Published in final edited form as:

Int J Cancer. 2010 May 15; 126(10): 2330–2340. doi:10.1002/ijc.24921.

Evidence for tissue factor phosphorylation and its correlation with protease activated receptor expression and the prognosis of primary breast cancer

Lisa Rydén¹, Dorte Grabau², Florence Schaffner³, Per-Ebbe Jönsson⁴, Wolfram Ruf³, and Mattias Belting^{5,#}

¹Department of Clinical Sciences, Section of Surgery, Lund University, Sweden

²Department of Clinical Sciences, Section of Pathology, Lund University, Sweden

³Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, U.S.A.

⁴Department of Surgery, Helsingborg Hospital, Helsingborg, Sweden

⁵Department of Clinical Sciences, Section of Oncology, Lund University, Sweden

Abstract

Tissue factor (TF)-mediated protease activated receptor (PAR)-2 signaling is associated with a pro-migratory, invasive and pro-angiogenic phenotype in experimental models of breast cancer, and has been mechanistically coupled to phosphorylation of the TF cytoplasmic domain (pTF). However, the clinical relevance of these findings are unknown. Here, we provide first *in vivo* evidence of TF phosphorylation in experimental as well as clinical breast cancer tumors. pTF was demonstrated in MDA-MB-231 xenografts and in tumors from the MMTV-PyMT transgene model of spontaneous murine breast adenocarcinoma. Tumors from PAR-2-deficient transgenic mice were negative for pTF, thus linking pTF to PAR-2 signaling. The clinical correlation between TF, pTF, PAR-1, PAR-2, and VEGF-A was determined by IHC on tumors from a cohort of 172 consecutive primary breast cancer patients with a median follow-up time of 50 months. In 160 evaluable patient tumors, pTF was associated with TF ($p=0.01$) and cancer cell expression of PAR-1 ($p=0.001$), PAR-2 ($p=0.014$) and VEGF-A ($p=0.003$) using χ^2 test. PAR-2 and VEGF-A were co-expressed ($p=0.013$) and associated with a more aggressive phenotype. Interestingly, all patients experiencing recurrences had tumors expressing pTF and PAR-2, and pTF alone as well as co-expression of pTF and PAR-2 were significantly correlated with shorter recurrence-free survival (log rank test, $p=0.04$ and $p=0.02$, respectively). This study provides first evidence to link PAR-2 expression and TF phosphorylation to clinical data in human breast cancer. In conjunction with experimental tumor models, these data support an important role of TF-PAR-2 signaling in breast cancer recurrence.

Keywords

Tissue factor; protease activated receptors; VEGF-A; breast cancer

[#]To whom reprint requests should be sent: Mattias Belting, Department of Clinical Sciences, Division of Oncology, Lund University, Barnagatan 2:1, SE-221 85, Lund, Sweden; Fax: +4646-176023; Mattias.Belting@med.lu.se.

Introduction

Cancer is associated with a hypercoagulable state, and the overall risk of venous thromboembolism is increased approx. 7-fold in patients with any malignancy compared with individuals without malignancy (1,2). Tissue factor (TF) plays a fundamental role in the initiation of the coagulation cascade and several experimental models of cancer progression link TF to the aggressiveness of tumor disease (3,4). Accordingly, immunohistochemical studies have found a correlation between high TF expression on tumor cells and an unfavourable clinicopathological phenotype as well as poor clinical outcome in several malignancies (5–7). However, the molecular mechanisms of these findings remain to be elucidated.

TF-initiated coagulation generates thrombin, and this protease plays a central role in platelet activation and fibrin formation during tumorigenesis (8). Importantly, more recent experimental studies provide evidence that TF promotes tumor progression independently of blood clot formation via cleavage-activation of a unique class of G protein-coupled protease activated receptors (PARs), in particular PAR-1 and PAR-2 (3). Both upstream TF-mediated signaling (TF-fVIIa and TF-fVIIa-fXa protease complexes) and downstream coagulation events (thrombin) may contribute to cancer biology through PAR-dependent regulation of tumor cell proliferation, migration, and metastasis, as well as angiogenesis via induction of *e.g.* VEGF-A and IL-8 (9–14). *In vitro* data support an intricate interplay between TF and PAR-2, *i.e.* TF-fVIIa or TF-fVIIa-fXa protease complex mediated activation of PAR-2 has been shown to induce down-stream phosphorylation of the cytoplasmic domain of TF, in particular residue Ser 258 in human TF (15). It has been suggested that PAR-2 dependent TF phosphorylation shuts off the negative regulatory function of the intracellular domain of TF in tumor cell migration and angiogenesis (16,17). It can thus be hypothesized that the phosphorylation status of TF is a marker for an activated TF signaling pathway in tumor cell biology.

The role of TF and PARs in breast cancer is of particular interest. Several experimental studies implicate PAR-2 as a positive regulator of breast cancer cell migration and invasion. Both TF-fVIIa and TF-fVIIa-fXa activation of PAR-2 have been shown to stimulate breast cancer cell migration, in the former case through induction of the pro-migratory cytokine IL-8 (12,13,18,19). The role of PAR-1 appears less clear, as previous studies have reported both stimulatory and inhibitory effects of PAR-1 activation in breast cancer cells (20–22). A specific role of PAR-2 in breast cancer lends support from recent animal studies, showing that PAR-1 deficiency had no effect on the development of palpable tumors, tumor expansion and metastasis in a transgenic model of metastasizing breast cancer (MMTV-PyMT), whereas PAR-2 deficiency significantly delayed the vascularization of spontaneously developing adenomas (23). Mechanistically, these data are consistent with *in vitro* data showing that TF-fVIIa mediated PAR-2 signaling induces pro-angiogenic and immune modulating cytokines and growth factors (24). Moreover, delayed tumor development resulted in decreased metastasis in PAR-2^{-/-} mice, and PAR-2^{-/-} tumors established from MMTV-PyMT mice were shown to grow slower than wild-type tumors, both when re-transplanted into wild-type and PAR2^{-/-} mice. These data indicate that PAR-2 on the tumor cell rather than the host cells is crucial (23,25). The fact that MMTV-PyMT animals deficient in PAR-2 showed delayed tumor expansion and decreased metastasis but still did develop tumors, implies that PAR-2 is not an oncogene required for the initiation and induction of breast cancer development but rather a regulator of tumor aggressiveness. Further support of this notion comes from xenograft studies showing that blocking antibodies directed at either TF or PAR-2 attenuate tumor growth and metastasis (26,27). Notably, it was shown that the presence of purified Fab'2 fragments of a cleavage blocking anti-PAR-2 antibody efficiently inhibited tumor growth of MDA-MB-231mfp

breast cancer xenografts, whereas anti-PAR-1 antibody and IgG controls had no significant effect (27).

In the present study, we have investigated the *in vivo* association of PAR-2 and TF phosphorylation in experimental and clinical breast tumors.

Materials and Methods

Experimental breast tumor models

All animal experiments were performed under approved protocols of the institutional animal use and care committee, The Scripps Research Institute. Human breast cancer xenografts were established by injection of MDA-MB-231mfp cells (2×10^6 cells in 100 μ L PBS) into the mammary fat pad of 6 week-old, female C.B-17 SCID mice (Taconic Farms, Germantown, NY), and allowed to grow for a period of 26 days. The MDA MB 231mfp cell-line was established from the parental MDA MB 231 line by *in vivo* selection in the mammary fat pad of immune deficient mice (28), resulting in increased aggressiveness. MDA MB 231mfp tumor growth was inhibited by antibodies that block TF-VIIa or PAR-2 signaling, validating this cell-line as relevant for studies of TF-dependent breast cancer progression (27). Mammary tumor polyoma middle T antigen (MMTV-PyMT) mice were crossed with PAR-2^{-/-} and TF^{ΔCT} mice. These mice develop breast adenocarcinomas with age in all of the mammary glands. Tumors were harvested from 14–18 week old mice and cryo-preserved. Frozen tumor sections were fixed with methanol, washed with PBS and blocked with PBS containing 5% BSA and 0.1% Tween (mouse tumors). For human breast cancer xenografts, the Mouse-on-mouse kit (Vector laboratories) was used as described by the manufacturer. Tumor sections were incubated with mouse anti-human TF extracellular domain antibody (10H10) directly labelled with Alexa Fluor-547, and either mouse anti-human TF intracellular domain antibody (4H4, non-phospho specific) or a phospho specific mouse anti-human TF antibody (4G6). Mouse tumors were incubated with rat anti-mouse TF antibody (11F6) raised against recombinant mouse TF extracellular domain, or with a rabbit polyclonal antibody raised against a synthetic peptide containing phosphorylated Ser and Thr residues adjacent to the conserved Pro residue of cytoplasmic TF. After several washes, the secondary antibodies were applied (goat anti-rat-FITC [Alexa Probes] or goat anti-rabbit-biotin [Vectastain]). Where appropriate, sections were extensively washed and incubated with streptavidin-Alexa 546 or -488 (Alexa Probes). Staining of mouse tumors with a pan phospho-Tyrosine antibody (Santa Cruz) was performed using the Mouse-on-mouse kit (Vector laboratories). Staining with rabbit anti-Smad2 phosphoserine antibody (Cell Signaling) was performed on paraformaldehyde fixed sections, followed by a goat anti-rabbit Alexa Fluor 546 secondary antibody. DAPI was added for visualization of nuclei and the slides were mounted with special mounting medium for fluorescence (DAKO). Images were acquired with the Bio Rad Radiance 2100 Rainbow laser scanning confocal microscope (Nikon TE 2000-U) and processed with ImageJ and Photoshop CS3.

Patient characteristics

Patients with unifocal primary breast cancer (T < 3cm) and a clinical negative axilla were included prospectively from March 2001 to March 2003 at the department of Surgery in Helsingborg within the National Swedish Sentinel Node Study including a protocol for evaluation of prospective use of immunohistochemistry (IHC) for diagnosis of sentinel lymph node metastasis (29). The present study included a protocol for consecutive immunohistochemical evaluation of all examined sentinel lymph nodes and was closed when the National Guidelines recommended IHC only in doubtful cases. The details of the protocol have been described elsewhere (29). The study was approved by the Ethics Committee at Lund University, Lund, Sweden and written informed consent was obtained

from all patients. The cohort originally included 184 patients; however, patients with predominantly ductal carcinoma *in situ* were excluded from further analysis and therefore no detailed scoring of pure *in situ* cases was performed for any of the analyzed markers. Breast-conserving surgery was offered to 76% of the patients, and patients with a metastatic sentinel lymph node had axillary dissection harvesting on average 13 nodes. After breast-conserving surgery, radiotherapy (50 Gy) was given to the breast, and in patients with three or more axillary lymph node metastases, loco-regional radiotherapy was delivered. Adjuvant treatment was administrated according to the National Guidelines including 5 years of tamoxifen to hormone responsive patients and poly-chemotherapy to hormone receptor negative patients. Hormone responsive patients <50 years with lymph node metastasis had chemo-endocrine treatment. No patient received preoperative treatment. After definitive histopathological examination of the sentinel lymph nodes, 43 patients were categorized as node positive (including 16 with micrometastases and 27 with macrometastases) and 129 as node negative (123 without any nodal involvement and 6 with submicrometastases). Patients were followed by annual mammogram and clinical investigation during a median follow-up time of 50 months.

Immunohistochemistry

Surgical specimens were formalin-fixed and representative parts of the breast carcinomas were paraffin-embedded. Consecutive sections were cut at 4 µm and deparaffinized and rehydrated in graded alcohols. Expression of pTF, TF, PAR-1, PAR-2, and VEGF-A was determined using the DAKO Envision kit K 5007 (an indirect polymer reinforcement technique) in a TechMate 500Plus, (DAKO, Copenhagen, Denmark). Antigen retrieval was performed by treatment in a microwave oven in target retrieval solutions pH 6 or pH 9 (PAR-1 and VEGF-A). Sections were incubated with the primary antibody for 25 min or 2 h (PAR-2). Antibodies used included rabbit anti-human pTF antibody (30) (1:500 dilution), mouse anti-human TF (1D10, American Diagnostica; 1:800 dilution), mouse anti-human PAR-1 (sc-13503, Santa Cruz; 1:150 dilution), mouse anti-human PAR-2 (sc-13504, Santa Cruz; 1:50 dilution), and rabbit anti-human VEGF-A (rb-9031, Neomarkers/Labvision; 1:200 dilution). Diaminobenzidine (DAB) was used for visualization. Negative control sections were performed by omitting the primary antibody in each staining batch, and sections were counter-stained with haematoxylin.

Slides were reviewed by a pathologist (D.G.) blinded to clinical and pathological information. A homogenous staining of tumors for pTF, TF, PAR-1 and PAR-2 was observed; therefore, a scoring system based on percentage of positive cells had no added value. Scoring was performed semi-quantitatively according to staining intensity on a scale as follows: 0 = total negative slide, 1 = weak, 2 = moderate, and 3 = strong intensity. Magnifications ranging from 4x to 40X were used during scoring, and nuclear, cytoplasmic and membranous staining were initially determined separately. For statistical analyses dichotomized scoring was used and tumors with 0 scoring were considered negative and 1–3 positive. Data on staining evaluation on a linear scale is also provided.

Statistical analysis

The association between immunohistochemical expression of pTF, TF, PAR-1, PAR-2, VEGF and clinicopathological parameters (age, tumor size, pathological grade, lymph node status, histopathological type, and hormone receptor status) were calculated by Chi-square test for categorized variables and Chi-square test for trend of variables with more than two categories when analyzing the staining intensity on a linear scale. Recurrence-free survival was determined by the occurrence of local, regional or distant recurrences and contra lateral breast cancer as primary event, and estimated according to Kaplan-Meier method. The log

rank test was used to compare survival in different cohorts of the patients. All calculations were performed in SPSS version 15.0. (SPSS inc., Ill., USA).

Results

PAR-2-dependent tissue factor phosphorylation in experimental breast carcinomas

The phosphorylation status of the TF cytoplasmic domain in breast cancer cells was studied *in vivo*. Human MDA-MB231mfp xenograft tumors grown in immunodeficient mice expectedly stained positive for TF, using antibodies directed at both the extracellular and the cytoplasmic domain (Fig. 1a). Interestingly, a significant fraction of TF-positive tumor cells showed positive staining with an antibody that recognizes pTF (Fig. 1b). In most cases, pTF co-localized with TF in the cytoplasm and plasma membrane, but also in the nuclei of tumor cells. A similar pTF staining pattern was observed in spontaneously formed mammary adenocarcinomas of the MMTV-PyMT mouse (Fig. 2a). To investigate the specificity of the different pTF locations, we next performed pTF stainings on tumors established in mice lacking the TF cytoplasmic domain (MMTV-PyMT/TF^{ΔCT} mice). No cytoplasmic/membranous pTF was detectable in these mice, providing evidence for specificity of the pTF antibody (Fig. 2b). However, punctuate background staining was seen in the nuclei and judged non-specific. Similar punctuate staining was also seen in human tumor samples and because of specificity concerns was not further considered in this study.

Previous *in vitro* studies have demonstrated a specific link between TF-dependent PAR-2 signaling and pTF (15–17). To investigate the significance of PAR-2 in signaling-induced posttranslational modification of TF *in vivo*, pTF expression was studied in tumors from PAR-2-deficient MMTV-PyMT mice (MMTV-PyMT/PAR-2^{-/-}). Consistent with the *in vitro* data, PAR-2^{-/-} tumors were devoid of pTF cytoplasmic staining (Fig. 2c). This result could not be attributed to a total decrease of TF, as there was no apparent difference in the level of non-phosphorylated TF in PAR-2-deficient as compared with wild-type MMTV-PyMT mice. Moreover, the results could not be attributed to a general deficiency of phosphorylation in PAR-2^{-/-}, as stainings for pSMAD2 (serine phosphorylation) as well as pTyr (pan phospho-tyrosine) was comparable in tumors from wild-type and PAR-2^{-/-} mice (Fig. 2d). These results provide the first *in vivo* evidence that the TF cytoplasmic domain can be phosphorylated in breast cancer cells through PAR-2-dependent mechanisms.

TF, pTF, PAR, and VEGF expression in clinical breast carcinomas

We next set out to determine the clinical relevance of the above data by performing IHC analysis of TF and pTF, as well as PAR-1 and PAR-2, *i.e.* the major PARs associated with coagulation protease signaling in tumor cell biology, on whole sections of tumors from a cohort of 172 primary breast cancer patients (29) (see Materials and Methods section and Table I for clinicopathological characteristics). In line with the results from experimental breast tumors (Fig. 1 and Fig 2), pTF stained positive in neoplastic cells with both nuclear and cytoplasmic localisation (Fig. 3a, upper right panel). TF was found mainly in neoplastic cells with membranous and cytoplasmic localization, but in some cases also nuclear and nucleolar localization (Fig. 3a, upper left panel). Only extra-nuclear staining was further considered for scoring, as nuclear TF staining was judged non-specific from experiments in MMTV-PyMT/TF^{ΔCT} mice (Fig. 2). Whereas PAR-2 was found almost exclusively in neoplastic cells with a diffuse and/or characteristic dotted cytoplasmic staining (Fig. 3a, mid panels and Fig. 3b, left panel), PAR-1 was visualized in both neoplastic cells and stromal cells (Fig. 3a, lower left panel).

In vitro studies have suggested a specific correlation between TF and VEGF expression in malignant cells through mechanisms that involve the TF cytoplasmic domain (10,31). It has

further been shown that TF-dependent activation of PAR-2 but not PAR-1 induces the expression of VEGF in human breast cancer cells (14). VEGF showed a diffuse and/or dotted cytoplasmic staining in neoplastic cells, but also in vessels and inflammatory cells of the stroma (Fig. 3a, lower right panel). In order to study whether PAR-2 and VEGF are expressed in the same areas of the tumors, serial sections of the same tumor were stained for PAR-2 and VEGF (Fig. 3b). The findings that PAR-2 and VEGF expression were significantly correlated (Table I) and were present in the same areas of the tumors (Fig. 3b) are consistent with previous studies on the putative role of PAR-2 in promoting VEGF induction (14). pTF was significantly co-expressed with TF, PAR-2 and VEGF, but also with PAR-1. TF was associated with pTF and with borderline significance with PAR-2 and VEGF-A. PAR-1 was strongly correlated with PAR-2, but not with TF or VEGF-A (for details, see Table I).

TF, pTF, PAR, and VEGF expression and relation to clinicopathological characteristics and outcome

In this cohort of primary breast cancer, TF did not correlate with any of the baseline clinicopathological data as given in Table I. Although pTF and PAR-1 showed a tendency of association with high Nottingham histological grade (NHG), it did not reach statistical significance ($p=0.1$). However, PAR-2 and VEGF-A significantly correlated with a more aggressive tumor type with high NHG (VEGF-A and PAR-2) and ER negativity (VEGF-A). High NHG is an independent predictor of both breast cancer-specific survival and disease-free survival in operable, invasive breast cancer as a whole as well as in different subgroups (*e.g.* independent of tumor size and lymph node stage) (32–33). When using a linear scale for expression of the evaluated markers similar results were achieved (Table II). VEGF-A correlated to ER-negativity ($p<0.001$) and high NHG ($p<0.001$) as well as to PR-negativity ($p=0.05$), and PAR-2 to high NHG ($p=0.01$). Interestingly, in this type of analysis, higher levels of TF expression correlated to PR-negativity ($p=0.04$).

The recurrence-free survival (RFS) in the whole cohort was approx. 90% during a median follow-up time of 50 months; a total of eighteen recurrences were encountered including thirteen distant recurrences. All patients with recurrences had tumors expressing pTF ($p=0.03$) and PAR-2 ($p=0.2$), Table I. Furthermore, co-expression of pTF and PAR-2 was found in all evaluable tumors from recurring patients, whereas none of the patients experiencing a recurrence had a tumor negative for co-expression of pTF and PAR-2 ($p=0.02$). The results support a role for PAR-2 induced TF phosphorylation in the prognosis of human breast cancer. Node status ($p=0.003$) and tumor size ($p=0.002$) were significant predictors of RFS by log rank test, whereas NHG $p=0.09$, TF ($p=0.6$), PAR-1 ($p=0.1$), PAR-2 ($p=0.2$) and VEGF-A ($p=0.2$) were not. Interestingly, pTF alone (Fig. 4) as well as co-expression of pTF and PAR-2 (data not shown) was significantly linked to a shortened RFS ($p=0.04$ and 0.02 , respectively).

Discussion

Targeted therapy of cancer is a rapidly expanding field in clinical oncology; however, a major draw-back with these strategies, *e.g.* tyrosine kinase inhibitors and VEGF-blocking antibodies, is lack of markers to predict who will benefit from a given treatment. TF-mediated activation of G-protein coupled PAR receptors emerges as an interesting target for the treatment of breast cancer disease (3,23–27). In this context, phosphorylation of TF may constitute an important determinant of malignant disease accessible for intervention of the TF-PAR signaling pathway.

Although experimental data convincingly show that TF plays a crucial role in tumor growth and metastasis, studies investigating the localization and prognostic significance of TF in

breast carcinoma have produced variable results and patterns of TF expression were frequently found to be inconclusive (34–36). Vrana *et al.* (34) showed no significant difference in TF expression levels between normal and malignant breast epithelia. In contrast, there was a significant correlation between increasing TF levels in the stromal compartment and malignant progression. In another study with 213 specimens of primary breast cancer (36) tumor TF was scored positive (classified as >10% TF-positive cells/tumor area) in 193 (approx. 90%) of cases, and was strongly correlated to stromal TF expression. In the present study, only ~40% of 152 evaluable tumors were positive for TF. The patient groups studied likely explain the different frequency of TF expressing primary tumors, because during a similar follow-up period of ~50 months only 10% of our patients versus 40% in the previous study (34) showed relapse.

However, the antibody reagents to detect TF are not standardized at present and detection of the TF extracellular domain is a technical challenge. Of the available monoclonal antibody reagents in our laboratory, only one antibody reacted reproducibly with formalin-fixed tissues. We have epitope information for the antibody and know that it is not reactive with TF when its ligand VIIa is bound. Under hypoxia, tumor cells ectopically synthesize VIIa (37), which can mask TF detection and thus result in non-homogenous staining for TF. Indeed, our analysis found that the fraction of tumors positive for pTF (~80%) was far greater than tumors that scored positive for TF with antibodies directed to the extracellular domain. Thus, detection of TF cytoplasmic domain phosphorylation avoids the technical difficulties of other approaches while providing a unique approach to identify tumor samples with an activated TF-VIIa-PAR-2 signaling pathway.

We provide the first evidence of TF phosphorylation in neoplastic cells of human xenografts, primary mouse tumors, as well as clinical breast cancer specimens. Similar analysis in PAR-2-deficient mice shows that TF phosphorylation indeed requires PAR-2 signaling in breast adenocarcinomas of the MMTV-PyMT mouse. In the clinical cohort, pTF and PAR-2 were co-expressed in malignant cells of primary tumors ($p=0.014$), and co-expression of the biomarkers was significantly correlated to recurrences ($p=0.02$). In line with this, no relapsing patient had a pTF negative ($p=0.03$) or PAR-2 negative tumor ($p=0.1$). pTF was significantly linked to RFS of primary breast cancer ($p=0.04$), and all patients with pTF-negative tumors were recurrence free during the 50 months follow-up period. Survival data showed a shortened RFS for patients with tumors expressing pTF as well as for patients with tumors co-expressing pTF and PAR-2. Thus, pTF expression correlates with increased PAR-2 signaling and unfavourable outcome in clinical breast cancer. The low number of events did not allow for multivariate analysis, and future studies with *e.g.* a larger cohort of more aggressive breast cancer are clearly needed to confirm the clinical data.

PAR-2 and VEGF-A were related to a more aggressive phenotype as indicated by correlation to higher histological grade (PAR-2 and VEGF-A), and oestrogen receptor negativity (VEGF-A). The correlation of PAR-2 and VEGF expression is in agreement with *in vitro* studies that position PAR-2 upstream of VEGF induction and the documented pro-angiogenic role of PAR-2 in tumor growth (23,27). The role of PAR-1 in breast cancer appears less clear, as previous studies have reported both stimulatory and inhibitory effects of PAR-1 activation in breast cancer cells (20–22), *e.g.* deregulated PAR-1 intracellular trafficking has been linked to persistent PAR-1 signalling (21) and trans-activation of the EGF receptor and ErbB2/HER2 in invasive breast carcinoma, but not in normal mammary epithelial cells (22); however, PAR-1 deficiency had no effect on the occurrence of palpable tumors, tumor expansion and metastasis in the MMTV-PyMT model of spontaneous breast cancer (23), and a PAR-1 blocking antibody did not significantly inhibit tumor growth of MDA-MB-231mfp breast cancer cells (27). The presented results show that PAR-1 is

largely co-expressed with PAR-2 ($p=0.001$) in breast carcinoma, and PAR-1 expression also correlated with pTF ($p=0.001$). However, PAR-1 expression did not correlate with any of the clinicopathological parameters in breast cancer (Table II). While these data do not exclude a role for PAR-1 in clinical breast cancer, it should be emphasized that TF phosphorylation is known to occur specifically downstream of PAR-2 activation but not of PAR-1, and that the regulatory function of the cytoplasmic domain of TF has been specifically linked to PAR-2 signaling (15–17).

In summary, this study provides evidence of TF phosphorylation in experimental as well as clinical breast cancer tumors, and specifically link TF phosphorylation to PAR-2 signaling *in vivo*. The clinical data support an important role of TF-PAR-2 signaling in the prognosis of breast cancer, and identify pTF as a potential biomarker of deregulated TF-PAR-2 signaling in primary breast tumors. pTF staining of other tumor types should more generally clarify the role of TF as a signaling versus a pro-coagulant receptor in cancer.

Abbreviations

ER	oestrogen receptor
IHC	immunohistochemistry
NHG	Nottingham histological grade
PAR-1	protease activated receptor 1
PAR-2	protease activated receptor 2
PR	progesterone receptor
pTF	phosphorylated TF
RFS	recurrence free survival
TF	tissue factor
VEGF-A	vascular endothelial growth factor A

Acknowledgments

This work was funded by: The Medical Faculty and University Hospital at Lund University (ALF); The Swedish Cancer Fund; The Swedish Research Council; The Crafoordska, Gunnar Nilsson, and Berta Kamprad Foundations; The Swedish Society of Medicine (to M.B.); and NIH grant HL-60742, CBCRP (to W.R.).

This study provides first *in vivo* evidence of TF phosphorylation in experimental as well as clinical breast cancer tumors, and specifically link TF phosphorylation to protease activated receptor (PAR)-2 signaling in an experimental model of spontaneous breast adenocarcinoma. In conjunction with experimental tumor models, the clinical data support an important role of TF-PAR-2 signaling in breast cancer recurrence.

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Figure 1. Phosphorylation of TF in a human breast tumor xenograft model

Human breast cancer cells (MDA-MB 231mfp) were implanted into the mammary fat pad of SCID mice. After 26 days of incubation tumors were dissected and frozen sections were fixed and stained with (a) anti-TF extracellular domain (red) and mouse monoclonal non-phospho specific anti-TF cytoplasmic domain (TF-CD; green) antibodies, or (b) anti-TF extracellular domain (red) and mouse monoclonal phospho-specific anti-TF cytoplasmic domain (pTF; green) antibodies as described in the Materials and Methods section. Confocal microscopy analysis shows significant co-localization of TF and pTF in merged pictures. No staining was observed in adjacent mouse tissue. (c) Control staining was performed with isotype matched mouse anti-human IgG and anti-mouse secondary antibody. Blue, DAPI nuclear stain. Scale bar, 10 μ m.

Figure 2. Phosphorylation of TF in murine breast adenocarcinoma

Spontaneously formed mammary adenocarcinomas were dissected from 14–18 weeks old MMTV-PyMT mice. Frozen sections of tumors from wild-type (WT) (*a*), TF cytoplasmic domain deleted (TF Δ CT; *b*) and PAR-2-deficient (PAR-2 $^{-/-}$; *c*) mice were stained for TF (green) and pTF (red) and analyzed by confocal microscopy. The two columns on the right represent magnifications of the indicated areas (white boxes). Scale bar, 20 μ m. Tumors from wild-type mice show cytoplasmic pTF staining (arrows) and punctuate nuclear staining (arrowheads) whereas TF Δ CT and PAR-2 $^{-/-}$ tumors displayed nuclear staining only. Shown are representative stainings of least 3 tumors per group. (*d*) Stainings for pan phospho tyrosine (left column: pTyr, green) and serine phosphorylation (mid column: pSmad2, red) on tumors from wild-type (WT) and PAR-2 $^{-/-}$ mice show comparable phosphorylation in tumors from the two mouse strains. Right column: Control staining with fluorophor-conjugated secondary antibody alone. Blue, DAPI nuclear stain. Scale bar, 10 μ m.

Figure 3. TF, pTF, PAR-2, PAR-1, and VEGF protein expression in human breast cancer surgical specimens

(a) Representative IHC images of the antigens as indicated in the respective panel stained with antibodies and visualized by diaminobenzidine as described under Materials and Methods. Nuclei were counterstained with hematoxylin and appear blue. Positive staining is indicated as a brown precipitate. Original magnification $\times 20$, except for PAR-2, right panel that represents $\times 40$ magnification of the indicated area to visualize the distinct dotted appearance of PAR-2 cytoplasmic staining (arrows). Nuclear and cytoplasmic staining of TF and pTF are indicated by arrows and arrowheads, respectively. Whereas PAR-2 was found almost exclusively in neoplastic cells, PAR-1 was visualized in both neoplastic cells and

stromal cells, which also was the case with VEGF-A, as indicated (“S”). (b) Serial sections of the same tumor were stained for PAR-2 and VEGF-A, confirming co-expression in the same areas of the tumor (magnification $\times 20$). (c) Negative control staining in the absence of primary antibody of the same tumor as in panel b.

Figure 4. pTF predicts poor prognosis in breast cancer

Kaplan-Meier estimates for recurrence-free survival according to pTF expression. The p-value was calculated using the log rank test.

Table 1
Relationship between clinicopathological variables and pTF, TF, PAR-1, PAR-2 and VEGF-A expression

Category	All cases N (%)	pTF neg N (%)	pTF pos N (%)	p- value *	TF neg N (%)	TF pos N (%)	p- value *	PAR-1 neg N (%)	PAR-1 pos N (%)	p- value *	PAR-2 neg N (%)	PAR-2 pos N (%)	p- value *	VEGF neg N (%)	VEGF pos N (%)	p- value *
Age (years)	172	32 (20)	129 (80)		96 (61)	61 (31)		20 (12)	141 (88)		10 (6)	150 (94)		42 (26)	118 (74)	
≤ 50	18	10	14 (11)	0.8	12 (12)	5 (8)	0.4	2 (10)	15 (11)	0.9	1 (10)	16 (11)	0.9	3 (7)	14 (12)	0.4
> 50	154	29 (91)	115 (89)		84 (88)	56 (92)		18 (90)	126 (89)		9 (90)	134 (89)		39 (93)	104 (88)	
Tumour size																
≤ 20mm	141	25 (78)	108 (84)	0.5	78 (81)	52 (85)	0.5	18 (90)	115 (82)	0.3	6 (60)	127 (85)	0.044	31 (74)	100 (85)	0.1
> 20mm	31	7 (22)	21 (16)		18 (19)	9 (15)		2 (10)	26 (18)		4 (40)	23 (15)		11 (26)	18 (15)	
NHG																
I	35	5 (16)	25 (21)	0.11	20 (21)	11 (18)	0.9	3 (15)	29 (21)	0.1	0	32 (21)	0.026	4 (10)	28 (24)	0.001
II	99	24 (75)	72 (56)		56 (58)	38 (62)		16 (80)	80 (57)		10 (100)	85 (57)		35 (83)	59 (50)	
III	38	3 (9)	30 (23)		20 (21)	12 (20)		1 (5)	32 (23)		0	33 (22)		3 (7)	31 (26)	
Node status																
neg	129	23 (72)	98 (76)	0.6	75 (78)	42 (69)	0.2	12 (60)	109 (77)	0.09	7 (70)	114 (76)	0.7	31 (74)	89 (75)	0.8
pos	43	9 (28)	31 (24)		21 (22)	19 (31)		8 (40)	32 (23)		3 (30)	36 (24)		11 (26)	29 (25)	
ER status																
neg	17	1 (3)	13 (10)	0.2	5 (5)	6 (10)	0.3	1 (5)	13 (9)	0.5	1 (10)	13 (9)	0.9	0	12 (10)	0.032
pos	155	31 (97)	116 (90)		91 (95)	55 (90)		19 (95)	128 (91)		9 (90)	137 (89)		42 (100)	106 (90)	
PR status																
neg	55	12 (38)	38 (30)	0.4	26 (27)	21 (34)	0.3	6 (30)	44 (31)	0.9	5 (50)	45 (30)	0.2	13 (31)	36 (31)	1.0
pos	117	20 (62)	91 (70)		70 (73)	40 (66)		14 (70)	97 (69)		5 (59)	105 (70)		29 (69)	82 (69)	
Histopathol. type																
Ductal carcinoma	124	17 (53)	99 (77)	0.001	66 (69)	49 (80)	0.3	9 (45)	107 (76)	0.001	3 (30)	112 (75)	0.002	26 (62)	90 (76)	0.1
Lobular carcinoma	36	15 (47)	19 (15)		23 (24)	10 (16)		11 (55)	23 (16)		7 (70)	27 (18)		14 (34)	19 (16)	
Tubular carcinoma	9	0	8 (6)		6 (6)	1 (1)		0	8 (6)		0	8 (5)		1 (2)	7 (6)	
Mixed type	4	0	3 (2)		1 (1)	1 (1)		0	3 (2)		0	3 (2)		1 (2)	2 (2)	
pTF																
neg	32	20	X	X	26 (27)	6 (10)	0.01	10 (50)	22 (16)	0.001	5 (50)	27 (18)	0.014	15 (26)	17 (14)	0.003

Category	All cases N (%)	pTF neg N (%)	pTF pos N (%)	p- value *	TF neg N (%)	TF pos N (%)	p- value *	PAR-1 neg N (%)	PAR-1 pos N (%)	p- value *	PAR-2 neg N (%)	PAR-2 pos N (%)	p- value *	VEGF neg N (%)	VEGF pos N (%)	p- value *
pos	129 (80)				71 (73)	55 (90)		10 (50)	119 (34)		5 (50)	124 (82)		27 (64)	101 (86)	
TF																
neg	96 (61)	X	X		X	X		15 (75)	82 (59)	0.18	8 (89)	88 (60)	0.08	21 (50)	75 (65)	0.08
pos	61 (39)							5 (25)	56 (41)		1 (11)	60 (40)		21 (50)	40 (35)	
PAR-1																
neg	20 (12)	X	X		X	X		X	X		5 (50)	15 (10)	0.001	5 (12)	15 (14)	0.8
pos	141 (88)										5 (50)	135 (90)		37 (88)	102 (86)	
PAR-2																
neg	10 (6)	X	X		X	X		X	X		X	X		6 (14)	4 (3)	0.013
pos	150 (94)													36 (86)	113 (97)	
Recurrence																
Yes	18 (10)	0	16	0.03	9	7	0.7	1	15	0.4	0	15	0.2	2	15	0.2
No	154 (90)	30	104		80	50		19	115		10	124		37	95	

Abbreviations: TF, tissue factor; pTF, phosphorylated TF; PAR-1, protease activated receptor 1; PAR-2, protease activated receptor 2; VEGF-A, Vascular endothelial growth factor A; N0, node negative; N1, nodal metastases; NHG, Nottingham Histological Grade; ER, oestrogen receptor; PR, progesterone receptor.

* Comparisons between groups by Chi-square test for 2x2 tables and Chi-square test for trend for variables with more than two categories.

Table II
pTF, TF, PAR-1, PAR-2, and VEGF-A on a linear scale in relation to clinicopathological characteristics

Score	pTF No.			TF No.			PAR-1 No.			PAR-2 No.			VEGF-A No.			p-value
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	
ER +	31	83	33	2	91	39	13	3	19	101	14	13	42	84	22	<0.001
ER-	1	9	4	0	5	4	2	0	1	8	2	3	0	6	5	1
PR+	20	64	27	2	70	33	6	1	14	78	10	9	29	68	14	0.05
PR-	12	28	10	0	26	10	9	2	6	31	6	7	13	22	13	1
NHG I	5	22	5	1	20	6	4	1	3	26	1	2	4	24	4	<0.001
NHG II	24	51	21	1	56	28	9	1	16	57	11	11	35	48	11	0
NHG III	3	19	11	0	20	9	2	1	1	26	4	2	3	18	12	1
T1	25	75	33	2	78	38	11	3	18	90	11	14	31	79	20	0.15
T2	7	17	4	0	18	5	4	0	2	19	5	2	11	11	7	0

Abbreviations: See Table I. The p-value indicates comparisons between groups by Chi-square test for trend of variables with more than two categories.