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Original Article

Tissue factor (TF) regulates cell viability, proliferation and invasion of human gastric cancer

Jing Zhang*, Donghong Ma*, Sujuan Zhang, Dongxu Wang, Hongbin Zhu

*Department of Gastroenterology and Hepatology, Chinese PLA NO.254 Hospital, Tianjin 300142, China. *Equal contributors.*

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Abstract: To investigate the effect of tissue factor (TF) on the biological behaviors of human gastric tumor tissues and cell line. The recombinant pcDNA3.1-antisense TF and pcDNA3.1-sense TF plasmids were constructed and transfected into SGC7901 cells with high TF expression or MKN45 cells with deficiency of TF, respectively. MTT, colony formation, FCM and transwell chamber methods were used to evaluate the effect of TF on cell viability, proliferation, cell cycle distribution and invasion abilities of gastric cancer cells. We first found an increased expression of TF in 24 pairs of human gastric cancer tissues. Functionally, the expression of TF was inhibited in SGC7901 cells transfected with the recombinant plasmid expressing antisense TF. Compared with parental cells and empty vector cells, the proliferation of TF-inhibited SGC7901 cells was significantly decreased ($P<0.05$). Proliferation index (PI) declined from 0.357 in parental cells to 0.286, and the invasion ability was decreased significantly ($P<0.001$). However, TF was overexpressed in MKN45 cells transfected with sense TF recombinant plasmid. Compared with parental cells and empty vector cells, the PI value was increased from 0.277 in parental cells to 0.366. Colony formation assay showed the consistent results. Moreover, the invasive ability of the TF-overexpressing MKN45 cells was significantly increased ($P<0.001$). TF may be a regulator of viability, proliferation and invasion of gastric cancer cells.

Keywords: Gastric cancer, tissue factor, viability, proliferation, invasion

Introduction

Tissue factor (TF), also known as tissue thromboplastin, is a transmembrane protein with a relative molecular mass of 47×10^3 Da [1]. It is a cell surface receptor of factor VII. Following binding to the coagulation factor VII and making it activated (FVIIa), and the resulting TF/FVII complex has a cascade effect that triggers exogenous coagulation [1]. TF is highly expressed in hemangiopericyte, some tissues and organs, but it is absent in normal vascular endothelial cells. TF can promote the metastasis of tumor cells [2]. It has been reported that TF is highly expressed in many tumor cells and plays an important role in tumor growth and metastasis [3]. However, there are few studies on the effect of TF on gastric cancer. Therefore, the aim of this study is to investigate the role of TF in the growth and metastasis of gastric cancer cells.

Materials and methods

Patients and tissues

A total of 24 gastric cancer patients hospitalized in Chinese PLA NO.254 Hospital from July 2016 to April 2017 were used in this study. The paired normal tissues and tumor tissues and were collected during surgery at the same time, followed by immediately frozen in liquid nitrogen and stored at -80°C. Informed consents were obtained according to the guideline of the ethics committee of Chinese PLA NO.254 Hospital.

Cell culture

Human gastric cancer cells (SGC7901, MKN45 and AGS) and immortalized gastric mucosa GES cells were obtained from the Department of Gastroenterology and Hepatology, Xijing Hospital, the Fourth Military Medical University.

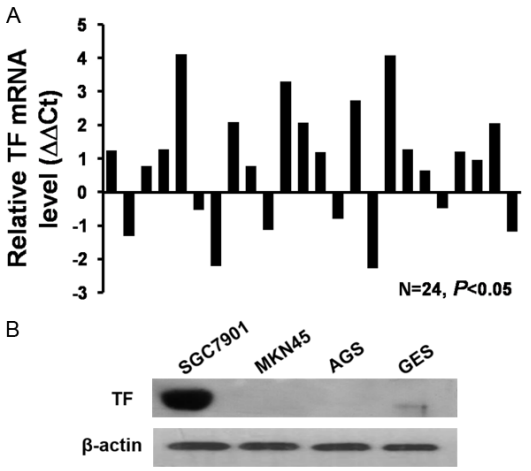

Figure 1. The expression of TF in GC tissues and cell lines. A. TF expression in 24 paired gastric cancer tissues by qRT-PCR. DDCt: DCtN-DCtT. B. TF expression in different human gastric cell lines by Western blotting. Lane 1, SGC7901; lane 2, MKN45; lane 3, AGS; lane 4, GES.

The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Melbourne, Australia) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Gastric cancer cell lines were split to low density (30% confluence) 24 hours before treatment.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from 30 mg tissues by Trizol reagent (Life Technology) as per the manufacturer's instructions. Reverse transcription was performed by using oligo$(dT)_{12\text{-}18}$ methods. QRT-PCR was performed in a total of 10 μl of reaction mixture by ABI-7500 fast system using SYBR Premix Ex Taq II reagent (Takara). GAPDH were used as an internal control. The primers are as belows: TF: Forward: 5'-GCCAGGAGAAAGGGGAAT-3', Reverse: 5'-CAGTGCAATATAGCATTTGCAGTAGC-3', GAPDH: Forward: 5'-ACGGTCAGGTCAACAACT-3', Reverse: 5'-GTGCTTCCAGTCCTTAACG-3'.

Reagents and constructs

The plasmids pcDNA3.1 (+) and pcDNA3.1 (-) were gifts from Dr. Hao Zhimin. The sense and antisense TF cloning primers (Sangon Biotech Co., Ltd., Shanghai, China) were 5'-CGG GAT CCA TGG AGA CCC CTG CCT GGC CC-3' and 5'-CCC TCG AGT TAT GAA ACA TTC AGT GGG GAG TTC-3', respectively, which contain BamH I/Xho I endorestriction sites. The desired fragment was obtained by Polymerase Chain Reaction (PCR). The obtained fragment was subcloned into pcDNA3.1 (+) and pcDNA3.1 (-), respectively. Trypsin and TRIzol reagents were from the Gibco (United States).

Western blotting

Equal amount of proteins were separated on the sodium dodecyl sulfate (SDS)-polyacrylamide gel and electro-transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The primary antibodies were: mouse anti-human TF antibody (Haematologic Technologies) and β-actin (Beyotime, Nanjing, China). β-actin was used as the loading control.

Cell growth assay

The cells (5×10^3) were seeded in 96-well plates. Each well was made in triplicate. Cells were tested on the second, fourth, sixth and eighth day respectively by MTT. Before test, 20 μl of MTT solution (5 g/L) was added into each well following a 4 hours of incubation at 37°C, and then the supernatant was aspirated. 150 μl of DMSO was added to dissolve the crystal for 10 min in a vibrator. The absorbance value of each well was measured at the 490 nm wavelength in the microplate reader. Cell growth curve was plotted by taking the DMSO solution without cells as the blank control.

Colony formation assay

The proliferation of SGC7901 and MKN45 cells was determined by colony formation assay followed by crystal violet staining. The transfected cells were seeded in 6-well plates at a density of 5×10^3 cells and grown for 14 days. Cells were fixed in 4% paraform aldehyde for 10 minutes. After washing with PBS, cells were stained with 1% crystal violet staining buffer for another 15 minutes. The 6-well plates were then aspirated, washed and allowed to air dry, followed by photographed in digital camera.

Flow cytometry assay (FCM)

FCM was performed with the standard method, using propidium iodide (PI; Sigma-Aldrich) staining of cellular DNA. Each sample was analyzed by flow cytometry with a FACScan Flow

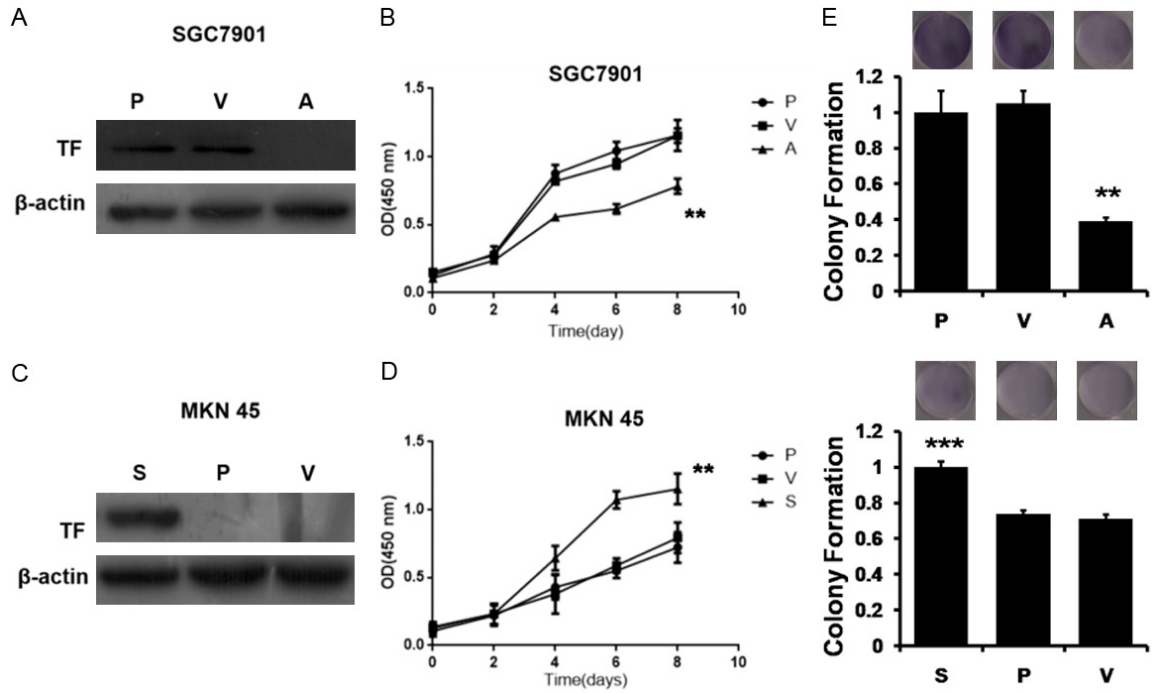

Figure 2. The effect of TF on cell viability and cell proliferation. A, C. Expression of TF in the parental (P), the empty vector-transfected (V), the antisense TF-transfected (A) and the sense TF-transfected (S) cells by Western blot analysis. β-actin was used as a loading control. B, D. In vitro cell growth rate determined by MTT assay in the cell lines as described above. E. Colony formation assay was performed in the cell lines as described above. The data are presented as the mean ± SD. **: P<0.01; ***: P<0.001, compared to V group.

Cytometer (Becton-Dickinson Biosciences, Mansfield, MA) using a 488 nm laser. The data were processed with the ModFit v. 2.0 (Verity Software House, Topsham, ME).

Transwell assay

The 24-well transwell chambers with an aperture of 8 um pre-coated with Matrigel (BD Bioscience, USA) were adopted. Cells were harvested and washed with serum-free medium. 1×10^5 cells were added in the upper chamber with a total volume of 200 μl of serum-free medium, while 300 μl of serum containing (10%) medium was added in the lower chamber. Cells were allowed to invade towards the medium containing 10% FBS for 24 h. The cells that reached the lower surface were fixed with methanol and stained with hematoxylin. The cells were counted in 5 randomly selected microscopic fields (×200) from each chamber.

Statistical analysis

The data was expressed with mean ± standard deviation (SD). Statistical analyses were performed using SPSS 10.0 statistical software (SPSS Company, USA). Student t test was used to analyze the TF expression in paired tissues. One way ANOVA was used to analyze the differences between groups. Statistical significance was assigned at P<0.05 (*), P<0.01 (**) or P<0.001 (***). All the experiments of cell lines were performed at least three times with triplicate samples.

Results

Upregulation of TF expression in human gastric cancer samples and cell lines

First, we test the expression of TF in paired GC samples. The results showed that TF was upregulated in GC tissues, compared to paired normal tissues (**Figure 1A**). We next examined the expressions of TF in different gastric cancer cell lines and immortalized gastric mucosa GES cells. As shown in **Figure 1B**, the results demonstrated that TF was highly expressed in SGC7901 cells, which was with hypermalignancy; but weakly expressed in MKN45, AGS and GES cell lines. Therefore, we performed gain-of-

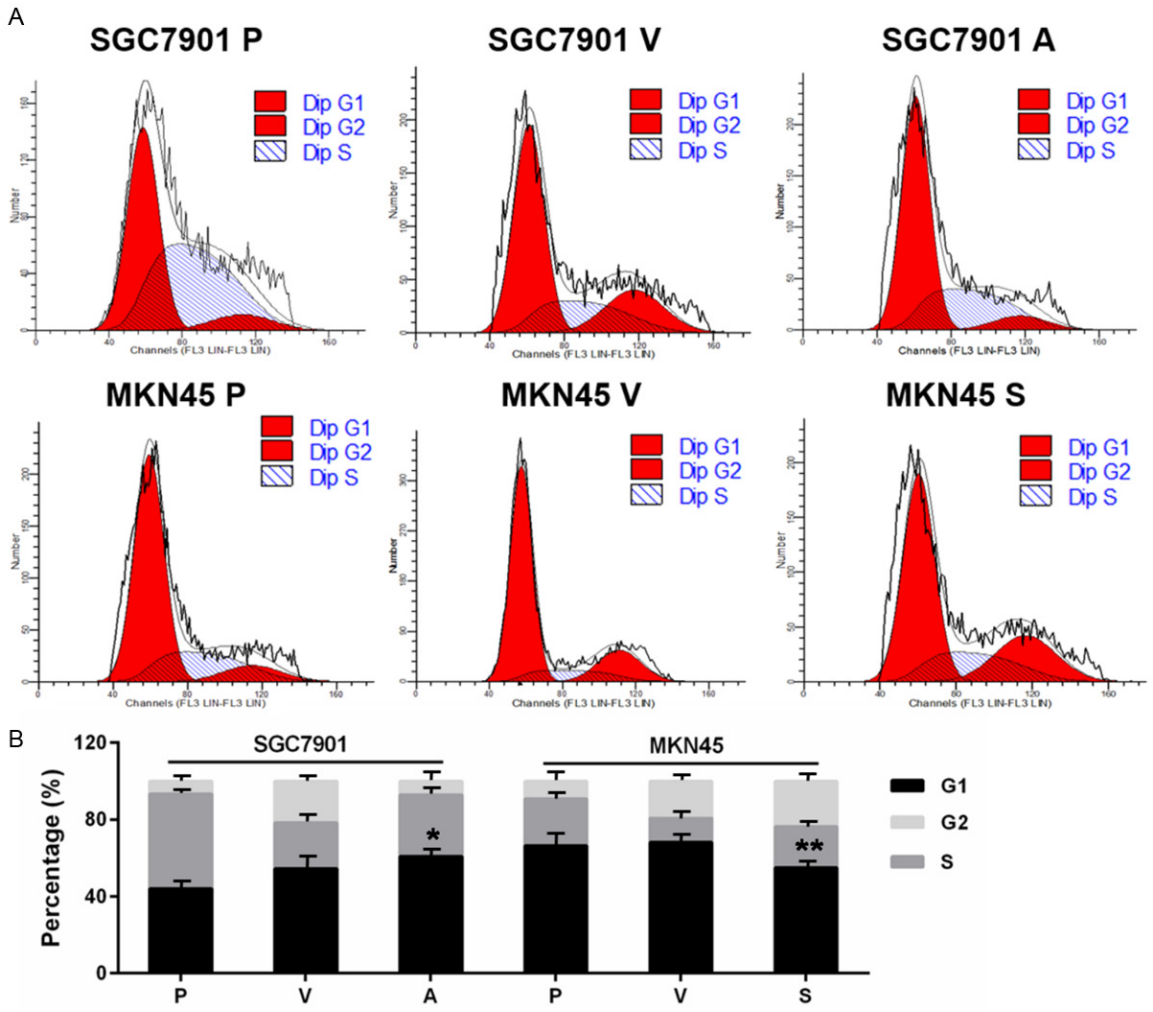

Figure 3. The effect of TF on cell cycle distribution. Representative images was shown (A) and percentage of each phase cells (B) in the indicated cells assessed by FCM. P: parental, V: the empty vector-transfected, A: the antisense TF-transfected, S: the sense TF-transfected. The data are presented as the mean ± SD. *: P<0.05; ***: P<0.001, compared to V group.

function studies below in MKN45 cells, whereas loss-of-function studies in SGC7901 cells.

TF contributed on gastric cancer cells viability and proliferation

Sense and antisense TF expression plasmids were firstly constructed and stably introduced into MKN45 or SGC7901 cells, respectively. As shown in **Figure 2A** and **2C**, TF-silenced SGC7901 cells and TF-overexpressed MKN45 cells were successfully obtained. Then MTT method was applied to analyze the cell growth rate of these cell lines. **Figure 2B** showed that compared with the parental cells and empty vector transfected cells, the proliferation of TF-silenced SGC7901 cells was reduced significantly (P<0.05). On the other hand, **Figure 2D** showed that proliferation of TF-overexpressed MKN45 cells was accelerated markedly (P< 0.05), in comparison with the parental and empty vector transfected MKN45 cells. Moreover, colony formation assay showed the consistent results (**Figure 2E**). Overall, the results demonstrated that TF may contribute on GC cells viability and proliferation.

Silencing of TF contributed to G1 arrest on cell cycle

FCM was performed to analyze the cell cycle and cell proliferation index (PI) was calculated for each cell line. In the SGC7901 cell cycle analysis, results showed that the G1-phase per-

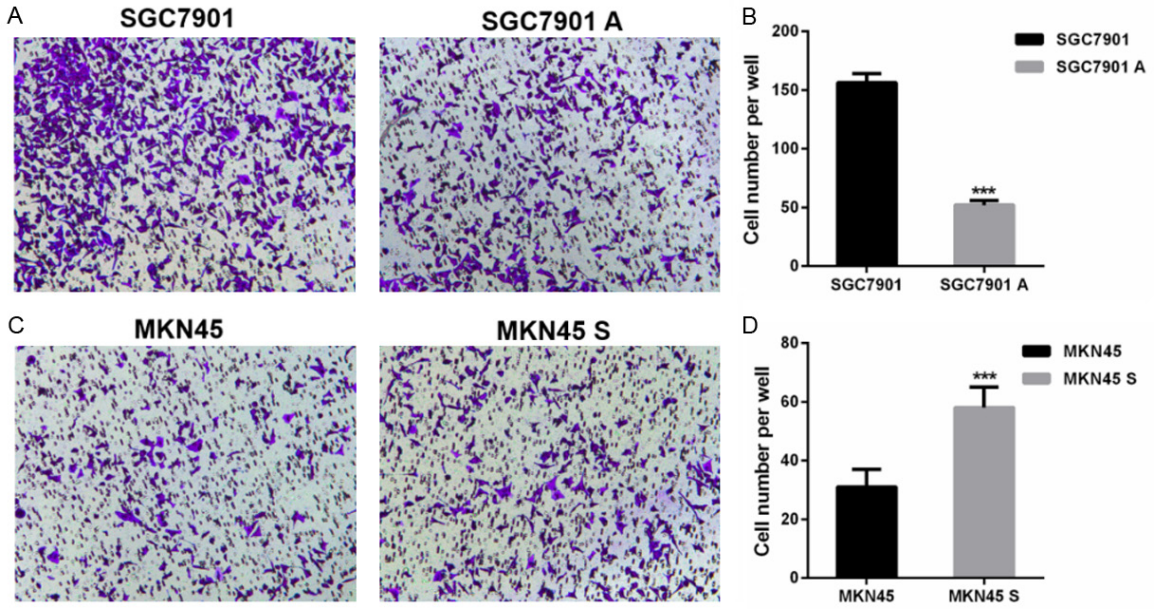

Figure 4. The effect of TF on cell invasion. Representative images (A, C) and numbers (B, D) of the indicated invasive cells analyzed by transwell assay. The data are presented as the mean ± SD. A: the antisense TF-transfected, S: the sense TF-transfected. The data are presented as the mean ± SD. ***: P<0.001, compared to control group.

centage was increased from 44.18% in the parental cells to 60.87% in the TF-silenced cells (**Figure 3**). PI of TF-silenced SGC7901 cells declined from 0.508 in the parental cells to 0.339. In the MKN45 cell cycle analysis, results showed that the G1-phase percentage was decreased from 66.45% in the parental cells to 55% in the TF-overexpressed cells (**Figure 3**). PI of TF-overexpressed MKN45 cells was increased from 0.276 in the parental cells to 0.412.

TF promoted the gastric cancer cell invasion

The cell invasion capacity was determined by counting the cells passing through the matrigel. The results showed that invasion of TF-silenced SGC7901 cells was significantly inhibited compared with the parental cells (P<0.001; **Figure 4A** and **4B**). As for MKN45 cells, TF overexpression led to an obviously elevated invasion ability (P<0.001; **Figure 4C** and **4D**).

Discussion

TF, an extrinsic coagulation system promoter, is highly expressed in many tumor cells and plays an important role in the tumor growth and metastasis [4-7]. In vitro experiments suggest that TF may affect the tumor cell proliferation and other biological behaviors of malignant tumor [4, 8]. In colon cancer, studies prove that in comparison with negative TF-expressed cell, positive TF-expressed colon cancer cells contain higher levels of VEGF and more microvessel. This finding suggests that TF can promote colon cancer cell growth and metastasis by enhancing angiogenesis [9]. Another study shows that in small cell lung cancer, expressions TF and VEGF are closely correlated with each other, thus indicating that TF can impact the tumor angiogenesis [10]. Our preliminary studies have also shown that TF can affect the tumor angiogenesis [11]. Wang X et al utilized the siRNA technology to knockdown TF expression in the metastatic melanoma LOX-L and found that lung metastasis was reduced in the nude mice by TF-knockdown [12]. All these reports suggest that TF can not only regulate VEGF expression in certain tumor cells [13], affect the proliferation and tumor formation, but also control the movement and metastasis of tumor cells.

Our preliminary study has shown that TF is highly expressed in gastric cancer [14]. Therefore, we hypothesized that TF might play an important role in the regulation of gastric cancer cell growth and metastasis. We found that TF was highly expressed in GC tissues and SGC7901 cells, while weakly expressed in MKN45 cells. In order to further explore the effect of TF on

the biological behaviors of gastric cancer cells, sense and anti-sense TF cDNA were adopted to stably transfect MKN45 (no TF expression) or SGC7901 (high expression of TF) cells, respectively. Compared with the parent cells and empty vector transfected cells, the viability and proliferation capacity of TF-silenced SGC7901 cells was inhibited while that of TF-overexpressed MKN45 cells was increased, by using MTT and colony formation assay. This means that TF could increase gastric cancer cell viability and proliferation.

Unlimited viability and proliferation, local invasion and distant metastasis are the characteristics of tumor cells. The unrestricted proliferation of tumor cells is mainly due to the loss of cell cycle control. The cell cycle analysis revealed that TF knockdown resulted in G1 phase arrest while TF overexpression led to reduction in G1 phase. In addition, TF overexpression produced a higher cell proliferation index. These findings illustrated that TF could really affect cell proliferation, and this proliferation control mainly attributed to the cell cycle regulation.

Tumor cell metastasis is a process involved the degradation of extracellular matrix (EMC) mediated by the main growth factor, permitting the tumor cells to penetrate into vascular endothelial and spread to the distance, and eventually form new lesions. Our study applied the transwell method to simulate EMC and the results shown that the number of invasive cells declined significantly after TF expression was inhibited, while TF overexpression caused the opposite effect. These data demonstrated that TF can promote the gastric cancer cell invasion.

In summary, our study revealed that TF promoted the viability, proliferation and invasion of gastric cancer cells. TF might be a useful therapeutic candidate for human gastric cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dongxu Wang and Hongbin Zhu, Department of Gastroenterology and Hepatology, Chinese PLA NO.254 Hospital, #60 Huangwei Road, Tianjin 300142, China. Tel: +86-22-84683046; Fax: +86-22-84683024; E-mail: dongxuwang36@yahoo.com (DXW); hongbinzhu@126.com (HBZ)

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