Universiteit
Leiden
The Netherlands

Improving breast cancer outcome by preoperative systemic therapy and image-guided surgery

Mieog, J.S.D.

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Chapter 7

Alternatively spliced and full-length tissue factor reveal a non-identical relationship to clinicopathological parameters in a large cohort of human breast cancer

van den Berg JW[1], Mieog JSD[1], de Kruijf EM, Wang J, Sajet A, Kuppen PJK, van de Velde CJH, Reitsma PH, Osanto S, Bogdanov VY, Versteeg HH, Liefers GJ

[1] Shared first authorship

ABSTRACT

Introduction

Alternatively spliced tissue factor (asTF) - a soluble variant of TF - influences tumor growth and angiogenesis in an integrin-dependent fashion, while full-length TF (flTF) stimulates angiogenesis via protease-activated receptor-2 (PAR2). Therefore, we investigated how TF isoforms relate to clinicopathology in human breast cancer.

Methods

Tumor material and matched normal tissue of 574 breast cancer patients was obtained after primary surgery and assembled in tissue micro arrays, which were stained with antibodies specific for asTF and flTF. The percentage of positive tumor cells was independently scored by two observers. Patients in the first quartiles were deemed negative. Associations of TF expression with clinicopathological parameters and survival were calculated. Subcellular localization of asTF and flTF was investigated in transfected baby hamster kidney cells and human breast cancer sections.

Results

asTF and flTF expression was found in virtually all breast cancer samples (> 95%), whereas normal tissue showed limited expression (asTF = 4%; flTF = 38%). asTF positive tumors were associated with poor histological differentiation and large tumors. flTF positive tumors were associated with poor histological differentiation and expression of the breast cancer stem cell marker ALDH1. asTF and flTF positivity did not influence clinical outcome. However, co-expression of flTF and ALDH1 resulted in poor relapse-free survival in patients younger than 65 years (P = .004), whereas the opposite effect was noted in older patients (P = .08). Unexpectedly, nuclei stained positive for asTF and confocal microscopy and biochemical fractionation confirmed that asTF, but not flTF, was localized in the perinuclear region.

Conclusion

We demonstrated, in the largest cohort to date, expression of both TF isoforms to be common in human breast cancer and to be associated with unfavorable tumor characteristics. flTF and the VII:PAR2 pathway seems to play a role in breast cancer stem cell population. The differences in cellular localizations of asTF and flTF further support the notion that asTF acts through mechanisms distinct from flTF in cancer.

INTRODUCTION

In the mid-nineteenth century, activation of coagulation was already observed to be tightly interwoven with cancer progression.[1] Patients suffering from various types of cancer frequently display Trousseau's syndrome, which is characterized by venous thrombosis of the upper and lower extremities. This thrombotic tendency is putatively caused by tumoral overexpression of full-length tissue factor (flTF), a 47 kDa transmembrane protein, on the surface of cancer cells.[2] Under physiological circumstances, flTF expression is limited to the surface of extravascular cells. Upon damage of the endothelium, flTF becomes exposed to the bloodstream and binds the blood-borne zymogen factor VII (FVII) and subsequently factors Xa, thrombin and fibrin are formed, thus resulting in a blood clot.[3] In a reciprocal manner, activation of coagulation also influences various stages of cancerous disease. The TF:FVIIa complex and thrombin have been implicated in tumor growth and metastasis.[4] For instance, flTF expression in colon cancer is associated with an increased risk of hepatic metastasis.[5] flTF expression in both colon and breast cancer has been suggested to promote tumor growth through modulation of angiogenesis.[6] Extensive research has uncovered that coagulation proteases activate so-called protease-activated receptors (PARs). The TF:FVIIa complex specifically activates PAR2 resulting in altered cell behavior such as proliferation, gene expression and migration.[7] In a spontaneous murine breast cancer model, activation of PAR2 by the TF:FVIIa complex was shown to enhance primary tumor growth via activation of the angiogenic switch.

In 2003, a natural occurring splicing variant of TF, alternatively spliced TF (asTF), was described which lacks exon 5. Due to this exon skipping, a frame shift occurs and hence asTF lacks a transmembrane region, but has a unique C-terminus, rendering asTF soluble.[8] Ever since its discovery, the role of asTF in coagulation has been debated,[9-11] however, an increasing body of evidence indicates that asTF may be involved in determining tumor biology and angiogenesis. In cervical cancer samples, asTF has been detected both on protein and mRNA level.[12] High levels of asTF mRNA confer an unfavorable outcome in non-small cell lung cancer,[13, 14] but in esophageal cancer, asTF mRNA levels did not affect outcome nor clinicopathology.[15] Experiments with murine cancer models revealed that tumor size of asTF-expressing tumors is larger and that those tumors display an enhanced number of tumor vessels.[16] Recently, our group reported that asTF drives angiogenesis in a non-proteolytic, integrin-ligating fashion. Human asTF activates $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrins on endothelial cells, thereby contributing to enhanced cell alignment and migration, respectively, which are indispensable in angiogenesis. In summary, these results suggest that tumor-derived asTF ligates integrins on endothelial cells in a paracrine manner, thus contributing to tumor angiogenesis.[12]

To date, clinical studies investigating the relation between asTF expression at protein level in tumor tissues and clinicopathological characteristics are lacking.

Furthermore, the relative contribution to cancer progression of the protease-mediated flTF axis and the integrin-mediated asTF axis is unclear. Apart from the contribution to tumor angiogenesis, previous studies showed that cancer stem cells are abundant in expression of flTF suggesting that the flTF:FVII:PAR2 axis in cancer stem cells fuels tumor growth and contributes to the maintenance of a chemotherapy resistant subset of tumor cells.[17-19] However, the relationship between flTF and cancer stem cells has not been validated in large patient studies nor in human breast cancer. Moreover, whether asTF has a function in cancer stem cell biology has not been investigated.

In this study, we employ a large cohort of human breast cancer samples with matched normal tissue and a long follow-up in order to clarify the relative contributions of TF isoforms to human breast cancer. Apart from classic clinicopathological parameters, we also investigated the relationship between TF isoforms and cancer stem cells. Moreover, we employed biochemical and microscopic techniques to characterize the tumoral and subcellular localization of asTF and flTF.

MATERIAL AND METHODS

Reagents

Normal goat serum and envision anti-rabbit HRP conjugate was purchased from DAKO (Glostrup, Denmark). The specific rabbit polyclonal human asTF antibody used in this study has been previously described and characterized.[8] A murine monoclonal anti human TF with an epitope against the AA encoded by exon 5 was purchased from American Diagnostica. RL-90 PDI and Histone 3 antibodies were purchased from AbCam (Cambridge, UK). Polyclonal goat-anti-human TF was a kind gift of Dr. W. Ruf (Scripps Institute, La Jolla, CA). Secondary antibodies directed against rabbit or mouse IgG with Alexa Fluor® fluorescent dyes were purchased from Invitrogen (Carlsbad, CA). Vectashield DAPI-containing mounting medium was purchased from Vector Labs (Burlingame, CA).

Cell lines and vectors

Baby hamster kidney cells were kindly provided by Dr. W. Ruf. MCF-7 and MDA-MB-231 cells (ATCC, Manassas, VA) were cultured in DMEM (PAA GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (Bodinco, Alkmaar, the Netherlands) and 50 μg/ml penicillin and 50 μg/ml streptomycin (PAA GmbH). ORFs for asTF (Baseclear) and flTF kind gift from Dr. W. Ruf were subcloned pcDNA3.1 (Invitrogen, Carlsbad, CA) vectors and used when appropriate. Transfections were performed with lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Study cohort

The patient population comprised all non-metastasized breast cancer patients primarily treated with surgery in the Leiden University Medical Center between 1985 and 1994 with tumor material available (N = 574).[20] Patients with bilateral tumors or a prior history of cancer, other than basal cell carcinoma or cervical carcinoma *in situ*, were excluded. The following data were known: age, tumor grade, histological type, TNM stage, local and systemic therapy, locoregional or distant tumor recurrence, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and the breast cancer stem cell marker aldehyde dehydrogenase-1 (ALDH1). All tumors were graded according to current pathological standards. Of 371 patients (65%), normal mammary tissue was available for analysis. Median follow-up was 17.9 years (range: 0.01 to 23.5). Approval was obtained from the Leiden University Medical Center Medical Ethics Committee. All samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Assessment of expression of asTF and flTF

Tissue sections of 4 μm were cut from a previously constructed tissue microarray of formalin-fixed paraffin-embedded tumors of 574 patients from whom tumor material was available and a tissue microarray of formalin-fixed paraffin-embedded normal corresponding mammary tissue of 371 patients. Immunohistochemical staining was performed according to standard procedures. Briefly, sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in MetOH. For asTF stainings, antigen retrieval was omitted whereas sections for flTF staining were subjected to antigen retrieval in sodium citrate buffer for 10 minutes at 100 °C. Sections were blocked for 1 hour with 10% normal goat serum in PBS/BSA 1% at room temperature. Then, sections were incubated overnight at 4 °C with 1 μg/ml anti-asTF or 20 μg/ml anti-flTF. Sections were washed in PBS, incubated for 30 minutes with Envision, washed and visualized in DAB solution. After counterstaining with hematoxillin, sections were dehydrated and covered. Human placenta tissue slides served as positive control, whereas placenta also served as a no primary control tissue for each staining procedure. The percentage of asTF and flTF positive tumor cells was scored by two observers in a blinded manner. Patients in the first quartiles were deemed negative.

Immunofluorescent detection of asTF and flTF

Tissue sections were processed and incubated with primary antibodies as mentioned above. After washing in PBS, sections were incubated with Alexa-488-labeled anti-

rabbit IgG and Alexa-546-labeled anti-mouse IgG for asTF and flTF, respectively. Sections were washed and mounted with DAPI-containing mounting medium. Pictures of the slides were obtained with a confocal laser scanning microscope (LSM510; Carl Zeiss Meditec, Jena, Germany) in a multitrack setting in which the slide is scanned multiple times with a fixed laser–filter pair. Alexa-488 was excited at 488 nm and detected with a 505 to 530 nm band-pass filter. Alexa-546 was excited at 543 nm and detected with a 560 to 615 nm band-pass filter. DAPI was excited at 358 nM and detected with a 455 to 470 nm band-pass filter. The computerized scans presented each fluorochrome signal with an artificial color: green for Alexa-488, red for Alexa-546 and blue for DAPI. All pictures were 512 × 512 pixels, 8-bit depth, and stack size 368.5 × 368.5 µm. A ×25 objective was used (PH2 Plan-NEOFluor 25×/0.80 Imm Korr; Carl Zeiss Meditec). Images were viewed and saved as merged images or as a set of two separate panels in LSM files.

Cell fractionation

asTF- and flTF-transfected baby hamster kidney cells were grown to subconfluency, trypsinized and washed twice with PBS. 1.0 x 10^6 cells were pelleted by centrifugation for 10 minutes at 1200 RPM at room temperature. The cell pellet was resuspended in 1 ml hypotonic buffer and cells were left on ice for 5 minutes. Then, 0.05% nonidet p-40 was added and cells were left on ice for 5 minutes for lysis of non-nuclear phospholipid membranes. The unlyzed nuclei were pelleted by centrifugation at 2000

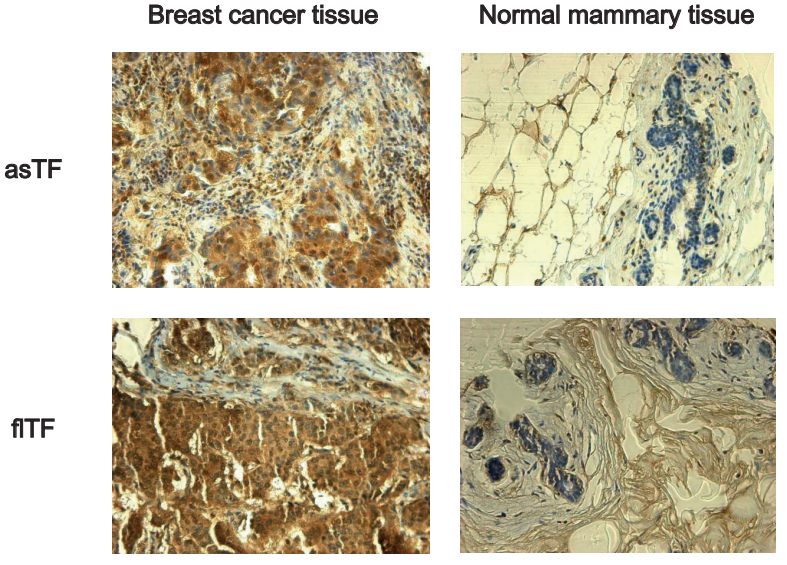

Figure 1. Representative photographs of tissue microarray punches of human breast cancer specimens (left panels) and matched normal mammary tissue (right panels) immunohistochemically stained for asTF (top panels) and flTF (bottom panels). A brown color indicates positive staining.

rpm for 10 minutes at 4 °C and washed three times with nonidet p-40. Proteins from the non-nuclear fraction were precipitated with 4 volumes of ice-cold acetone after which the obtained nuclear and non-nuclear pellets were resuspended in Laemmli buffer. Proteins were detected by western blot and purity of the fractions was assessed by blotting for protein disulfide isomerase (PDI) and Histone 3 as markers for the cytoplasmic and nuclear fractions, respectively.

Statistical analysis

Statistical analyses were performed using the statistical packages SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL) and Stata (version 10.0 for Windows, StataCorp, College Station, TX). Cohen's kappa coefficient was used to assess the inter-observer agreement in quantification of asTF and flTF expression. The Cohen's kappa coefficient was 0.85 and 0.87 for asTF and flTF, respectively. The χ^2 test was used to evaluate associations between various clinicopathological parameters and asTF and flTF expression. Relapse-free period was defined as the time from date of surgery until an event (locoregional recurrence and/or a distance recurrence, whichever came first). Relapse-free period is reported as cumulative incidence function, after accounting for death as competing risk.[21] Relative survival was calculated by the Hakulinen method as the ratio of the survival observed among the cancer patients and the survival that would have been expected based on the corresponding (age, sex, and year) general population. National life tables were used to estimate expected survival. Relative excess risks of death were estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of curves. Analyses were performed for all patients and stratified for age and ALDH1 status. Age of 65 years at time of diagnosis was chosen as the cut-off point for age stratification.[22] An interaction term with age and ALDH1 and flTF status was introduced in Cox proportional hazard model to assess the interaction in prognostic effects of ALDH1 status for the age groups.

RESULTS

asTF and flTF are abundantly, but differentially expressed in breast cancer

Expression of asTF and flTF was found in virtually all breast cancer samples, whereas normal mammary tissue showed limited expression (asTF = 4%, flTF = 38%; Figure 1). At the first quartile, 60% and 95% of tumor cells expressed asTF and flTF, respectively (Figure 2). The association of asTF and flTF expression with clinicopathological characteristics is shown in Table 1. Patients with an asTF positive tumor had tumors

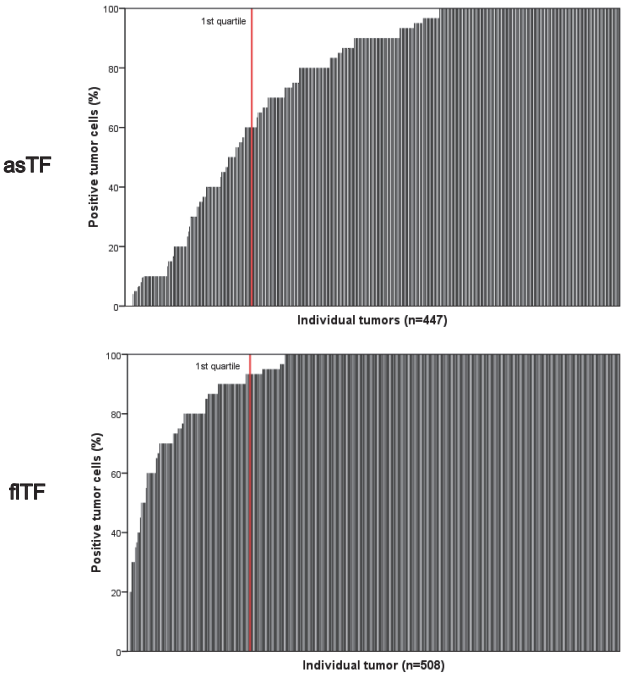

Figure 2. Bar chart indicating the number of positive tumor cells for each individual tumor for staining of asTF (top) and flTF (bottom). The red line marks the first quartile; these patients were deemed asTF or flTF negative.

with a higher histological grade compared to patients with an asTF negative tumor (odds ratio (OR) grade III vs. grade I = 3.88, 95% CI = 2.02 to 7.48, $P < .001$). Moreover, asTF positive tumors were larger in terms of tumor size (OR T3-4 vs. T1 = 2.18, 95% CI = 1.09 to 4.36, $P = .002$). Patients with a flTF positive tumor had tumors with a higher histological grade compared to patients with a flTF negative tumor (OR grade III vs. grade I = 3.12, 95% CI = 1.71 to 5.66, $P < .001$). However, flTF was not associated with tumor size (OR T3-4 vs. T1 = 1.6, 95% CI = 0.86 to 3.81, $P = .26$). flTF expression was highly associated with the expression of the breast cancer stem cell ALDH1 ($P = .001$), whereas asTF expression was not ($P = .84$). In addition, we unexpectedly noted a nuclear staining for asTF in breast cancer that was absent in placenta specimens and flTF-stained breast cancer specimens. As this unexpected staining pattern did not affect all nuclei, we regarded this to be a specific staining. High percentages of positive nuclei virtually coincided with high percentages of positive cytoplasm, which was reflected in a strong correlation ($P = .001$).

asTF and flTF do not affect relapse-free period nor survival

The association of asTF and flTF status with relapse-free period and relative survival is shown in Figure 3. Analysis of relapse-free period showed no association between

Table 1. Association of asTF and flTF with patient and tumor characteristics

	Total		asTF					flTF				
			Negative		Positive		*P*	Negative		Positive		*P*
	N	%	N	%	N	%		N	%	N	%	
Total	574	100	119	100	328	100		157	100	351	100	
Age							0.03					0.71
< 40	48	8.4	5	4.2	26	7.9		15	9.6	26	7.4	
40-60	227	48.3	72	60.5	153	46.6		75	47.8	171	48.7	
> 60	249	43.4	42	35.3	149	45.4		67	42.7	154	43.9	
Grade							<0.001					<0.001
I	80	14.2	26	22.2	28	8.6		30	19.6	41	11.8	
II	282	49.9	58	49.6	159	48.9		88	57.5	157	45.2	
III	203	35.9	33	28.2	138	42.5		35	22.9	149	42.9	
Histology							0.39					0.18
Ductal	513	90.6	104	88.9	300	92.3		133	86.9	320	92.2	
Lobular	53	9.4	13	11.1	25	7.7		119	13.1	27	7.8	
T status							0.002					0.26
T1	211	38.0	56	48.7	97	30.4		64	42.1	120	35.1	
T2	272	49.0	46	40.0	173	54.2		71	46.7	171	50.0	
T3-4	72	13.0	13	11.3	49	15.4		17	11.2	51	14.9	
N status							0.62					0.18
N0	307	55.1	64	54.7	166	52.0		93	60.0	182	53.5	
N1-3	250	44.9	53	45.3	153	48.0		62	40.0	158	46.5	
ER status												0.96
Negative	203	37.6	37	31.9	124	39.1	0.17	54	36.7	123	36.5	
Positive	337	62.4	79	68.1	193	60.9		93	63.3	214	63.5	
PgR status							0.10					0.06
Negative	223	41.6	40	34.2	136	42.9		50	34.0	144	43.0	
Positive	313	58.4	77	65.8	181	57.1		97	66.0	191	57.0	
HER2 status							0.14					0.28
Negative	435	80.9	86	93.5	236	88.1		105	92.1	244	88.4	
Positive	103	19.1	6	6.5	32	11.9		9	7.9	32	11.6	
ALDH1 status							0.84					0.001
Negative	201	40.5	44	39.3	123	38.2		74	50.7	105	34.1	
Positive	295	59.5	68	60.7	199	61.8		72	49.3	203	65.9	

asTF, alternatively spliced tissue factor; flTF, full length tissue factor; T, tumor; N, Axillary lymph node; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor 2; ALDH1, aldehyde dehydrogenase 1

asTF and flTF expression and clinical outcome (P = .38 and P = .23, respectively). In this cohort, 55% of patients with asTF positive tumors was relapse-free at 10 year follow-up compared to 61% of patients with asTF negative tumors (absolute difference = 6%, P = .38). 58% of patients with flTF positive tumors was relapse-free at 10 year follow-up compared to 63% of patients with flTF negative tumors (absolute difference = 5%, P = .23). Analysis of relative survival showed a similar pattern (Figure 3).

Age influences the prognostic role of flTF and ALDH1 co-expression

To further study the relationship between the breast cancer stem cell marker ALDH1 and the co- expression of flTF, the association of flTF status and ALDH1 status with relapse-free period was analyzed (Figure 4). Patients were grouped according to co-expression of ALDH1 and flTF versus one of both marker overexpressed or none overexpressed. Relapse-free period showed a trend towards a significant association between ALDH1 and flTF co-expression and poor clinical outcome for the whole

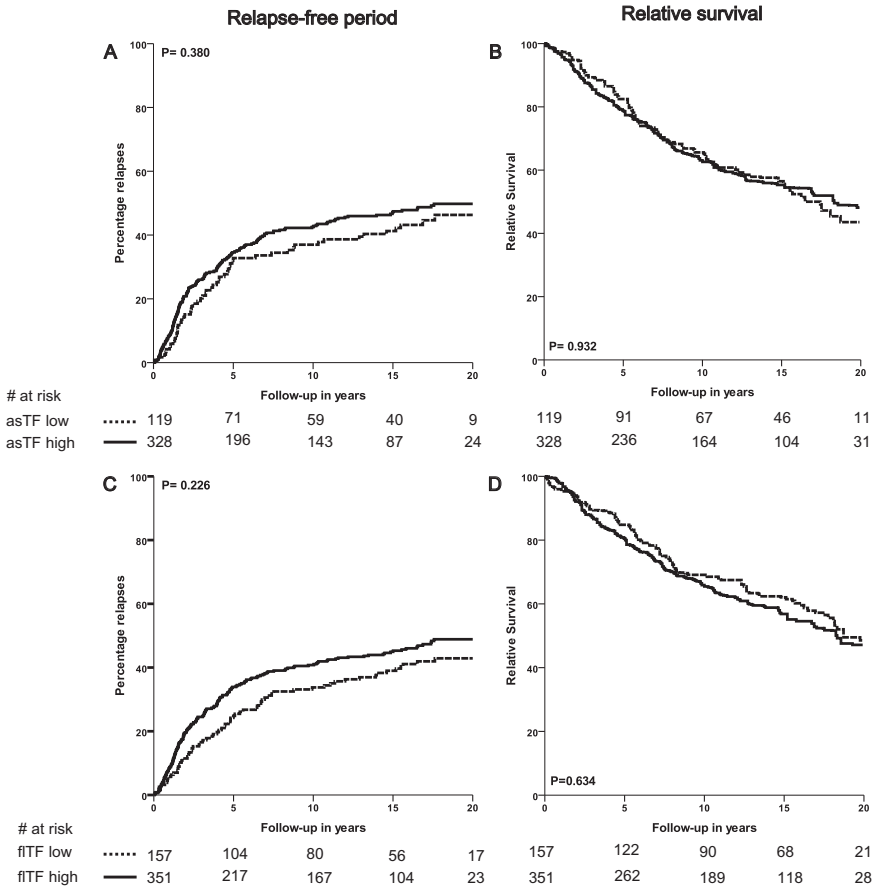

Figure 3. Expression of TF isoforms and association with relapse-free period (**A**, **C**) and relative survival (**B**, **D**). Log-rank *P*-values are shown in each graph.

population (*P* = .093; Figure 4A). In the group of patients aged younger than 65 years, a strong association was found between ALDH1 and flTF co-expression and poor relapse-free period (*P* = .004; Figure 4B) and 43% of patients with ALDH1 and flTF positive tumors was relapse-free at 10 year follow-up compared to 67% of patients without co-expressing tumors (absolute difference = 24%).

Conversely, in the group of patients aged older than 65 years, a trend towards an opposite effect of ALDH1 and flTF co-expression and improved clinical outcome was found (*P* = .084; Figure 4C). In this group, 71% of patients with ALDH1 and flTF positive tumors was relapse-free at 10 year follow-up compared to 53% of patients without co-expressing tumors (absolute difference = 18%; Figure 4C). Interaction analysis demonstrated a statistically significant difference in the prognostic effect of ALDH1 and flTF status in young and elderly patients (*P* = .003).

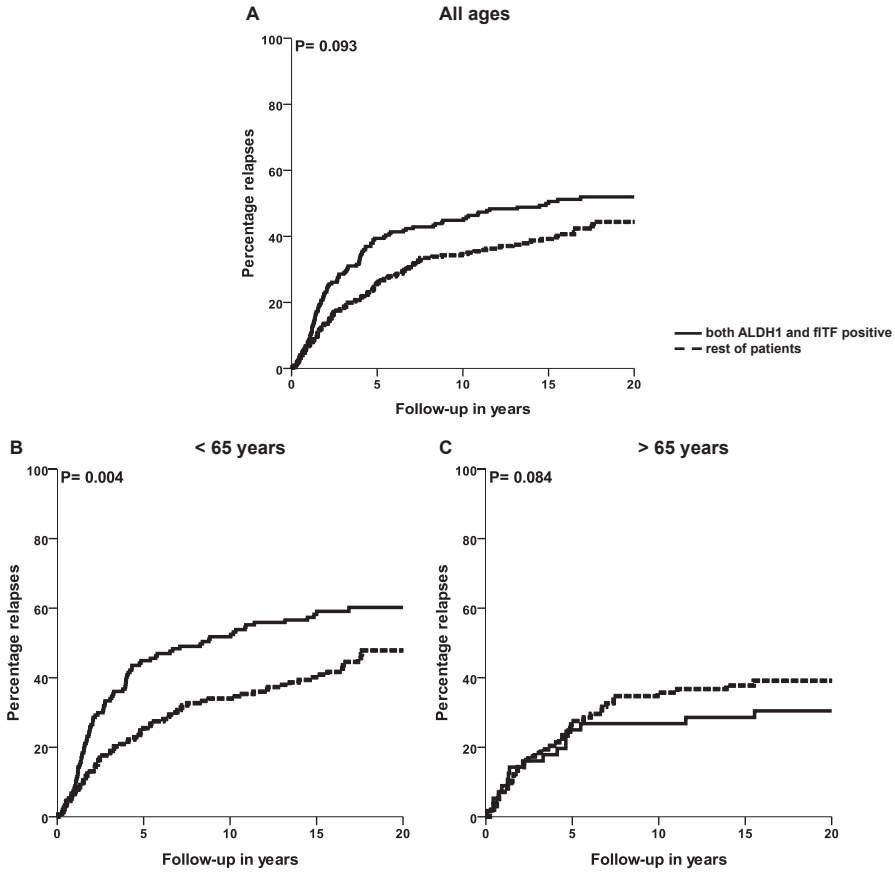

Figure 4. Relapse-free period according to flTF and ALDH1 co-expression for all patients (**A**), for patients aged < 65 years (**B**) and for patients aged > 65 years (**C**). Log- rank *P*-values are shown in each graph.

asTF and flTF display different subcellular localizations

The observation of asTF specific positive staining nuclei in breast cancer specimen prompted us to confirm this unexpected localization of asTF. Confocal microscopy revealed that TF isoforms in human breast cancer show limited co-localization and rather have a different subcellular localization (Figure 5A). flTF as expected is located in the cytoplasm and on the cell membrane, whereas the asTF signal is most prominent adjacent to the cell's nucleus. Although co-localization of both isoforms could be expected, the number of cells displaying co-localization is limited. In transfected baby hamster kidney cells, asTF similarly localized to the perinuclear areas (Figure 5B). We confirmed the findings with confocal microscopy by cellular fractionation of asTF- or flTF-transfected baby hamster kidney cells (Figure 5C). Indeed, asTF was detectable in the nuclear fraction at 40 kDa, which is the expected weight of fully glycosylated asTF, and at 36 kDa, which putatively represents underglycolyated asTF.

DISCUSSION

Since its discovery, the role of asTF in coagulation has been a matter of debate. However, a role for asTF in cancer has been suggested on basis of both experimental and small-scale patient studies. Recently, our group confirmed that asTF bears an angiogenic potential that acts through a different mechanism when compared to flTF. Earlier small-scale clinical studies indicated a relation between asTF mRNA levels and clinical outcome for several cancer types. Here, to our best knowledge, we are the first to report on the clinicopathologic associations of asTF at protein level in breast cancer using a large patient cohort with a long follow-up. Further, we were able to distinguish between TF isoforms by use of two isoform-specific antibodies. Moreover, we investigated the relative contribution of TF isoforms to breast cancer stem cells identified by ALDH1 expression.

In concordance with some previous small-scale clinical studies, we found both TF isoforms to be abundantly expressed in human breast cancer. A comparison with matched normal tissue from the same patients, led us to conclude that this expression pattern is specific for cancerous epithelia, since expression of both isoforms was considerably lower in normal epithelia. Moreover, asTF expression was almost absent in normal epithelia, further underlining that epithelial asTF expression may be a general phenomenon in breast cancer. Despite the near omnipresence of TF isoforms in breast cancer, we were able to correlate increasing percentages of asTF or flTF positive tumor cells with histological grade. Tumor size was only correlated with asTF, possibly reflecting that asTF has a differential effect on tumor size when compared to flTF.

As previous reports described striking effects of TF isoforms in murine cancer models on survival, we expected that the aforementioned correlations with clinicopathologic characteristics would result in a poor relapse-free and/or overall survival. Indeed, we found an impaired survival for patients with asTF or flTF positive tumors (absolute difference at 10 year follow-up was 6% and 5%, respectively), however, this finding did not reach statistical significance. A likely explanation for this finding is that TF isoforms heavily contribute to tumor growth in early tumorigenesis and are involved in the angiogenic switch, as reported in mouse studies. However, in the current group of patients, who were all operated in the pre-screening era, the process of tumor growth was already passed the stage of the angiogenic switch. Therefore, future research should focus on patients with small, non-palpable breast lumps to further elucidate the role of TF isoforms in human breast cancer tumorigenesis.

Unexpectedly, we observed a specific nuclear staining for asTF. To date, flTF has only been detected on the cell membrane and in the cytoplasm. The latter may reflect a large intracellular stock that can be employed for trafficking of flTF to the cell membrane in (patho)physiological situations when flTF is rapidly needed.[23] Further, the cellular secretion of asTF has been debated[11] and, although secretion of asTF is

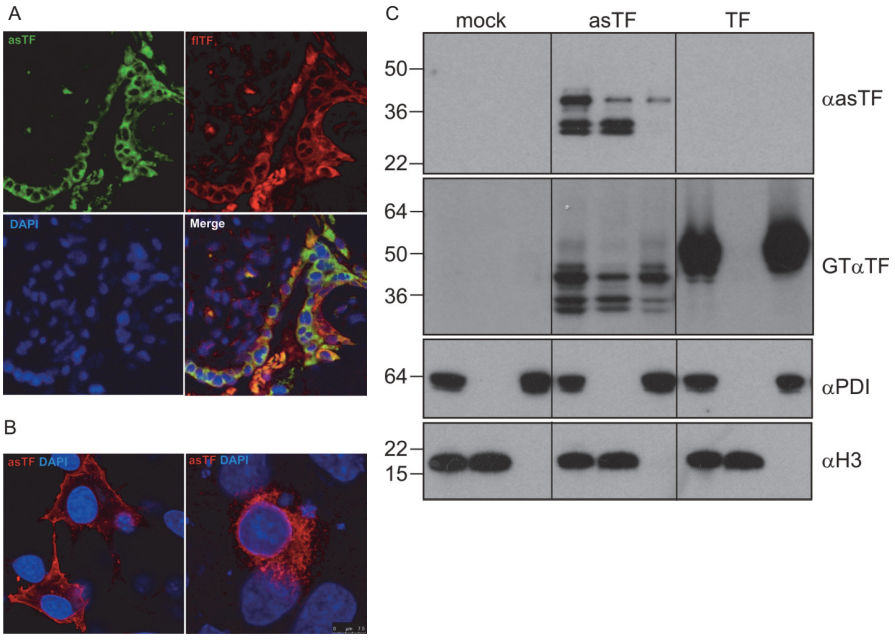

Figure 5. Subcellular localization of asTF and flTF. A. Confocal microscopy of TF isoforms in human breast cancer. Limited co-localization of asTF and flTF is found. flTF is located in the cytoplasm and on the cell membrane. asTF signal is most prominent adjacent to the cell's nucleus. **B.** In transfected baby hamster kidney cells, asTF is localized at the perinuclear areas. **C.** Western blot of cellular fractionated mock (left panel) and asTF (middle panel) and flTF (right panel) transfected baby hamster kidney cells demonstrates that asTF was detectable in the nuclear fraction and flTF in the cytoplasmic fraction. (40 kDa is expected weight of fully glycosylated asTF and 36 kDa is expected weight of underglycolyated asTF). Left lane, unfractionated cells. Middle lane, nuclear fraction. Right lane, cytoplasmic fraction. PDI, protein disulfide isomerase, marker of cytoplasmic fration. H3, histone 3, marker of nuclear fraction.

possible after proper stimuli,[24] it is not excluded that asTF accumulates intracellularly and promotes processes other than ligation of extracellular integrins. Future studies may prove an intracellular role for asTF other than its extracellular function of integrin ligation.

In this study, we demonstrated that tumors expressing the breast cancer stem cell marker ALDH1 are correlated with overexpression of flTF and not with asTF overexpression. Cancer stem cells, defined as a small subset of tumor cells with stem cell-like features including epithelial-to-mesenchymal transition, have the capacity of self-renewal and differentiation, giving rise to heterogeneous tumor cell population. Various studies have shown that cancer stem cells are relatively resistant to chemo- and radiotherapy, which might provide opportunities for understanding treatment resistance and tumor dormancy.[25, 26] The cancer stem cell marker ALDH1 expression has shown promise as a clinically relevant prognostic marker in breast cancer.[27-29] The current finding of the relation of flTF and ALDH1 sheds new light on the interaction between the tissue factor/protease activated receptor (TF:VII:PAR2) pathway and

the growth potential of the cancer stem cell subset. These observations are in line with others,[17-19] however, to the best of our knowledge, we are the first to investigate this relationship in breast cancer. Moreover, we demonstrated that asTF expression was not associated with ALDH1 expression. This finding suggests that the role of the asTF/integrin-dependent pathway is of limited, if any, role in the formation and maintenance of the breast cancer stem cell subset. Interestingly, co-expression of flTF and ALDH1 was shown to have a strong synergistic prognostic effect, which implies that flTF:FVII:PAR2 signaling but not asTF-mediated integrin ligation contributes to stem cell function. Recently, we found evidence for a strong age-dependent role of tumor stem cells in breast cancer and this synergistic relation between flTF and stem cells is even more dramatic in breast cancer patients younger than 65 years, whereas this relation inverses above 65 years. How age, the flTF:FVII:PAR2 axis and tumor stem cells mechanistically further relate is unclear, but the strong hormonal influence on flTF and FVII expression is likely to play a major role. In summary, our data suggest that the flTF:FVII:PAR2 pathway is important for the cancer stem cell population in early breast cancer. Therefore, modulation of the activity of this pathway might interfere with the cancer stem cell subset, which may subsequently improve patient survival.

In conclusion, differential TF isoform expression in breast cancer relates to different patient characteristics, which may reflect the different mechanisms of how TF isoforms modulate cancer biology. Targeting both TF isoforms may prove beneficial in early stages of tumor biology by counteracting tumor angiogenesis, especially by targeting asTF since its expression is rather tumor specific. On the other hand, targeting of the flTF:FVII:PAR2 axis may also improve patient outcome since this axis seems to be involved in the small subset of cancer stem cells, which are relatively resistance to current chemotherapeutic and radiotherapeutic regimens.

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