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Expression of flTF and asTF splice variants in various cell strains and tissues

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Abstract. Tissue factor (TF) expressed at the protein level includes two isoforms: The membrane-bound full-length TF (flTF) and the soluble alternatively spliced TF (asTF). flTF is the major thrombogenic form of TF, whereas asTF is more closely associated with tumor growth, angiogenesis, metastasis and cell growth. In order to further investigate the different expression and functions of TF splice variants, the expression of these two splice variants were detected in numerous cell strains and tissues in the present study. Quantitative polymerase chain reaction was used to measure the transcript levels of the TF variants in 11 human cell lines, including cervical cancer, breast cancer, hepatoblastoma, colorectal cancer and umbilical vein cells, and five types of tissue specimen, including placenta, esophageal cancer, breast cancer, cervical cancer (alongside normal cervical tissues) and non-small cell lung cancer (alongside adjacent and normal tissues). Furthermore, the effects of chenodeoxycholic acid (CDCA) and apolipoprotein M (apoM) on the two variants were investigated. The results demonstrated that flTF was the major form of TF, and the mRNA expression levels of flTF were higher than those of asTF in all specimens tested. CDCA significantly upregulated the mRNA expression levels of the two variants. Furthermore, overexpression of apoM promoted the expression levels of asTF in Caco-2 cells. The mRNA expression levels of asTF in cervical cancer tissues were significantly higher than in the corresponding normal tissues. To the best of our knowledge, the present study is the first to compare the expression of flTF and asTF in various samples. The results demonstrated that CDCA and apoM may modulate TF isoforms in different cell lines, and suggested that asTF may serve a role in the pathophysiological mechanism underlying cervical cancer development. In conclusion, the TF isoforms serve important and distinct roles in pathophysiological processes.

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Introduction

Tissue factor (TF) is a 47-kD transmembrane cell-surface glycoprotein, which is primarily known as the initiator of the blood coagulation cascade (1). Human TF is genetically encoded by the TF gene, which is transcribed to TF premature mRNA. Alternative splicing of TF results in three naturally occurring protein isoforms: Full-length (fl)TF, alternatively spliced (as)TF and TF-A. asTF and flTF serve important and distinct roles in various biological processes that involve vessel formation and maturation, and initiation of the blood coagulation cascade (2). asTF, which arises from exclusion of the fifth exon of the primary TF transcript, exhibits low prothrombogenic potential but is more closely associated with tumor growth, angiogenesis, metastasis and cell growth (3-5). TF-A, another splice variant, is only expressed at the mRNA level in a number of cancer cell lines and in endothelial cells (Fig. 1). To the best of our knowledge, the biological function of TF-A mRNA is currently unknown; therefore, this splice variant was not taken into account in the present study (6-8).

Few studies regarding TF expression have discriminated between flTF and asTF; therefore, it is necessary to investigate the expression status of these two isoforms in different diseases. Bile acids are strong signaling molecules that are capable of influencing various biological processes, including inflammation, apoptosis, cancer progression and atherosclerosis. Chenodeoxycholic acid (CDCA) is a bile acid that has been demonstrated to enhance ectopic vessel formation (9). Similarly, apolipoprotein M (apoM), which was discovered by Xu and Dahlbäck in 1999 (10), is mainly located in high-density lipoprotein in the blood and has been demonstrated to be associated with tumor growth, atherosclerosis and thrombosis (11-13). In order to investigate the association between TF variants and CDCA/apoM, the difference in the

expression of TF variants in various cell strains and tissues was examined in the present study. The results of this study may contribute to further studies on the function and mechanism of TF in associated diseases.

Materials and methods

Cell lines and cell culture. Human cervical cancer cell lines [C-33A, human papilloma virus (HPV)-negative; HeLa, HPV18-positive; and SiHa, HPV16-positive], human breast cancer cell lines (ZR-75-1, luminal A subtype; MCF-7, luminal A subtype; BT-474, luminal B subtype; MDA-MB-468, basal-like subtype; and MDA-MB-231, basal-like subtype), a human hepatoblastoma cell line (HepG2), a human colorectal cancer cell line (Caco-2) and a human umbilical vein cell line (EA.hy926) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured according to their respective conditions and maintained in the Comprehensive Laboratory of The Third Affiliated Hospital of Soochow University (Changzhou, China).

The C-33A, HeLa, SiHa, Caco-2 and HepG2 cells were cultured in minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the MCF-7 and EA.hy926 cells were maintained and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.). The BT-474 and ZR-75-1 cells were cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.), whereas the MDA-MB-468 and MDA-MB-231 cells were cultured in Leibovitz's L-15 medium (Gibco; Thermo Fisher Scientific, Inc.). All of the complete media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin, and 10% non-essential amino acid solution was added for the culture of cervical cancer cell lines. All cells were incubated at 37˚C in an atmosphere containing 5% CO_2, with the exception of the MDA-MB-468 and MDA-MB-231 cells, which were cultured at 37˚C in a humidified atmosphere containing 100% air.

The HepG2 and EA.hy926 cells were seeded in 6-well plates with the concentration of $2x10^5$ cells/well, and allowed to grow to 80-90% confluence. Subsequently, they were washed and incubated in serum-free medium containing 50 μM CDCA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 0.1% ethanol for 24 h at 37˚C. Total RNA was then extracted.

The apoM coding sequence was obtained by polymerase chain reaction (PCR) amplification from human genomic DNA (forward primer, 5'-GAGGATCCCCGGGTACCGGTCGCC ACCATGTTCCACCAAATTTGGGCAGC-3'; reverse primer, 5'-TCCTTGTAGTCCATACCGTTATTGGACAGC TCACAGGCCTC-3') and was inserted into the Ubi-MCS-3FLAG-CMVEGFP vector (cat. no. GV365; Shanghai Genechem Co., Ltd., Shanghai, China). Empty lentiviral vectors with green fluorescent protein (GFP) and lentivirus-mediated human apoM overexpression vectors with GFP were prepared by Shanghai GeneChem Co., Ltd. In brief, 20 μg/ml of GV365 vector, 15 μg/ml of pHelper 1.0 and 10 μg/ml of pHelper 2.0 (Shanghai Genechem Co., Ltd.) were cotransfected into 293T cells (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) with enhanced infection solution and polybrene (Shanghai Genechem Co., Ltd.,), cultured at 37˚C for 48 h at 70% confluence. Lentiviral particles [$1x10^9$ transducing units (TU)/ml] were obtained from supernatants following centrifugation at a speed of 75,000 x g for 2 h at 4˚C. Subsequently, Caco-2 and EA.hy926 cells at 50% confluence were incubated with the lentiviral vector (multiplicity of infection, 50; $3.75x10^8$ TU) in dishes. After 12 h, culture medium containing the lentivirus was aspirated from the wells and fresh complete medium was added. The expression intensity of GFP was observed 3 days later and apoM overexpression was confirmed using reverse transcription-quantitative PCR (RT-qPCR). Caco-2 and EA.hy926 cells transfected with empty vectors (multiplicity of infection=50) were set up as the corresponding negative control (NC) groups.

Tissue sample collection. Placenta tissue specimens from 20 women (age, 24-33 years); esophageal cancer tissue specimens from 18 men and two women (age, 56-72 years); 14 breast cancer tissue specimens (age, 46-74); 34 cervical cancer specimens (age, 35-75 years) and 16 normal cervical control samples (age, 38-65 years); and non-small cell lung cancer (NSCLC) tissues and adjacent/normal tissues from seven men and nine women (age, 48-76 years) were collected at the Third Affiliated Hospital of Soochow University between July 2014 and September 2016. The patients had not received preoperative radiotherapy and/or chemotherapy. The experimental protocols were approved by the Institutional Ethics Committee of the Third Affiliated Hospital of Soochow University and all patients provided written informed consent for this study. All patients had undergone modified radical operations. All tissue samples including placental tissues, esophageal cancer tissues, breast cancer tissues, cervical cancer tissues, normal cervical tissues, NSCLC tissues and their adjacent/normal tissues were excised and quickly frozen in liquid nitrogen until subsequent analysis.

RNA isolation and RT-qPCR. Total RNA was extracted from cells and tissues using the total RNA purification kit (Shenergy Biocolor Biological Science & Technology Company, Shanghai, China), according to the manufacturer's protocol. cDNA was synthesized, according to the manufacturer's protocol, using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). Primers and TaqMan probes (labeled with carboxyfluorescein) for human flTF and asTF were designed using Primer Premier version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table I). The mRNA expression levels of flTF and asTF were quantified relative to the mRNA expression levels of GAPDH, and quantification was performed using a LightCycler® 480 Instrument II (Roche Applied Science, Penzberg, Germany) in a final volume of 25 μl. PCR reactions, purchased from the Shenergy Biocolor BioScience and Technology Company (Shanghai, China), were performed using the following mixture: 2.5 μl MgCl2 (25 mM; with 4 μl MgCl2 in the asTF PCR reactions), 2.5 μl PCR buffer (10X), 0.5 μl 4X dNTP (10 mM), 0.25 μl Taq DNA polymerase (5 units), 0.04 μl each primer and probe (100 μM), 2 μl cDNA template (replaced by water in no template controls) and nuclease-free water to a final volume of 25 μl. Thermal cycling was performed under

Table I. Primers and probes for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'FAM, 3'BHQ-1)
flTF	TGATGTGGATAAAGGAGAA AACTACTGT	CTACCGGGCTGTCTGTA CTCTTC	TTCAAGCAGTGATTCCCTCCC GAACA
asTF	ATCTTCAAGTTCAGGAAAGA AATATTCTAC	GCTCTGCCCCACTCCT GCC	TTGGAGCTGTGGTATTTGTGG TCATCATC
GAPDH	CAGGGCTGCTTTTAACTC TGGT	CATGGGTGGAATCATATT GGAAC	TGGATATTGTTGCCATCAATGA CCCCT

asTF, alternatively spliced TF; flTF, full-length TF; TF, tissue factor.

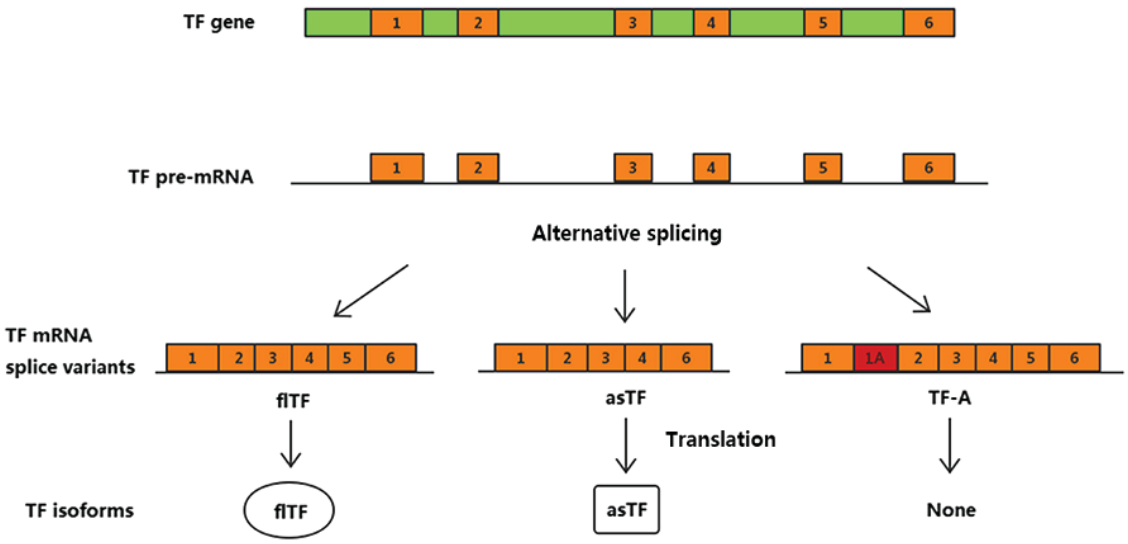

Figure 1. Schematic diagram of TF isoform expression. flTF consists of an intracellular domain, a transmembrane domain and an extracellular domain. asTF lacks the transmembrane domain and is therefore soluble. Retention of a part of intron 1 as alternative exon 1A in the mature transcript leads to the generation of the third variant, TF-A. asTF, alternatively spliced TF; flTF, full-length TF; TF, tissue factor.

the following conditions: 3 min of initial denaturation at 95˚C, followed by 40 cycles at 95˚C for 5 sec (temperature transition rate 4.4˚C/sec) and 60˚C extension for 25 sec. Samples were amplified simultaneously in triplicate in a single assay run. mRNA expression levels are presented as a ratio between the target gene and GAPDH gene expression; the fold-change was calculated using $2^{-\Delta\Delta Cq}$ (14).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data are expressed as the means ± standard deviation or standard error of the mean. Data were analyzed using Student's t-test or one-way analysis of variance (ANOVA). Tukey's multiple comparison test was conducted following ANOVA to compare multiple groupse (NSCLC tissues and their adjacent/normal tissues). $P<0.05$ was considered to indicate a statistically significant difference.

Results

mRNA expression levels of TF variants in human cell lines and tissues. As shown in Fig. 2A, the mRNA expression levels flTF were compared with those of asTF in 11 human cell lines, including human cervical cancer (C-33A, HeLa and SiHa), breast cancer (ZR-75-1, MCF-7, BT-474, MDA-MB-468 and MDA-MB-231), hepatoblastoma (HepG2), colorectal cancer (Caco-2) and umbilical vein (EA.hy926) cells. The expression levels were also compared in five types of tissue specimen, including placenta, esophageal cancer, cervical cancer, lung cancer and breast cancer tissues (Fig. 2B). The results demonstrated that flTF and asTF exist in a wide range of human tissues and cells, and the mRNA expression levels of flTF were significantly higher compared with asTF in all samples tested ($P<0.05$).

Effects of CDCA on the mRNA expression levels of TF variants in HepG2 and EA.hy926 cells. There was a significant increase in the expression levels of flTF in the HepG2 and EA.hy926 cells treated with CDCA (Fig. 3A). CDCA also promoted the expression of asTF in EA.hy926 cells, but had no significant effect on HepG2 cells (Fig. 3B).

Effects of apoM overexpression on the mRNA expression levels of TF variants in Caco-2 and EA.hy926 cells. Compared with in the NC (empty vector control) group,

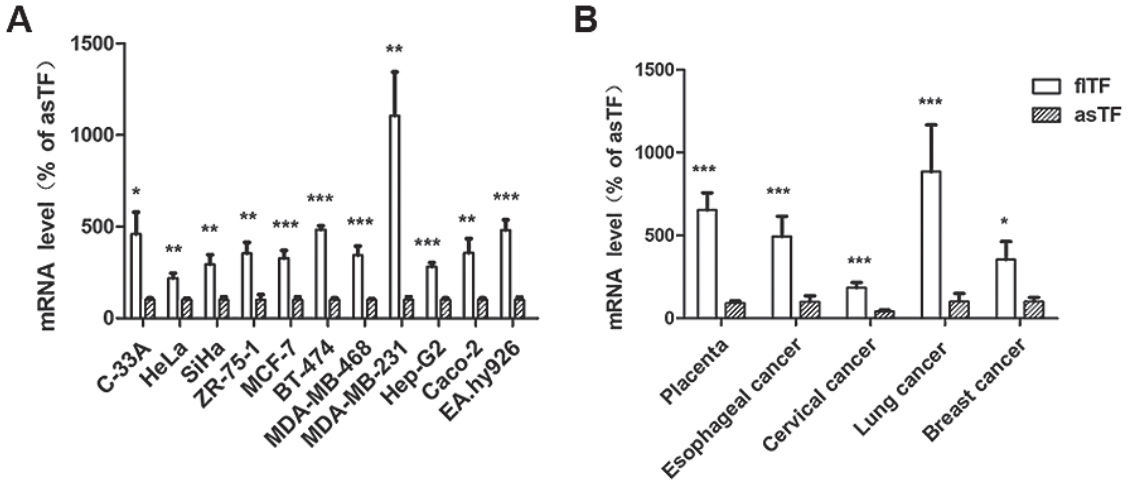

Figure 2. Expression levels of flTF and asTF in human cell lines and tissues. Quantitative polymerase chain reaction analysis revealed that flTF and asTF mRNA was expressed in (A) all 11 human cell lines tested and (B) all five types of tissue specimens tested. *P<0.05, **P<0.01 and ***P<0.001 vs. asTF. asTF, alternatively spliced TF; flTF, full-length TF; TF, tissue factor.

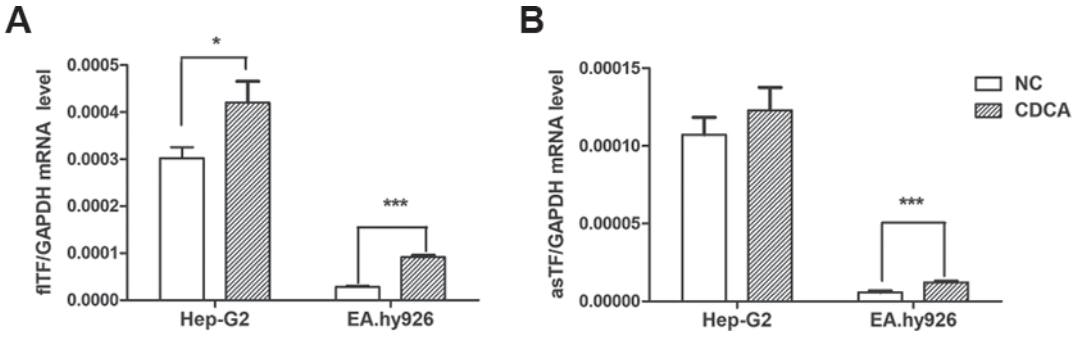

Figure 3. Expression levels of flTF and asTF in HepG2 and EA.hy926 cells treated with CDCA (50 μmol). mRNA expression levels of (A) flTF and (B) asTF in the CDCA and NC groups (treated with 0.1% ethanol) were quantified using quantitative polymerase chain reaction and normalized to those of GAPDH (n=6). *P<0.05 and ***P<0.001 vs. NC. asTF, alternatively spliced TF; CDCA, chenodeoxycholic acid; flTF, full-length TF; NC, negative control; TF, tissue factor.

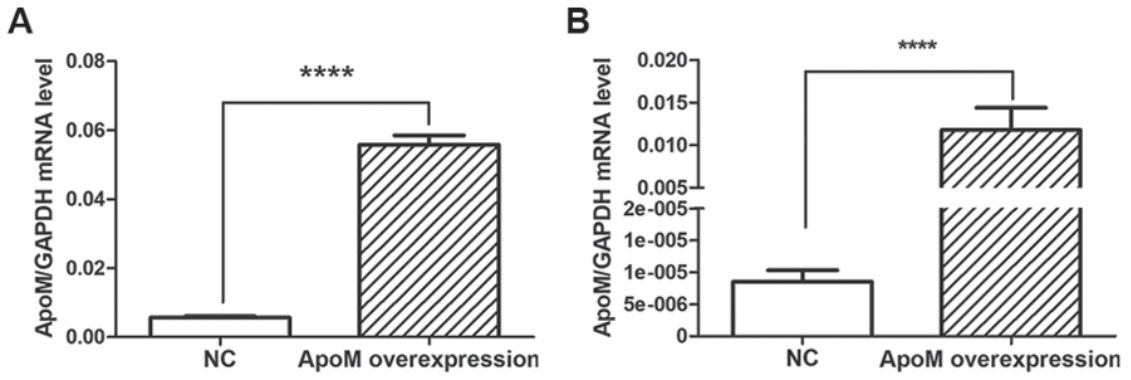

Figure 4. ApoM transduction efficiency. ApoM mRNA expression levels in NC (infected with empty vectors) and overexpression groups (infected with apoM lentivirus vectors) in (A) Caco-2 and (B) EA.hy926 cells, as quantified using quantitative polymerase chain reaction. The mRNA expression levels of apoM were higher in the apoM overexpression group compared with in the NC group. ****P<0.0001 vs. NC. apoM, apolipoprotein M; NC, negative control.

apoM lentivirus transduction increased apoM expression by 9.95-fold (P<0.0001) in Caco-2 cells (Fig. 4A) and 1,379.07-fold (P<0.0001) in EA.hy926 cells (Fig. 4B). As shown in Fig. 5, the results demonstrated that the mRNA expression levels of asTF were increased in Caco-2 cells overexpressing apoM compared with in the NC group (P<0.05); however, no significant effect was observed on flTF expression in those cells. In addition, apoM overexpression had no significant effect on flTF and asTF in EA.hy926 cells.

mRNA expression levels of TF variants in tumor, normal and adjacent tissues. To investigate whether the expression of individual splice variants differed during the tumor process, the expression levels of flTF and asTF were investigated in

Table II. Expression levels of flTF and asTF in cell lines and tissues.

		flTF		asTF	
Cell lines and tissues	Group	Expression levels	P-value	Expression levels	P-value
Caco-2	ApoM OE group	$4.73x10^{-4}±6.22x10^{-5}$	0.66	$2.29x10^{-4}±4.53x10^{-5}$	0.03[a]
	Control group	$4.23x10^{-4}±9.22x10^{-5}$		$1.19x10^{-4}±1.27x10^{-5}$	
EA.hy926	ApoM OE group	$1.40x10^{-4}±1.43x10^{-5}$	0.24	$4.17x10^{-5}±8.43x10^{-6}$	0.82
	Control group	$1.59x10^{-4}±3.86x10^{-6}$		$4.50x10^{-5}±1.14x10^{-5}$	
HepG2 cells	CDCA group	$4.20x10^{-4}±4.53x10^{-5}$	0.04[a]	$1.23x10^{-4}±1.48x10^{-5}$	0.42
	Control group	$3.02x10^{-4}±2.34x10^{-5}$		$1.07x10^{-4}±1.11x10^{-5}$	
NSCLC	Normal tissues	0.034±0.034	0.97	0.005±0.008	0.54
	Adjacent tissues	0.052±0.067		0.011±0.016	
	Tumor tissues	0.040±0.041		0.008±0.015	
Cervical cancer	Normal tissues	0.007±0.006	0.62	0.0003±0.0004	0.001[a]
	Tumor tissues	0.007±0.007		0.002±0.002	

[a]Statistically significant difference. ApoM, apolipoprotein M; asTF, alternatively spliced TF; CDCA, chenodeoxycholic acid; flTF, full-length TF; NSCL, non-small cell lung cancer; OE, overexpression; TF, tissue factor.

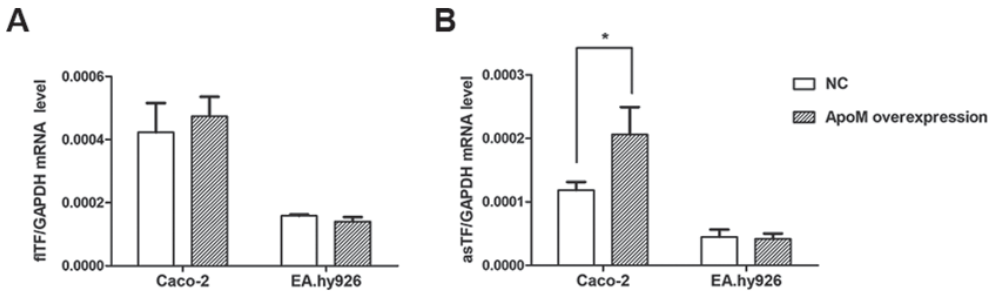

Figure 5. Effects of apoM overexpression on the mRNA expression levels of flTF and asTF in Caco-2 and EA.hy926 cells. (A) flTF and (B) asTF mRNA expression levels in the apoM overexpression and NC groups were quantified using quantitative polymerase chain reaction. No significant effect on flTF was observed in cells overexpressing apoM. In addition, apoM overexpression promoted the mRNA expression levels of asTF in Caco-2 cells, but had was no significant effect on asTF expression in EA.hy926 cells. *P<0.05 vs. NC. apoM, apolipoprotein M; asTF, alternatively spliced TF; flTF, full length TF; NC, negative control; TF, tissue factor.

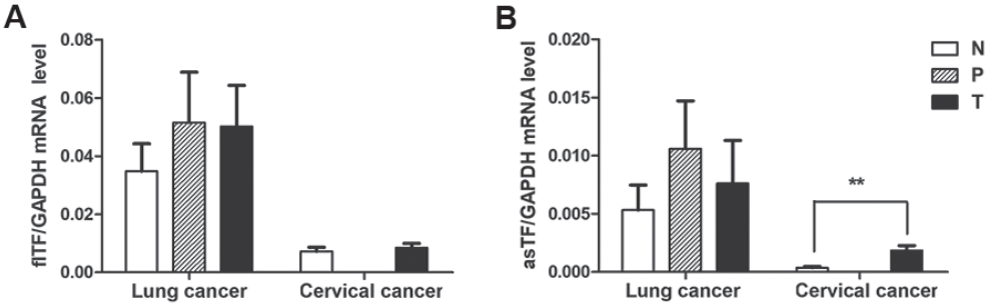

Figure 6. mRNA expression levels of flTF and asTF in NSCLC and cervical cancer tissues. NSCLC tissues were paired, whereas cervical tissues were unpaired due to the lack of paracancerous tissues. (A) flTF and (B) asTF mRNA expression levels in the lung and cervical cancer tissues and adjacent/normal tissues were quantified using quantitative polymerase chain reaction. No significant effect was observed on the mRNA expression levels of flTF among the lung and cervical tissues and their adjacent/normal tissues. The expression levels of asTF were higher in cervical cancer tissues compared with in the normal group, whereas there was no significant difference in asTF expression between lung cancer and adjacent/normal tissues. **P<0.01. asTF, alternatively spliced TF; flTF, full length TF; N, normal tissue; NSCLC, non-small cell lung cancer; P, paracancerous tissue; T, tumor tissue; TF, tissue factor.

cervical and lung cancer specimens. As shown in Fig. 6, there was no significant difference in the mRNA expression levels of flTF and asTF splice variants among any of the lung cancer tissues. There was also no significant difference in the mRNA expression levels of flTF between cervical cancer and normal cervical tissues; however, asTF was significantly increased

Table III. Information regarding the TF primers and amplified products in studies over the last decade (2008-2017).

Author, year	Forward primer (5'-3')	Reverse primer (5'-3')	Products	(Refs.)
Rossiello MR *et al*, 2008	AAACCCGTCAATCAAGTCTAC	GAAAGTGTTGTTCCTTCTGAC	flTF and asTF	(15)
Sovershaev MA *et al*, 2008	CCCCAGAGTTCACACCTTACCT	CACTTTTGTTCCCACCTGTTCA	flTF and asTF	(16)
Regina S *et al*, 2008	CCAAACCCGTCAATCAAGT	TGCTTCACATCCTTCACAATCTCG	flTF and asTF	(17)
Plotkowski MC *et al*, 2008	CCCGAACAGTTAACC GGAAGA	GCTCCAATGATGTAGAATATTTCTCTGA	flTF	(18)
Liu F *et al*, 2008	GCACTAAGTCAGGAGATTGG	CTGTCTGTACTCTTCCGGTT	flTF	(19)
Usui M *et al*, 2009	CAGTGCAATATAGCATTTGCAGTAGC	CTACTGTTTCAGTGTTCAAGCAGTGA	flTF	(20)
Lockwood CJ *et al*, 2009	GAAGCAGACGTACTTGGCACGG	CCGAGGTTTGTCTCCAGGTA	flTF and asTF	(21)
Di Stefano R *et al*, 2009	TGAATGTGACCGTAGAAGATGA	GGAGTTCTCCTTCCAGCTCT	flTF and asTF	(22)
Wang HJ *et al*, 2009	TCCCGAACAGTTAACCGGAA	GACCACAAATACCACAGCTCCA	flTF	(23)
Wang HJ *et al*, 2010	CAGGGAATGTGGAGAGCAC	GGCTGTCCGAGGTTTGTC	flTF and asTF	(24)
Teng Y *et al*, 2010	CCGAACAGTTAACCGGAAGA	TCAGTGGGGAGTTCTCCTTC	flTF	(25)
Valsami S *et al*, 2011	AATGTGGAGAGCACCGGTTCT	CGTTCATCTTCTACGGTCACATTC	flTF and asTF	(26)
Blank M *et al*, 2011	TTCACACCTTACCTGGAGACAAAC	AACATCCCGGAGGAGGCTTAGGA	--	(27)
Ben-Hadj-Khalifa S *et al*, 2011	CCGACGAGATTGTGAAGGATGT	AGAGGCTCCCCAGCAGAAC	flTF and asTF	(28)
Gerotziafas GT *et al*, 2012	AATGTGGAGACCGGTTCT	TCCGTTCATCTTCTACGGTCAC	--	(29)
Wang JG *et al*, 2012	AATGTGGAGAGCACCGGTTC	CGTTCATCTTCTACGGTCACATTC	flTF and asTF	(30)
Gebhard C *et al*, 2012	TCCCCAGAGTTCACACCTTACC	TGACCACAAATACCACAGCTCC	flTF and asTF	(31)
Lin M *et al*, 2012	AGAGTACAGACAGCCCGGTAGAGTG	GCCAGGATGATGACAAGGATGA	flTF	(32)
Maruyama K *et al*, 2012	CTCGGACAGCCAACAATTCAGAGT	TGTTCGGGAGGGAATCACTGCTTGAACACT	flTF	(33)
Sun L *et al*, 2013	GCCAGGAGAAAGGGGAAT	CAGTGCAATATAGCATTTGCAGTAGC	flTF	(34)
Yang HP *et al*, 2013	GAACCCAAACCCGTCAAT	TCTCATACAGAGGCTCCC	flTF and asTF	(35)
Eisenreich A *et al*, 2013	CTCGGACAGCCAACAATTCAG	CGGGCTGTCTGTACTCTTCC	flTF	(36)
Carneiro-Lobo TC *et al*, 2014	CAGGCACTACAAATACTGTGG	TGTAGACTTGATTGACGGGTT	flTF and asTF	(37)
Chen C *et al*, 2014	GTGATTCCCTCCCGAACAGTT	CTGGCCCATACACTCTACCG	flTF	(38)
Balia C *et al*, 2014	TTGGCAAGGACTTAATTTATACAC	CTGTTCGGGAGGGAATCAC	flTF	(39)
Liu LX *et al*, 2014	ACGCTCCTGCTCGGCTGGGT	CGTCTGCTTCACATCCTTCA	flTF and asTF	(40)
Bravo ML *et al*, 2015	CCAAACCCGTCAATCAAGTC	ACAATCTCGTCGGTGAGGTC	flTF and asTF	(41)
Sovershaev TA *et al*, 2015	GAATGTGACCGTAGAAGATG	CACTGAAACAGTAGTTTTCTCC	flTF	(42)
Wang B *et al*, 2015	CAAACCCGTCAATCAAGTCTAC	CTTCACATCCTTCACAATCTCG	flTF and asTF	(43)
Wang R *et al*, 2015	CACCGACGAGATTGTGAAGG	CGGAGGCTTAGGAAAGTGTTG	flTF and asTF	(44)
Orellana R *et al*, 2015	CCAAACCCGTCAATCAAGTC	ACAATCTCGTCGGTGAGGT	flTF and asTF	(45)
Jacobsen C *et al*, 2015	GCCAGGAGAAAGGGGAAT	CAGTGCAATATAGCATTTGCAG	flTF	(46)
Dong R *et al*, 2015	TGACCTCACCGACGAGATTGTGAA	TCTGAATTGTTGGCTGTCCGAGGT	flTF and asTF	(47)
Li W *et al*, 2016	AAGCAGTGATTCCCTCTCG	AACACAGCATTGGCAGCAG	--	(48)
Scalise V *et al*, 2016	TTGGCAAGGACTTAATTTATACAC	CTGTTCGGGAGGGAATCAC	flTF	(49)

Table III. Continued.

Author, year	Forward primer (5'-3')	Reverse primer (5'-3')	Products	(Refs.)
Krychtiuk KA *et al*, 2017	CAGACAGCCCGGTAGAGTGT	CCACAGCTCCAATGATGTAGAA	flTF	(50)
Gao H *et al*, 2017	GGCGCTTCAGGCACTACAA	TTGATTGACGGGTTTGGGTTC	flTF and asTF	(51)
Brambilla M *et al*, 2018	TGATGTGGATAAAGGAGAAAACTACTGT	TCTACCGGGCTGTCTGTACTCTT	flTF	(52)

asTF, alternatively spliced TF; flTF, full-length TF; TF, tissue factor.

in cervical cancer tissues compared with in normal cervical tissues (P<0.01).

Summary of the negative results. Overexpression of apoM had little effect on the mRNA expression levels of flTF in the Caco-2 and EA.hy926 cells. In addition, there was no significant difference between the mRNA expression levels of flTF in the adjacent/normal and tumor NSCLC and cervical cancer tissues (Table II). With regards to asTF mRNA expression, CDCA had little effect on HepG2 cells and apoM overexpression did not affect the levels of asTF in the EA.hy926 cells. Similarly, there was no statistically significant difference in asTF mRNA levels between the adjacent and tumor NSCLC tissues.

Discussion

Since asTF and flTF serve important and often distinct roles in various biological processes, it is appropriate to study them separately. However, as shown in Table III (15-52), total TF, including asTF and flTF, or flTF alone, has been investigated in studies over the last decade. Few studies have been conducted that have detected asTF and flTF levels separately, according to the primer sequences that have been designed. Furthermore, the primers used in three of these previous studies (27,29,48) are not completely matched with the TF sequence and therefore cannot be used to amplify asTF or flTF.

In terms of expression, as the major form of TF, the mRNA expression levels of flTF were higher than those of asTF in all specimens tested in the present study. Furthermore, in terms of function, CDCA increased the expression levels of flTF in HepG2 cells, whereas those of asTF were not affected. Therefore, it was hypothesized that flTF may contribute to the onset of liver cancer. The present study also demonstrated that CDCA increased the expression levels of flTF and asTF in EA.hy926 cells, which indicated that CDCA may be associated with vasoconstriction. Furthermore, Kundu *et al* (9) demonstrated that CDCA is capable of promoting vessel formation. In a previous study, both variants were revealed to mediate various physiological and pathological functions, including angiogenesis (2). Therefore, CDCA may promote vessel formation through the upregulation of these two variants; however, the possible mechanism requires further investigation.

asTF is associated with the development of numerous types of cancer. A previous study demonstrated that the mRNA expression levels of apoM in colorectal cancer tissues were significantly increased in patients with lymph node metastasis (53). The present study revealed that the expression levels of asTF were increased in Caco-2 cells that overexpressed apoM compared with the control cells. Conversely, overexpression of apoM had little effect on flTF. Consistent with this result, Yu *et al* (54) demonstrated that flTF expression in colorectal cancer has no influence on cell proliferation *in vitro*; however, asTF has been reported to promote cell proliferation *in vitro* and tumor growth *in vivo* (1,36,55). It may therefore be hypothesized that asTF, not flTF, contributes to the onset of cancer.

Venteclef *et al* (56) demonstrated that bile acids suppress apoM expression *in vitro* and *in vivo*. The results of the present study demonstrated that as one of the bile acids, CDCA increased the expression levels of the two TF variants in

the EA.hy926 cells and apoM increased as TF expression in Caco-2 cells. These data suggested that apoM may be involved in the regulation of TF expression induced by CDCA, but this requires further investigation.

The mRNA expression levels of flTF were not significantly different between the cervical cancer and normal tissues; however, those of asTF were markedly increased in cancer tissues. Previous studies regarding the coagulant properties of asTF have been fairly inconclusive (57,58), but they are likely to be essential during angiogenesis associated with the development of cancer (59,60). The present study demonstrated that the measurement of asTF mRNA may be associated with cervical cancer risk. The expression levels of flTF and asTF in lung cancer and paracancerous tissues were higher compared with in normal control tissues; however, the differences were not statistically significant. Rollin *et al* (61) demonstrated that the relative amount of asTF is low. This previous study also analyzed the levels of asTF in NSCLC tumors according to clinicopathological features; the results revealed that there is no association between asTF and sex, age, stage and differentiation grade, yet patients with high asTF tumor mRNA expression had a poorer prognosis. Therefore, further studies are required to investigate the correlation between TF and NSCLC tumors.

A limitation of the present study is that control or paired normal esophageal and breast cancer tissues samples were not analyzed. Therefore, the role of flTF and asTF in these two types of cancer could not be clearly identified.

In conclusion, TF isoforms are able to activate distinct signaling pathways (2), leading to the modulation of cancer-associated biological processes and nonhemostatic pathophysiological processes, including thrombosis, angiogenesis, tumor growth and metastasis. A previous study demonstrated that flTF mediates cell signaling via protease activated receptor 2 and downstream signaling proteins, including protein kinase C and extracellular signal-regulated kinase 1 and 2, whereas asTF exhibits activity via integrin ligation (2,62). To the best of our knowledge, the present study is the first to reveal that flTF expression may be increased compared with asTF in all tissue specimens tested, and suggested that overexpression of apoM and CDCA may affect the mRNA expression levels of the two variants. Furthermore, the expression levels of the two variants may be different in the same cancer tissues. These results provide further information regarding the TF system and emphasize the significance of flTF and asTF expression in tumor progression and other types of disease.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

GL and NX contributed to the design of the study and revision of the manuscript. LP performed the experiments, analyzed the data and contributed to writing the manuscript. YY and QM performed experiments. MY and SY were responsible for experiments and statistical analysis.

Ethics approval and consent to participate

The experimental protocols were approved by the Institutional Ethics Committee of the Third Affiliated Hospital of Soochow University and all patients provided written informed consent for this study.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

All authors declare that they have no competing interests.

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