Chapter 6 - Alternatively spliced and full-length tissue factor have dissimilar properties in breast cancer

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Part of this work has been published in

Kocatürk B*, **Van den Berg YW***, Tieken C*, Mieog JS, de Kruijf EM, Engels CC, van der Ent MA, Kuppen PJ, Van de Velde CJ, Ruf W, Reitsma PH, Osanto S, Liefers GJ, Bogdanov VY*, Versteeg HH*. Alternatively spliced tissue factor promotes breast cancer growth in a β1 integrin-dependent manner. *Proc Natl Acad Sci U S A*. 2013 Jun 25. [Epub ahead of print].

Abstract

Background: 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). We investigated the distribution of TF isoforms in human breast cancer specimens and its relationship with clinical oucome.

Methods: Tumor material and matched normal tissue of 574 breast cancer patients was obtained after primary surgery and assembled in tissue micro arrays (TMA), which were stained with antibodies specific for asTF and flTF. Associations of asTF and flTF expression with clinicopathological parameters and relapse-free period and relative survival were calculated.

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. Co-expression of flTF and ALDH1 was associated with poor relapse-free period in patients younger than 65 years (P = .004), whereas the opposite 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, but not asTF is associated with cancer stem cells in breast cancer. The differences in cellular localizations of asTF and flTF further support the notion that asTF acts through mechanisms distinct from flTF in cancer.

Introduction

The thrombotic tendency of cancer patients is believed to be caused by excessive expression of full-length tissue factor (flTF), a 47 kDa transmembrane protein, on the surface of cancer cells[1]. Under physiological circumstances, flTF expression is limited to the surface of cells that reside in subendothelial tissues. 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 formation of a blood clot[2]. In a reciprocal manner, activation of coagulation also influences various stages of cancerous disease. The flTF:FVIIa complex and thrombin have been implicated in tumor growth and metastasis[3]. For instance, flTF expression in colon cancer as well as in breast cancer has been suggested to promote tumor growth through modulation of angiogenesis[4]. Extensive research has uncovered that the effects of coagulation proteases on tumor biology are primarily mediated by activation of so-called protease-activated receptors (PARs). The flTF:FVIIa complex specifically activates PAR2 resulting in altered cell behavior such as proliferation, gene expression and migration[5], and in a spontaneous murine breast cancer model activation of PAR2 by the flTF:FVIIa complex was shown to enhance primary tumor growth via activation of the angiogenic switch[6].

In 2003, a natural occurring splicing variant of TF, alternatively spliced TF (asTF), was described which lacks exon 5, and due to this exon skipping, a frame shift occurs. The resulting asTF protein lacks a transmembrane region, and has a unique C-terminus, rendering asTF soluble[7]. Since its discovery, the role of asTF in coagulation has been debated[8-10], but an increasing body of evidence indicates that asTF may be involved in tumor biology and angiogenesis. In cervical cancer samples, asTF is detected both at the protein and mRNA level[11]. High levels of asTF mRNA confer an unfavorable outcome in non-small cell lung cancer[12;13]. Experiments in a xenograft model for pancreatic cancer revealed that tumor size of asTF-expressing tumors is larger and that those tumors display an enhanced number of tumor vessels[14]. Recently, our group reported that asTF drives angiogenesis in a non-proteolytic, integrin-ligating fashion. Human asTF activates α6β1 and αVβ3 integrins on endothelial cells, thereby promoting enhanced cell alignment and migration respectively, which are key processes in angiogenesis. Furthermore, we showed that asTF promotes monocyte-endothelium interactions through integrin β1-dependent upregulation of cellular adhesion molecules[15]. This eventually results in the influx of monocytes in tumor tissue that can differentiate into M2-type macrophages that secrete pro-angiogenic cytokines[16]. In summary, these data suggest that asTF promotes tumor angiogenesis via ligation of integrins on endothelial cells and influx of monocytes in tumor tissue that further promotes tumor angiogenesis.

To date, clinical studies that investigate the relation between tumor asTF expression at the protein level and clinicopathological characteristics are lacking. Furthermore, the relative contribution of the protease-mediated flTF axis and the integrin-mediated asTF axis to cancer progression is unclear. Apart from the contribution to tumor angiogenesis, previous studies showed that cancer stem cells abundantly express 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 clinical follow-up in order to clarify the relative contributions of TF isoforms to human breast cancer.

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[7]. A murine monoclonal anti human TF with an epitope against the amino acids encoded by exon 5 was purchased from American Diagnostica. PDI (RL90) 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). T75 Cell culture flasks and other cell culture disposables were purchased from Greiner Bio-One (Alphen a/d Rijn, the Netherlands).

Cell lines and vectors- Baby hamster kidney cells, 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 u/ml penicillin/ 50 µg/ml streptomycin (PAA GmbH, Pasching, Austria). ORFs for asTF (Baseclear, Leiden, the Netherlands) and flTF (kind gift from Dr. W. Ruf, Scripps Institute, La Jolla CA) were subcloned into pcDNA3.1 (Invitrogen, Carlsbad CA) vectors and used when appropriate. Transfections were performed with lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad CA) 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)21. All tumors were graded according to current pathological

standards. Of 266 patients (46%), 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).

Immunohistochemistry- 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 266 patients. Tumors from the murine xenograft experiment were fixed in a similar fashion. Immunohistochemical staining was performed according to standard procedures. Briefly, sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked with 0.3% H2O2 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 degrees Celsius. 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 degrees Celsius 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 using DAB solution. After counterstaining with hematoxillin, section were dehydrated and covered. The percentage of asTF and flTF positive tumor cells was scored by two observers in a blinded manner. Patients in the 1st quartiles were deemed negative.

Immunofluorescence– 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 respectively asTF and flTF. 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. AlexaFluor 488 was excited at 488 nm and detected with a 505- to 530-nm band-pass filter. AlexaFluor 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 Fluor 488, red for Alexa Fluor 546 and blue for DAPI. Images were viewed and saved as merged images or as a set of two separate panels in LSM files.

Cell fractionation– Cells were grown to subconfluency, trypsinized and washed twice with PBS. 1.0 x 106 cells were pelleted by centrifugation for 10 minutes at 1200 RPM at room

temperature. The cell pellet was resuspended in 1 ml hypotonic buffer (10 mM Hepes, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, pH 7.9 with complete protease inhibitor according to manufacturers instructions) 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 non-lysed nuclei were pelleted by centrifugation at 2000 rpm for 10 minutes at 4 degrees Celsius 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 respectively the cytoplasmic and nuclear fractions.

Statistical analysis- Statistical analyses were performed using the statistical packages SPSS (version 16.0 for Windows, Spps Inc, Chicago, IL, USA) and Stata (version 10.0 for Windows, StataCorp, College Station, TX, USA). 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 0.88 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[22]. 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[23]. 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). The first quartile included tumors in which 60% of the tumor cells expressed asTF and and 95% expressed

flTF (Figure 2). The association between the percentage of asTF and flTF positive cells per tumor specimen and clinicopathological characteristics is shown in Table 1. Tumoral asTF expression was associated with a higher histological grade in comparison with asTF negative tumors (odds ratio (OR) grade III vs. grade I =3.88, 95% CI = 2.017-7.476). Moreover, asTF positive tumors were larger in terms of tumor size (OR T3-4 vs. T1 = 2.18, 95% CI = 1.087-4.357). In contrast, tumoral flTF expression was associated with a higher histological grade compared to flTF negative tumors (OR grade III vs. grade I = 3.12, 95% CI = 1.713-5.663), but not with tumor size (OR T3-4 vs. T1 = 1.60, 95% CI = 0.855-2.996). flTF expression was associated with the expression of the breast cancer stem cell marker ALDH1 (OR = 1.99, 95% CI = 1.331-2.967), whereas asTF expression was not (P = .84). In addition, we noted nuclear staining for asTF in breast cancer that was absent in placenta specimens and flTF-stained breast cancer specimens. As this staining pattern did not affect all nuclei, we regarded this to be a specific staining. High percentages of positive nuclei coincided with high percentages of positive cytoplasm. No associations were found between as/flTF expression and the hormone receptor status, either estrogen or progesterone, or Her2-Neu mutation.

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 asTF and flTF expression and clinical outcome (log-rank P = .38 and P = .23, respectively). Fifty-five % of patients with asTF-positive tumors was relapse-free at 10 years follow-up compared to 61% of patients with asTF-negative tumors (absolute difference = 6%, P = .38). Fifty-eight % of patients with flTF-positive tumors was relapse-free at 10 years 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 each markers overexpressed or none overexpressed. Relapse-free period showed a trend towards association between ALDH1 and flTF co-expression and poor clinical outcome for the whole 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 short relapse-free period (P = .004; Figure 4B) and 43% of patients with ALDH1 and flTF-positive tumors was relapse-free at 10 years 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 years 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).

asTF and flTF display different subcellular localizations– The notion that nuclei stain positive for asTF in the present immunohistochemical studies prompted us to further characterize this phenomenon. Confocal microscopy revealed that TF isoforms in human breast cancer specimens on the same TMA showed limited co-localization and display a different subcellular localization (Figure 5A). flTF is located in the cytoplasm and on the cell membrane, whereas asTF most prominently localizes near or in the perinuclear region. In transfected BHK cells, asTF similarly localized to the perinuclear space (Figure 5B). We confirmed these findings biochemically by cellular fractionation of asTF- or flTF-transfected BHK cells (Figure 5C). Indeed, asTF was detectable in the nuclear fraction at 40 kDa, which is the expected molecular weight of fully glycosylated asTF, and at 36 kDa, which putatively represents underglycolyated asTF.

Discussion

Since its discovery, a role for asTF in coagulation has been a matter of debate, but 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 harbors angiogenic potential that acts through a different mechanism when compared to flTF. Earlier studies indicated a relation between asTF mRNA levels and clinical outcome for several cancer types. Here, we report on the clinicopathologic associations of asTF at the protein level in breast cancer using a large patient cohort with a long follow-up. Moreover, we investigated the relations of TF isoforms with breast cancer stem cells identified by ALDH1 expression.

We found both asTF and flTF 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 epithelial cells, since expression of both isoforms was considerably lower in normal epithelium. Although we found that expression of TF isoforms in breast cancer almost is a general phenomenon, we were able to find an association between increasing percentages of asTF or flTF positive tumor cells and histological grade. Only asTF expression was associated with tumor size, possibly reflecting an effect that is asTF specific.

Previous reports described effects of the two TF isoforms on survival in murine cancer models. On the basis of these results we had expected that positivity of tumor specimens for asTF and/or flTF would be associated with a shorter relapse-free and/or overall survival. Indeed, we found shortened survival for patients with asTF or flTF positive tumors, but this difference was small and not statistically significant (absolute difference at 10 year follow-up was 6% and 5%, P = .38 and P = .23, respectively). A possible explanation for this lack of a strong association between as/flTF and survival may be that the two TF isoforms contribute to tumor growth primarily by their involvement in the angiogenic switch early in tumorigenesis (as reported in mouse studies). In the current group of patients the process of tumor growth was already advanced and well beyond the stage of the angiogenic switch. Perhaps future studies should focus on patients with small, non-palpable breast lumps to further elucidated 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 used for trafficking of flTF to the cell membrane in situations when flTF is rapidly needed[24]. Further, the cellular secretion of asTF has been debated[10] and, although asTF is readily secreted after proper stimuli[25], it cannot be excluded that asTF accumulates intracellularly and promotes processes other than ligation of extracellular integrins.

We demonstrated that tumors expressing the breast cancer stem cell marker ALDH1 also overexpress flTF but not asTF. 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, 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[26;27]. The cancer stem cell marker ALDH1 expression has shown promise as a clinically relevant prognostic marker in breast cancer[28-30]. The current finding of the relation between flTF and ALDH1 sheds new light on the interaction between the tissue factor/protease activated receptor (flTF:VII:PAR2) pathway and the growth potential of the cancer stem cell subset. We further 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. 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 stronger in breast cancer patients younger than 65 years, whereas it inverses above 65 years[21]. In

summary, our data suggest that flTF, and possibly the flTF:FVII:PAR2-axis, is important for the cancer stem cell population in early breast cancer. Therefore, modulation of the activity of flTF 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 by which TF isoforms modulate cancer biology. Targeting both TF isoforms may prove beneficial in early stages of tumor biology by counteracting two different pathways of tumor angiogenesis. Moreover, interference with the flTF:FVII:PAR2 axis may also improve patient outcome since this axis seems to be involved in the small cancer stem cells subset, which is relatively resistance to current chemotherapeutic and radiotherapeutic regimens.

	Total		asTF				P	flTF				P
			Negative		Positive			Negative		Positive		
	N	%	N	%	N	%		N	%	N	%	
Total	574	100	119	100	328	100		157	100	351	100	
Age												
<40	48	8,4	5	4.2	26	7.9	0.03	15	9.6	26	7.4	0.71
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												
I	80	14,2	26	22.2	28	8.6	<0.001	30	19.6	41	11.8	<0.001
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	
Histological type												
Ductal	513	90,6	104	88.9	300	92.3	0.39	133	86.9	320	92.2	0.18
Lobular	53	9,4	13	11.1	25	7.7		119	13.1	27	7.8	
T status												
T1	211	38,0	56	48.7	97	30.4	0.002	64	42.1	120	35.1	0.26
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												
N0	307	55,1	64	54.7	166	52.0	0.62	93	60.0	182	53.5	0.18
N1-3	250	44,9	53	45.3	153	48.0		62	40.0	158	46.5	
ER status												
Negative	203	37,6	37	31.9	124	39.1	0.17	54	36.7	123	36.5	0.96
Positive	337	62,4	79	68.1	193	60.9		93	63.3	214	63.5	
PgR status												
Negative	223	41,6	40	34.2	136	42.9	0.10	50	34.0	144	43.0	0.06
Positive	313	58,4	77	65.8	181	57.1		97	66.0	191	57.0	
Her2 status												
Negative	435	80,9	86	93.5	236	88.1	0.14	105	92.1	244	88.4	0.28
Positive	103	19,1	6	6.5	32	11.9		9	7.9	32	11.6	
ALDH1 status												
Negative	201	40.5	44	39.3	123	38.2	0.84	74	50.7	105	34.1	0.001
Positive	295	59.5	68	60.7	199	61.8		72	49.3	203	65.9	

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

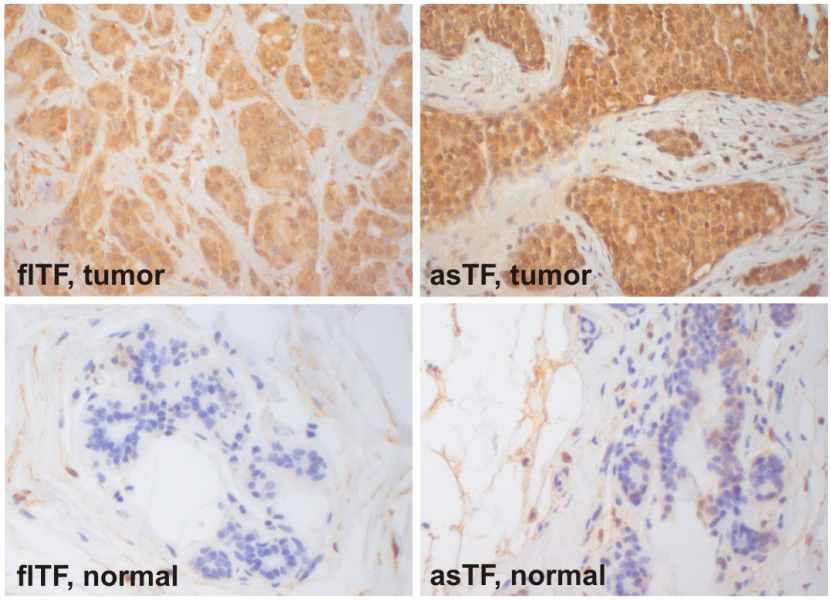

Figure 1. Representative photographs of tissue microarray punches of human breast cancer specimens (left panels) and matched normal mammary tissue (right panels) immunohistochemically stained for asTF (top panels) and flTF (bottom panels). Brown color indicates positive staining.

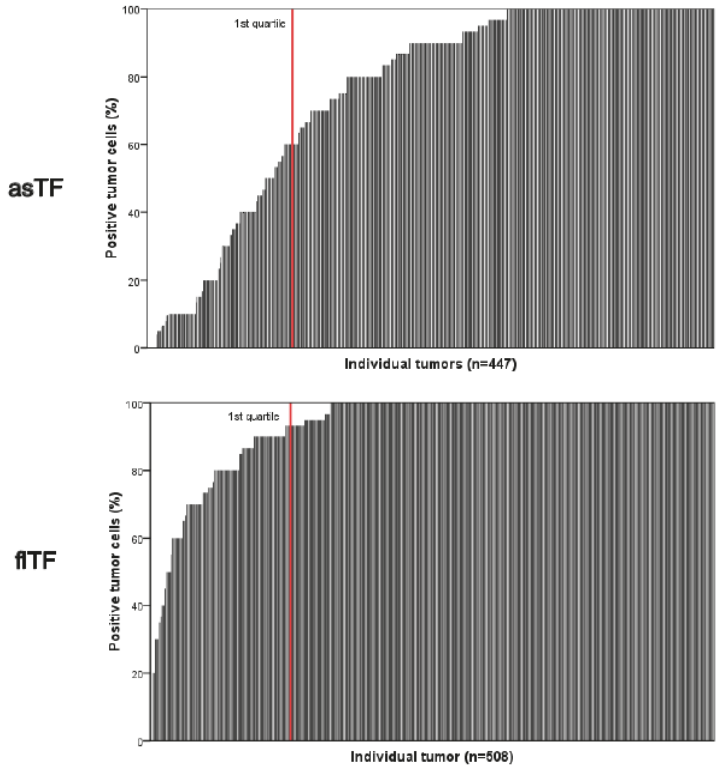

Figure 2. Bar chart indicating the number of positive tumor cells for each individual tumor for asTF (top) and flTF (bottom) staining. The red line marks the first quartile; these patients were deemed asTF or flTF negative.

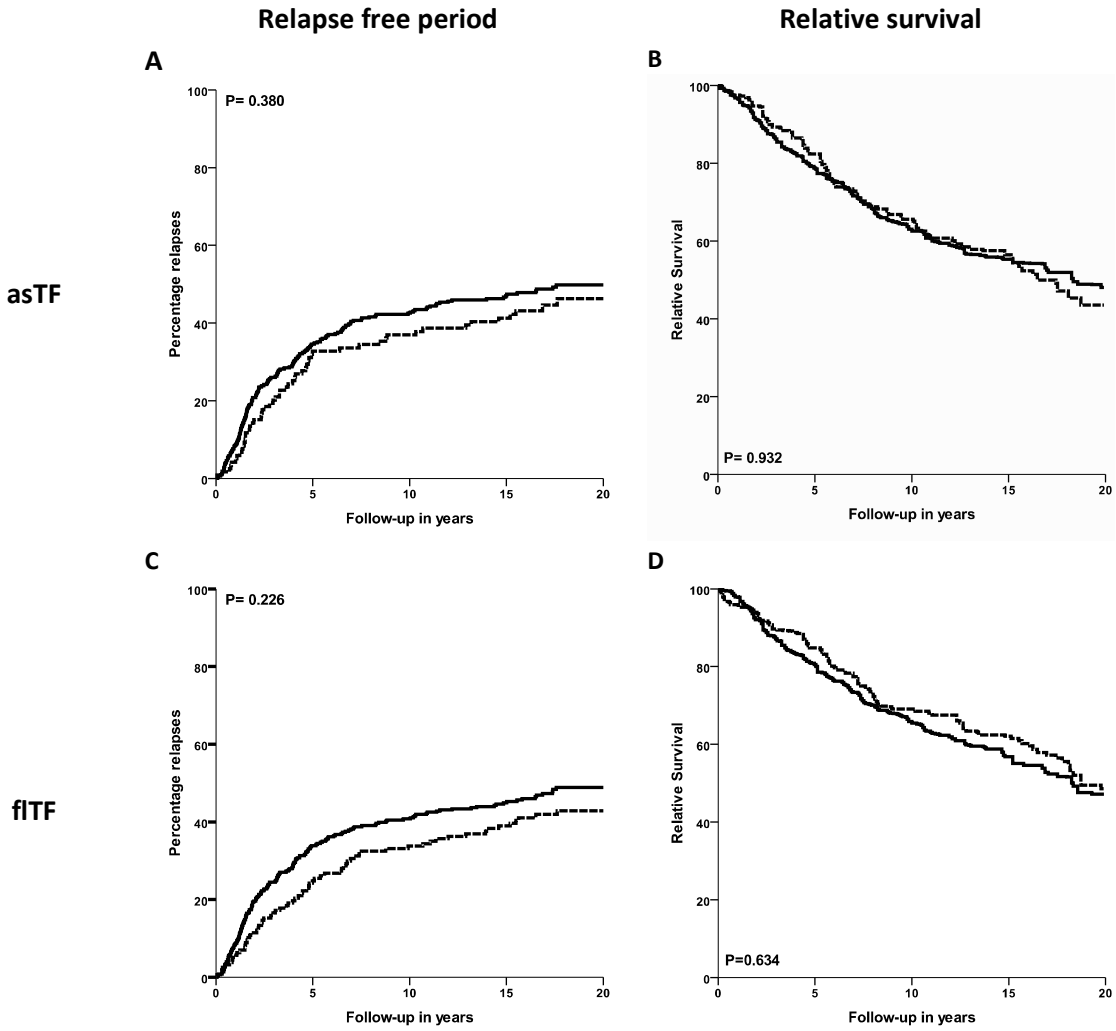

Figure 3. Expression of TF isoform and the association with relapse-free period (A,C) and relative survival (B, D). Log-rank P-values are shown in each graph. Dotted lines indicate low expression, continuous lines indicate high expression.

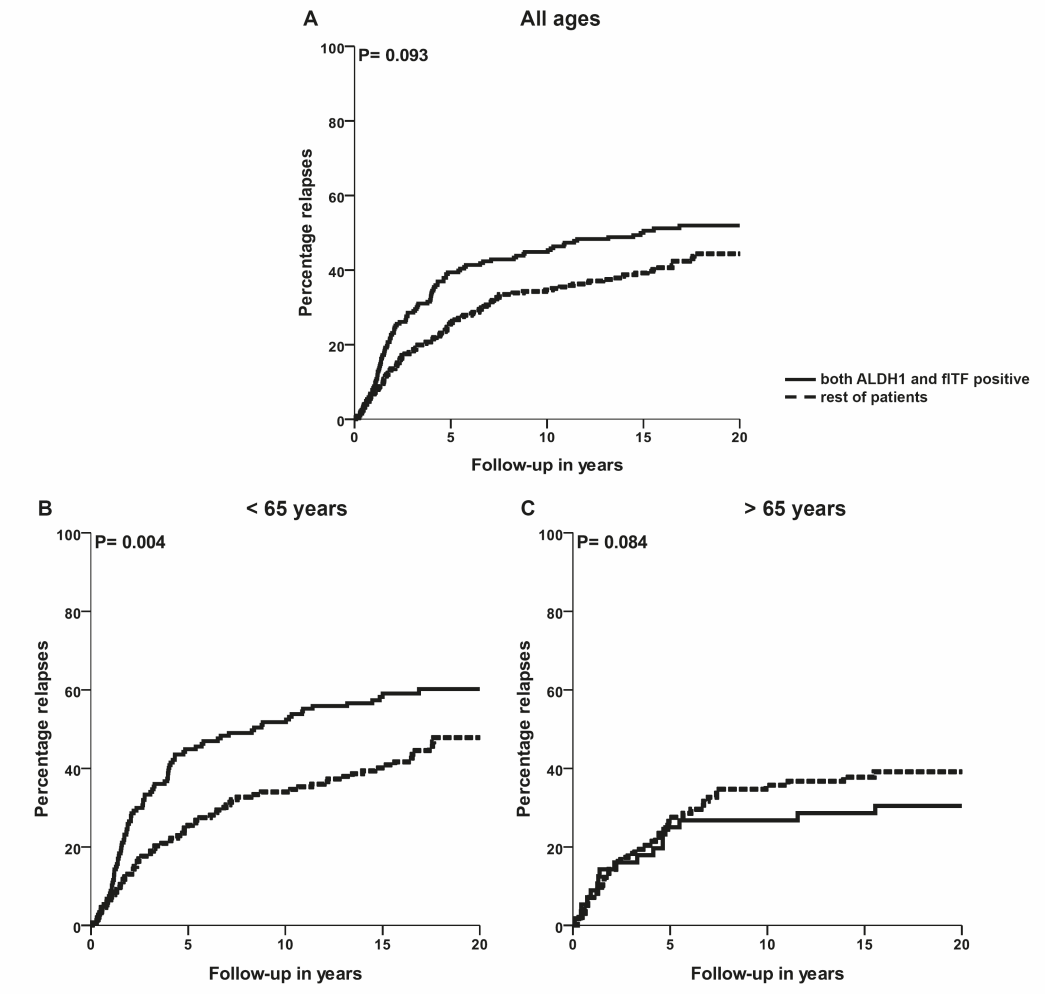

Figure 4. Relapse-free period according to flTF and ALDH1 co-expression for all patients (A), for patients aged < 65 years (B) and for patients aged > 65 years (C). Logrank P-values are shown in each graph.

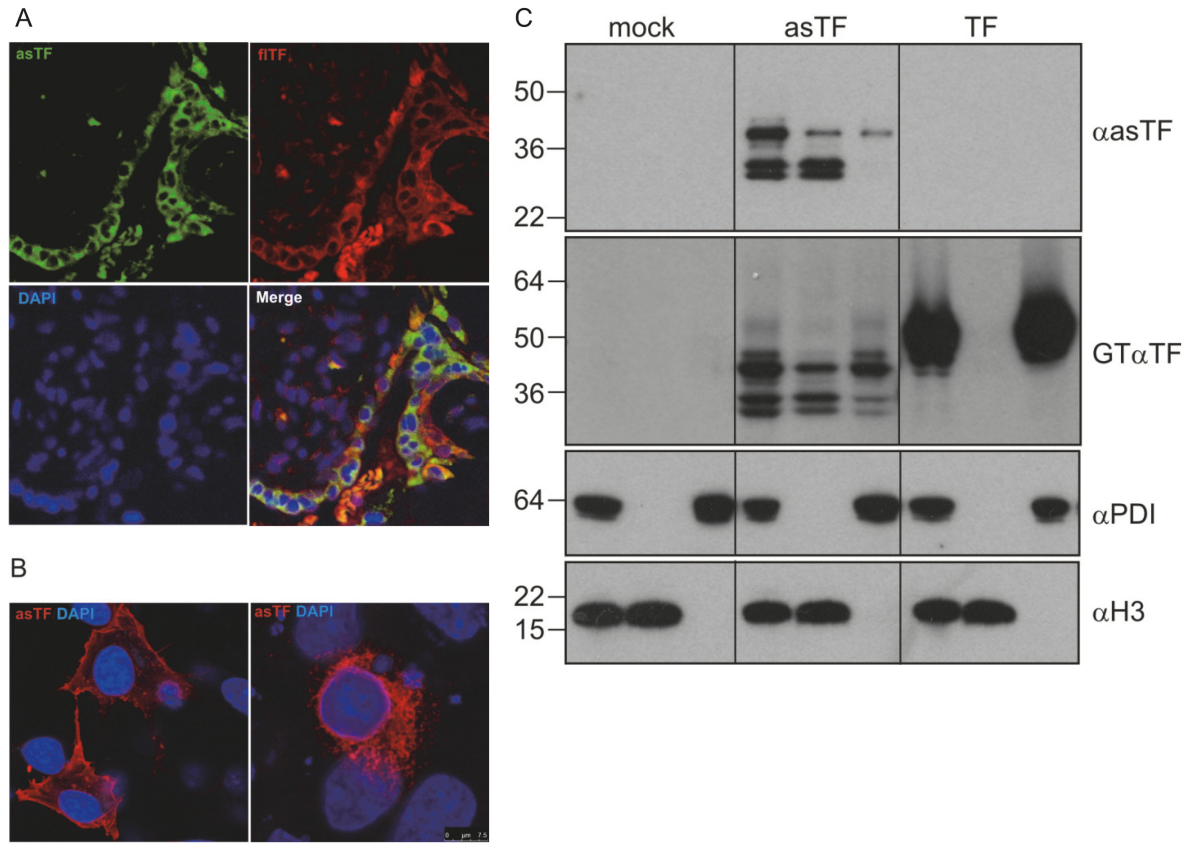

Figure 5. Subcellular localization of asTF and flTF. A. Confocal microscopy of TF isoforms in human breast cancer. Limited co-localization is found. flTF is located in the cytoplasm and on the cell membrane. asTF signal is most prominent adjacent to the cell's nucleus. **B.** In transfected baby hamster 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.

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