ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis

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Abstract

Extracellular signal-regulated kinase (ERK)1/2 signalling mediates communication between growth factor receptors and the cell nucleus and has been linked to several key events in the transformation process such as proliferation and invasion. We therefore sought to delineate the degree of phosphorylated ERK1/2 in breast cancer and potential links to upstream receptors such as VEGFR2, ErbB2, downstream targets, such as Ets-2, as well as clinico-pathological parameters, clinical outcome and response to tamoxifen. ERK1/2 phosphorylation was assessed by immunohistochemistry using a phospho-specific ERK1/2 antibody in three breast cancer cohorts including a total of 886 tumours arranged in tissue arrays. Cohort I consisted of 114 patients, cohort II of 248 post-menopausal patients randomized to either two years of tamoxifen or no adjuvant treatment and cohort III of 524 patients. Surprisingly, ERK1/2 phosphorylation correlated inversely with tumour size. Phosphorylated ERK1/2 was further associated with the presence of VEGFR2 (cohort II and III) and the degree of phosphorylated Ets-2, indicating *in vivo*, a signalling cascade from VEGFR2 via ERK1/2 to Ets-2 phosphorylation. Interestingly, ERK1/2 phosphorylation correlated with better survival in untreated patients independently of lymph-node status and tumour size indicating that ERK1/2 signalling might be associated with a less aggressive phenotype. Finally, patients with oestrogen receptor positive and ERK1/2 phosphorylated tumours also had an impaired tamoxifen response.

Introduction

Mitogen-activated protein kinases (MAP kinases) are a family of highly conserved serine/threonine protein kinases whose activity is regulated by reversible phosphorylation in response to a wide variety of extracellular signals (Santen et al., 2002; Wetzker & Böhmer, 2003). The functions of the proteins in the cascade are to transduce as well as amplify extracellular signalling from upstream regulators. Three major MAPK signalling cascades exist, the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) cascade, the p38 kinase cascade and the extracellular signal-regulated kinase (ERK) cascade, including ERK1 ($p42^{MAPK}$) and ERK2 ($p44^{MAPK}$). ERK activity has been implicated in diverse cellular activities including cell proliferation, differentiation, migration and cell death (Cobb & Goldsmith, 1995; Schaeffer & Weber, 1999). The ERK1/ERK2 isozymes (ERK1/2) become activated by phosphorylation on both threonine and tyrosine residues by MEK1/2 (Map kinase kinase). Up-stream of MEK1/2 is the signal transducer Raf-1 (Map kinase) and further up-stream is Ras, a low molecular weight G-protein. Receptor tyrosine kinases, activated by extracellular ligands such as growth factors, activate Ras via Shc, Grb2 and Sos. Besides activation as a down-stream target by tyrosine kinase receptors, ERK1/2 can also be activated by signalling cascades initiated by ligands binding to other receptors for example integrins (Chen et al., 1994).

Activated ERK1/2 control a variety of nuclear transcription factors by phosphorylation. The Ets and AP-1/ATF families of transcription factors have been identified as key nuclear mediators of MAP kinase pathway activation (Santen et al., 2002). For example, Ets-2 is a target for activated ERK1/2 and it has been shown that ERK1/2 phosphorylate Ets-2 on threonine 72 in Ras activated NIH3T3 and RAW264 cells (Yang et al., 1996). Further, ERK1/2 are the major enzymes that phosphorylate Ets-2 in macrophages stimulated with colony stimulating factor (CSF) –1 (Fowles et al., 1998). Other enzymes such as Akt (Protein

kinase B) and jun N-terminal kinase (JNK) can also activate Ets-2 through phosphorylation (Smith et al., 2000).

Up to date there are only a few reports studying ERK1/2 in primary breast cancers (Adeyinka et al., 2002; Gee et al., 2001; Mueller et al., 2000; Sahl et al., 1999; Sivaraman et al., 1997). It has nevertheless been shown that ERK1/2 are elevated both at the mRNA and protein level in primary breast carcinomas and in lymph node metastases compared to normal tissue, benign fibroadenoma and fibrocystic disease (Sivaraman et al., 1997) and that ERK1/2 activity is elevated in malignant breast cancer tissue compared to benign fibroadenoma and fibrocystic disease. Whether ERK1/2 expression correlates with ERK1/2 activity remains unclear but in one study ERK2 expression correlated with ERK1/2 kinase activity (Sahl et al., 1999), while others have shown that ERK1/2 expression cannot be linked to the activity (Mueller et al., 2000). Increased ERK1/2 activity also correlated significantly to breast cancer relapse within 40 months of follow-up time compared to patients with low ERK1/2 activity (Mueller et al., 2000) and decreased survival for patients treated with anti-hormonal therapy with advanced primary carcinoma or metastatic disease (Gee et al., 2001).

Vascular endothelial growth factor A (VEGF–A) is expressed by tumour cells and exhibits pro-angiogenic functions by binding to the tyrosine kinase receptor VEGFR2 on endothelial cells (Cross et al., 2003). Furthermore, VEGFR2 is co-expressed with VEGF-A in several malignant epithelial cells (prostate, breast, pancreas and gastric) (Kollermann & Helpap, 2001; Ryden et al., 2003; Takahashi et al., 1996; von Marschall et al., 2000). The expression of VEGFR2 in tumour cells implicates that tumour cells are targets for VEGF-A action via autocrine loops besides pro-angiogenic functions in endothelial cells. The intracellular pathways for VEGFR2 in tumour cells have not been fully detailed. In pancreatic tumour cell lines, VEGF-A stimulation caused up-regulation of phosphorylated ERK1/2 indicating an intracellular pathway via ERK1/2 (von Marschall et al., 2000). It has also been shown that

VEGF-A induces activation of p38 MAPK pathway in mesothelial cells (Catalano et al., 2003). In endothelial cells the intracellular signalling of VEGFR2 is well characterized and upon VEGF-A activation, VEGFR2 dimerizes and is autophosphorylated in the cytoplasmic kinase domain (Kendall et al., 1999). In primary endothelial cells VEGFR-2 also associates with phospholipase C γ (PLCγ) and activate ERK1/2 via PKC in a Ras independent manner (Takahashi & Shibuya, 1997; Takahashi et al., 1999; Takahashi et al., 2001).

In order to better understand the importance of ERK1/2 signalling in breast cancer and specifically the relevance regarding key features of aggressiveness and proliferation we delineated ERK1/2 phosphorylation using immunohistochemistry in three different cohorts of breast cancer patients including randomized untreated or tamoxifen treated patients with long-term follow-up as well as a mixture of patients with different tumour aggressiveness. One surprising finding was an association between ERK1/2 phosphorylation and small tumours. We also showed that both Ets-2 phosphorylation and VEGFR2 were associated with activated ERK1/2 with some discrepancies between the three analyzed series. Finally, ERK1/2 phosphorylation was significantly associated with good prognosis in untreated post-menopausal breast cancer, whereas patients with oestrogen receptor positive ERK1/2 phosphorylated tumours did not respond to tamoxifen treatment.

Materials and methods

Patients and tumour samples

Breast cancer cohort I included 114 patients diagnosed with breast carcinoma. The median age was 60 (range 30-88), median tumour size 22 (8-100) and 53 patients were node negative and 48 node positive. Nodal status was missing in 13 cases. The median follow-up time was 79 months. Eighty-two tumours were oestrogen receptor positive and 31 tumours were negative. Oestrogen receptor status was missing in one case.

Breast cancer cohort II included 248 post-menopausal patients (> 55 years old). All patients included in the study were part of a clinical trial (1980-1987) and were randomized to either two years of treatment with tamoxifen (40 mg daily) or no adjuvant treatment (Collaborative, 1998). The median age was 66 (range 55-75), median tumour size 25 (1-76) and 159 patients were node negative and 86 node positive. Nodal status was missing in three cases. The median follow-up time was 18 years. In this cohort 140 tumours were oestrogen receptor positive and 35 tumours were negative. Oestrogen receptor status was missing in 73 cases.

A reference breast cancer cohort (III) included 524 consecutive breast cancer cases diagnosed at the department of Pathology, Malmö University Hospital, between 1988-1992. The median age was 65 (range 27-96), median tumour size 16 (1-100) and 298 patients were node negative and 168 node positive. Nodal status was missing in 58 cases. In this cohort 417 tumours were oestrogen receptor positive and 72 tumours were negative. Oestrogen receptor status was missing in 35 cases.

Tissue microarray and immunohistochemistry

Representative areas of the paraffin embedded tumours in cohorts I-III were marked on the corresponding H&E slides and tissue microarrays then constructed. In brief, two 0.6 mm

tissue cores were taken from each donor block and mounted in a recipient block using a manual arrayer (MTA-1, Beecher Inc, WI). The number of tissue cores per recipient block was limited to approximately 200. Four μm sections were dried, deparaffinized, rehydrated and microwave treated for 2 x 5 minutes in a 10 mM citrate buffer (pH 6.0). All sections were stained in a Dako Techmate^{TM} machine (DAKO A/S, Glostrup Denmark) and visualized using DAB. The antibodies used were an anti-human phospho-p42/p44 MAP kinase (Thr202/Tyr204) antibody (1:100, Cell Signaling Technology^{TM}, Beverly, MA), an anti-human Ets-2 PT72 provided by M.C. Ostrowski (1:500) (Fowles et al., 1998), an anti-human VEGF (A-20) recognizing VEGF 165, 189 and 121 (1:400, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and a rabbit polyclonal Flk-1 VEGFR2/KDR antibody (1:1000, Santa Cruz Biotechnology Inc.). The phospho-ERK and phospho-Ets-2 stainings were scored as percent positive tumour cell nuclei, 0 as negative, 1 as 1-30%, 2 as 30-70% and 70-100% positive tumour cell nuclei. For the phospho-ERK antibody both stromal and tumour cell nuclei were separately evaluated whereas only tumour cell nuclear reactivity was assessed with the phospho-Ets-2 antibody. VEGF-A and VEGFR2 localised to the cytoplasm of tumour cells and scored according to staining intensity from 0 representing lack of staining, 1 as low staining intensity, 2 as intermediate staining and 3 as intense staining. All tumour arrays were examined by two investigators and all disconcordant results re-evaluated and a mutual conclusive decision was made. Evaluation of Ki-67, cyclin E, D1, p16, p21 in cohorts I-II has been described elsewhere (Lodén et al., 1999; Lodén et al., 2003; Nielsen et al., 1997; Stendahl et al., 2004)

Cell line array

The breast cancer cell lines MDA-MB-468, T-47D and MCF7 (American Type Culture Collection, Rockville, MD) were used to verify the anti-human phospho-p42/p44 MAP kinase

antibody. MDA-MB-468, CAMA-1 and MCF7 were grown in RPMI 1640 supplemented to contain 10% FCS and 1 mM sodiumpyruvate. T-47D was grown in DMEM supplemented to contain 10% FCS, 10 mM HEPES and 0.2U/ml insulin. Cells were harvested and fixed in 1 ml 4% paraformaldehyde for 30 minutes and when 5 minutes remained 20 µl of Mayer´s hematoxylin was added. The cells were centrifuged at 100 × g for 2 minutes and the paraformaldehyde was removed, 1 ml 70% ethanol was added and the cells were left over night. The cells were then dehydrated using increasing concentrations of ethanol and finally xylen. After dehydration the cell pellets were embedded in paraffin and arranged in a cell line array.

Western Blotting

Cultured cells were washed in 10 ml PBS, harvested and lysed in buffer containing 50 mM Tris-HCl pH 7.5, 0.5% NP-40, 0.5% sodiumdeoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF and 0.1 mg/ml PMSF supplemented with the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). The protein lysates were centrifuged at 19 000 × g for 30 minutes and the supernatants were collected. For detecting phosphorylated ERK1/2, 40 µg protein was run on a 12% polyacrylamide gel and transferred onto nitrocellulose membranes Hybond™ ECL™ (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The membranes were probed with anti-human phospho-p42/p44 MAP kinase (1:500, Cell Signaling Technology) antibodies followed by a peroxidase-conjugated anti-rabbit antibody (1:5000, Amersham Life Science, Alesbury, U.K). The proteins were detected by an enhanced chemiluminescence detection system plus (ECL+ plus™) reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions and exposed on ECL™ Hyper Film™ (Amersham Pharmacia Biotech). After detection of phosphorylated ERK1/2 membranes were re-probed with an anti-human actin antibody (Santa

Cruz Biotechnology Inc.) followed by an anti-goat antibody (1:5000, Sigma) and the bands were detected as above.

Statistical methods

Spearman's rho and chi-square linear by linear association were used for comparison between phosphorylated ERK1/2 and other factors. Log-rank tests and Kaplan-Meier analysis were used for survival analysis and a Cox regression model was used for multivariate analysis. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 11.0 software (SPSS Inc., IL).

Results

Specificity of ERK1/2 antibodies and distribution in tumour tissue microarrays

The specificity of the phospho-ERK1/2 antibodies was first validated using Western blotting in parallel with immunohistochemical analyses of formalin fixed, paraffin embedded cell lines mimicking the handling of the primary tumours. As shown in figure 1, the phospho-ERK1/2 antibodies produced two strong bands of 42 and 44 kDa respectively as well as nuclear and cytoplasmic staining in two of the analysed cell lines (MCF-7 and MDA-MB-468) whereas the phospho-ERK1/2 antibodies produced very faint bands using Western blot and cytoplasmic staining for the two other cell lines (T-47D and CAMA-1) clearly validating the specificity of the phospho-specific ERK1/2 antibody in formalin fixed material. The ERK1/2 phosphorylation status was initially assessed in two different breast cancer cohorts (I and II) arranged in tissue microarrays and it were possible to evaluate tumour cell nuclei for ERK1/2 phosphorylation in a total of 293 out of 362 of the breast carcinoma specimens. Breast cancer material I included 114 patients and in 68 out of 99 tumours there was presence of nuclear phosphorylated ERK1/2 in various amounts (Figure 2). In cohort II, consisting of tumours

from post-menopausal breast cancer patients, nuclear staining was seen in 68 out of 194 specimens. Besides staining in tumour cells, nuclear ERK1/2 staining was also observed in stromal cells (Figure 2). All ERK1/2 data from cohort I and II in tumour cells and in stromal cells are summarised in table 1.

ERK1/2 and relation to other clinico-pathological parameters

We then analyzed potential associations between ERK1/2 phosphorylation and general clinico-pathological parameters such as tumour type, size, grade, stage, hormone receptor status and presence of lymph-node and distant metastases (Table 2). Surprisingly, there was a strong inverse association ($p = 0.001$) between ERK1/2 phosphorylation and tumour size in both cohort I and II (Figure 3). No other parameters were linked to tumour specific ERK1/2 phosphorylation. The presence of stromal ERK1/2 phosphorylation was not linked to any of the clinico-pathological parameters.

ERK1/2 and clinical outcome

Clinical follow-up data were available for all patients in cohorts I and II and they were analysed separately. In cohort I, 27 out of 114 patients died from breast cancer with a median follow-up time of 79 months and in cohort II 164 out of 248 patients with a median follow-up of 18 years. When all patients were included neither tumour specific nor stromal ERK1/2 phosphorylation was associated with survival in cohort I or in cohort II. Cohort I consisted of a mixture of patients with different treatments and any potential prognostic information could not be separated from predictive information regarding response to a specific treatment. We therefore analyzed patients randomized to no treatment in cohort II separately in order to focus on this issue. In untreated patients, tumour specific phosphorylated ERK1/2 correlated significantly with overall survival ($p = 0.017$; Figure 4). Also in a Cox multivariate-analysis

including patient age, ERK1/2 phosphorylation, tumour size and lymph-node status, tumour specific ERK1/2 phosphorylation was significantly associated with survival (p = 0.012, Table 3).

ERK1/2 and tamoxifen response

The potential effect of ERK1/2 phosphorylation status on tamoxifen response could also be delineated by comparing randomised untreated and tamoxifen treated patients in cohort II. Overexpression of cyclin D1 was strongly associated with an impaired tamoxifen response in this patient cohort and, as shown earlier, the strongest tamoxifen response was observed in tumours with more than 90% oestrogen receptor α (ER) positive cells (Stendahl et al., 2004). In ER positive tumours (>90% positive cells) stratified according to ERK1/2 phosphorylation, there was a clear difference in tamoxifen response (Figure 5a, b). Patients with > 1% ERK1/2 phosphorylated tumour cells did not respond to tamoxifen despite the presence of ER (p = 0.662) whereas patients with ER positive but phospho-ERK1/2 negative tumours responded as expected (p = 0.042). The interaction between ERK1/2 phosphorylation status and tamoxifen treatment was analyzed in a Cox multivariate analysis including variables for ERK status, tamoxifen treatment, tumour size, age at diagnosis and an interaction variable (ERK1/2 phosphorylation (+/-) x tamoxifen treatment (+/-)). The p-value for the interaction-variable was 0.083, indicating that there was a trend towards a difference in tamoxifen response between patients with tumours positive for phosphorylated ERK1/2 and patients negative for phosphorylated ERK1/2.

ERK1/2 and relation to other tumour biological markers

Increased ERK1/2 activity has been shown to induce both cell proliferation and cell cycle arrest depending on the duration of the ERK1/2 phosphorylation. In different models and cell

line systems, sustained ERK1/2 activation led to increased levels of cyclin D1 mRNA (Roovers & Assoian, 2000; Roovers et al., 1999; Weber et al., 1997) whereas strong levels of ERK1/2 activation instead was followed by an upregulation of p21 mRNA levels, thus inhibiting cell cycle progression (Bottazzi et al., 1999; Sewing et al., 1997). We therefore studied the relationship between ERK1/2 phosphorylation and proliferative activity as well as links to selected cell cycle regulators. We further wanted to delineate any potential activating events for ERK1/2 such as VEGF-A, VEGFR2 and ErbB2. Many of these parameters had previously been analyzed in cohort I (Lodén et al., 2003; Ryden et al., 2003) and potential associations could therefore easily be determined. Interestingly, increased ERK1/2 phosphorylation correlated significantly but weakly with decreased Ki-67 index ($p = 0.04$). There was no association to protein levels of cyclin D1 determined by Western blotting nor was there any association to p21 or p16. Regarding activating events, no associations were found to VEGF-A, ErbB2 or VEGFR2 in cohort I (Table 4). In cohort II however, strong correlations were observed between ERK1/2 phosphorylation and VEGF-A ($p = 0.017$) and VEGFR2/KDR ($p < 0.001$). In cohort II there was also an association between ERK1/2 phosphorylation and the nuclear intensity of cyclin D1 ($p = 0.004$) determined by immunohistochemistry. The discrepancy between associations with cyclin D1 in the two first cohorts might be due to the different detection methods used.

ERK1/2 and relation to Ets-2 phosphorylation

In order to further validate the ERK1/2 phosphorylation in the tumours we analyzed the presence of Ets-2 phosphorylation in cohorts I and II using a phospho-specific antibody. Ets-2 is a transcription factor downstream of ERK1/2 that has been experimentally linked to ERK1/2 phosphorylation but not yet been studied in larger tumour series. The specificity of the phospho-Ets-2 antibody was determined in a similar setting as for the phospho-ERK1/2

antibody by comparing Western blotting with immunohistochemical staining of corresponding cells cultured on glass slides. Strong bands correlated with positive nuclear cell staining whereas negative cells produced no bands (Smith et al. 2000). In cohort I 69 out of 88 tumours and in cohort II 106 of 165 tumours were positive for phosphorylated Ets-2. Among the analyzed clinico-pathological data and cell cycle regulators as well as ERK1/2 phosphorylation there was a strong inverse association between Ets-2-phosphorylation and tumour size ($p = 0.006$ cohort I, $p = 0.012$ cohort II) as well as a positive link to ERK1/2 phosphorylation ($p < 0.001$ cohort I and II).

Erk1/2 in cohort III

Due to some inconsistencies in the results obtained when analysing cohort I and II regarding ERK1/2 phosphorylation and links to upstream and downstream targets we next analysed a third cohort consisting of 524 patients representing a consecutive series of breast cancer. It was possible to evaluate tumour cell nuclei for ERK1/2 phosphorylation in 365 of the 524 tumour specimens. In concordance with the first two cohorts we observed that ERK1/2 phosphorylation correlated inversely with tumour size ($p < 0.001$) whereas there was no correlation to proliferation. ERK1/2 phosphorylation further correlated positively to the upstream receptor VEGFR2 ($p = 0.046$) but not the activator of VEGFR2, VEGF-A. Additionally, ERK1/2 phosphorylation correlated significantly with phosphorylation of the downstream target Ets-2 ($p < 0.001$) and also to cyclin D1 ($p = 0.029$) (Table 4). These data clearly illustrates and validates a signalling cascade from VEGFR2 to ERK1/2 phosphorylation and Ets-2 phosphorylation in breast cancer and also links this pathway to tumour size as summarised in a schematic model in figure 6.

Discussion

In this study we have used three different breast cancer cohorts in order to outline the relevance for MAP kinase activation and specifically ERK1/2 phosphorylation in breast cancer. Key events such as proliferation and invasion are closely linked to ERK1/2 in an experimental setting and we therefore wanted to study ERK1/2 in primary tumours including the whole spectrum of breast cancer and not a limited and selected population represented by many cell lines. The proliferative ability in primary tumours and cell lines also differ considerably, with a large resting cell population in primary tumours in contrast to very few resting tumour cells in cell lines. Breast cancer cohort I included 114 patients in a variety of clinical stages of disease, from very aggressive metastasizing tumours to small, slow growing tumours. Breast cancer cohort II, on the other hand, included 248 postmenopausal breast cancer patients defined as "low risk" at the start of the randomized trial in 1980 including both T1-3 and N0-1 tumours. These patients would not have been characterized as low risk with a more modern definition but were nevertheless randomized to two years of tamoxifen treatment or no adjuvant treatment. The randomized setting allows for more reliable information on the prognostic value of tumour biological parameters, without the interference of adjuvant treatment, as well as the predictive value regarding treatment response. In addition, breast cancer cohort III was used to validate the interrelationship between the study markers.

For the untreated patients in cohort II a strong positive correlation was found between ERK1/2 phosphorylation and overall survival independent of lymph-node status, tumour size and age. It is remarkable that ERK1/2 phosphorylation, besides lymph-node status and tumour size, produced independent prognostic information after 18 years of follow-up. Our results are not consistent with an earlier publication (Mueller et al., 2000) analyzing ERK1/2 activity and

relapse free survival in a group of 131 patients with primary breast cancer with rather short follow-up time. These results showed that high ERK1/2 activity in cytosolic fractions of tumour lysates was linked to increased risk of relapse. Nevertheless, the enrolled patients had received different adjuvant therapies, producing a mixture of prognostic and predictive information and this, in combination with differences in the methods of analysis, could potentially explain the observed discrepancies.

The observed link between ERK1/2 phosphorylation and an impaired response to tamoxifen could be useful when designing new treatment approaches in order to increase the effect of tamoxifen. Our data is further supported by a previous non-randomised small study without an untreated control patient group by Gee *et al* (Gee et al., 2001). ERK1/2 can affect the phosphorylation status of the ER either directly (Kato et al., 1995) or through activation of the 90 kDa ribosomal S6 kinase (Joel et al., 1998), which could result in increased ER activity and might explain the impaired tamoxifen response despite the existence of the ER in the tumours. Phosphorylated ERK1/2 may also affect other proteins associated with the ER pathway and the exact mechanism for the possible interference with tamoxifen response has to be detailed in the future. In addition, upstream signals triggering ERK1/2 phosphorylation, such as ErbB2 and VEGFR2, have been associated with tamoxifen resistance (De Placido et al., 1998, Ryden *et al. submitted*).

When examining the relationship between activated ERK1/2 in cohort I-II and known prognostic variables, there was no association between phosphorylated ERK1/2 and tumour type, grade, stage, hormone receptor status and presence of lymph-node and distant metastases. On the other hand, a strong and inverse association between phosphorylated ERK 1/2 and tumour size was observed in cohorts I-III.

In cohort II we observed a strong positive correlation between phosphorylation of ERK1/2, VEGF-A and VEGFR2 which was not detected in cohort I. We therefore analysed cohort III

and found a significant correlation between VEGFR2 and phosphorylated ERK1/2, but no correlation to VEGF-A. ERK1/2 are reversibly phosphorylated by a panel of different protein kinases and upstream signalling molecules as a result of activation of different receptor tyrosine kinases and G-protein coupled receptors. The intracellular signalling pathways for VEGFR2 in tumour cells is not fully explored, but results from VEGF-A stimulation *in vitro* of different types of tumour cells expressing VEGFR2 (pancreas and mesothelial tumours) indicate that MAPK signalling is activated both via p38 and ERK1/2 cascade (Catalano et al., 2003). This is, however, the first report demonstrating a significant association between phosphorylated ERK1/2 and VEGFR2 *in vivo.*

Ets-2 is a downstream target of ERK1/2 that has been shown to interact with co-regulatory transcription factors e.g. basic helix loop helix transcription factors and the jun family of proteins. Depending on the interaction partner, Ets-2 might activate or repress the transcription of numerous of target genes involves in invasion such as urokinase plasminogen activator (uPA) and matrix metallo proteinase -9 (MMP-9) (Watabe et al., 1998) as well as cell cycle regulatory proteins for example $p16^{INK4a}$ (Ohtani et al., 2001). In this study, we have found a close link between Ets-2 phosphorylation and ERK1/2 phosphorylation in breast cancer. To our knowledge, such a link has never been reported previously in primary tumours. We also observed a strong inverse association between Ets-2 phosphorylation and tumour size in all cohorts, potentially linking Ets-2 in breast cancer with processes influencing limitations in tumour size as proliferation, apoptosis and infiltrative growth properties. The downstream target genes activated by Ets-2 in primary breast carcinoma remain to be elucidated.

Taken together, we have used three different cohorts consisting of 886 tumours and successfully analyzed a total of 694 primary breast tumours for ERK1/2 phosphorylation in order to investigate the relationship between phosphorylated ERK1/2 and clinico-pathological factors such as tumour type, size, grade, stage, hormone receptor status and presence of

lymph-node and distant metastases. We have shown that activated ERK1/2 correlated with smaller tumours and a less aggressive tumour type. These results were further supported by the fact that activated ERK1/2 correlated with a better prognosis in untreated patients. Further, we have shown that activated ERK1/2 and phosphorylated Ets-2 are strongly associated in breast cancer. Phosphorylated Ets-2 is one of the downstream transcription factors that, together with other transcription factors, execute the effects of an activated ERK1/2 pathway. In summary, by analyzing tumour material from three different patient cohorts, we have been able to outline an entire signalling cascade from VEGFR2 via ERK1/2 to Ets-2 phosphorylation in primary breast cancer.

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Table 1.
Summary of data obtained from the evaluation of phospho-ERK1/2 staining in cohort I and II.

	Cohort I		Cohort II	
Number of cells with nuclear positivity	Tumour cells	Stromal cells	Tumour cells	Stromal cells
70-100%	11	19	11	17
30-70%	20	32	17	30
1-30%	37	29	40	38
0%	31	16	126	108
Total evaluated	99	96	194	193
Missing	15	18	54	55
Total	114	114	248	248

Table 2.
Associations between tumour specific ERK1/2 phosphorylation and clinico-pathological parameters (cohort I).

Parameter (n = number of patients)		Nuclear phospho-ERK staining				*P-value*[a]
		0	+	+ +	+++	
Tumour size						0.001
(<22mm)	(n = 47)	9	17	11	10	
(≥22mm)	(n = 46)	20	19	7	1	
Disease stage						0.604
I	(n = 25)	6	11	5	3	
II	(n = 57)	22	19	11	5	
III	(n = 0)	0	0	0	0	
IV	(n = 8)	1	4	1	2	
Histological typ						0.349
Ductal	(n = 87)	29	29	19	9	
Lobular	(n = 6)	1	3	0	2	
Lymph node status						0.132
pN0	(n = 48)	12	20	11	5	
pN+	(n = 40)	18	13	5	4	
Oestrogen receptor						0.079
Negative	(n = 28)	8	17	3	0	
Positive	(n = 70)	23	20	17	10	
Progesterone receptor						0.090
Negative	(n = 37)	11	20	5	1	
Positive	(n = 60)	19	17	15	9	
NHG						0.113
1	(n = 19)	4	7	7	1	
2	(n = 38)	12	12	6	8	
3	(n = 42)	15	18	7	2	

[a] Correlations were calculated using chi-square linear by linear association.

Table 3.
Multivariate Cox analysis for overall survival in relation to tumour size, nodal status, age at diagnosis and ERK1/2 phosphorylation (cohort II).

Prognostic Factor	*Relative risk*	95% CI Lower	95% CI Upper	*P-value*
Tumour size	1.82	1.07	3.11	0.027
Nodal status	3.36	1.96	5.77	<0.001
Age at diagnosis	2.73	1.60	4.65	<0.001
ERK1/2 phosphorylation	0.49	0.28	0.86	0.012

Table 4.
Associations between tumour specific ERK1/2 phosphorylation and potential activators as well as downstream effectors.

Activators/Effectors	Cohort I n	Cohort I *R-value*[a]	Cohort I *P-value*[a]	Cohort II n	Cohort II *R-value*[a]	Cohort II *P-value*[a]	Cohort III n	Cohort III *R-value*[a]	Cohort III *P-value*[a]
VEGFR2	95	-0.01	n.s.	191	0.23	<0.001	279	0.12	0.046
VEGF-A	96	-0.15	n.s.	192	0.17	0.017	282	0.01	n.s.
Cyclin D1 (WB[b])	95	-0.09	n.s.	n.d.			n.d.		
Cyclin D1 (IHC[c])	n.d.			164	0.23	0.004	353	0.12	0.029
P16	95	-0.01	n.s.	n.d.			n.d.		
Phospho-Ets-2	80	0.59	<0.001	159	0.33	<0.001	343	0.51	<0.001

n.s. non significant, n.d. not done.
[a] Associations were calculated using Spearman´s rho.
[b] Western blotting.
[c] Immunohistochemistry.

Legends to figures

Figure 1.

Validation of the phospho-ERK1/2 antibody using Western blotting and immunohistochemistry. Phosphorylated ERK1/2 was present in MCF7 and MDA-MB-468 as shown by positive nuclear and cytoplasmic staining, cytoplasmic staining were present in CAMA-1 and T-47D. Correspondingly, protein extracts form MCF7 and MDA-MB-468 gave two intense bands of approximately 42 and 44 kDa using Western blotting and protein extracts from CAMA-1 and T-47D produced very faint bands.

Figure 2.

A breast cancer tissue microarray stained immunohistochemically with the phospho-ERK1/2 antibody (brown). Nuclear staining of different intensities and fractions were observed in both tumour cells and non-tumour cells.

Figure 3.

Box plot showing an inverse relationship between in tumour size and fraction of ERK1/2 phosphorylation (cohort I).

Figure 4.

Overall survival for 100 untreated postmenopausal women with breast cancer according to phospho-ERK1/2 staining. The tumours were divided into three groups, high and moderate (100-30%, n = 16), low (30-1%, n = 21) and negative staining (0-1%, n = 63).

Figure 5.

Overall survival showing the response to tamoxifen treatment versus no treatment for patients with more than 90% oestrogen positive tumour cells in (a) phospho-ERK1/2 negative patients or (b) phospho-ERK1/2 positive patients.

Figure 6.

A schematic model based on the significant associations between parameters observed in this study (+ denotes positive correlations and – denotes negative correlation).

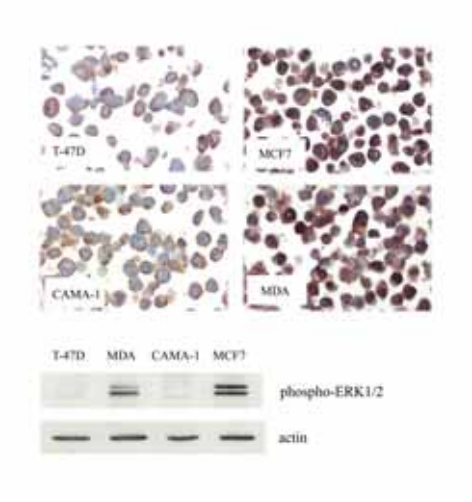

Figure 1

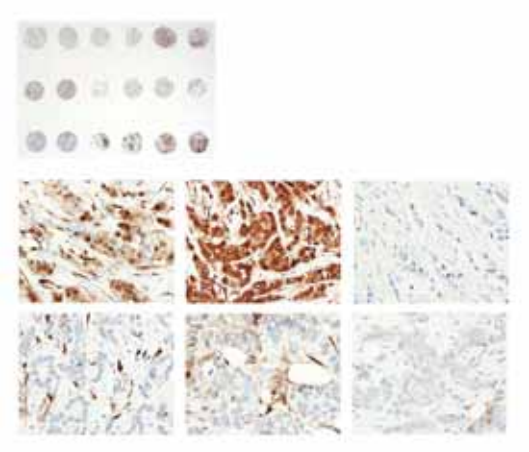

Figure 2

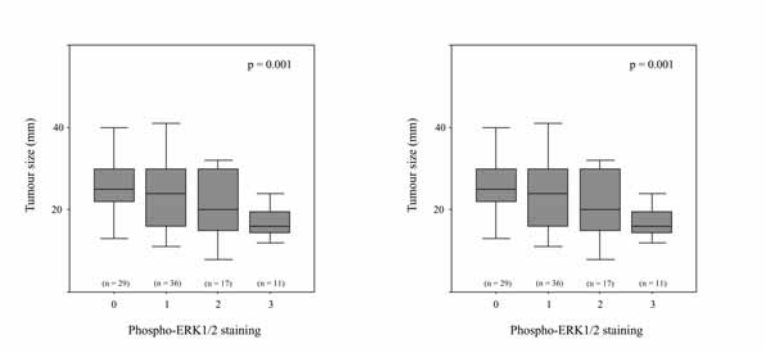

Figure 3

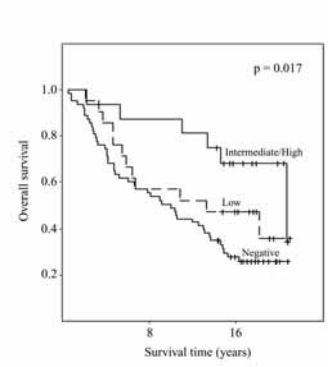

Figure 4

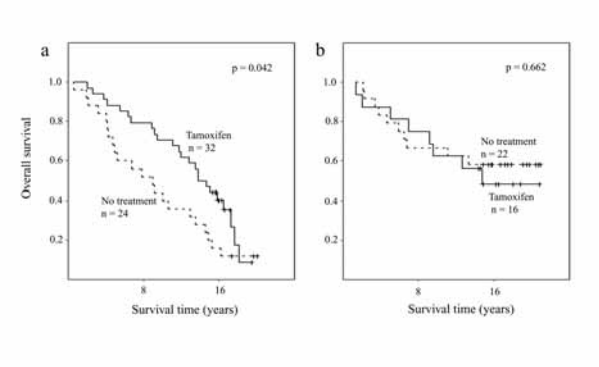

Figure 5

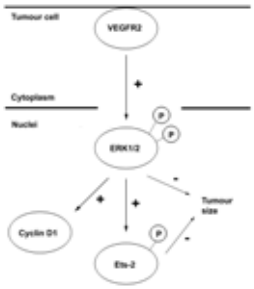

Figure 6