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*Original Article*

# Plasma tissue factor levels and microparticle-associated tissue factor activity in patients with gastric cancer

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**Abstract:** Tissue factor (TF), which has an important role in blood coagulation, was reported to be abnormal expressed, associated with microvascular density and poor prognosis in patients with different types of cancers. This study showed that TF expression was elevated in gastric cancer tissues compared with adjacent normal tissues and health gastric mucosa tissues by the method of real-time reverse transcriptase-polymerase chain reaction analysis. Furthermore, plasma TF levels in patients with gastric cancer (median, 65.5 pg/mL) was higher than health subjects (median, 13.0 pg/mL) using enzyme-linked immunosorbent assay. Plasma TF levels was increasing with advancing stage of gastric cancer and significantly associated with tumor differentiation ($P<0.01$). MP-associated TF activity in gastric cancer patients (median, 0.955 pg/mL) was also increasing compared with health subjects (median, 0.11 pg/mL) ($P<0.01$). It is also associated with tumor stages and tumor differentiation. Therefore, plasma TF level and MP-TF activity may add useful information regarding tumor stage or differentiation in gastric cancer screening.

**Keywords:** Tissue factor, microparticles, gastric cancer, blood coagulation

## Introduction

Gastric cancer (GC) is one of the most prevalent types of cancer and is the second leading cause of global cancer deaths [1, 2]. Curative resection is the most effective treatment for GC, and the 5-year survival rate is more than 90% when GC is detected early. The most frequently used tumor marker in GC is carcinoembryonic antigen (CEA) and CA199, but only a proportion of patients have high level of this marker [3]. It is also reported that serum soluble E-cadhein, MYC cell-free plasma DNA or gastric juice LncRNA-AA174084 may be used for early GC diagnosis [3-5].

After Trousseau's description of thrombophlebitis as a complication of pancreatic cancer in the 19th century, the notion that increased expression of tissue factor (TF) underlies the connection between coagulation and cancer has become generally known [6]. TF is a 47-kDa transmembrane glycoprotein that is a major physiologic initiator of blood coagulation. Abnormal expression of TF was reported in different types of cancers, including gastric cancer and hematologic malignancies [7, 8]. TF binds and activates factor VIIa, the TF-VIIa complex, then activates factor X, leading eventually to the generation of thrombin required for physiologic hemostasis. In addition clotting-dependent pathway, TF may also contribute to angiogenesis by up-regulating vascular endothelial growth factor (VEGF) and down-regulating the angiogenesis inhibitor thrombospondin a mechanism independent of coagulation activation [9]. Khoran *et al* correlated TF expression with vascular endothelial growth factor (VEGF) expression, microvessel density, and venous thromboembolism (VTE) in pancreatic cancer

[10]. The process of inducing angiogenesis by TF is essential for tumor growth and metastasis.

Active circulating TF was detected on small, negatively charged membrane vesicles, as named microparticles (MPs) [11, 12] and elevated levels of active circulating TF within the membrane of MPs have been found in a number of malignancies [13]. Furthermore, the MP-associated TF (MP-TF) activity has been brought into focus in recent years, as high activity in plasma have been found in cancer patients with venous thromboembolism as compared with those without thrombosis [14-16]. But, other reports have not found a correlation between circulating MP-TF activity and coagulation activation in a prospective research involving with various types of cancer [17]. Therefore, the utility of MP-TF activity as a predictive marker of thrombotic complications in cancer remains controversial.

In this study, we detected TF expression in gastric cancer tissues by the methods of Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) and western blot. Furthermore plasma TF levels and MP-TF activity were measured in gastric cancer patients. The correlations between plasma TF levels or MP-TF activity and clinical features were also shown in our research.

## Patients and methods

### *Patients' selection and sample collection*

The study protocol was approved by the local ethics committee and the study was conducted in accordance with the Declaration of Helsinki. A total of 106 patients with GC underwent gastric resection at the second affiliated Hospital of Harbin Medical University, between November 2013 and June 2014. The 106 GC tissues and the paired, adjacent, non tumors tissues located 6 cm away from the edge of tumor were obtained from surgical excision. The 34 healthy gastric mucosa (HGM) samples were also collected as control. All tissues were treated with liquid nitrogen and stored in -80°C until use. Tissues also were preserved in RNA fixer (Tiangen, Beijing, China) for RNA extraction at -80°C. In addition, venous blood samples were collected from the 106 patients with gastric cancer and 39 health subjects. The protocol is listed as follows. Especially, in all patients blood samples were collected before receiving any chemotherapy to prevent these agents affecting experimental results.

The diagnosis of each case was confirmed histopathologically. Tumors were staged according to the American Joint Committee on Cancer (AJCC) using tumor-node-metastasis (TNM) classification. Histological grade was assessed following the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines for Oncology (V.1.2011).

### *Quantitative reverse transcriptase polymerase chain reaction*

Total RNA was extracted from tissues with using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Invitrogen). The quality of total RNA was detected with 1% agarose gel electrophoresis. Total RNA (1 μg) was reverse transcribe with an oligo dT primer and reverse transcriptase (ReverTra Ace, TOYOBO) in a final reaction 10 μL. Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was achieved using THUNDERBIRD qPCR Mix (TOYOBO) with 20 pmol of each gene-specific primer in a total volume of 50 μL on lightCycler 480 systems (Roche). Reaction was performed using the following conditions 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, for 45 cycles. The primer sets used were as follows: TF forward, 5-ATGGAGACCCCTGCCTGGC-3; TF reverse, 5-TGAAACATTCAGTGGGGAGTTCTCCT-3; GAPDH forward, 5-ACACCCACTCCTCCACCTTT; GAPDH reverse, 5-TGACAAAGTGGTCGTTGAGG-3. The expression levels of TF were calculated using the ΔCt method using GAPDH as the control to normalize the data. Higher ΔCt values indicate lower expression of TF.

### *Western blotting*

Total protein was extracted from tissues. Protein 50 μg was subjected to SDS-PAGE on 10% acrylamide gels using a discontinuous buffer system and transferred to a nitrocellulose filter membrane. The filter was blocked with phosphate-buffered saline (PBS) containing skim milk, and then incubated with 1:400 diluted rabbit anti-human TF antibody (Sekisui, USA) for the detecting TF. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG

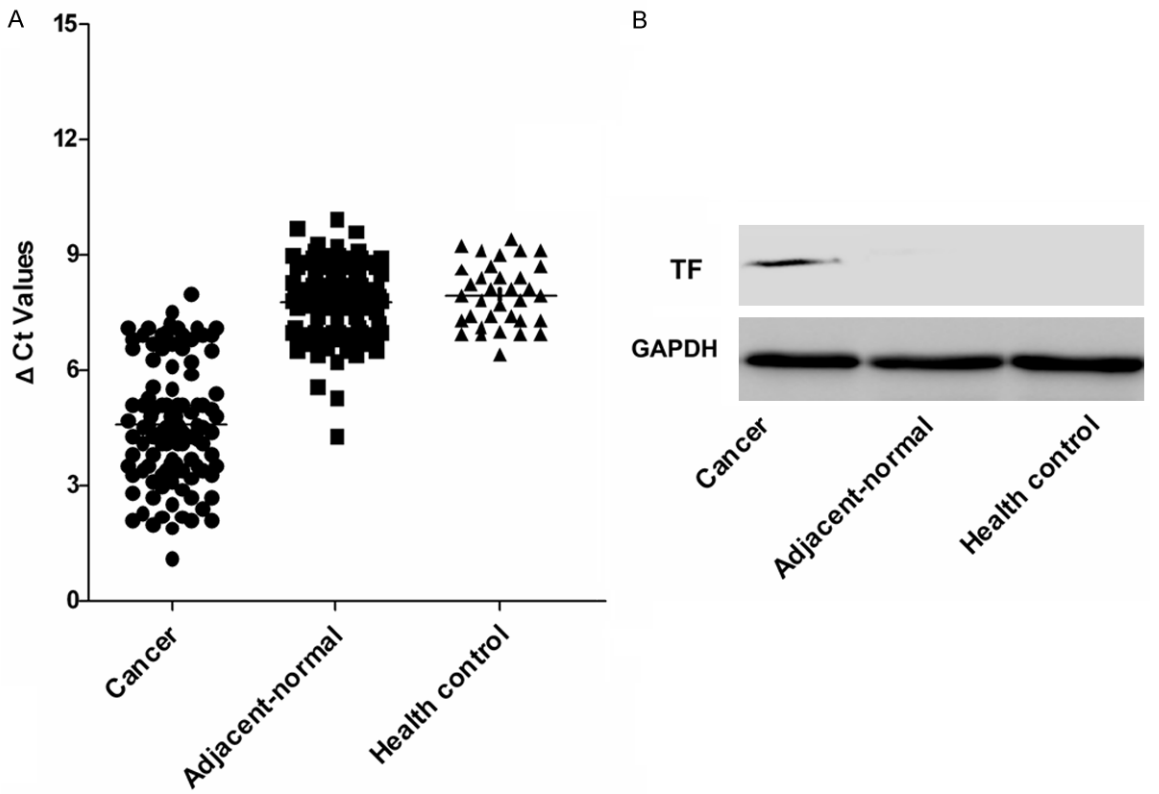


**Figure 1.** TF expression levels elevated in gastric cancer tissues. A. The TF expression levels in gastric cancer tissues detected by QRT-PCR including gastric cancer tissues (n=106), adjacent normal tissues (n=106) and health control (n=34). TF expression levels were calculated using the ΔCt method. Data are expressed as the mean from three independent experiments. Asterisks indicate $P<0.01$. B. Western blot of TF expression in gastric cancer tissues, adjacent normal tissues and health control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) bands indicate equal loading. All experiment repeated three independent experiments.

antibody (Jackson ImmunoResearch, USA) were then applied. Protein was visualized with ECL substrate (Millipore, Germany) according to the manufacturer's instructions.

*Detection of plasma TF level with enzyme-linked immunosorbent assay*

Plasma TF levels were measured using a commercially enzyme-linked immunosorbent assay kit (American Diagnostica Inc, USA) according to the manufacturer's instruction, as previously reported [18]. SpectraMax Microplate Reader (BIO-RAD, iMark) was used to read absorbance on the plate.

*MPs isolation and MP-TF activity measurement*

Venous blood samples for measurements of MP-TF activity were collected into citrate vacuum tubes 0.105 mol/L on the day of study entry. Cells were cleared by centrifugation at 1550 g about 20 min and 2 min at 13,000 g for obtaining platelet-free plasma (PFP) [19]. Then the plasma was immediately snap frozen in liquid nitrogen and stored at -80°C for the isolation of MPs. To obtain MPs, PFP was thawed on ice for 60 min and then centrifuged at 20,000 g for 45 min at room temperature. After each centrifugation and removed supernatant, MPs were washed two times with filtered Hanks' balanced salt solution (HBSA) to reduce contamination with plasma proteins. Finally 25 μL MPs pellet was resuspended with 75 μl of Tyrode's buffer [20-22].

The measurement of MP-TF activity was performed according to a standardized protocol as previously published [23-25]. After preparation of MPs, the pellet was incubated with either an antibody for human TF or a control antibody for 15 minutes, and then 50 μL aliquots were added to duplicate wells of a 96-well plate. In the next step, 50 μL HBSA containing 10 nM

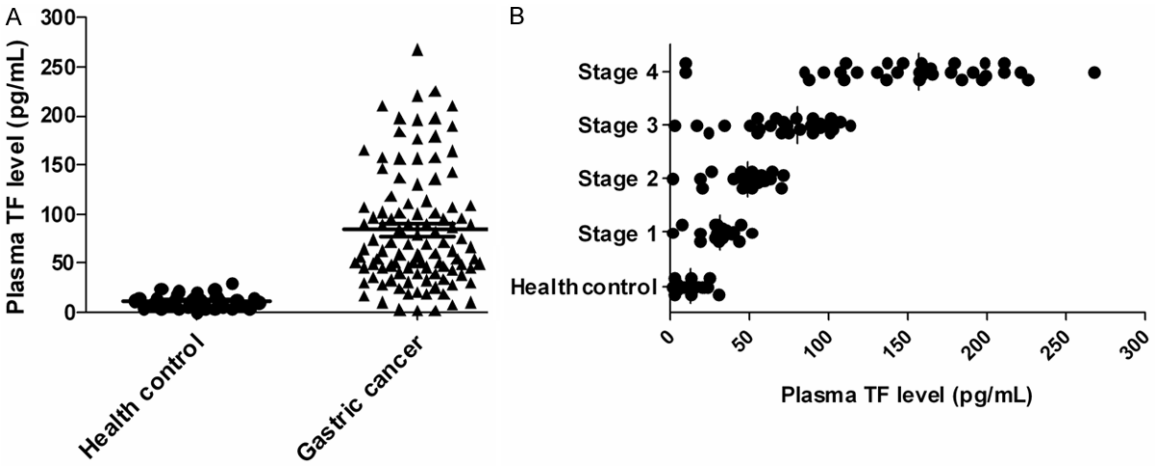


**Figure 2.** The plasma TF level in patients with gastric cancer and healthy subjects. A. The plasma TF expression levels in patients with gastric cancer were detected by ELISA including gastric cancer patients (n=106) and health control (n=32) $P<0.01$. B. Increasing plasma TF expression levels through all stages with gastric cancer progress $P<0.01$.

**Table 1.** Plasma TF levels and patient

| Characteristics | Number | Median TF (pg/mL) | Range (pg/mL) | *P*-value |
|---|---|---|---|---|
| Age, (y) | | | | |
| >60 | 45 | 60.10 | 10.0-220.0 | |
| <60 | 61 | 69.7 | 2.0-267.8 | 0.77 |
| Gender | | | | |
| Men | 48 | 71.95 | 18.90-220.0 | |
| Women | 58 | 58.0 | 2.0-267.8 | 0.11 |
| Differentiation | | | | |
| Moderate | 40 | 50.5 | 2.0-225.6 | |
| Poor | 66 | 88.2 | 2.0-267.8 | 0.007 |
| Tumor stage | | | | |
| 1-2 | 44 | 44.6 | 2.0-71.04 | |
| 3-4 | 62 | 101.3 | 2.9-267.8 | 0.001 |
| Lauren type | | | | |
| Intestinal | 63 | 60.1 | 2.0-267.8 | |
| Diffuse and mixed | 43 | 70.9 | 2.0-225.6 | 0.72 |
| Grade | | | | |
| Low | 48 | 55.8 | 2.0-220.6 | |
| High | 58 | 76.6 | 2.0-267.8 | 0.35 |
| Histology | | | | |
| Serious | 50 | 71.1 | 10.0-267.8 | |
| Others | 56 | 61.2 | 2.0-225.6 | 0.41 |

factor VIIa (FVIIa), 300 nM factor X (FX), and 10 mM $CaCl_2$ were added to each sample and the mixture was incubated for 2 hours at 37°C, after which FXa generation was determined. Last, absorbance at 405 nm was measured using a SpectraMax Microplate Reader (BIO-RAD, iMark). The TF-dependent FXa generation which represents the MP-TF activity, was calculated by subtracting the amount of FXa generated in the presence of TF from the amount of FXa collected in the presence of the control antibody [19].

*Statistical methods*

Data were collected and analyzed by SPSS 20.0 statistical software program (SPSS Inc, Chicago, IL). One-way analysis of variance, Mann-Whitney-Wilcoxon test, Students' t test, chi-square and Jonckheere's test for ordered medians (JTOM) were used according the actual conditions. Results were considered to be statistically significant at $P<0.05$.

## Results

*TF expression is elevated in gastric cancer tissues*

High expression of TF was reported in different types of cancers, gastric cancer and hematologic malignancies by immunohistochemical staining [7, 8]. In our study, we used QRT-PCR to detect TF levels in 106 gastric cancer tissues compared with adjacent normal tissues and 34 healthy gastric mucosa tissues. Higher ΔCt values indicate lower expression of TF. The results showed that

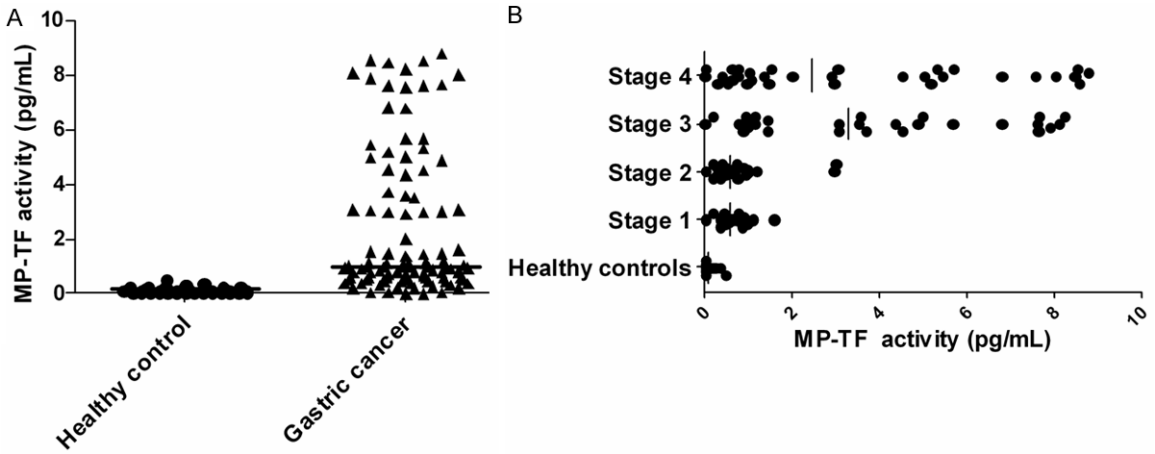


**Figure 3.** The microparticle-associated tissue factor activity for patients with gastric cancer and healthy controls. A. The microparticle-associated tissue factor activity in patients with gastric cancer tissues were detected including gastric cancer tissues (n=106) and health control (n=35) *P*<0.01. B. Microparticle-associated tissue factor activity was analyzed through all stages with gastric cancer progress.

**Table 2.** MP-TF activity and patient

| Characteristics | Number | Median TF (pg/mL) | Range (pg/mL) | *P*-value |
|---|---|---|---|---|
| Age,( y) | | | | |
| >60 | 45 | 0.97 | 0.01-8.21 | |
| <60 | 61 | 0.96 | 0.01-8.76 | 0.66 |
| Gender | | | | |
| Men | 48 | 0.94 | 0.02-8.21 | |
| Women | 58 | 1.00 | 0.01-8.76 | 0.44 |
| Differentiation | | | | |
| Moderate | 40 | 0.69 | 0.01-7.89 | |
| Poor | 66 | 1.40 | 0.02-8.76 | 0.01 |
| Tumor stage | | | | |
| 1-2 | 44 | 0.59 | 0.02-3.01 | |
| 3-4 | 62 | 3.05 | 0.01-8.76 | 0.001 |
| Lauren type | | | | |
| Intestinal | 63 | 0.91 | 0.01-8.76 | |
| Diffuse and mixed | 43 | 1.11 | 0.21-8.56 | 0.16 |
| Grade | | | | |
| Low | 48 | 0.95 | 0.19-7.62 | |
| High | 58 | 0.97 | 0.01-8.76 | 0.69 |
| Histology | | | | |
| Serious | 50 | 1.25 | 0.01-8.56 | |
| Others | 56 | 0.89 | 0.01-8.76 | 0.10 |

TF expression was elevated in gastric cancer tissues compared with adjacent normal tissues and health gastric mucosa tissues (healthy control) (*P*<0.01) (**Figure 1A**). The 89 of 106 gastric cancer tissues was up-regulated compared with paired, adjacent, normal tissues. The average expression level in gastric cancer tissues was more ten times than the average level in adjacent normal tissues or healthy control. TF expression levels between adjacent normal tissues and health control tissues have no significant difference.

In order to validate the results of QRT-PCR detection, western blot was applied to detect TF expression of tissues in protein levels. Random selection of three up-regulated gastric cancer tissues, paired adjacent normal tissues and health gastric mucosa tissues were detected by western blot. As shown in **Figure 1B**, the expression levels of TF increased in gastric cancer tissues compared with adjacent normal tissues and health gastric mucosa tissues. TF expression can hardly detect in adjacent normal tissues and health gastric mucosa tissues by western blot. These results demonstrated that the TF expression is elevated in gastric cancer tissues.

*Plasma TF levels in healthy subjects and gastric cancer patients*

We evaluated plasma TF levels in the 106 patients with gastric cancer and 32 health subjects by ELISA. TF levels were significantly higher in patients with gastric cancer (median, 65.5 pg/mL; range: 2.0-267.8 pg/mL) when compared with those with health individuals (medi-

an, 13.0 pg/mL; range: 1.0-30.1 pg/mL) ($P$< 0.01) (**Figure 2A**). In addition, there was a trend for increased plasma TF levels along with the gastric cancer stages. The increasing trend for TF expression against overall stage 1-4 gastric cancers was significant by taking into account the health subjects to examine plasma TF levels ($P$<0.001 by JTOM) (**Figure 2B**).

Furthermore, the association between plasma TF levels and clinicopathological parameters is presented in **Table 1**. No significant differences were detected between TF levels and some of the clinicopathological features including age, gender, histology, Lauren type and grade. But the TF levels of overall TNM staging haven significant difference as grouped 1-2 vs. 3-4 stages ($P$<0.01). Moreover, Tumor differentiation also showed significant difference ($P$<0.01).

*MP-TF activity in healthy subjects and gastric cancer patients*

The median MP-TF activity in 106 pancreatic cancer patients was 0.95 pg/mL (range: 0.01-8.76 pg/mL). In 35 healthy subjects, the median MP-TF activity was 0.11 pg/mL (range: 0.02-0.37 pg/mL) and significantly lower than in patients, as shown in **Figure 3A** ($P$<0.01).

MP-TF activity levels according to stage from gastric cancer stages are given in **Figure 3B**. MP-TF activity was not significantly elevated along with overall the gastric cancer stages. However, the MP-TF activity of classified by TNM staging haven significant difference as grouped 1-2 vs. 3-4 stages ($P$<0.01). Moreover, the relation between TF expression and clinicopathological features is tested as shown in **Table 2**. No significant differences were detected between MP-TF activity and some of the clinicopathological features (age, gender, histology, Lauren type, grade) with the exception of tumor differentiation ($P$<0.01).

## Discussion

Up-regulation of *TF* gene expression appears to be characteristic of malignant cells and normal host cells responding to inflammatory or remodeling signals (e. g., tumor-associated endothelial cells, monocytes, macrophages, neutrophils and fibroblasts). Via both clotting-dependent and -independent pathways, aberrant expression of TF and thrombin are capable of inducing angiogenesis, the process of generating new blood vessels from preexisting vessels, which is essential for tumor growth and metastasis [25]. In the clotting-dependent pathway of TF induced angiogenesis, TF activates factor VII, and initiates the clotting cascade that generates thrombin, which in turn induces endothelial proliferation and stimulates the release of cytokines such as interleukin 8 (IL-8) and VEGF [26, 27]. In addition, the clotting cascade is responsible for the activation of platelets that releases stored VEGF and facilitates the extravasation of tumor emboli [28]. This study showed that higher levels of TF expression in tissues could be detected using real-time PCR and western blot in gastric cancer patients compared with health subjects or adjacent normal tissues in gastric cancer patients which is in accordance with immunohistochemical analysis of TF expression reported by others [7, 29]. These results suggest that abnormal expression TF may play a key role in thrombin involving in gastric tumor growth or metastasis and real-time PCR may be a alternative tool for the detection of TF expression in gastric cancer tissues.

TF expression and its relationship with treatment outcome in gastric adenocarcinoma had been reported previously in Japan and European population [7, 29]. It also reflects that the different immunohistochemistry approach in scoring can influence outcome from recording both the strongest intensity of staining (Is) and the intensity for the majority of cells stained (Im). Thus, detection of plasma TF levels is a alternative tool for detection TF expression. The key findings from our study were plasma TF levels significantly elevated in patients with gastric cancer when compared with health controls. It is also showed that a trend for increased plasma TF levels along with the gastric cancer stages. The increasing trend was very significant throughout all stages of gastric cancer. These results were in accordance with other immunohistochemical analysis results [29]. The significant association was detected between plasma TF levels and tumor differentiation and stages. But the association between plasma TF levels expression and survival outcome were not shown in this article. Previous reports showed that TF expression was no relation to survival outcome, and not a good predictor of survival outcome [29]. But it is also

reported tissue factor is a clinical Indicator of Poor Prognosis [7]. Thus, whether plasma TF levels are a potentially valuable prognostic factor in patients with gastric cancer can also need further validation. However, in patients with gastric cancer, a significant inverse relationship between preoperative elevated plasma CEA levels and patient survival has also been reported despite disagreement among various reports regarding the relationship between preoperative CEA and prognosis [30].

MP-associated TF activity is a quantitative estimate of the concentration of TF in the MP preparation, which can act as cofactor of FVIIa in FX activation. As MP-associated TF activity is a functional form of TF compared with plasma TF, MP-associated TF activity has more meaningful application. The important finding of our study is that MP-TF activity is highly elevated in patients with gastric cancer. Moreover, a increasing trend for high MP-TF activity as TNM stages classified two groups (1-2 and 3-4 stages). The association of MP-TF activity with the risk of VTE in many cancer types is controversial [19, 31, 32]. But a strong association between high MP-TF activity and increased risk of mortality was found. MP-TF activity might represent a biomarker for an aggressive, poorly differentiated and invasive pancreatic cancer phenotype [33, 34]. Thus, the relationship of MP-TF activity in gastric cancers with VTE and mortality also need further investigation.

In conclusion, our study found TF expression in gastric cancer tissues is elevated by the methods of QRT-PCR and western blot. In addition, we confirmed that the plasma TF levels were increasing with advancing stage of gastric cancer. Furthermore, plasma TF levels and MP-TF activity are correlation with tumor stages and differentiation. Plasma TF levels and MP-associated TF activity may add new useful information regarding tumor stage or differentiation in gastric cancer screening.

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## Disclosure of conflict of interest

None.

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