24–72% staining reported by other investigators.[16–18] The results of the cited studies of pERK1/2 expression in breast cancer patients are conflicting, and the populations that were examined in those investigations were not characterised as extensively and were more heterogeneous compared with our cohort of patients. Furthermore, we did not use the same methods to evaluate pERK1/2. We noted that tamoxifen-treated patients whose tumours were stained intensely for phosphoERK1/2 had better RFS and BCCS, although this was not confirmed by the multivariate analyses. In contrast to our findings, other researchers[17,18] have suggested that pERK1/2 positivity is a negative prognostic factor associated with a

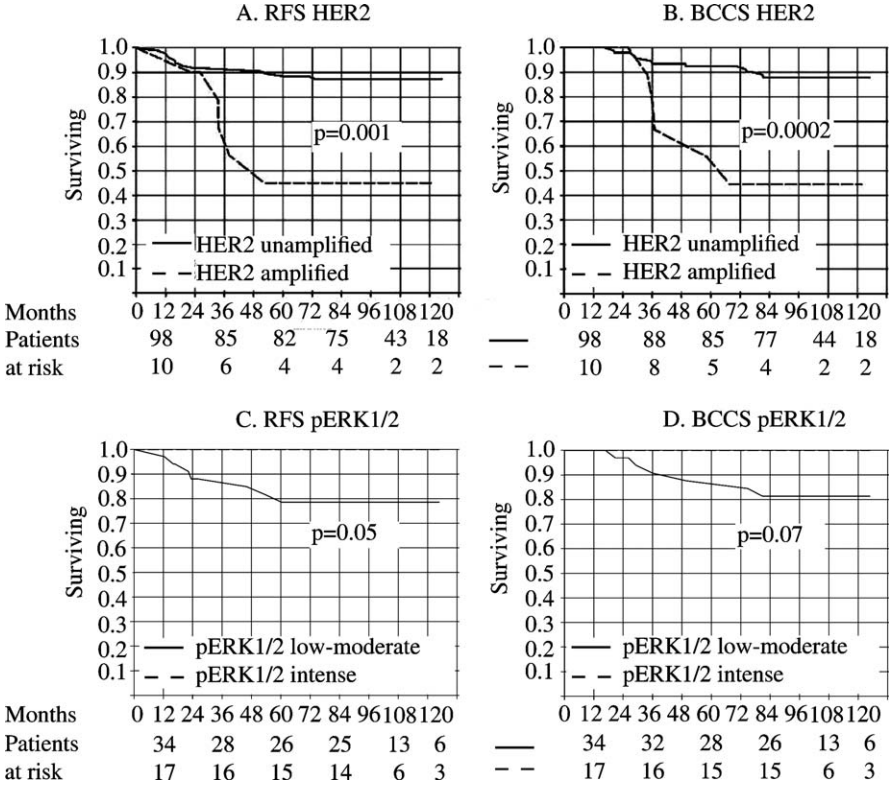

Fig. 3 – Estimates of RFS and BCCS in the 108 tamoxifen-treated patients with respect to expression of HER2 and pERK1/2 (Kaplan–Meier log-rank). (A) RFS of women with unamplified vs. amplified expression of HER2 ($P = 0.001$). (B) BCCS of women with unamplified vs. amplified expression of HER2 ($P = 0.001$). (C) RFS of intense vs. low to moderate expression of pERK1/2 ($P = 0.05$). (D) BCCS of intense vs. low to moderate expression of pERK1/2 ($P = 0.07$).

Table 3 – Univariate (Kaplan–Meier log-rank) and multivariate (Cox proprotional hazard model) analyses of relapse-free survival (RFS) in tamoxifen-treated women (n = 108)

Factor	Univariate RFS		Multivariate RFS		
	Patients without relapse (%)	P	HR	(95% CI)	P
pERK1/2 positive vs. negative	–			–	
pERK1/2 weak vs. intense staining	74 vs. 100	0.05		–	
pERK1/2 in surrounding tissue positive vs. negative	–			–	
ERα^{S118} positive vs. negative	–			–	
HER2 amplified vs. unamplified	50 vs. 88	0.0010	5	(1.54–16.67)	0.007
PgR positive vs. negative	–			–	
Histological grade[a] 1 vs. 2 vs. 3	100 vs. 80 vs. 65	0.007		–	
Tumour size ⩽20 mm vs. >20 mm	90 vs. 74	0.02		–	
0–3 vs. ⩾4 lymph node metastases	87 vs. 50	0.05		–	
Age ⩽50 vs. >50 years	–			–	

–, Not significant.
a Histological grade according to Elston–Ellis. Estimates at median follow-up of 104 months (8.7 years).

Table 4 – Univariate (Kaplan–Meier log-rank) and multivariate (Cox proprotional hazard model) analyses of breast cancer-corrected survival (BCCS) in tamoxifen-treated women (n = 108)

Factor	Univariate BCCS		Multivariate BCCS		
	Patients surviving (%)	P	HR	(95% CI)	P
pERK1/2 positive vs. negative	–			–	
pERK1/2 weak vs. intense staining	–			–	
pERK1/2 in surrounding tissue positive vs. negative	–			–	
ERα^{S118} positive vs. negative	–			–	
HER2 amplified vs. unamplified	50 vs. 89	0.0002	5.26	(1.56–16.67)	0.007
PgR positive vs. negative	–			–	
Histological grade[a] 1 vs. 2 vs. 3	100 vs. 82 vs. 65	0.004		–	
Tumour size ⩽20 mm vs. >20 mm	90 vs. 77	0.03		–	
0–3 vs. ⩾4 lymph node metastases	88 vs. 50	0.02	1.82	(1.12–1.96)	0.034
Age ⩽50 vs. >50 years	–			–	

–, Not significant.
a Histological grade according to Elston–Ellis. Estimates at median follow-up of 104 months (8.7 years).

shortened response to endocrine therapy and shorter survival after initiation of anti-oestrogen therapy. However, it has also been reported that it is unlikely that pERK1/2 can be used as a marker of endocrine sensitivity.[16] In our tumour material the ones with pERK1/2-positive staining have tumourbiologic prolifes similar with HER2 negative tumours which agree with their better survival compared with pERK1/2 negative tumours. The contradictory data regarding the use of pERK1/2 as a prognostic marker might be partly explained by the use of different analytical methods and a lack of standardisation in the evaluation of the activated protein. In addition, it is possible that cells respond differently to different degrees and temporal modes of ERK1/2 activity.[14,19] Preclinical reports have suggested that ERK1/2 activity is involved in both cell proliferation,[19] apoptosis[20] and differentiation.[14] Alblas and colleagues found that MCF-7 cells proliferated in response to short bursts of ERK1/2 activity, but only responded by differentiation to more prolonged ERK1/2 phosphorylation. From this standpoint, it might be argued that microenvironmental/external circumstances are important in determining whether the effects of pERK1/2 will result in survival, apoptosis, differentiation or proliferation.[14]

Similar to previous studies, ERα^{S118} was associated with ERα and pERK1/2, and both ERα^{S118} and pERK1/2 were significantly correlated with other markers of good prognosis, such as low Elston grade and small tumour size.[21,22] Also in the tamoxifen-treated (ERα-positive) women, we found a significant association between ERα^{S118} and pERK1/2, which suggests that pERK1/2 participates either directly or indirectly in the activation of ERα^{S118} in human breast cancer.[22]

The population we studied comprised relatively few HER2-positive tumours (9% and 11% according to FISH and IHC, respectively), although the rate we detected is not lower than the previously described range of 5–55%.[23–25] The number of ERα-positive and lymph node-positive tumours was statistically higher in our study population than in the patients that were initially identified in the cancer registry but subsequently excluded from our investigation. Nonetheless, although we cannot rule out selection bias, the two groups did not differ with respect to age at diagnosis or tumour size

or grade. After correction for multiple tests with the Bonferroni–Holm method, the only significant difference that remained concerned the frequency of ERα-positive tumours. There was no selection bias between the total patient population and the subgroup of tamoxifen-treated women with ERα-positive tumours ($n = 108$).

Modification of endocrine therapy strategies based on HER2 status is still a controversial approach due to the inconsistent results regarding a correlation between response to hormone therapy and expression/amplification of *HER2*, even though the majority of reports indicate worse response on tamoxifen in HER2 positive breast cancers.[4,5,7,8,26,27] Love and colleagues[28] have reported that overexpression of HER2 may favourably influence the response to adjuvant endocrine therapy with oophorectomy and tamoxifen, and such treatment is potentially comparable to the action of aromatase inhibitors in postmenopausal women. Data from two clinical trials with neoadjuvant endocrine therapy reinforces the hypothesis that aromatase inhibitors may be more effective than tamoxifen in ER-positive early breast cancer concurrently overexpressing HER2.[29,30] In the 108 tamoxifen treated women our multivariate survival analyses showed *HER2* amplification to be significantly associated with worse RFS, BCCS and OAS.

The worse outcome in HER2-positive women given adjuvant tamoxifen in our material cannot be explained only with the crosstalk that is suggested to occur between HER2 and ERα via activation of ERK1/2. On the contrary, we found that intense expression of activated ERK1/2 in both breast cancer cells and connective tissue surrounding breast tumours indicates prolonged survival. This finding implies that pERK1/2 in our study has a pro-apoptotic function in human breast cancer. In conclusion, our results indicate the need for further studies using exogenous inhibitors of HER2 signalling and other mitogenic pathways to elucidate tamoxifen resistance.

Conflict of interest statement

None declared.

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