

RESEARCH COMMUNICATION

Effect of Tissue Factor on Invasion Inhibition and Apoptosis Inducing Effect of Oxaliplatin in Human Gastric Cancer Cell

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Abstract

Objective: Tissue factor (TF) is expressed abnormally in certain types of tumor cells, closely related to invasion and metastasis. The aim of this study was to construct a human gastric cancer cell line SGC7901 stably-transfected with human TF, and observe effects on oxaliplatin-dependent inhibition of invasion and the apoptosis induction. **Methods:** The target gene TF was obtained from human placenta by nested PCR and introduced into the human gastric cell line SGC7901 through transfection mediated by lipofectamine. Stably-transfected cells were screened using G418. Examples successfully transfected with TF-pcDNA3 recombinant (experimental group), and empty vector pcDNA3 (control group) were incubated with oxaliplatin. Transwell chambers were used to show change in invasive ability. Caspase-3 activity was detected using a colorimetric method and annexin-V/PI double-staining was applied to detect apoptosis. **Results:** We generated the human gastric cancer cell line SGC7901/TF successfully, expressing TF stably and efficiently. Compared with the control group, invasion increased, whereas caspase-3 activity and apoptosis rate were decreased in the experimental group. **Conclusion:** TF can enhance the invasive capacity of gastric cancer cells in vitro. Its increased expression may reduce invasion inhibition and apoptosis-inducing effects of oxaliplatin and therefore may warrant targeting for improved chemotherapy.

Keywords: Tissue factor - gastric cancer - oxaliplatin - invasion - apoptosis

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Introduction

Gastric cancer is one of the most common cancers in the world. Many Asian countries, including Korea, China, and Japan, have very high incidences of gastric cancer. Besides surgery, chemotherapy is the main adjuvant treatment for postoperative and advanced gastric cancer. Oxaliplatin, a third-generation platinum, followed by C-DDP and carboplatin, coordination complex of the 1, 2-diaminocyclohexane families, generates covalent adducts between platinum and two adjacent guanines or guanine and adenine in cell DNA, which lead to a disruption in DNA replication and transcription (Boudny et al., 1992; Kollmannsberger et al., 2000). Oxaliplatin has been used widely in advanced gastric cancer, and shows great prospect (Raymond et al., 1998).

Tissue factor (TF), a 47-kDa transmembrane protein, binds plasma FVII/FVIIa and triggers coagulation cascade. TF is higher in a variety of tumors, and TF-FVIIa complex plays an important role in tumor cell growth, invasion, angiogenesis, as well as metastasis, through a variety of signal transduction pathways (Rao and Pendurthi, 2005; Forster et al., 2006). However, the effect of TF on antitumor efficacy of oxaliplatin in human gastric cancer has not been reported. In the present study, human gastric cancer cell line SGC7901, which was transfected

successfully with TF-pcDNA3 recombinant and empty vector pcDNA3 incubated with oxaliplatin, was used to investigate the invasion and apoptosis in human gastric cancer. TF reduces oxaliplatin in human gastric cancer cell invasiveness' in vitro inhibitory effect, and also reduces oxaliplatin-induced apoptosis.

Materials and Methods

TF gene cloning

Total DNA was extracted from human placenta tissue based on Trizol's specification and on amplified human TF full-length cDNA by RT-PCR. The nest PCR primer was designed and synthesized in accordance with full-length human TFmRNA in Genbank. Upstream outer primer 5'-TTCAGCGCAACCTCCCGAGC-3'; downstream outer primer 5'-AGCTCTAGATTATGAATCATTGAGTG-3'; upstream inner primer 5'-GTCAAGCTTATGGAGACC GCTGCCTG-3'; downstream inner primer 5'-AGCTCT AGATTATGAAACCTTCAGTG-3'; and Hind III, Xba I were introduced at the ends of the inner primer. PCR reaction condition was followed by 35 cycles each, with denaturation at 98 °C for 2 min, 98 °C for 10 s, and 68 °C for 1 min, which was extended to 72 °C for 5 min, and then kept at 4 °C. Specific PCR products were recovered. Nest PCR was started, with the first PCR products as template,

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followed by nest PCR amplification procedure, which was followed further by 35 cycles each with denaturation at 98 °C for 2 min, 98 °C for 10 s, and 52 °C for 1 min, which was extended to 72 °C for 5 min, and then kept at 4 °C. The products were detected by agarose electrophoresis, and then purified and recovered with a gel extraction kit (Beijing Solarbio Science and Technology Co., Ltd.).

Plasmid construction

The recovered TFcDNA was ligated into the pMD18-T (pMD-TF). The pMD-TF and pcDNA3.1 were double digested with Xba I/Hind III. The products were separated by agarose electrophoresis. TFcDNA fragments were subcloned into the pcDNA3.1 vectors, and then transformed into *E. coli* DH5 α competent cells. The sequence of the mini-preparation plasmid was confirmed by enzyme digestion and DNA sequencing. Gene sequencing was accomplished by Sangon Biotech Shanghai Co., Ltd. The plasmid with correct sequences was prepared for transfection.

Cell culture and transfection of cell line SGC7901

Human gastric cancer cell line SGC7901 was provided by the Molecular Biology Lab of Lanzhou University. RPMI 1640 DMEM (Sangon Biotech Shanghai Co., Ltd.), which contained 10% fetal bovine serum, was used to subculture SGC7901 in 5% CO₂ at 37 °C. The single-cell suspension was prepared and planted in the tissue culture plate with 24 wells. When cells reached 90% adhesion, transfection was performed in accordance with Lipofectamine™ 2000's specification (Invitrogen, Inc.), was conducted and screened with G418 (300 μ g/ml, maintenance concentration: 150 μ g/ml). Positive ones were obtained after 4 weeks. Divide the cells into 3 groups, namely, experimental group (SGC7901/TF): SGC7901 cells transfected by recombinant plasmid TF-pcDNA3.1, screened by G418; negative control (SGC7901/pcDNA3.1) SGC7901 cells transfected by pGenesil-NP pcDNA3.1 and screened by G418; and blank control (nSGC7901): SGC7901 passage cells, not transfected and not screened by G418.

RT-PCR

According to the specification of the kit, total RNA was extracted from three specimens and detected as nest PCR. β -actin was used as GAPDH. Forward primer was 5'-CATGTAGGTTGCTATCGAGGC -3', reverse primer was 5'-CTGCTTAATGTCACGCACCAT -3', and amplification was 250 bp. PCR reaction condition was followed by 35 cycles each, with denaturation at 98 °C for 2 min, 98 °C for 10 s, and 60 °C for 1 min, which was extended to 72 °C for 5 min, and then kept at 4 °C. After 1.5% agarose gel electrophoresis, it was detected with the image analysis system to obtain integrate optic density. Results were expressed as TF IOD/ β -actin IOD, and further semi-quantitative analysis was made.

Flow cytometry

Immunofluorescence technique was used. A total of 2 μ g/ml rabbit anti-human TF polyclonal antibody and goat anti-rabbit IgG-FITC (Boster Corporation) was added;

1 \times 10⁴ cells were collected by flow cytometry, and the percentage of positive cells was then analyzed.

Invasive ability

Cells were incubated overnight at 37 °C. After placing 15 μ l Matrigel gel (BD Biosciences) on the basal membrane, all cells were cultured overnight in the serum-free RPMI 1640 DMEM of the previous day's experiment. They were then made into a single-cell suspension (concentration: 1 \times 10⁵/ml) that contained 0.5% serum, and then RPMI1640, which contained 10% serum in the tissue culture plate with 24 wells, with 600 μ l in each well, was planted. Cell suspension in the Transwell chamber (100 μ l in each well) was added. The chamber was put into the complete medium, and three wells in each group were multiple cropped, and then cultured in 5% CO₂ at 37 °C for 24 h. The remaining cells on the surface of the membrane were cleaned, and the membrane in carbinol was fixed for 1 min. The amount of invasion cells was observed using a microscope (200 \times) after HE staining. The cells of each membrane under five different views were counted, and then the average was calculated.

The experimental group (SGC7901/TF) and the negative control (SGC7901/pcDNA3.1) were both incubated with 10.0 μ g/ml oxaliplatin. The invasive ability of cells, in accordance with method above, was detected. Oxaliplatin was bought from Jiangsu HengRui Medicine Co., Ltd.

Detect the apoptosis of associated proteins

The experimental group and the negative control to the tissue culture plate with 6 wells (hole density: 1 \times 10⁶) were inoculated. They were incubated with 40.0 μ g/ml oxaliplatin for 4 h, 8 h, 16 h, and 24 h. The cells were then collected. Caspase-3 activity was detected by Caspase-3 activity assay buffer solution. PS was detected by Annexin combing fluid, and apoptosis rate (the number of apoptotic cells/the number of total cells \times 100%) was calculated. A Caspase-3 Activity Detection Kit was bought from Shanghai BiYunTian Biological Technology Co., Ltd. An Annexin V-EGFP Apoptosis Assay Kit was bought from Glasgow's Biological Technology Co., Ltd.

Results

Plasmid construction

The constructed TF-pcDNA3.1 recombinant was identified by double digestion with XbaI and HindIII, and yielded two fragments of 5.4 kb and 900 bp. It was what we expected. Meanwhile, Sangon Biotech Shanghai Co., Ltd. accomplished the gene sequencing. Results indicated a correct sequence (Forster et al., 2006). Construction of the vector was successful (Figure 1).

TF expression

The relative expression of TFmRNA from each group was assayed. The successfully transferred SGC7901/TF cells' TFmRNA expression level was obviously higher. On the condition that β -action did not express any statistical difference, the experimental group (4.28 \pm 0.37) was obviously higher than the negative control (1.12 \pm 0.32)

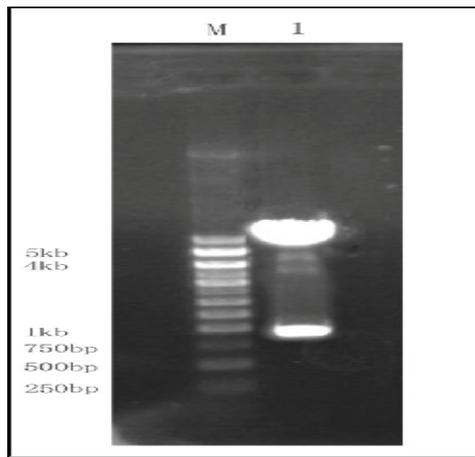


Figure 1. Recombinant Plasmid of TF-pcDNA 3 after Double Enzyme Digestion of Xba I/Hind III

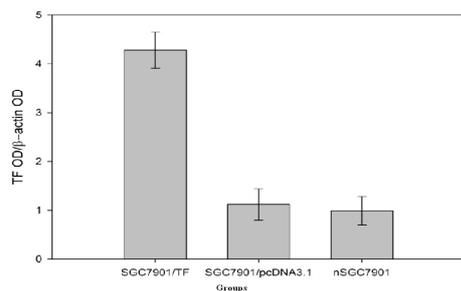


Figure 3. Expression Level of TF mRNA

Table 1. the Changes of caspase-3's Absorbance after Effected by Oxaliplatin ($\bar{X} \pm s$)

Group	4h	8h	16h	24h
TF				
10 μ g	0.036 \pm 0.002	0.042 \pm 0.003	0.059 \pm 0.005	0.072 \pm 0.004
20 μ g	0.048 \pm 0.004	0.056 \pm 0.003	0.077 \pm 0.004	0.099 \pm 0.008
30 μ g	0.057 \pm 0.004	0.065 \pm 0.003	0.091 \pm 0.009	0.130 \pm 0.010
40 μ g	0.065 \pm 0.007	0.090 \pm 0.009	0.151 \pm 0.011	0.207 \pm 0.011
Sham				
10 μ g	0.043 \pm 0.003	0.055 \pm 0.005	0.072 \pm 0.005	0.091 \pm 0.009
20 μ g	0.057 \pm 0.003	0.068 \pm 0.006	0.089 \pm 0.005	0.122 \pm 0.005
30 μ g	0.064 \pm 0.002	0.077 \pm 0.004	0.123 \pm 0.009	0.160 \pm 0.012
40 μ g	0.076 \pm 0.006	0.116 \pm 0.011	0.168 \pm 0.012	0.286 \pm 0.012

The experiment group and control group had statistic differences in each timing ($P < 0.05$)

and the blank control (0.99 ± 0.29) ($P < 0.01$) (Figures 2 and 3).

Antigen TF expression ($66.12 \pm 5.09\%$) on the SGC7901/TF cell surface was obviously higher compared with the negative control and the blank control, having a statistical difference ($P < 0.01$) (Figure 4).

Invasive ability

Transwell chamber was used to show change in the invasive ability of in vitro cells. Compared with the negative control and the blank control, the in vitro invasiveness of the experimental group increased ($F = 16.8$, $P < 0.01$).

Cell nucleus was observed under the high power lens of an optical microscope on the lower surface

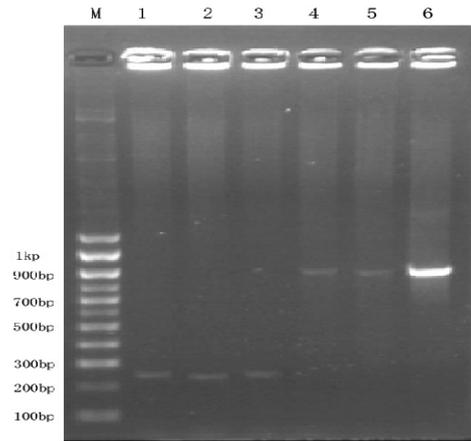


Figure 2. PCR Products of TF Gene. Lane1: β -actin mRNA in nSGC7901, Lane2: β -actin mRNA in SGC7901/pcDNA3.1; Lane3: β -actin mRNA in SGC7901/TF; Lane4: TF mRNA in nSGC7901; Lane5: TF mRNA in SGC7901/pcDNA3.1; Lane6: TF mRNA in SGC7901/TF

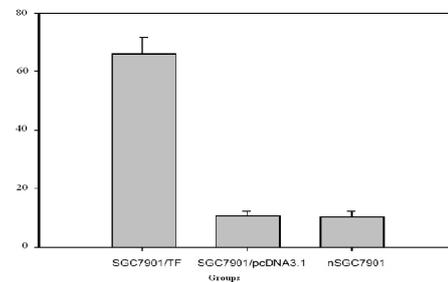


Figure 4. The Expression Rate of Antigen TF

Table 2. the Changes of Apoptosis Rate after Effected by Oxaliplatin ($\bar{X} \pm s$)

group	4h	8h	16h	24h
TF				
10 μ g	1.01 \pm 0.30	1.78 \pm 0.29	2.72 \pm 0.45	3.56 \pm 0.23
20 μ g	2.14 \pm 0.49	3.54 \pm 0.70	5.21 \pm 0.59	8.13 \pm 0.89
30 μ g	4.04 \pm 0.52	5.81 \pm 0.41	7.31 \pm 0.78	15.20 \pm 1.20
40 μ g	6.93 \pm 0.71	8.67 \pm 0.66	13.40 \pm 1.32	22.77 \pm 1.95
Sham				
10 μ g	1.74 \pm 0.31	2.65 \pm 0.38	3.93 \pm 0.58	5.57 \pm 0.65
20 μ g	3.85 \pm 0.68	5.95 \pm 1.07	8.81 \pm 0.98	14.52 \pm 1.41
30 μ g	5.32 \pm 0.55	7.05 \pm 0.62	10.33 \pm 1.09	21.10 \pm 1.00
40 μ g	8.03 \pm 0.90	11.03 \pm 1.36	16.90 \pm 1.59	28.00 \pm 2.41

The apoptosis rates showed significant differences between experiment group and control group (For 4h, 8h $P < 0.05$, for 16h, 24h, $P < 0.01$)

of Transwell's polyester film. Results of the in vitro invasion assay indicated significant differences between the experimental group (38.7 ± 3.6) and the control group (32.3 ± 2.1) ($P < 0.05$).

Caspase-3 activity and apoptosis rate

Incubated with Oxaliplatin for 4 h, 8 h, 16 h, and 24 h, Caspase-3 activity and apoptosis rate both decreased in the experimental group and the control group by spectrophotometer detection. Results showed significant differences ($P < 0.05$) (Tables 1, 2).

Discussion

Patients with malignant tumors often experience various coagulopathy. This phenomenon has caught the attention of many scholars. The incidence of phlebothrombosis in patients with malignant tumor was about 4%-20% (Khorana et al., 2007). In autopsy, the number is as high as 50% among patients with tumor (Gomes and Deitcher, 2003). Phlebothrombosis is one of the main complications in patients with malignant tumor and the second cause of death in cancer patients (Donati, 1994). Gallus et al. (1997) found that after the same surgical procedures, patients with tumor were three times more likely to suffer from fatal pulmonary vein embolization than those without. At the same time, Prandoni and Piccioli, (2006) found that spontaneous phlebothrombosis patients were ten times more likely to suffer from malignant tumor than secondary patients. A recent system review also suggested that the incidence of malignant tumor among spontaneous phlebothrombosis patients was 10% (95% confidence intervals: 8.6%-11.3%) during the first year. This incidence is obviously higher than that of secondary patients' (2.6%, 95% confidence intervals: 1.6%-3.6%) (Carrier et al., 2008).

Malignant tumor patients' hypercoagulable state is mainly mediated by the extrinsic pathway. These patients' serum TF level was about 67% higher than normal (Kakkar and DeRuvo, 1995). The increase in the TF level in circulated blood is responsible for the formation of malignant tumor patients' hypercoagulable state and is an important reason for phlebothrombosis (Manly et al., 2010).

TF is the initiation factor of the extrinsic pathway and the only blood coagulation factor expressed on the cell surface. Recent research shows that TF involved in cell signal transduction, supplying important protection to embryonic development and maintaining body functions, has certain effects on tumor angiogenesis, tumor growth, and metastasis (Toomey et al., 1997). TF expression level increases in certain types of malignant tumors, including gastric cancer, colon cancer, and pancreatic cancer (Nakasaki et al., 2002; Nitori et al., 2005; Yamashita et al., 2007). Level increase was closely related to MVD, malignant tumor staging, and prognosis (Jiao et al., 2005). Mueller et al. (1992) found that the TF expression level of metastatic melanoma cells was 1000 times higher than the metastatic-free ones.

Amirkhosravi et al. (2007) inhibited the lung metastasis of invasive B16 cells successfully by injecting recombinant TFPI into a laboratory rat through its caudal vein. Meanwhile, compared with untransfected B16 cells and antisense-transfected cells, transfected cells' lung metastasis decreased obviously. This study showed that TF participates in a malignant tumors invasion process.

To further study the influence of TF pairs of gastric cancer cell invasiveness, we obtained human TF cDNA from human placenta by nest PCR and then inserted it into a eukaryotic expressive vector to construct the recombinant. This recombinant gene was introduced into human gastric cell line SGC7901 through transfection

mediated by lipofectamine. Transwell experiments were carried out to observe the effects of TF on the invasive ability of human gastric cancer cell SGC7901 in vitro. TF expression levels in successfully transferred SGC7901/TF cells by liposome TF-pcDNA3.1 were obviously higher, and so was the antigen expression level on the cell surface. These two indices had no statistical differences between the negative control and the blank control.

The human gastric cancer cell line SGC7901/TF, which expressed TF stably and efficiently, was built successfully. We carried out the Transwell experiments to observe changes in in vitro cell invasion ability. Compared with the control group and the blank group, invasion ability increased significantly in the SGC7901/TF cells ($F=16.8$, $P<0.01$). TF could enhance the invasive ability of gastric cancer cells in vitro.

Based on a previous study, the experimental group (SGC7901/TF) and the control group (SGC7901/pcDNA3.1) were incubated respectively. The migration of SGC7901 was increased significantly, but Caspase-3 activity and apoptosis rate decreased when transferred TF-pcDNA3 was compared with the cells transferred pcDNA3, indicating that, through cell apoptosis and invasion pathway, TF could influence oxaliplatin chemotherapy in vitro. As such, its efficiency is decreased, thereby affecting gastric cancer patients' prognosis. However, its mechanism of action was not clarified. Camere et al. (1999) screened changes in human Keratinocytes' gene expression profile by gene chips before and after the combination of TF and FVIIa. They found that 24 genes increased, including *cfos*, *EGF*, and *IL 1*. This activation of cytokine or oncogene could promote invasive ability and MMP expression, whereas MMPs could lead to a cancer cell's extracellular matrix degradation, thus participating in the regulation of cancer cell invasion (Camerer et al., 1999). Researchers have found that TF/FVIIa complex could inhibit the apoptosis of a baby hamster's kidney cells induced by serum deprivation by activating PI3K/Akt signal transduction (Sorensen et al., 2003; Versteeg et al., 2004). This phenomenon probably indicates that, by complex TF/FVIIa formation, TF activates signal transduction, which influences cancer cell apoptosis and invasion.

A high-level TF expression could form a local hypercoagulable state in a tumor, where a great quantity of fibrin and tumor thrombus appears. This procedure could separate cancer cells from a less favorable survival environment and shelter the cancer cells from the surveillance of the immune system and from antitumor drug attacks (Lykke and Nielsen, 2003).

Through the thrombin generated from the TF activation in the coagulation pathway, the cancer cells could degrade and change the extracellular matrix. The cancer cells would therefore survive, proliferate, adhere, invade, and metastasize. TF can reduce invasion inhibition and the apoptosis-inducing effect of oxaliplatin in in vitro human gastric cancer cell, and probably affect the prognosis of patients with cancers. However, there is still a need to further study the detailed TF mechanisms of action in the future.

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