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Original Article

TEM7 (PLXDC1), a key prognostic predictor for resectable gastric cancer, promotes cancer cell migration and invasion

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Abstract: Tumor endothelial marker 7 (TEM7) is a new candidate of molecular target for antiangiogenic therapy. This study aims to evaluate its expression in gastric cancer (GC) and to explore the correlation between its expression and the clinical outcome of patients. Expression of TEM7 was analyzed in both tumor tissues and cell lines of GC by real-time quantitative RT-PCR (qRT-PCR) and Western blot. RNA interference (RNAi) approaches were used to investigate the biological functions of TEM7. The effects of TEM7 on cell migration and invasion were evaluated by Transwell assays. In vitro experiments revealed that TEM7 was significantly overexpressed in GC cell lines (N87, AGS and SGC-7901) by 2-fold to 4-fold, and knockdown of TEM7 could significantly inhibit cancer cell migration and invasion. For GC patients, TEM7 gene expression was elevated in tumors in most cases (25/31), and its expression was closely correlated with tumor differentiation, depth of cancer invasion, lymphatic metastasis and TNM stage. The overall survival of TEM7 (-) group was significantly higher than that of TEM7 (+) group (P = 0.048) and TEM7 (++) group (P = 0.003). TEM7 is highly expressed in GC and is likely correlated with tumor invasion and migration, and thus its expression is closely related to the clinical outcome of patients.

Keywords: TEM7, gastric cancer, prognosis, migration, invasion

Introduction

The worldwide incidence of gastric cancer (GC) is gradually declining. However it is a major public health problem, especially in East Asian countries, and the age-standardized incidence rate is over 20 per 100,000 persons [1, 2]. It is also the second leading cause of cancer death (10.4% of cancer deaths) [3]. Despite improvements in preoperative staging, surgical techniques, and normative adjuvant chemotherapy, the outcome of patients with gastric cancer remains poor, especially for advanced stage [4, 5]. The five-year survival rate of patients with stage I and II gastric cancer after curative resection is 83-99% and 48-70%, while that is 25% for patients with stage IV cancer [6].

Researches about tumor biological behavior, biomarkers identification and individualized targeted therapy are the most fundamental steps to improve the survival of gastric cancer patients [7, 8]. For example, trastuzumab plus chemotherapy can improve the poor survival of patients with positively expressed human epidermal growth factor receptor 2 (HER2) [9-11]. In addition, several potential molecular targets, such as vascular endothelial growth factor receptor (VEGF) and mammalian rapamycin (mTOR), have been explored [12-14].

Tumor endothelial marker 7 (TEM7), also known as plexin domain-containing 1 (PLXDC1), initially identified as a highly expressed protein in the vascular endothelium of human tumors [15], is a new candidate of molecular target for antiangiogenic therapy. Elevated expression of TEM7 in tumor endothelial cells and its role in tumor angiogenesis have been well demonstrated [16-18]. However, the relation between TEM7

expression and gastric cancer has rarely been addressed. In 2007, Fuchs B et al confirmed that TEM7 is a cell surface protein and it is involved in metastasis of osteogenic sarcoma [19]. Based on these, we are wondering that if TEM7 is also expressed on the surface of gastric cancer cells and whether it may be a potential biomarker for the individualized therapy.

Therefore, in this work, TEM7 expression in GC cell lines was examined by both western blotting and qRT-PCR. TEM7 expression was inhibited via siRNA inference and its influence on tumor cell migration and invasion was studied. In addition, TEM7 expression in tumor tissues was analyzed by immunohistochemistry and the correlations of its expression with clinicopathological characteristics and the outcome of gastric cancer patients were also studied.

Materials and methods

Patients and sample collection

This study was designed according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guideline [20]. The inclusion and exclusion criteria used in this study were: 1) patients with a distinct pathologic diagnosis of gastric cancer; 2) patients with no history of other malignant tumors; 3) patients underwent radical gastrectomy with D2 lymph node dissection; 4) patients with inoperable, metastatic or recurrent gastric cancer were excluded; 5) patients received no radiotherapy, chemotherapy, or other anti-cancer therapies prior to surgery; 6) patients with complete clinicopathological and follow-up data. Informed consent was obtained and this study was approved by the ethics committee of Renji Hospital, Shanghai Jiaotong University School of Medicine.

Fresh samples of matched tumor and adjacent normal tissues from 5 gastric cancer patients (Supplementary Table 1) were used for microarray analysis. Thirty-one consecutive patients with gastric cancer admitted to Renji Hospital (Eastern Branch), Shanghai Jiaotong University School of Medicine from Sep 2012 to Dec 2012 were recruited as the discovery cohort to validate the results of microarray analysis. The fresh tumor tissue samples were used for real-time PCR. As the test cohort, 289 gastric cancer patients hospitalized from during the same period were enrolled retrospectively. In addition, we assessed another retrospectively validation cohort comprising 96 patients during the same period. The paraffin-embedded tissue samples were used for tissue microarray and immunohistochemical staining (Supplementary Figure 1).

The database is comprised of parameters including patients' age, gender, Lauren classification, tumor differentiation degree, gross appearance, TNM stage, tumor vascular thrombus, perineural invasion and the special pathological type (signet ring cell cancer). Characteristics of patients in test and the validation cohort were shown in Supplementary Table 2.

Physical examination, blood test, abdomen ultrasonography and chest X-ray were performed for all patients each 6 months during the first year after surgery and every 6-12 months thereafter. Gastroscope and computed tomography (CT) or magnetic resonance imaging (MRI) were performed at 6-month intervals during the first year after surgery, and subsequently at 12-months intervals or immediately after a recurrence was suspected.

Complete follow-up data of patients in test and validation cohort were available. Patients were followed up until March 2013. Overall survival (OS) was defined as the period from surgery to death or the last follow-up examination. The median follow-up of the test cohort was 47 months (range, 3-80 months) and the 1-, 3- and 5-years survival rates were 92.0%, 72.0% and 60.3%, respectively. In the validation cohort, the median follow-up was 46 months (range, 6-80 months) and the 1-, 3- and 5-years survival rates were 90.6%, 69.6% and 58.0%, respectively.

Microarray analysis

Briefly, samples of 5 matched tumor and adjacent normal tissues were used to synthesize complementary molecules of double-stranded DNAs (cDNAs), and labeled using Agilent Quick Amp Labeling Kit. The labeled cDNAs were hybridized onto the Human LncRNA/mRNA Array v2.0 (8 × 60 K, Arraystar, Rockville, MD). After the slides washed, the arrays were scanned by the Agilent Scanner G2505B (Agilent Technologies, Santa Clara, CA). Agilent Feature Extraction software (version 10.7.3.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the Gene-

Spring GX v11.5.1 software package (Agilent Technologies, Santa Clara, CA).

Cell culture

Gastric cancer cell lines MGC-803, HGC-27, NCI-N87, AGS, SGC-7901 were provided by the Shanghai Cancer Institute, Renji Hospital, School of Medicine, Shanghai Jiaotong University. The immortalized human gastric mucosal cell line GES-1 was purchased from the Cell Bank of Shanghai. Cells were routinely cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2.

qRT-PCR

RNA expression was measured by qRT-PCR by the SYBR-Green method according to the manufacturer's instructions. qRT-PCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The forward and reverse TEM7 primer sequences were 5'-ACACGCTGCCAGATAACAGG-3' and 5'-TCGGCCACATCTACCCACA-3', respectively. β-actin was used as the internal control. Each sample was analyzed in triplicate. The 2-ΔCt method was used to quantify the relative gene expression levels.

Western blot analysis

Total protein was extracted from the micro-dissected cells and the concentration was estimated using a Bio-Rad protein assay kit (BioRad, Hercules, CA, USA). Fifty μg total proteins were loaded onto 10% sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. β-actin was used as the loading control. The membranes were blocked with 5% skimmed milk and 0.1% Tween-20 for 1 h, followed by incubation with the TEM7 primary antibody (1:500, Abcam Biotechnology, Cambridge, UK) overnight at 4°C. The membranes were subsequently incubated for 1 h at room temperature with the anti-rabbit secondary antibody (Dako, Carpinteria, CA, USA). Antibody binding was detected using an Odyssey infrared scanner (Li-Cor Biosciences Inc, Lincoln, NE, USA).

Small interfering RNA (siRNA) transfection

Three TEM7-targeted siRNAs and a negative control siRNA were purchased from Genpharma Biotech Company (Genpharma, Shanghai, China). The siRNAs were 21 nucleotides long and synthesized chemically with standard purification. The siRNA-1 sequence was 5'-AACCGGCCUAUCGGAUGCCTT-3' and antisense RNA 5'-GGCAUCCGAUAGGCCGGUUTT-3'. The siRNA-2 sequence was 5'-GAAGGAGCAUCUUUGAAUATT-3' and antisense RNA 5'-UAUUCAAAGAUGCUCCUUCTT-3'. The siRNA-3 sequence was 5'-UCUCGGCGAAGGAGCAUCUTT-3' and antisense RNA 5'-AGAUGCUCCUUCGCCGA GATT-3. Synthetic sequence-scrambled siRNA was used as a negative control siRNA. Cells were plated in 6-well plates (10 cm^2) and cultured in growth media until cell density reached 50% prior to siRNA transfection using Lipofectamine RNAi MAX (Invitrogen, CA).

Migration and invasion assays

Cell migration and invasion assays were performed using the BD Falcon™ FluoroBlock™ 24-Multiwell insert plates (8 micron pore size) (BD Biosciences, San Jose, USA). For the invasion assay, the insert membranes were precoated with diluted BD Matrigel™ Matrix (BD Biosciences, San Jose, USA). Cell suspension (2×10^5 cells) was added to the upper chamber. Then 1 ml of chemoattractant (10% FBS) was added to the basal chambers. The plates were incubated for 48 h. For the migration assay, the insert membranes were not coated with Matrigel, but they were cultured under the same conditions. Finally, the insert membranes were cut and stained with crystal violet (0.04% in water; 100 ml), and the migrated cells were counted under an inverted microscope and were photographed.

Tissue microarray construction

Tissue microarrays were constructed by Suzhou Xinxin Biotechnology Co., Ltd (Xinxin Biotech-pnology Co, Suzhou, China). Tissue paraffin blocks of gastric cancer samples from test and validation cohort were stained with hematoxylin-eosin to confirm the diagnoses and marked at fixed points with most typical histological characteristics under a microscope. Two 1.6-mm cores per donor block were transferred into a recipient block tissue microarray, and each dot array contained fewer than 160 dots. Three-micron-thick sections were cut from the recipient block and transferred to glass slides with

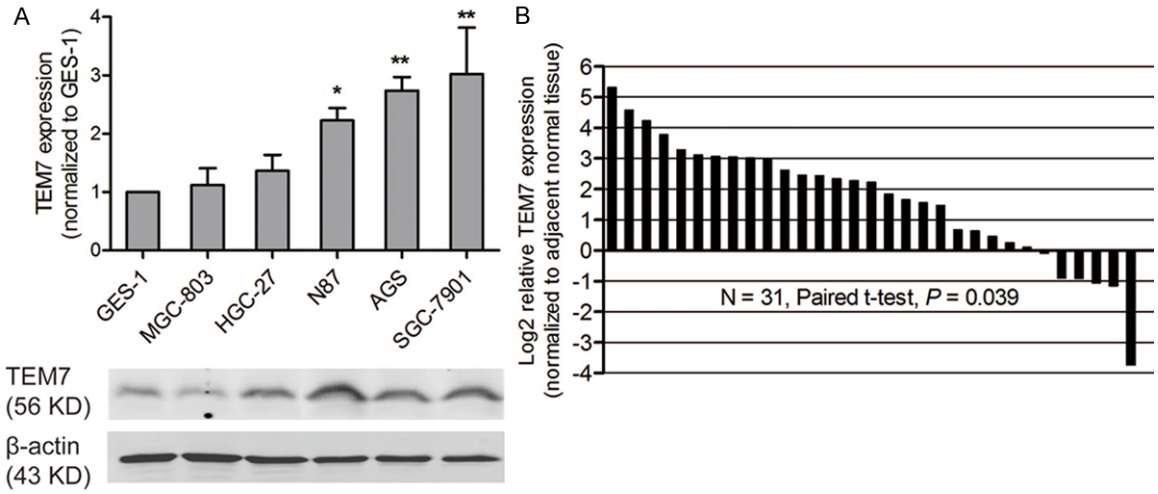

Figure 1. TEM-7 expression levels in gastric cancer cell. A. qRT-PCR analysis of TEM-7 expression levels in GC cell lines (MGC-803, HGC-27, NCI-N87, AGS, SGC-7901) compared with the normal human gastric epithelial cell line (GES-1). B. TEM7 expression in 31 matched tumors and adjacent normal tissues from 31 GC patients were determined by qRT-PCR. *P < 0.05, **P < 0.01.

an adhesive tape transfer system for ultraviolet cross linkage.

Immunohistochemistry analysis

The slides were baked at 56°C for 1 h, deparaffinized in xylene for 20 min, and rehydrated through a graded series of ethanol concentrations (5 min in 100% ethanol followed by 5 min in 70% ethanol). Antigen retrieval was performed in a pressure cooker for 5 min with Target Retrieval Solution (Dako, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked using a peroxidase blocking reagent (Dako, Carpinteria, CA, USA) for 5 min. Next, an TEM7 antibody (1:200, Abcam Biotechnology, Cambridge, UK) was applied to cover the specimens for 1 h at room temperature, and this was followed by incubation with a labeled polymer-HRP anti-rabbit secondary antibody (Dako, Carpinteria, CA, USA) for 30 min at room temperature. Thorough rinsing with Tris-Buffered Saline and Tween-20 was performed after incubation. The slides were visualized using the diaminobenzidine substrate-chromogen system (Dako, Carpinteria, CA, USA) and washed with deionized water before hematoxylin counterstaining. The slides were then dehydrated through a series of increasing ethanol solutions, cleared in xylene and coverslipped with Digital Picture Exchange mounting medium (Leica Biosystems, Wetzlar, Germany).

The staining intensity and the percentage of positive cells were recorded. Immunohistochemical results were categorized as follows: (-), if no staining was observed; (+), if > 25% of the tumor cells had weak or moderate staining intensity; (++), if the tumor cells had strong staining intensity. Given the heterogeneity of protein expression in the tumor cells, the highest scoring from either of the TMA cores was considered the final result.

Statistical analysis

Statistical analyses were conducted using SPSS 17.0 software (Chicago, IL, USA). For comparisons, two-tailed Student t test, one-way analyses of variance and chi-squared tests were applied when appropriate. OS were calculated according to the Kaplan-Meier method. The log-rank test was used to compare the survival distributions. Only variables of significance in univariate analysis were entered into the multivariate analysis. All statistical tests were 2-sided. Differences with a *P*-value of < 0.05 were considered statistically significant.

Results

Increased TEM7 expression in gastric cancer by microarray analysis

The microarray data discussed in this article have been deposited in National Center for

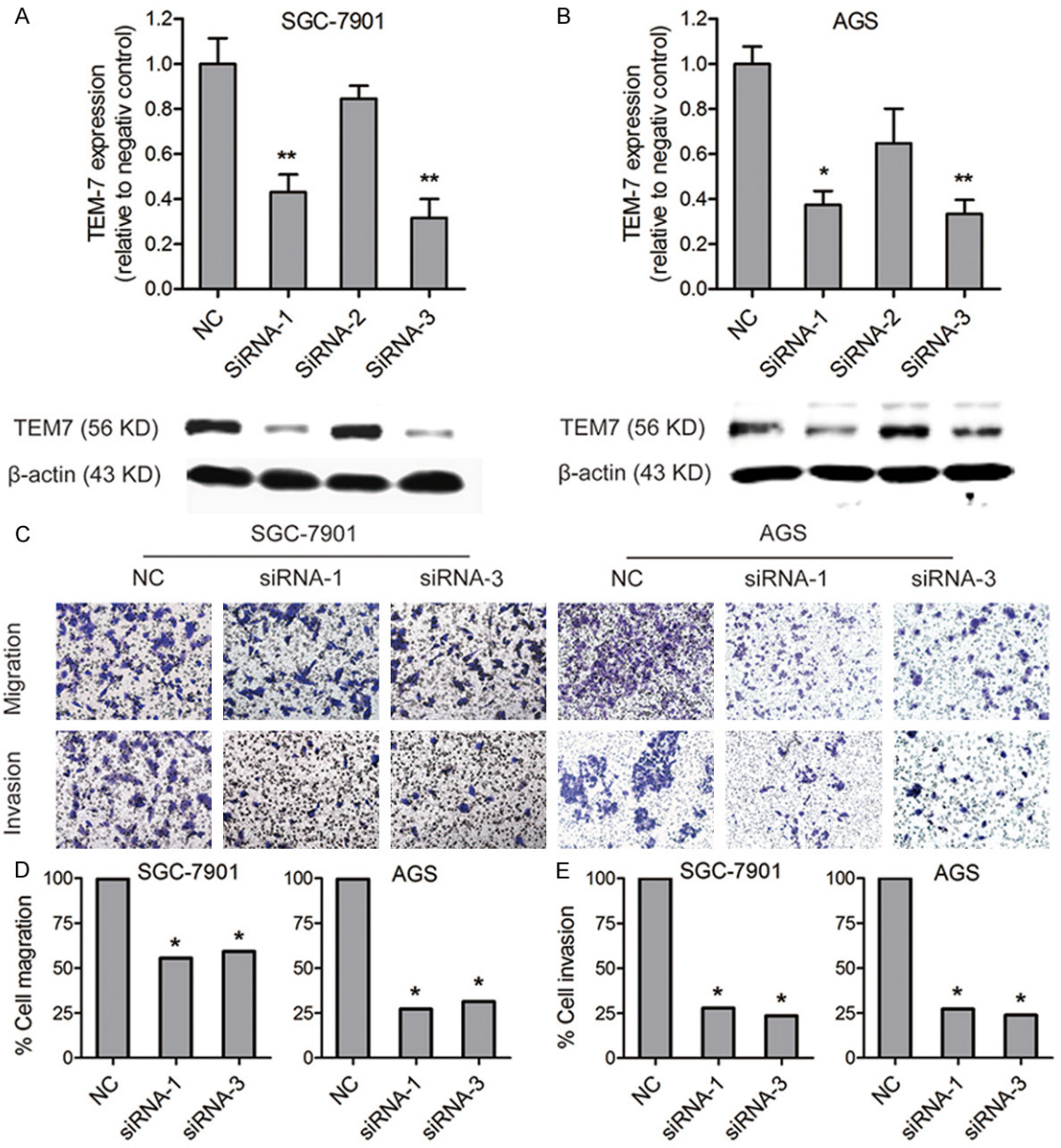

Figure 2. qRT-PCR analysis of TEM-7 expression following treatment of (A) SGC-7901 or (B) AGS cells with three individual siRNAs targeting TEM-7. Effects of TEM-7 on cell migration, invasion and metastasis. (C) SGC-7901 or AGS cells were transfected with TEM-7 siRNA or si-NC. (D, E) Transwell assays were performed to investigate the migratory and invasive ability of gastric cancer cells. *P < 0.05, **P < 0.01.

Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE51308. Among the 30,215 mRNAs detected in this microarray analysis, 47 unique mRNAs were markedly elevated in GC tissues compared to normal tissues (fold change > 3, $P < 0.05$). The result showed that TEM7 mRNA was overexpressed in gastric cancer (fold change = 3.16, $P = 0.034$).

Validation of TEM7 overexpression in gastric cancer cell lines

Then we proceeded to validate differential expression of TEM7. qRT PCR results of 5 gastric cancer cell lines versus gastric mucosal cell line GES-1 revealed that TEM7 was significantly over expressed in N87, AGS, SGC-7901 cell lines by 2-fold to 4-fold. The result of western blot analysis also showed that TEM7 expres-

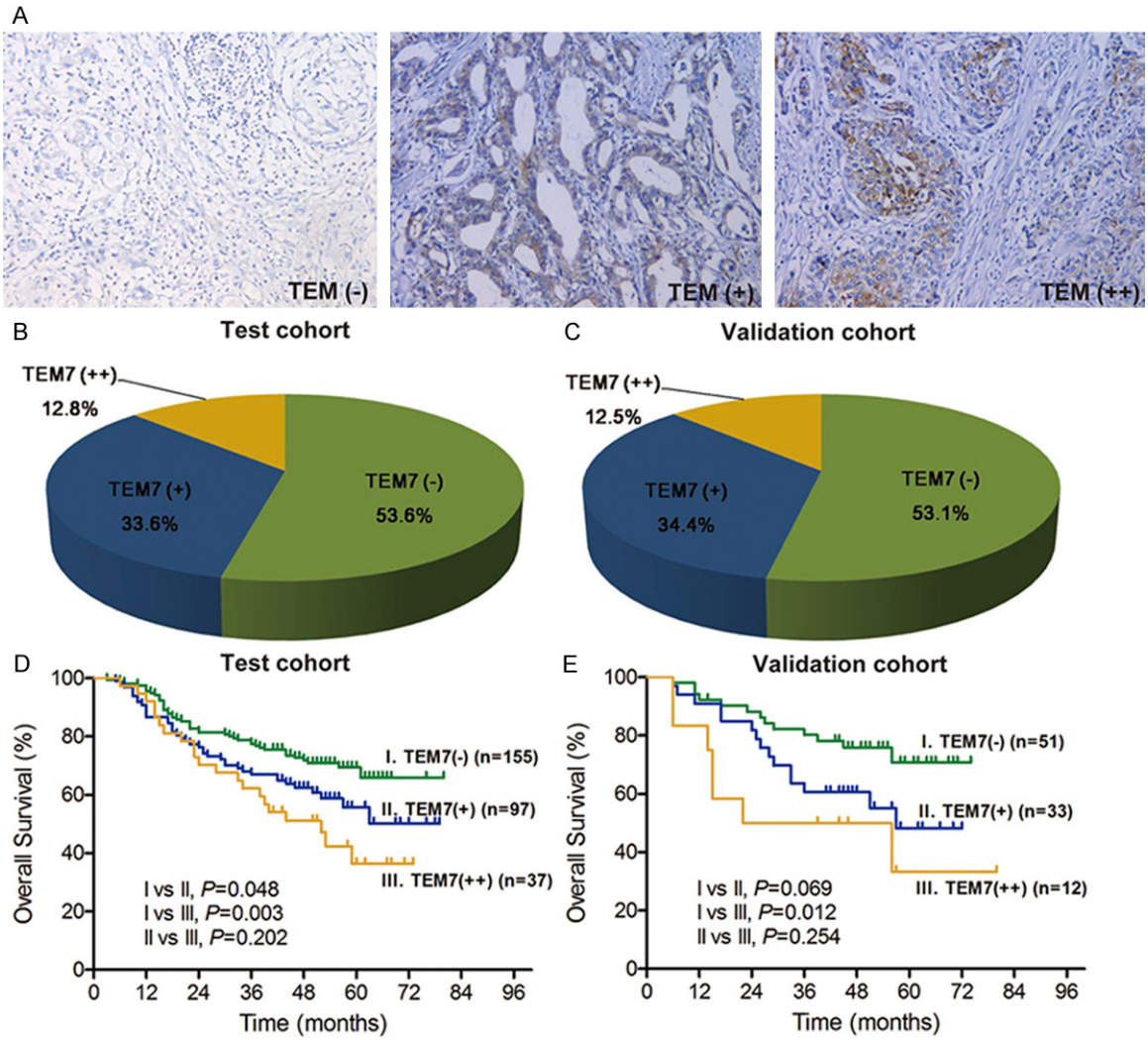

Figure 3. TEM-7 protein expression and clinicopathologic characteristics. A. TEM-7 was detected in test and validation cohort by immunochemical staining. B, C. TEM-7 expression in test cohort was similar in validation cohort. D, E. Patients with high levels of TEM-7 expression showed reduced overall survival times compared with patients with low TEM-7 ($P < 0.05$, log-rank test).

sion levels in gastric cancer cell lines were much higher (**Figure 1A**). Subsequently, TEM7 expression in 31 matched tumors and adjacent normal tissues from 31 gastric cancer patients were determined by Real-time PCR. TEM7 gene expression was elevated in tumors in most cases (25/31, average fold change 3.1, paired t test *P* value = 0.039; **Figure 1B**).

siRNA-mediated knockdown of TEM7 inhibits GC cell migration and cell invasion

To investigate the functional role of TEM7 in gastric carcinogenesis, siRNA was used to downregulate TEM7 expression in the gastric cancer cell lines AGS and SGC-7901. Of the 3 different siRNA sequences tested, 2 with more knockdown efficiency (siRNA-1 and siRNA-3) were selected for subsequent functional analysis in vitro (**Figure 2A**, **2B**).

The results of transwell migration and invasion assays were shown in **Figure 2C**. The migration assay results revealed that knockdown of TEM7 could significantly inhibit gastric cancer cell migration. The migration of SGC-7901 cell was inhibited by nearly 50%, and that of AGS by more than 60% (**Figure 2D**). The results of the invasion assay showed that knockdown of TEM7 inhibited cell invasion by greater than

Table 1. Relationship between TEM7 expression and clinicopathological features of gastric cancer patients in test and validation cohort

Variable	Test cohort (n = 289)				Validation cohort (n = 96)			
	TEM7			*P* value	TEM7			*P* value
	(-)	(+)	(++)		(-)	(+)	(++)	
Age at diagnosis (years)[a]								
≤ 61	79	54	15	0.292	30	20	4	0.228
> 61	76	43	22		21	13	8	
Gender								
Male	107	62	23	0.592	34	19	10	0.267
Female	48	35	14		17	14	2	
Lauren classification								
Intestinal	51	26	10	0.515	17	6	5	0.196
Diffuse/mixed/not classified	104	71	27		34	27	7	
Differentiation								
Well	59	26	5		27	8	2	
Moderate	45	31	13	0.038*	14	16	4	0.018*
Poor	51	40	19		10	9	6	
Gross appearance								
Borrmann I	10	5	4		3	2	0	
Borrmann II	38	16	1	0.093	9	4	0	0.199
Borrmann III	101	72	30		38	22	11	
Borrmann IV	6	4	2		1	5	1	
Tumor size (cm)								
≤ 5	103	67	25	0.911	39	20	7	0.219
> 5	52	30	12		12	13	5	
Depth of tumor invasion (T)								
T1	43	15	4		18	3	0	
T2	23	17	6		9	5	0	
T3	0	4	0	0.017*	1	1	1	0.014*
T4a	74	55	21		19	21	7	
T4b	15	6	6		4	3	4	
Lymphatic metastasis (N)								
N0	80	42	9		29	13	3	
N1	26	17	10		8	7	1	
N2	29	18	7	0.022*	4	6	5	0.055
N3a	14	17	9		6	4	0	
N3b	6	3	2		4	3	3	
TNM stage								
Ia	36	12	4		15	2	0	
Ib	21	11	1		7	4	0	
IIa	6	10	4		4	2	1	
IIb	31	20	6	0.130	10	9	2	0.009**
IIIa	15	13	4		3	3	0	
IIIb	19	11	6		1	6	5	
IIIc	27	20	12		11	7	4	
Vascular tumor thrombus								
No	137	83	31	0.682	47	28	10	0.491
Yes	18	14	6		4	5	2	
Perineural invasion								

70% in both SGC-7901 and AGS gastric cancer cell lines (**Figure 2E**).

TEM7 is closely associated with tumor aggressiveness of gastric cancer

Paraffin-embedded tissues from test and validation cohort were examined by immunochemical staining. In the test cohort, TEM7 (-), (+) and (++) expressions were observed in 151 (53.6%), 97 (33.6%) and 37 (12.8%) cases respectively (**Figure 3A**, **3B**). And the TEM7 expression status was similar in the validation cohort (**Figure 3C**). The correlation between TEM7 expression and the clinical pathological features was shown in **Table 1**. Through statistical analysis, TEM7 expression was not correlated with age, gender, Lauren classification, Borrmann classification, tumor size, tumor vascular thrombus, perineural invasion and signet ring cell cancer, while it was closely correlated with tumor differentiation (in the test cohort, $P = 0.038$; in the validation cohort, $P = 0.018$) and depth of cancer invasion (in the test cohort, $P = 0.017$; in the validation cohort, $P = 0.014$). In

No	144	90	34	0.977	49	29	12	0.201
Yes	11	7	3		2	4	0	
Signet ring cell carcinoma								
No	134	91	34	0.156	43	25	12	0.150
Yes	21	6	3		8	8	0	

[a]Median age of total 395 patients was 61 years; *P < 0.05; **P < 0.01.

addition, it was observed that TEM7 expression was correlated with lymphatic metastasis in the test cohort (P = 0.022) and TNM stage in the validation cohort (P = 0.009). These results strongly implied that gastric cancer cases with high TEM7 expression were more likely to exhibit aggressive clinicopathological features.

TEM7 is a dismal prognostic factor for overall survival of resectable gastric cancer patients

The correlation between TEM7 expression status and OS of gastric cancer patients was further investigated. Our results showed that a low expression level of TEM7 was related to a superior OS. In the test cohort, the OS of TEM7 (-) group was significantly higher than that of TEM7 (+) group (5-year survival rates, 69.4% vs 55.7%, P = 0.048) and TEM7 (++) group (5-year survival rates, 69.4% vs. 36.4%, P = 0.003) (**Figure 3D**). The results were also validated in the validation cohort. A significant difference in OS was found between TEM7 (-) and (++) groups (5-year survival rates, 70.8% vs. 33.3%, P = 0.012) (**Figure 3E**).

Discussion

TEM7, a cell surface transmembrane protein, was first identified as highly elevated in the vasculature of a human colon cancer tissue compared to that of normal adjacent colon tissue in 2000 [15]. TEM7 consists of 500 amino acids and is located on the cell surface as a single pass transmembrane protein with a conserved cytoplasmic tail [16, 17]. Under normal and pathological conditions, TEM7 has been found to be involved in angiogenesis [21, 22]. It was expected as a hopeful target for anti-angiogenic therapy because of a relatively easy access to drugs in the previous research [23]. In 2007, it was reported that TEM7 protein was expressed on the surface of tumor cells and involved in metastasis of osteogenic sarcoma [19]. This suggests that TEM7 could be possibly expressed on tumor cells other than endothelial cells, which means that TEM7 has the potency to be a target for individual therapy.

In this study, we firstly analyzed the data of microarray analysis (GSE513-08) and validated the results by real time PCR in gastric cancer cell lines and matched tumor/adjacent normal tissues. The results confirmed that TEM7 mRNA expression was increased in gastric cancer. Then, further immunochemistry analysis showed that nearly 50% gastric cancer samples had TEM7 positive expression in both test and validation cohort, and the positive signals were observed in both cytomembrane and cytoplasm. Similar to Fuchs' findings, the endothelial mark is indeed expressed in some kinds of tumor cells, including osteogenic sarcoma and gastric cancer [19].

There is no clear answer to the question why TEM7 is expressed on the surface of gastric cancer cells, while its transmembrane region contains a plexin-like as well as a weak nidogen-like domain, which is speculated to be involved in mediating protein-protein interactions [17]. Recently, two functional binding partners of TEM7 have been reported. Cortactin, found to bind TEM7 in 2004 [17], was reported to be present in lamellipodia and mediate cancer progression such as migration and invasion [24, 25]. Nidogen, another putative binding partner for TEM7, was an extracellular basement membrane protein with strong affinity to laminin and collagen IV [26, 27]. The interaction between the extracellular matrix and the cells in solid malignancies can influence the tumor microenvironment and thus leads to tumor development like migration and invasion [28]. Therefore, we speculate that TEM7-expressed GC cell is likely to represent a cell subtype, and TEM7 expression is correlated with cell proliferation and invasion. Similar to Fuchs' findings in osteogenic sarcoma, an inhibitory effect of TEM7 siRNA on gastric cancer cell migration and invasion was observed in this study. This suggests that TEM7-expressed GC cells possess stronger invasiveness and migration ability, and high expression of TEM7 can promote cancer development.

Through immunohistochemistry analysis of tumor tissues (296 in test cohort and 96 in vali-

dation cohort) and comparative analysis of TEM7 expressions in subgroups with different clinicopathological characteristics, we found that TEM7 expression is closely correlated with tumor differentiation and depth of cancer invasion. In the TEM7 (++) group, more than 50% patients were with poor differentiation, and about 80% patients were at stage T4. TEM7-positive GC cells have higher degree of malignancy and invasiveness. Further, the prognosis analysis showed that TEM7 expression is closely related to the outcome of gastric cancer patients, and patients with TEM7 positively expressed in tumor tissue have lower OS than those with TEM7 negative expressed.

In summary, TEM7 is highly expressed in GC cells and is likely correlated with tumor invasion and migration. It may act as a significant prognostic indicator and as a potential target for individualized treatment. Future study on its binding proteins and associated signaling pathways will probably provide the new candidate for targeted therapy of gastric cancer.

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Disclosure of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Table 1. Information about 5 gastric cancer cases used in microarray analysis

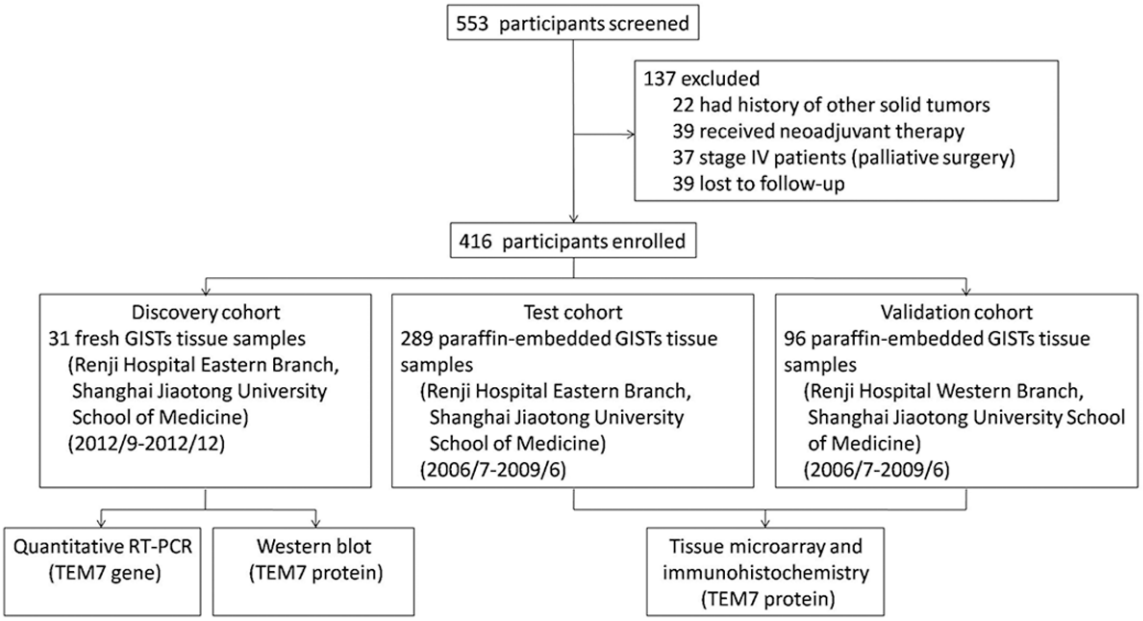
ID	Gender	Age (years)	Pathological type	TNM stage
N197507 T197507	Female	59	adenocarcinoma	T4aN2M0
N196955 T196955	Male	61	adenocarcinoma	T3N0M0
N197046 T197046	Male	41	signet ring cell carcinoma	T4bN2M0
N197359 T197359	Male	62	adenocarcinoma	T4aN3aM0
N197728 T197728	Female	56	adenocarcinoma	T2N0M0

Supplementary Table 2. Background disposition and clinical characteristics of gastric cancer patients in test and validation cohort

	NO. (%)		*P* value
	Test cohort (n = 289)	Validation cohort (n = 96)	
Age at diagnosis (years)[a]			
≤ 61	148 (51.2%)	54 (56.3%)	0.411
> 61	141 (48.8%)	42 (43.8%)	
Gender			
Male	192 (66.4%)	63 (65.6%)	0.901
Female	97 (33.6%)	33 (34.4%)	
Lauren classification			
Intestinal	87 (30.1%)	28 (29.2%)	0.898
Diffuse/mixed/not classified	202 (69.6%)	68 (70.8%)	
Differentiation			
Well	90 (31.1%)	37 (38.5%)	0.098
Moderate	89 (30.8%)	34 (35.4%)	
Poor	110 (38.1%)	25 (26.0%)	
Gross appearance			
Borrmann I	19 (6.6%)	5 (5.2%)	0.388
Borrmann II	55(19.0%)	13 (13.5%)	
Borrmann III	203 (70.2%)	71 (74.0%)	
Borrmann IV	12 (4.2%)	7 (7.3%)	
Tumor size (cm)			
≤ 5	195 (67.5%)	66 (68.8%)	0.900
> 5	94 (32.5%)	30 (31.3%)	
Depth of cancer invasion (T)			
T1	62 (21.5%)	21 (21.9%)	0.962
T2	46 (15.9%)	15 (15.6%)	
T3	10 (3.5%)	4 (4.2%)	
T4a	150 (51.9%)	47 (49.0%)	
T4b	27 (9.3%)	11 (11.5%)	
Lymphatic metastasis (N)			
N0	131 (45.3%%)	45 (46.9%)	0.139
N1	53 (18.3%)	16 (16.7%)	
N2	54 (18.7%)	15 (15.6%)	
N3a	40 (13.8%)	10 (10.4%)	
N3b	11 (3.8%)	10 (10.4%)	
TNM stage			
Ia	52 (18.0%)	17 (17.7%)	0.911
Ib	33 (11.4%)	11 (11.5%)	
IIa	20 (6.9%)	7 (7.3%)	
IIb	57 (19.7%)	21 (21.9%)	
IIIa	32 (11.1%)	6 (6.3%)	
IIIb	36 (12.5%)	12 (12.5%)	
IIIc	59 (20.4%)	22 (22.9%)	
Vascular tumor thrombus			
No	251 (86.9%)	85 (88.5%)	0.727
Yes	38 (13.1%)	11 (11.5%)	

Perineural invasion			
No	268 (92.7%)	90 (93.8%)	0.822
Yes	21 (7.3%)	6 (6.3%)	
Signet ring cell carcinoma			
No	259 (89.6%)	80 (83.8%)	0.105
Yes	30 (10.4%)	16 (16.7%)	

[a]Median age of total 385 patients was 61 years.



Supplementary Figure 1. Study design.