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Original Article

Silencing of WISP3 suppresses gastric cancer cell proliferation and metastasis and inhibits Wnt/β-catenin signaling

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Abstract: CCN6/Wnt1-inducible signaling protein-3 (CCN6/WISP3) is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, which are often dysregulated in cancers. However, the functional role and clinical significance of WISP3 in gastric cancer remain unclear. In this study, we found that silencing of WISP3 suppressed gastric cancer cell proliferation, migration and invasion. Cell adhesion to collagens (collagen I and IV), but not to fibronectin, were significantly inhibited by silencing of WISP3. Furthermore, silencing of WISP3 prevented β-catenin transferring from cell cytoplasm to nuclear, and suppressed canonical Wnt/β-catenin signaling and its downstream target genes, cyclin D1 and TCF-4. By immunohistochemical analysis of 379 patients, we found that the expression of WISP3 is closely associated with gastric cancer size and tumor invasion, and indicates a poor prognosis in both test cohort (253 patients) and validation cohort (126 patients). Moreover, the expression of WISP3 was positively correlated with the expression of cyclin D1 and TCF-4 in gastric cancer tissues. Taken together, our data suggests that WISP3 might be a promising prognostic factor and WISP3-Wnt/β-catenin axis may be a new therapeutic target for the intervention of gastric cancer growth and metastasis.

Keywords: WISP3, gastric cancer cell, proliferation, invasion, canonical Wnt signaling

Introduction

Gastric cancer is one of the most common malignancies worldwide [1]. It is the second cause of the most cancer deaths in the world [2]. Although its incidence has declined, the incidence remains high in Asian countries [1, 3]. Gastric cancer is heterogeneous, so it is difficult to predict the outcomes of patients using classical histological and molecular classifications [4]. Although gastric cancer can be curable if detected early, but most patients are diagnosed with late-stage disease, when current therapeutic strategies are far from optimal [3]. Two strategies have been conducted in gastric cancer treatment, chemotherapy and surgery. Adjuvant chemotherapy has been shown to benefit some patients with early gastric cancer, whereas conventional chemotherapy has limited efficacy for advanced gastric cancer. Surgery is the only curative treatment strategy [3]. Surgery and combination chemotherapies have been shown to confer only modest survival benefits in advanced gastric cancer, resulting in an overall 5 year survival rate of 24% [5, 6]. Therefore, to improve prognosis of these high-risk patients, it is urgent to identify predictive biomarkers and to develop refined treatment strategies.

CCN6/Wnt1-inducible signaling protein-3 (CCN6/WISP3) is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, which consists of six

Table 1. WISP3 expression in test group and validation group

variable	Test (253 cases)	Validation (126 cases)	*P*
Age (year)			
< 60	112	55	0.909
≥ 60	141	71	
Gender			
Male	163	89	0.228
Female	90	37	
Lauren type			
Intestinal type	143	71	0.975
Diffuse type	110	55	
Tumor size (cm)			
< 5	130	71	0.361
≥ 5	123	55	
Depth of invasion (T)			
T1+T2	82	49	0.212
T3+T4	171	77	
Lymph node metastasis (N)			
No	105	54	0.801
Yes	148	72	
TNM stage			
I	62	37	
II	64	32	0.587
III	107	45	
IV	20	12	
Early gastric cancer			
No	210	98	0.219
Yes	43	28	
Neural invasion			
No	232	122	0.058
Yes	21	4	
vessel carcinoma embolus			
No	219	109	0.989
Yes	23	10	

members, CCN1 (CYR61/cysteine-rich61), CCN2 (CTGF/connective tissue growth factor), CCN3 (NOV/nephroblastoma/overexpressed gene), CCN4 (WISP1/Wnt1-inducible signaling protein-1), CCN5 (WISP2/Wnt1-inducible signaling protein-2) and CCN6 (Wnt1-inducible signaling protein-3) [7-12]. Apart from CCN5, all CCN proteins are composed of 4 conserved cysteine-rich modular domains which exhibit homology to conserved regions in a variety of extracellular proteins [17], it contains an N-terminal secretory signal, module I analogous to an insulin-like growth factor-binding protein-like module (IGFBP); module II for a von Willebrand factor type C repeat module (VWC) that may participates in protein complex formation; module III analogous to a thrombospondin1 (TSP1) domain that is involved in the binding to sulfated glycosaminoglycans either on the cell surface or in the extracellular matrix, and module IV of a cysteine-knot-containing module (CT) [13-17] is recognized in several growth factors such as TGF-β, PDGF and NGF cytokines like endothelin 1, and events that result in cellular stress such as hypoxia, may participate in dimerization and receptor binding [17].

CCN proteins can bind to integrin receptors and heparin sulfate proteoglycans by different modules of CCN proteins, thereby trigger a wide range of biological functions such as mitosis, cell adhesion, proliferation, migration, extracellular matrix, growth arrest, production, apoptosis, differentiation, survival [14, 18, 19]. Overexpression of CTGF/CCN2 can induce cell death [20]. The multimodular architecture of CCN proteins has also been associated with tumor progression. For example, WISP3/CCN6 acts as a tumor suppressor in breast cancer [21], but it is oncogenic in colorectal cancer. While WISP2/CCN5 acts as a tumor suppressor in colorectal cancer [22]. The presence of variants of the CCN proteins has been associated with the development and progression of some tumor types, by lacking one or more modules and generated by alternative splicing or gene mutations [23-25].

CCN6 is a secreted cysteine rich matricellular protein, which is 36.9 KD. It has been suggested that CCN6/WISP3 played an important role in the progression and development of cancer.

Table 2. Relationship between WISP3 expression and clinicopathologic features of gastric cancer patients

Variable	WISP3 (Test)		*P*	WISP3 (Validation)		*P*
	-	+		-	+	
Age (year)						
< 60	59	53	0.731	31	23	0.321
≥ 60	71	70		35	36	
Gender						
Male	86	77	0.555	45	44	0.362
Female	44	46		22	15	
Lauren type						
Intestinal type	68	75	0.039	32	39	0.038
Diffuse type	56	36		35	20	
Tumor size (cm)						
< 5	80	50	< 0.001	46	25	0.003
≥ 5	50	73		21	34	
Depth of invasion (T)						
T1+T2	60	22	< 0.001	32	17	0.029
T3+T4	70	101		35	42	
Lymph node metastasis (N)						
No	71	34	< 0.001	40	14	< 0.001
Yes	59	89		27	45	
TNM stage						
I	49	13	< 0.001	27	10	0.002
II	34	30		20	12	
III	41	66		16	29	
IV	6	14		4	8	
Early gastric cancer						
No	95	115	< 0.001	46	52	0.009
Yes	35	8		21	7	
Neural invasion						
No	123	109	0.084	65	57	0.897
Yes	7	14		2	2	
vessel carcinoma embolus						
No	116	103	0.201	59	50	0.587
Yes	14	20		8	9	

Unlike all other known CCN proteins, 4 of 10 conserved cysteine residues are not recognized in the VWC module of WISP3 that is frequently mutated in patients with progressive pseudorheumatoid dysplasia [26], CCN6/WISP3 is a tumor suppressor gene which decreases invasion in inflammatory breast cancer as well as in non-inflammatory breast cancers with lymph node metastasis [27-34], CCN6/WISP3 may locate in the downstream of Wnt-1 signaling and that aberrant levels of WISP expression in colon cancer may play a role in colon tumorigensis [35]. Different alternative forms may contribute to the development of hepatocellular carcinoma (HCC) [36]. CCN6/WISP3 can stimulate the proliferation of lung fibroblasts as a profibrotic mediator [37]. But it has not been studied in gastric cancer.

In this study, to investigate the functional role of WISP3 in gastric cancer, we silenced WISP3 expression by RNA interference and found that silencing of WISP3 significantly suppressed gastric cancer cell migration, invasion and adhesion to collagens (collagen I and IV), but not to fibronectin. Furthermore, we uncovered that silencing of WISP3 prevented β-catenin transferring from cell cytoplasm to nuclear and inhibited canonical Wnt/β-catenin signaling. We further explored the clinical significance of WISP3 in gastric cancer by immunohistochemical staining of tissue microarray. The results showed that WISP3 expression status is closely associated with clinicopathological characteristics of gastric cancer, including tumor size, lymph node metastasis, infiltrating depth, and TNM stage in both test and validation cohort. Further analysis showed that the expression status of WISP3 is closely correlated with patient prognosis in both test and validation cohort, suggesting that WISP3 might be a promising prognostic factor.

Materials and methods

Cell lines and cell culture

Human gastric cancer cell lines AGS, HGC27, SGC7901 and others were maintained in RPMI

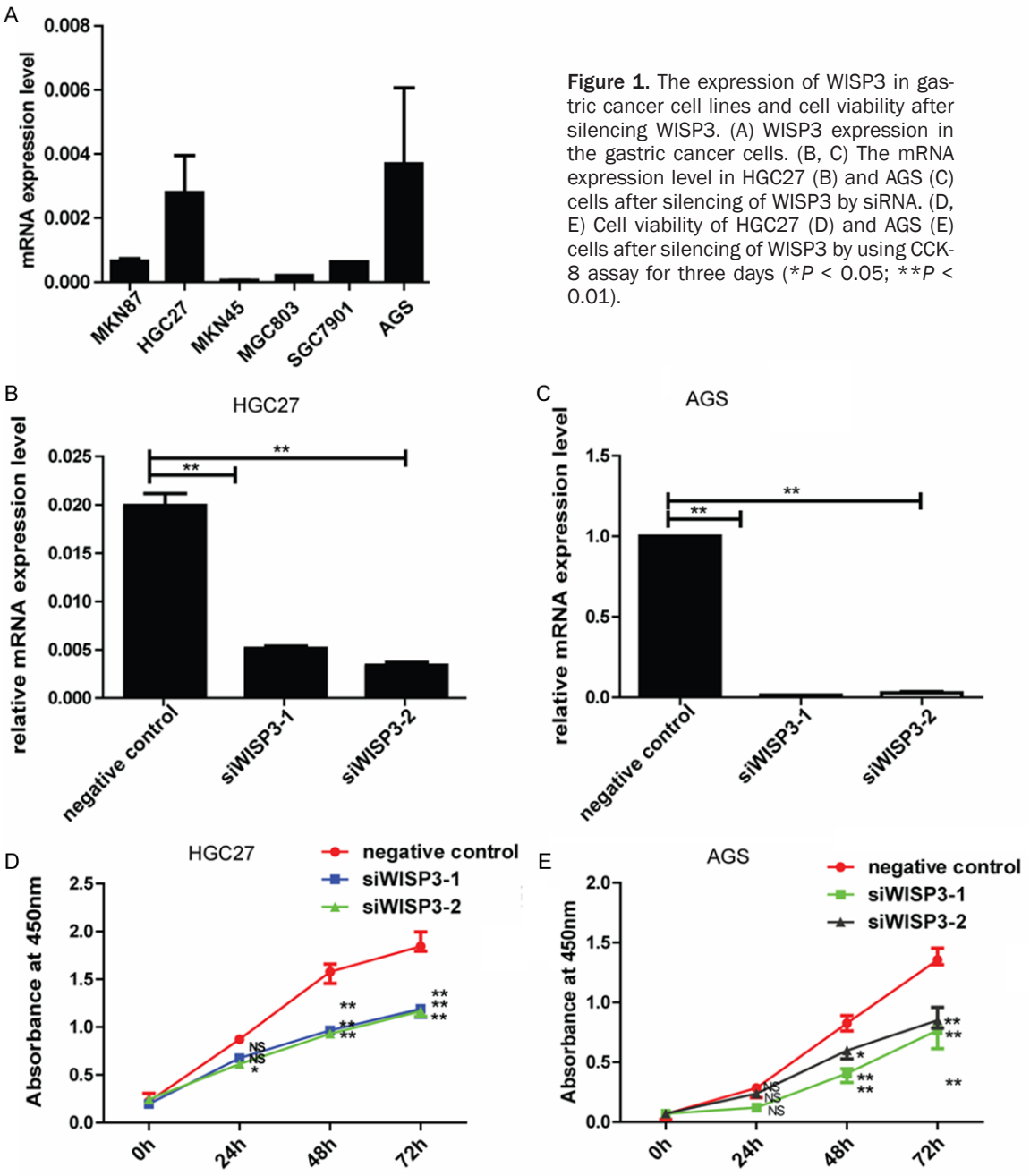

Figure 1. The expression of WISP3 in gastric cancer cell lines and cell viability after silencing WISP3. (A) WISP3 expression in the gastric cancer cells. (B, C) The mRNA expression level in HGC27 (B) and AGS (C) cells after silencing of WISP3 by siRNA. (D, E) Cell viability of HGC27 (D) and AGS (E) cells after silencing of WISP3 by using CCK-8 assay for three days (*$P < 0.05$; **$P < 0.01$).

1640 (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C, 5% CO_2. The medium was changed at alternate days and cells were split before they reached confluency.

Study population

The inclusion criteria used in current study were defined as follow: (1) histologically confirmed an adenocarcinoma of the stomach and (2) the date of death or survival data were available. Patients were excluded when (1) histology identified a tumour type other than adenocarcinoma, (2) histopathological data were incomplete, (3) patients had previously undergone a resection stomach with cancer in the gastric remnant cancer, (4) date of patient death or survival had not been recorded, and (5) patients who received perioperative chemotherapy. TNM stage of all study patients was determined according to the seventh edition of the UICC guidelines.

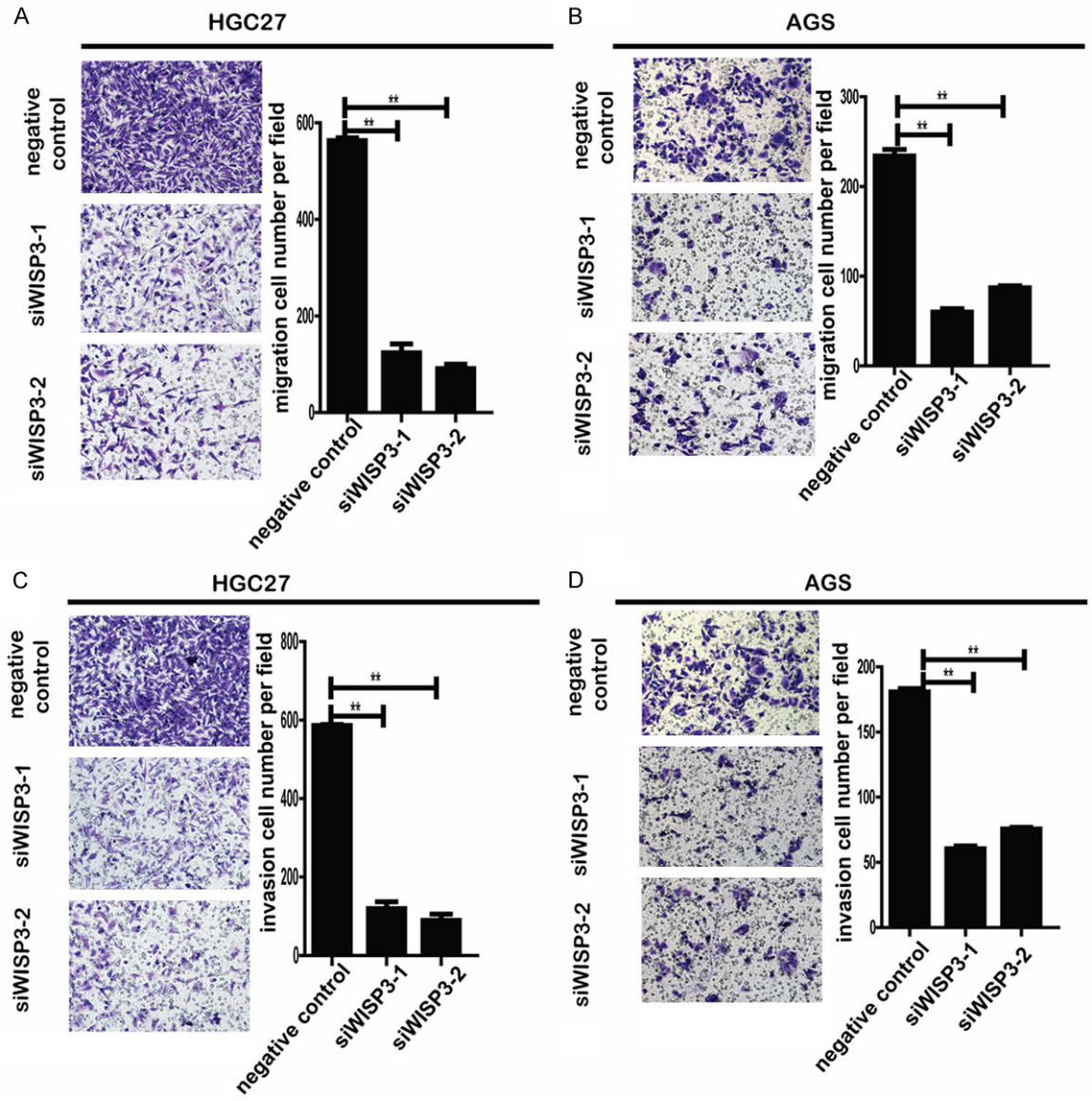

Figure 2. Silencing of WISP3 suppresses gastric cancer cells migration and invasion. (A, B) Representative images (left) of HGC27 (A) and AGS (B) cells after silencing of WISP3 that migrated to the bottom of Transwell filter (8 μm, pore diameter) and statistical analysis (right) of the cell migration. (C, D) Representative images (left) of HGC27 (C) and AGS (D) cells after silencing of WISP3 that invaded through Matrigel to the bottom of Transwell filter (8 μm, pore diameter) and statistical analysis (right) of the cell invasiveness. The results shown are mean ± SD of migration, and invading cells were photographed at 200× magnification per field. (*, $P < 0.05$; **, $P < 0.01$).

According to inclusion and exclusion criteria, a total of 715 and 408 eligible cases were identified from east and west surgery departments of Renji Hospital between January 2004 and December 2008. Using computer-generated random numbers via SPSS software, 253 patients in east department were selected for follow-up and used as test group of prognosis. In parallel, 126 patients in west department were selected as validation group. The clinicopathological characteristics of test and validation group were shown in **Table 2**.

Tissue microarray construction

Tissue microarrays were constructed by Suzhou Xinxin Biotechnology Co., Ltd (Xinxin Biotechnology Co, Suzhou, China). Tissue paraffin blocks of WISP3 samples from 379 cases were stained with hematoxylin-eosin to confirm the diagnoses and marked at fixed points with

most typical histological characteristics under a microscope. Two 2-mm cores per donor block were transferred into a recipient block tissue microarrayer, 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 an adhesive tape transfer system for ultraviolet cross linkage.

Immunohistochemical stain

The tissue microarray glass slides were baked at 55°C for 1 hour, and then de-paraffinized gradually through xylene, 50% xylene, gradient concentrations of ethanol until immersed into tap water. Tissue sections were blocked for peroxidase activity with 0.3% Hydrogen peroxide at 37°C for 30 mins. Antigen retrieval was carried out via boiling in 10 mmol/L citrate buffer (pH 6.0) for fifteen mins. Then the tissues were incubated with WISP3 antibody (rabbit monoclonal antibody, 1:200 dilution, Abcam), cyclin D1 (rabbit monoclonal antibody, 1:150 dilution, Millipore), TCF-4 (rabbit monoclonal antibody, 1:150 dilution, Millipore) overnight at 4°C. Next day, the tissues were washed with 1×phosphate buffer solution (PBS) for three times and incubated with HRP-labeled anti-rabbit secondary antibody (1:200 dilutions, Dako, Carpinteria, CA, USA) for 1 hour at room temperature. Immunostaining was carried out using diaminobenzidine substrate chromogen (Dako, Carpinteria, CA, USA) method and chromogenic reaction was controlled under microscope. After immunostaining, tissues were immersed into hematoxylin for nuclear staining. The TMA slides were then dehydrated through gradient concentrations of ethanol, cleared with xylene, and coverslipped with neutral balsam (Sangon, Shanghai, China).

IHC staining of WISP3, cyclin D1, and TCF-4 was evaluated according to both of the proportion of stained cells and their intensity. The extent of WISP3, cyclin D1, and TCF-4 staining was semi-quantitatively scored as follows: 0 = negative, 1 = 1-25% of cells, 2 = 26-50% of cells, 3 = 51-75% of cells and 4 = 76-100% of cells were stained. Staining intensity was scored as 0 = negative, 1 = weak, 2 = medium and 3 = strong. The sum of the intensity and extent score was used as the final staining score. A score of ≥ 4 was considered as WISP3, cyclin D1, and TCF-4 high expression level (+). Others were considered as WISP3, cyclin D1, and TCF-4 low expression level (-).

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from gastric cancer cell lines using Trizol reagent (Takara, Dalian, China) followed the manufacturer instructions. The reverse-transcription reactions were carried out with random primers and M-MLV Reverse Transcriptase (Takara, Dalian, China). The gastric cancer cell lines of cDNA were used for templates of quantitative real-time PCR reaction in SYBR-Green method. All the qPCR reactions were performed on a StepOneTM real-time PCR System (Applied Biosystems, Foster City, CA, USA). 18S RNA was used as an internal control. The 2-ΔCt method was used to quantify the relative WISP3 expression levels. The forward and reverse WISP3 primer sequences were: 5'-CTCAAGTACTCAGAGTTACA-3' and 5'-TGACCCCTCATTTGGGCA-3' respectively.

In vitro migration and invasion assays

For the Transwell migration assay, 5×10^4 gastric cells were placed on the top chamber of each insert with the non-coated membrane (Millicell). Cells were trypsinized and resuspended in RPMI 1640 and 600 μL of medium supplemented with 10% fetal bovine serum were injected into the lower chamber. After 24 hours for gastric cells in the migration assays, any cells remaining in the top chambers or on the upper membrane of the inserts were carefully removed. After fixation and staining in a dye solution containing 0.1% crystal violet and 20% methanol, cells adhering to the lower membrane of the inserts were counted and imaged through an I×71 inverted microscope (Olympus Corp. Tokyo, Japan). We carried out invasion assay by adding 100 μl matrigel (BD Bioscience, Franklin Lakes, NJ) into top chamber of transwell and placed 1×10^5 gastric cells onto the matrigel. 48 hours later, the transwell for invasion was ceased and staining.

Cell viability assay

Cell viability was detected using a standard Cell Counting Kit-8 assay. Gastric cells were seeded into 96-well plates (100 μl per well) at a density of 3×10^4 cells per ml. We added 10 μl of reagent from Cell Counting Kit-8 (Dojindo, Kumamoto,

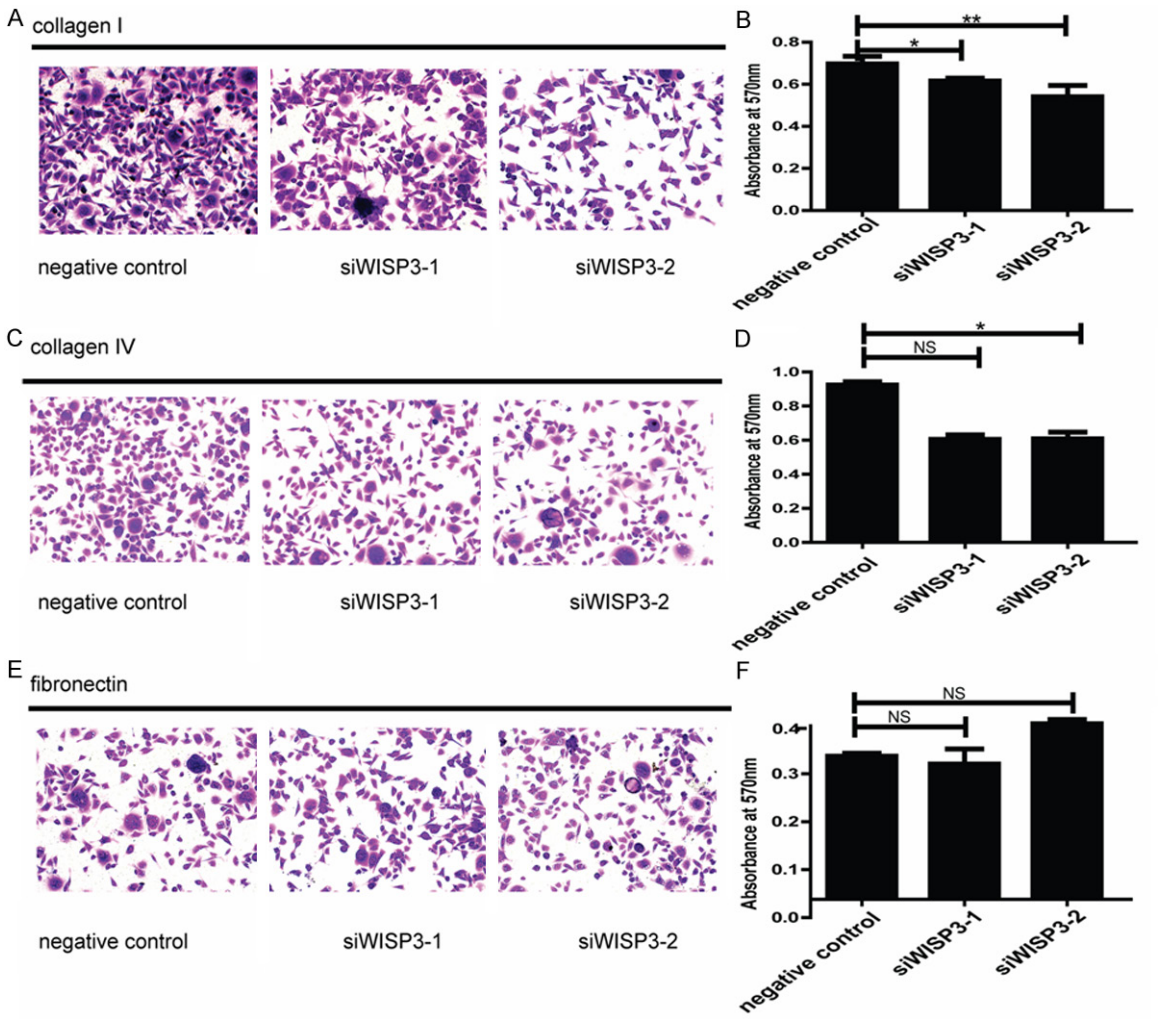

Figure 3. Silencing of WISP3 reduces cell adhesion to collagens. A: Silencing of WISP3 suppresses AGS cell adhesion to collagen I. C: Silencing of WISP3 suppresses AGS cell adhesion to collagen IV. E: Silencing of WISP3 has no effects on AGS cell adhesion to fibronectin. B, D, F: The results shown are mean ± SD of adhesion cells were photographed at 200× magnification. (*, $P < 0.05$; **, $P < 0.01$).

Japan) to each well for detection at day 1, 2, 3. After ninety minutes of incubation at 37°C, the optical density was measured using microplate reader at a wavelength of 450 nm.

Luciferase reporter assay

Gastric cells were seeded in 96-well plates and transfected with mixture of 100 ng TCF/catenin reporter plasmid (Wnt/β-catenin signaling), or 100 ng PFR reporter plasmid (Wnt/PCP signaling), 5 ng ATF2 reporter (plasmind Wnt/PCP signaling), and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 20 hours, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

Cell adhesion assay

Cells were trypsinized, counted and then plated at a density of 1-5×10^4 cells per well in 96-well plates coated with 100 ng/ml fibronectin or 100 ng/ml collagen I, 100 ng/ml collagen IV. The cells were incubated at 37°C for 1 h in a CO_2 incubator. Non-attached cells were removed by three washings with 1×phosphate-buffered saline (PBS). Fix immediately with a fresh solution of 1% glutaraldehyde in PBS. Stain cells with crystal violet (0.1% H_2O). Wash excess of dye with tap water. Add 50 µl per well of 0.2% Triton X-100. Read in ELISA reader wavelength

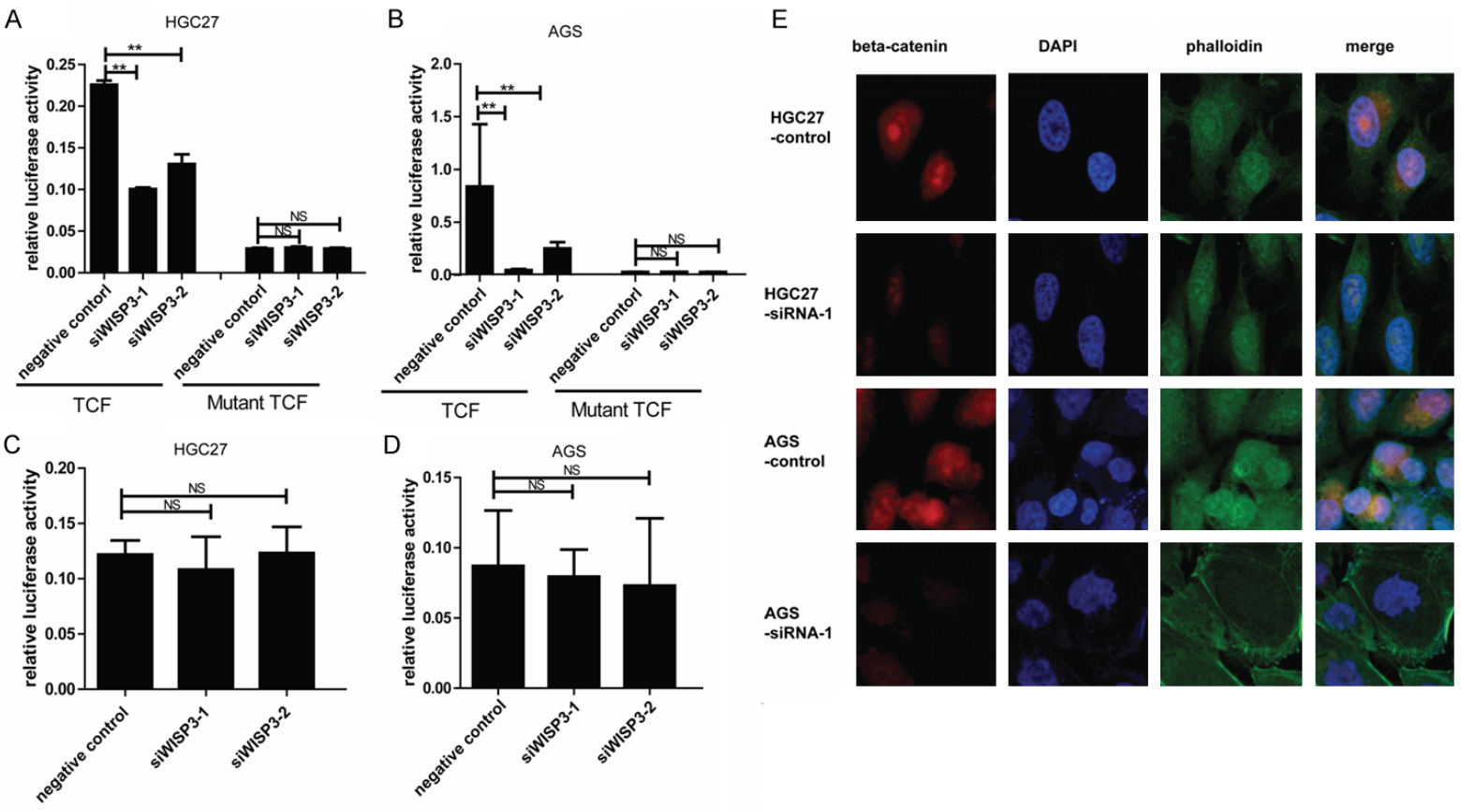

Figure 4. Silencing of WISP3 suppresses Wnt/β-catenin signaling. (A, B) Dual-luciferase reporter assay showed that silencing of WISP3 inhibited Wnt/β-catenin signaling of HGC27 (A) and AGS (B) cells. The results shown are mean ± SD of relative firefly/Renilla ratio. (C, D) Noncanonical Wnt/PCP signaling has not been changed after silencing of WISP3 in HGC27 (C) and AGS (D) cells. (E) Immunofluorescent staining showed that silencing of WISP3 prevented β-catenin transferring from cell cytoplasm to nuclear. (*, $P < 0.05$; **, $P < 0.01$).

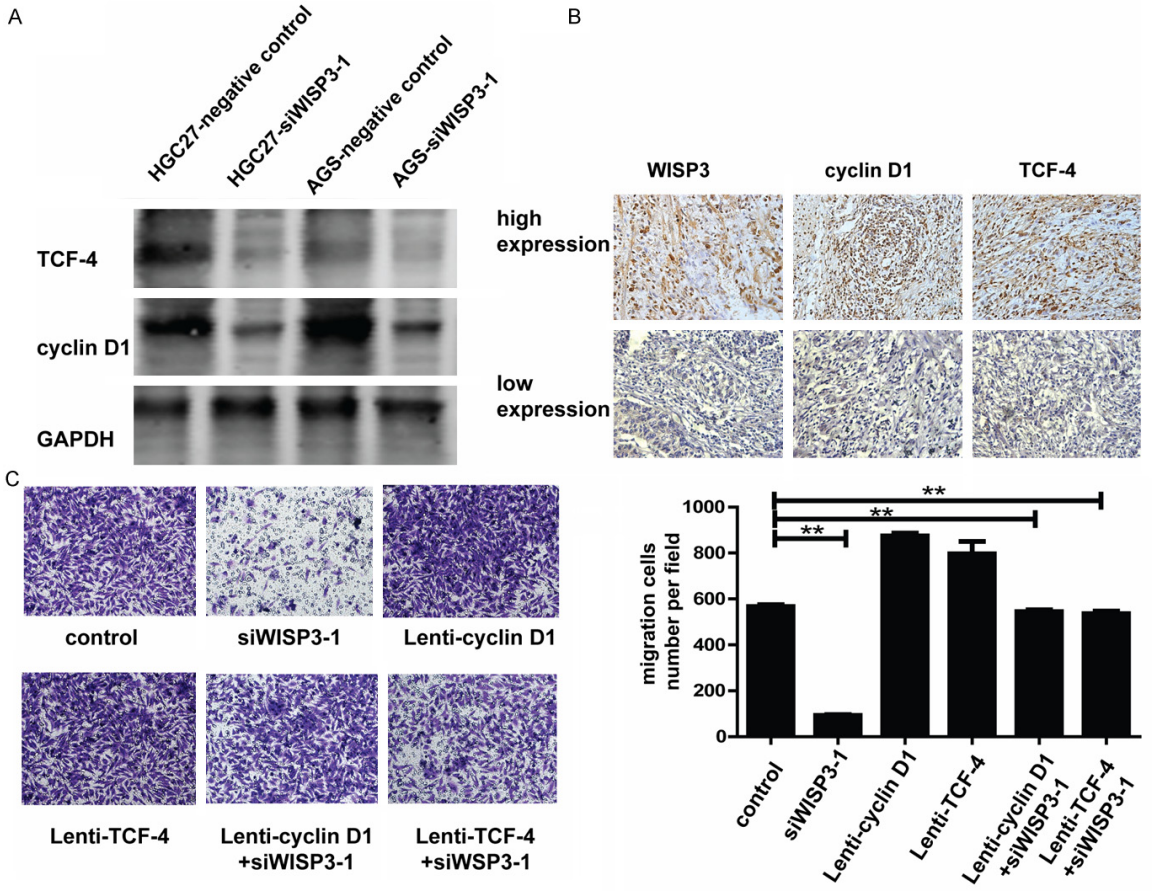

Figure 5. Silencing of WISP3 inhibits the expression of downstream target genes of Wnt/β-catenin and restoration of downstream target genes counteracts the effects of WISP3 silencing on cell migration. A: The expression of cyclin D1 and TCF-4 were significantly reduced after silencing WISP3. B: Immuohistochemistry staining revealed the correlations of WISP3 with cyclin D1 or TCF-4 in gastric cancer tissues by Pearson correlation coefficient. Representative images of immunostaining for WISP3, cyclin D1 and TCF-4 (upper panel). There are significant correlations between the expression of WISP3 and cyclin D1 ($R = 0.59$, $P < 0.001$), and WISP3 and TCF-4 ($R = 0.63$, $P < 0.001$) (lower panel). C: Forced expression cyclin D1 or TCF-4 counteracted the effects of WISP3 silencing on cell migration.

of 570 nm. These experiments were performed in triplicate and repeated twice.

Immunofluorescence stain

Remove medium, followed h 3 washes of 1× PBS. Fix sample in 2% paraformaldehyde made in PBS for 15 minutes at room temperature. Wash 2 times in PBS to remove residual paraformaldehyde. Permeabilize cells with 0.02% Triton X-100 in distilled water for 1 min. Wash monolayer 3 times with PBS. Block with 1% BSA for 60 minutes. Primary antibody of WISP3 (1:200) incubated for 60 minutes at room temperature. Wash monolayer 5 times with PBST (PBS supplement with 0.1% Tween-20). Incubated with secondary antibody (1:200) (Donkey anti Rabbit, Jireurope, USA) for 60 minutes. Wash coverslip 5 times with PBST. DAPI stain for 20 minutes (1 μg/ml) (Sigma-Aldrich, USA) away from light. Wash coverslip 3 times with PBS. Adhere coverslip to a slide and store samples horizontally at -20°C.

SiRNA transfection

SiRNA duplexes targeted at WISP3 and scramble control siRNA duplex were obtained from GenePharma (Shanghai, China). Small interfering RNAs duplexes for WISP3 were as follows: siRNA1 sense, 5'-GCGACAGCAAUAUAUUAAATT-3', anti-sense, 5'-UUUAAUAUAUUGCUGUCGCTT-3'; siRNA2 sense, 5'-CCAGGUACAUUAUCAUAAUTT-3', anti-sense, 5'-AUUAUGAUAAUGUACCUGGTT-3'. The scramble control siRNA duplex was as sence, 5'-UUCUCCGAACGUGU-

Table 3. Relationship between WISP3 expression and cyclin D1, TCF-4 in gastric cancer patients

Variable expression	Total n	WISP3 expression (n = 253)		*P* Value[b]
		High[a] (%)	Low[a] (%)	
cyclin D1				
high	132	99 (75.0)	33 (25.0)	< 0.001
low	121	24 (20.2)	97 (81.5)	
TCF-4				
high	145	110 (75.9)	35 (24.1)	< 0.001
low	108	13 (12.0)	95 (88.0)	

[a]Values inparentheses indicate percentage values. [b]Evaluated by χ^2 test.

CACGUTT-3', anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfection was performed by using the Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Statistical analysis

Statistical analyses were conducted using SPSS 16.0 software (Chicago, IL, USA). We performed chi-squared tests in cross tables to assess the relationships between expression levels of WISP3 and clinicalpathological factors. Overall survival (OS) was calculated using Kaplan-Meier method (**Table 2**). The survival distributions were compared through log-rank test. One-way analysis of variance (ANOVA, Post-hoc testing) was used to compare groups (**Table 1**). *P* value less than 0.05 was considered statistically significant.

Ethics statement

This project was approved by committee of Renji Hospital, Shanghai Jiaotong University School of Medicine for the use of samples. All patients' data were pseudonymised before study inclusion.

Results

Silencing of WISP3 suppresses gastric cancer cell proliferation in vitro

WISP3 expression level was detected in six gastric cancer cell lines, including AGS, MKN87, HGC27, SGC7901, MKN45 and MGC803 cells (**Figure 1A**). To investigate the functional role of WISP3 in gastric cancer progression, we silenced the expression of WISP3 by transient transfection of siRNA into two gastric cancer cells, HGC27 and AGS cells, which have relatively higher expression of WISP3. The expression of WISP3 was significantly downregulated by transfection of siRNAs. The knockdown efficiency was confirmed by qRT-PCR (**Figure 1B** and **1C**). We then examined the role of WISP3 on gastric cancer cell proliferation by CCK8 assay .The results showed that silencing of WISP3 significantly suppressed HGC27 and AGS cells proliferation (**Figure 1D** and **1E**).

Silencing of WISP3 suppresses gastric cancer cell migration and invasion in vitro

To further explore the role of WISP3 in gastric cancer metastasis, we performed an in vitro migration and invasion assay. As shown in **Figure 2A** and **2C**, the number of HGC27 cells passing through the transwell without or with Matrigel in the negative control group was significantly higher than those in the siRNA silenced groups. And the number of AGS cells passing through the transwell without or with Matrigel in the negative control group was also significantly higher than those in the siRNA silenced groups (**Figure 2B** and **2D**). These results indicate that silencing of WISP3 can significantly reduce the in vitro migration and invasion ability of gastric cancer cells.

Silencing of WISP3 suppresses gastric cancer cell adhesion to collagen I and IV, but not to fibronectin

Cell adhesion to extracellular matrix (ECM) plays important roles in cancer migration and invasion. To further explore the role of WISP3 in gastric cancer cell adhesion, we used three common ECM proteins, including collagen I, collagen IV and fibronectin, to examine whether silencing of WISP3 could affect adhesive capacity of gastric cancer cells. The results showed that silencing of WISP3 significantly suppressed gastric cancer cell adhesion to collagen I and collagen IV (**Figure 3A-D**), but not to fibronectin (**Figure 3E** and **3F**).

Silencing of WISP3 suppresses canonical Wnt signaling in gastric cancer cells

To uncover the underlying mechanism by which silencing of WISP3 suppresses gastric cancer cell migration and invasion, we examined the activation of the canonical Wnt/β-catenin pathway and the non-canonical Wnt/PCP pathway.

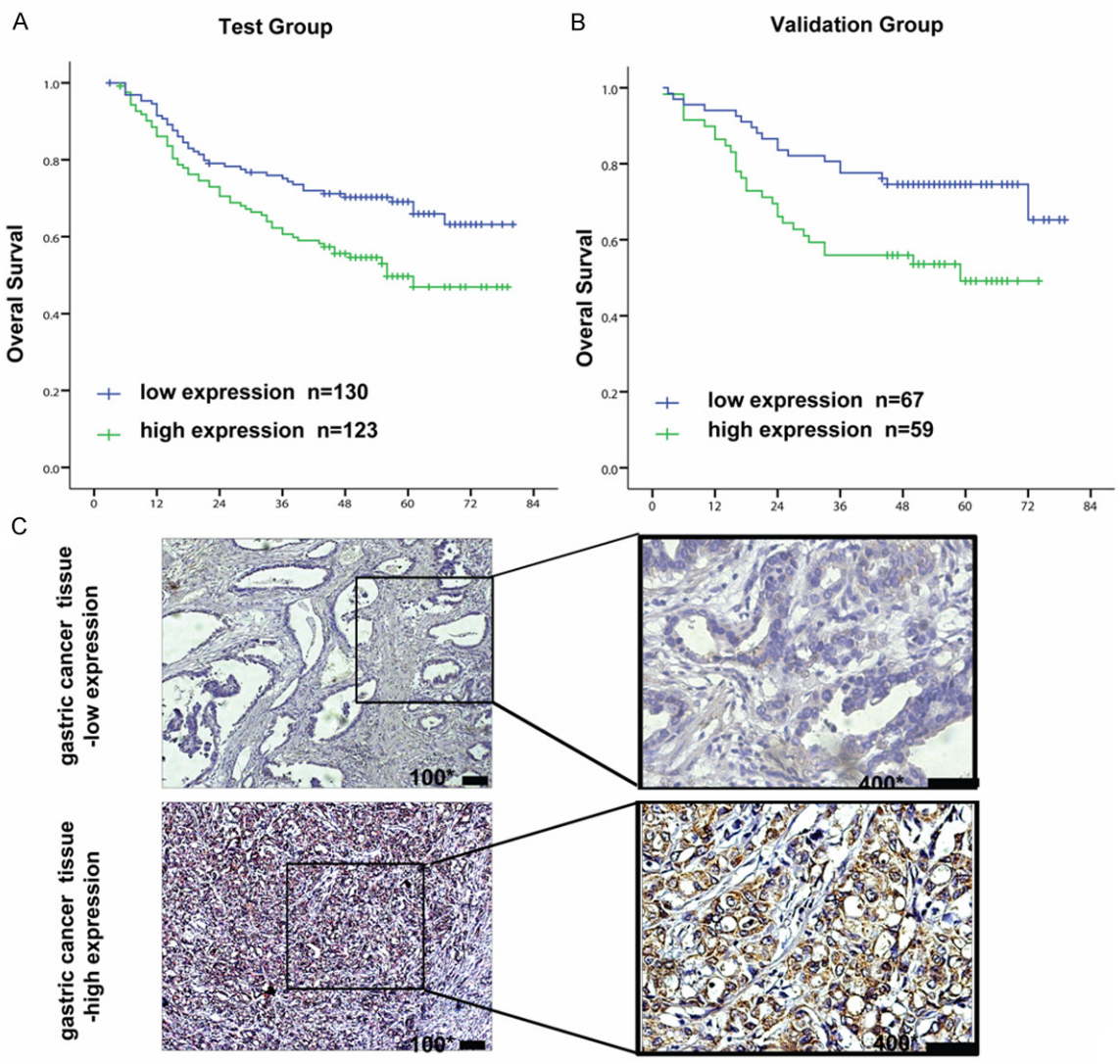

Figure 6. Clinicalpathological correlation of WISP3 expression in gastric tumor patients (A, B) Kaplan-Meier analysis of overall survival related to the expression of WISP3 in the test (A) and validation (B) cohort. (C) Immunohistochemical staining of WISP3 in gastric cancer tissue.

Gastric cancer cells were transfected with a Wnt/β-catenin reporter plasmid (TCF/catenin plasmid) and negative control counterpart plasmid or non-canonical Wnt/PCP pathway reporter plasmid (ATF2 plasmid). The results showed that silencing of WISP3 significantly suppressed canonical Wnt pathway (**Figure 4A** and **4B**), while non-canonical Wnt/PCP pathway was not altered by silencing of WISP3 (**Figure 4C** and **4D**). Furthermore, we found that silencing of WISP3 significantly reduced nucleus location of β-catenin, indicating silencing of WISP3 prevent β-catenin transferring from cell cytoplasm to nuclear, which leads to the suppression of Wnt/β-catenin signaling (**Figure 4E**).

The downstream target gene of Wnt/β-catenin, cyclin D1 and TCF-4, was also examined. The results showed that the expression of cyclin D1 and TCF-4 was down-regulated after silencing of WISP3 (**Figure 5A**). Because cyclin D1 and TCF-4 are key downstream target genes of Wnt/β-catenin, we speculated that forced expression of these two genes could rescue the effects of WISP3 knockdown. The results showed that forced expression cyclin D1 or TCF-4 counteracted the effects of WISP3 knockdown on cell migration (**Figure 5C**).

We further investigated the correlations between the expression of WISP3 and the expres-

sion of cyclin D1 or TCF-4 in gastric cancer tissues with tissue microarray that contained 253 gastric cancer tissue samples (**Figure 5B**; **Table 3**). The results showed that 123 gastric cancer tissues with high expression level of WISP3 showed high expression level of cyclin D1 (99/123) and high expression level of TCF-4 (110/123). And 130 gastric cancer tissues with low expression level of WISP3 showed low expression level of cyclin D1 (97/130) and low expression level of TCF-4 (95/130). The results showed that the expression of WISP3 was positively correlated with the expression of cyclin D1 ($R = 0.59$, $P < 0.001$), and TCF-4 ($R = 0.63$, $P < 0.001$).

WISP3 is closely correlated with clinicopathological parameters and patient prognosis in gastric cancer

Above results showed that WISP3 play important roles in gastric cancer progression by affecting cancer cell proliferation, migration, invasion and adhesion to ECM. To further explore the clinical significance of WISP3, we performed immunohistochemical staining of tissue microarray which contains 397 cases of gastric cancer tissues (**Figure 6**). The patients were divided into two cohorts: test cohort (253 patients) and validation cohorts (126 patients). The clinicopathological characters of these two cohort were in **Table 1**. χ^2 analysis showed that WISP3 expression level was correlated with Lauren classification (intestinal-type, gastric cancer), tumor size (≥ 5 cm), infiltrating depth (T3+T4), lymph node metastasis ($P < 0.05$), TNM stage ($P < 0.05$), early gastric cancer ($P < 0.05$) in both test and validation cohorts (**Table 2**). Kaplan-Meier survival analysis with log-rank test showed gastric cancer patients with WISP3 high expression suffered poorer prognosis in both test and validation cohorts. The correlation between WISP3 expression and overall survival (OS) of gastric cancer patients was analyzed in test and validation groups. The OS in the WISP3 low expression group was remarkably superior to that in the WISP3 high expression group ($P = 0.08$ in test cohort, $P = 0.07$ in validation cohort), the 1, 3, 5 year survival rates of low expression group were 90.7%, 73.6%, 70.3% in test cohort and 91.8%, 73%, 57.4% in validation cohort. The 1, 3, 5 year survival rates of high expression group were 82.1%, 74.6%, 65.2% in test cohort and 83.1%, 53.6%, 49.1% in validation cohort. Taken together, WISP3 expression in gastric cancer tumor tissues was closely correlated with OS of gastric cancer patients, suggesting that WISP3 might be a promising prognostic factor for gastric cancer.

Discussion

Metastasis is a multifactorial process, including tumor cells capable of escaping their normal microenvironment, traversing into and out of lymphatic or blood vessels and proliferating. Implicit in these stages, invasion is the critical ability for tumor cells to metastasis [30]. The high mortality of gastric cancer is mainly due to cancer metastasis. Secreted factors in cancer microenvironment are important players in modulating cancer cell invasion and migration [38-40]. CCN6/WISP3 is a secreted protein that has been reported to associate with cancer metastasis and progression [27-34]. In colon cancer, CCN6/WISP3 is up-regulated and may play an important role in tumorigenesis [22]. It has been reported that CCN6/WISP3 decreases EMT and invasion by attenuation of IGF-1 receptor signaling and E-Cadherin through snail and ZEB1 in breast cancer [28, 30]. However, the functional role and clinical significance of WISP3 in gastric cancer have not been investigated.

In this study, we found that WISP3 closely associated with indicators of cancer metastasis, such as lymph node metastasis and infiltrating depth, suggesting that WISP3 may play an important role in regulating gastric cancer invasion and metastasis. The Kaplan-Meier curves analysis revealed that WISP3 expression was closely correlated with OS of gastric patients. Patients with WISP3-high expression level had shorter OS than those with WISP3-low expression level. Therefore, we identified that WISP3 is an available predictor of poor prognosis including OS in gastric patients. In addition, the expression level of WISP3 is high in the advanced gastric cancer patients (**Table 2**), further study of the clinical value of WISP3 in predicting the recurrence risk of postoperative WISP3 patients may contribute to improving the clinical therapeutic effects.

Silencing of WISP3 could suppress gastric cancer cells proliferation, invasion, metastasis and adhesion to ECM. These data suggest that WISP3 can affect gastric cancer from multiple aspects and it may be a key regulator of gastric

cancer progression. Cell-matrix interaction is crucial for cancer metastasis. Silencing of WISP3 could suppress AGS cell adhesion to collagens, collage I and IV, but not to fibronectin. Cell adhesion to these ECM proteins is mediated by the different integrin subfamilies. Integrin α1β1 and α2β2 both bind a range of collagens including types I, IV and VI [41]. Silencing of WISP3 suppressed gastric cancer cell adhesion to collagens, indicating collagen-binding integrins, likely α1β1 or α2β2, may be involved in WISP3-induced gastric cancer cell behaviors.

CCN6/WISP3 is a component of Wnt1 signaling pathway [43]. Wnt family is aberrantly expression in many tumor cancers. It has been reported that Wnt/β-catenin signaling was often activated in gastric cancer [45], and this manifestation of cancer mostly results from many genes activation mediated by β-catenin. In our study, we have showed that silencing of WISP3 in AGS and HGC27 gastric cancer cells suppressed Wnt/β-catenin signaling, while it had no effect on Wnt/PCP signaling. Furthermore, we found silencing of WISP3 prevented β-catenin transferring from cell cytoplasm to nuclear, and inhibited the expression of downstream target genes, cyclin D1 and TCF-4. Wnt/β-catenin signaling has been implicated in regulating cancer progression by promoting cancer cell growth and metastasis. A recent study has indicated that Wnt signaling is associated with the self-renewal of gastric cancer stem cell [46]. In this study, we also found that silencing of WISP3 in HGC27 and AGS gastric cancer cells could suppress gastric cancer cell proliferation, which might be due to the inhibition of Wnt/β-catenin signaling.

Taken together, our study has demonstrated for the first time that WISP3 could be used as a prognostic factor for gastric cancer. WISP3-Wnt/β-catenin axis may be a new therapeutic target for the intervention of gastric cancer growth and metastasis.

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Disclosure of conflict of interest

None.

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