Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer; a predictive role for SRC-1

F J Fleming, E Myers, G Kelly, T B Crotty, E W McDermott, N J O’Higgins, A D K Hill, L S Young

Background: In human breast cancer, the growth factor receptor HER2 is associated with disease progression and resistance to endocrine treatment. Growth factor induced mitogen activated protein kinase activity can phosphorylate not only the oestrogen receptor, but also its coactivator proteins AIB1 and SRC-1.

Aim: To determine whether insensitivity to endocrine treatment in HER2 positive patients is associated with enhanced expression of coactivator proteins, expression of the HER2 transcriptional regulator, PEA3, and coregulatory proteins, AIB1 and SRC-1, was assessed in a cohort of patients with breast cancer of known HER2 status.

Methods: PEA3, AIB1, and SRC-1 protein expression in 70 primary breast tumours of known HER2 status (HER2 positive, n = 35) and six reduction mammoplasties was assessed using immunohistochemistry. Co-localisation of PEA3 with AIB1 and SRC-1 was determined using immunofluorescence. Expression of PEA3, AIB1, and SRC-1 was correlated with clinicopathological parameters.

Results: In primary breast tumours expression of PEA3, AIB1, and SRC-1 was associated with HER2 status (p = 0.0486, p = 0.0444, and p = 0.0012, respectively). In the HER2 positive population, PEA3 expression was associated with SRC-1 (p = 0.0354), and both PEA3 and SRC-1 were significantly associated with recurrence on univariate analysis (p = 0.0345; p = 0.0001). On multivariate analysis, SRC-1 was significantly associated with disease recurrence in HER2 positive patients (p = 0.0066).

Conclusion: Patients with high expression of HER2 in combination with SRC-1 have a greater probability of recurrence on endocrine treatment compared with those who are HER2 positive but SRC-1 negative. SRC-1 may be an important predictive indicator and therapeutic target in breast cancer.

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MATERIALS AND METHODS

Patient selection

HER2 positive tumours from patients who received tamoxifen treatment were selected for our study. Each case was matched with a HER2 negative patient of similar age and tumour size at time of diagnosis. HER2 status was evaluated using the Dako (Glostrup, Denmark) HercepTest immunocytochemical assay. Scoring was assessed according to the manufacturer’s instructions. A score was assigned according to the intensity and pattern of cell membrane staining: 0 to +, no staining, or staining in < 10% of cells; +2, weak to moderate staining in > 10% of cells; +3, strong staining in > 10% of cells. In tumour samples scoring +2 with the Hercep test, HER2 status was confirmed by fluorescent in situ hybridisation using the Vysis kit (Abbott Laboratories, Maidenhead, Berkshire, UK) according to the manufacturer’s instructions. Patients who received neoadjuvant chemotherapy or endocrine treatment were not included. Histologically normal breast tissue specimens were obtained from six patients who underwent reduction mammoplasty. All patients received chemotherapy and tamoxifen (20 mg/day) from 0.824 to 0.914 and from 0.743 to 0.871, respectively. These coefficients and the confidence bounds ranged

Immunohistochemistry

Tissue sections (5 µm thick) were cut from paraffin wax embedded breast tumour tissue and reduction mammoplasty blocks and mounted on Superfrost Plus slides (BDH, Poole, Dorset, UK). Sections were dewaxed, rehydrated, and washed in phosphate buffered saline (PBS). Endogenous peroxidase was blocked using 3% hydrogen peroxidase in PBS for 10 minutes. The sections were incubated with the following primary antibodies: rabbit antihuman ERα (1 µg/ml; Novocastra, Newcastle upon Tyne, UK), goat antihuman SRC-1 (1 µg/ml), mouse antihuman PEA3 (10 µg/ml), and rabbit antihuman AIB1 (1 µg/ml; Santa Cruz Technology, Santa Cruz, California, USA) for 60 minutes at room temperature. Sections were subsequently incubated with the corresponding biotin labelled secondary antibody (1/2000 dilution) for 30 minutes, followed by peroxidase labelled avidin–biotin complex. Sections were developed in 3,3’-diaminobenzidine tetrahydrochloride and counterstained with haematoxylin. Negative controls were performed using matched IgG controls (Dako). Sections were examined under a light microscope. Immunostained slides were scored for ERα, SRC-1, PEA3, and AIB1 using the Allred scoring system. Each slide was evaluated using light microscopy. A proportion score was assigned, which represented the estimated proportion of positively stained tumour cells (none, 0; < 1%, 1; > 1% to < 10%, 2; > 10% to < 33%, 3; > 33% to < 66%, 4; > 66%, 5). An intensity score was assigned that represented the average intensity of the positive tumour cells (none, 0; weak, 1; intermediate, 2; strong, 3).

Statistical analysis

Continuous variables were assessed using a two sample t test. Fisher’s exact test was used in the comparison of two proportions throughout or equivalently to test for association in 2 x 2 tables. Where comparisons were made on variables that were correlated, the p value for each comparison was adjusted using Bonferroni’s inequality. A Cox proportional hazards model was used to find significant predictors of disease free survival time. The predictors included in the model were: HER2, axillary status, size of tumour, grade of tumour, PEA3, AIB1, ERα, ERβ, and SRC-1. A stepwise procedure was used to find the best model. Kaplan–Meier

<table>
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<th>Table 1 Clinicopathological parameters of the HER2 positive and HER2 negative groups</th>
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<td>Mean (SE) age</td>
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Continuous variables were analysed using the two sample t test. Nominal variables were analysed using Fisher’s exact test.
Figure 1 PEA3, AIB1, and SRC-1 protein expression in paraffin wax embedded invasive breast carcinoma and normal breast tissue specimens. (A) Immunohistochemical localisation of the HER2 transcription factor PEA3 and the p160 coactivators AIB1 and SRC-1 in primary breast cancer counterstained with haematoxylin and matched IgG controls (original magnification, ×200). (B) PEA3, AIB1, and SRC-1 protein expression was absent in normal breast tissue (original magnification, ×200).

Figure 2 Colocalisation of PEA3 with the coactivators AIB1 and SRC-1 in invasive breast carcinoma. Immunofluorescent colocalisation of PEA3 with AIB1 (original magnification, ×100) and PEA3 with SRC-1 (original magnification, ×200).
estimates of survival functions were used to illustrate significant predictors of survival time. A logistic regression model was fitted to analyse the presence or absence of disease recurrence. SAS statistical software, Version 6, Fourth Edition (SAS Institute Inc 1989) was used to perform these analyses.

RESULTS
Patient characteristics
Table 1 shows the patient characteristics for the entire population and for HER2 positive and HER2 negative groups. The mean (SD) probability of survival beyond five years for the overall group was found to be 0.6146 (0.0825). The two groups were matched for age and tumour size. There was no difference in terms of axillary lymph node status between the two groups; however, patients who expressed HER2 were found to have higher grade tumours (grade III v non-grade III; p = 0.0162; table 1).

PEA3, SRC-1, and AIB1 expression in breast tumours of known HER2 status
The transcription factor PEA3 and the p160 coactivator proteins, AIB1 and SRC-1, were localised within paraffin wax embedded human breast tissue using immunohistochemistry. Strong positive staining for PEA3 was detected within the nuclei of invasive ductal and invasive lobular breast tumour epithelial cells, whereas expression within the cytosol was negligible (fig 1A). Both AIB1 and SRC-1 were expressed in the nuclei of breast epithelial cells, predominantly in those of the duct (fig 1A). Expression of AIB1 was more widespread within the tumour compared with that of SRC-1; this was reflected in the Allred scoring for the coactivators, where the median score over two observers for AIB1 was 5 (0–8), compared with a median score of 1.5 (0–4) for SRC-1 (p < 0.0001). Normal breast tissue did not express PEA3, AIB1, or SRC-1 (fig 1B). Immunofluorescence was undertaken to determine whether the HER2 transcription factor PEA3 could be localised to the same breast tumour cell as the steroid coregulators AIB1 and SRC-1. PEA3 colocalised with both AIB1 and SRC-1 within a subset of breast tumour epithelial cells (fig 2). Where PEA3 and AIB1 or SRC-1 were expressed within the same breast tissue, cellular coexpression of the transcription factor and the coregulators was seen in more than 70% of the positive tumour epithelial cells.

There was no relation between ERα and HER2 status, although ERβ was found to associate inversely with HER2. The rate of expression of PEA3 was significantly higher in HER2 positive patients than in those who did not express the oncogene (69% v 34%; p = 0.0486). AIB1 and SRC-1 were both more frequently expressed in HER2 positive than in HER2 negative patients (p = 0.0444 and p = 0.0012, respectively; table 2).

DISCUSSION
The transcriptional activity of ER is dependent both on its recruitment and interaction with coregulatory proteins. Selective ER modulators, such as tamoxifen, inhibit cancer growth through the competitive antagonism of the ER. Altered concentrations and perhaps activity of coactivator proteins at the ER–oestrogen response element complex may be important in the alteration of the agonist/antagonist profile of ER modulators in resistant tumours. In the breast, the p160 family of coactivators, including AIB1 and SRC-1,
has been implicated in ER signalling and tumour progression.\textsuperscript{15} Expression of AIB1 has been previously demonstrated exclusively in tumour versus normal breast tissue, whereas expression of SRC-1 has been shown in a subset of normal breast tissues adjacent to the tumour.\textsuperscript{17} In our study, no expression of either of these coactivator proteins was seen in normal reduction mammaplasties. There is little consensus within the literature, however, with regard to correlations between the expression of these coactivator proteins and resistance to endocrine treatment.\textsuperscript{26, 27} The specificity and activity of the coactivator proteins is thought to be regulated by intracellular signalling cascades, including phosphorylation via the MAP kinase pathway.\textsuperscript{16} In our study, we have demonstrated significant correlations between coactivator proteins and resistance to endocrine treatment in patients who overexpress the tyrosine kinase receptor HER2. However, based on our restrictive patient selection criteria, definitive clinical implications should be interpreted with caution.

Overexpression of the EGFR family member HER2 has been associated both experimentally and in clinical studies with tamoxifen resistance. Transcriptional regulation of this growth factor receptor is thought to be controlled, at least in part, by the Ets family of transcription factors, including PEA3. In the breast, PEA3 expression has previously been observed exclusively in tumour tissue.\textsuperscript{20} Consensus binding sites for PEA3 have been identified on the promoter region of the HER2 gene.\textsuperscript{21} Although the precise role of PEA3 in the transcription of HER2 remains to be fully elucidated, molecular and clinical studies have suggested a positive role for PEA3 in HER2 mediated breast tumorigenesis.\textsuperscript{3} Consistent with this, we found that the expression of the PEA3 transcription factor was associated with that of HER2 in our cohort of patients with breast tumours. Furthermore, in our cohort of HER2 positive patients with breast cancer, PEA3 was significantly associated with disease recurrence in patients on endocrine treatment.

“We have described a positive correlation between HER2 and its transcriptional mediator PEA3 and the coactivator protein SRC-1, implicating SRC-1 in HER2 mediated resistance to endocrine treatment”

It has been suggested that HER2 plays an essential role in endocrine sensitivity by activating the MAP kinase cascade, resulting in the phosphorylation, not only of ER, but also of coregulatory proteins. Previous studies by Bouras et al have described a significant correlation between AIB1 mRNA and HER2 status.\textsuperscript{22} Here, the expression of the p160 coactivators AIB1 and SRC-1 was associated with HER2 expression; moreover, expression of the HER2 transcription factor PEA3 was also associated with SRC-1 in the HER2 positive population. Immunofluorescence studies revealed PEA3 coexpression with AIB1 and SRC-1. Although this observation does not provide direct evidence of linkage between these factors, it does provide support for an association between PEA3 and p160 proteins. A recent study by Osborne et al found that tumours with high expression of both AIB1 and HER2 have a poor response to tamoxifen.\textsuperscript{10} In our study, we failed to find a correlation between AIB1 and recurrence on endocrine treatment in HER2 positive patients. This discrepancy may be caused, at least in part, by our restricted patient population and the nature of our patient selection—patients with HER2 positive tumours were matched with HER2 negative patients, which resulted in a relatively high recurrence rate. We found that the SRC-1 protein was significantly associated with insensitivity to endocrine treatment; this observation contrasts with that previously reported by Berns et al, who described an association between SRC-1 and a favourable response to tamoxifen.\textsuperscript{23} However, that study examined SRC-1 mRNA values in a limited number of tumour samples. In our cohort of patients with breast cancer, we found that the probability of tumour recurrence on endocrine treatment was significantly higher in patients who expressed HER2 in combination with SRC-1 than in those who expressed HER2 alone. These clinical ex vivo observations support previous molecular in vitro data linking SRC-1 and HER2 in human breast cancer.\textsuperscript{24} We have previously reported that SRC-1 can interact with both isoforms of ER to modulate transcription.\textsuperscript{25} However, in our present study, an inverse relation was seen between ER$^\beta$ and SRC-1 expression, and there was no association detected between ER$^\alpha$ expression and SRC-1 in our HER2 patient population. These observations raise the possibility that the p160 proteins may interact with transcription factors other than ER to modulate gene transcription. Of interest, recent reports have described an interaction between the p160 protein and the MAP kinase activated Ets transcription factor family.\textsuperscript{25} These interactions may be relevant to the development of endocrine resistance, although in depth clinical and molecular studies are required to elucidate this complex transcription factor–coactivator association.

The data reported here have described a positive correlation between HER2 and its transcriptional mediator PEA3 and the coactivator protein SRC-1, implicating SRC-1 in HER2 mediated resistance to endocrine treatment. These results suggest that coactivator proteins, including SRC-1, may serve as essential mediator proteins, bridging the crosstalk between the growth factor/kinase and steroid signalling pathways. These coactivator proteins may serve not only as a complement to HER2 as a predictor of response to endocrine treatment, but may also prove attractive therapeutic targets, lying as they do at the crossroads of the kinase and steroid pathways.

Take home messages

- Patients with breast cancer who have high expression of both HER2 and SRC-1 have a greater probability of recurrence on endocrine treatment compared with those who are HER2 positive but SRC-1 negative
- SRC-1 may be an important predictive indicator and therapeutic target in breast cancer
activator PEA3 are co-ordinately up-regulated in human breast cancer.

Introduction. In:

The design and analysis of clinical experiments

The PEA3 subfamily of ets genes

Ets regulation of the erbB2 promoter.


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