

Distinct prognostic values and antitumor effects of tumor growth factor $\beta 1$ and its receptors in gastric cancer

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Abstract. Gastric cancer (GC) is one of the most common malignancies and is the second leading cause of cancer-associated mortality world-wide. In the present study, the prognostic value and antitumor effects of transforming growth factor $\beta 1$ (TGF $\beta 1$) and its receptors in GC were explored. The online Kaplan-Meier plotter database was used to investigate the prognostic values of TGF $\beta 1$ and its receptors. The present study demonstrated that low mRNA expression levels of TGF $\beta 1$ and its 3 receptors, transforming growth factor $\beta 1$ (TGF $\beta 1$), TGF $\beta 2$ and TGF $\beta 3$, was associated with improved overall survival time in patients with GC. Cell Counting Kit-8 and Transwell assays were used to confirm the effects of TGF $\beta 1$, TGF $\beta 1$, TGF $\beta 2$ and TGF $\beta 3$ on the proliferation, migration and invasiveness of the AGS and MKN45 GC cell lines. It was found that the knockdown of these genes blocked cell proliferation, migration and invasion in GC cells. To the best of our knowledge, the present study is the first to determine the role of TGF $\beta 1$ and TGF $\beta 3$ in GC cells. The results indicate that in addition to TGF $\beta 1$ and TGF $\beta 2$, TGF $\beta 1$ also plays a specific role in the occurrence and development of tumors. Thus, these markers may be considered as potential prognostic indicators in human GC. The findings of the present study indicate that not only TGF $\beta 1$ and TGF $\beta 2$, but also TGF $\beta 1$ is involved in the progression of GC. The findings of the present study provide new ideas and approaches for the treatment of patients with GC.

Introduction

Gastric cancer (GC) is one of the most common malignancies with ~1 million new cases reported globally every year according to the GLOBOCAN (2002) and Cancer Incidence

in Five Continents databases (1). The mortality rate of GC is the second highest amongst all malignant tumors (2). A good prognosis in patients with GC requires a timely diagnosis and is also associated with different pathological characteristics, genetic background and the treatment method used (3,4). Due to developments in cellular and molecular biology, understanding of the pathogenesis of GC has gradually increased over the past 20 years, but the overall survival rate of patients remains unchanged (5). Tumor related molecules, signaling pathways, proteases and their inhibitors are all involved in the process of tumor development (6,7). Therefore, the molecular analysis of these processes has important significance in the development of therapeutics and the prognosis of GC in clinical practice (8,9).

Transforming growth factor $\beta 1$ (TGF $\beta 1$) is a type of polypeptide cytokine with multiple functions in humans (10). Almost all cells in the body can produce TGF $\beta 1$ and its receptors, including epithelial, endothelial, hematopoietic, nerve and connective tissue cells (11). Assoian *et al* successfully extracted TGF $\beta 1$ from human platelets for the first time in 1983 (8,12). TGF $\beta 1$ has since been reported to play an important role in the regulation of cellular proliferation (12). TGF β receptors (TGF β R) are high affinity binding proteins of TGF $\beta 1$ located on the cell membrane (13). These receptors have been categorized into 3 isoforms according to electrophoretic mobility; TGF $\beta 1$, TGF $\beta 2$ and TGF $\beta 3$ (14). By binding to TGF β R, TGF $\beta 1$ exerts a wide range of biological effects (14). Previous studies have focused on the relationship of TGF $\beta 1$ and TGF β R with cancer (14,15). TGF $\beta 1$ demonstrates diverse functions in tumors, such as the inhibition of cell proliferation, differentiation and apoptosis in the early stages of tumor development (14). In advanced stage cancer, TGF β promotes angiogenesis, induction of extracellular matrix production, invasion and metastasis (16,17). TGF $\beta 1$ and TGF β R are important members of the TGF β /SMAD signaling pathway, which is involved in the regulation of cell proliferation and differentiation. The TGF β /SMAD pathway is one of the most frequently altered signaling pathways in tumors, including GC (18-20).

The online Kaplan-Meier plotter (K-M plotter) is capable of assessing the effect of any gene or gene combination on survival in breast, ovarian, lung and gastric cancer, using patient samples on gene chips or RNA-seq data (21). To date, the K-M plotter has been used to identify and validate

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a number of genes in these cancer types (22-27). The K-M plotter database contains the prognostic and mRNA mapping information of 876 patients with GC (21). In the present study, the K-M plotter was used to determine the prognostic value of mRNA expression of TGF β 1 and its receptors in patients with GC, and the effects of TGF β 1 were validated in GC cell lines.

Materials and methods

Prognostic analyses of patients with GC. Using the K-M plotter (kmplot.com/analysis/) the association between the mRNA expression of TGF β 1 and its receptors, and overall survival (OS) time was analyzed. Using the K-M plotter online software, gene expression, relapse free and OS time data can be downloaded from the Gene Expression Omnibus (Affymetrix microarrays only), the European Genome-Phenome Archive and The Cancer Genome Atlas databases (kmplot.com/analysis/index.php?p=service&cancer=gastric). Clinical data were collected from 876 patients with GC, including sex, perforation history, Tumor Node Metastasis (TNM) stage (28), Lauren classification (29), HER2 status, pathological grade and treatment method. The mRNA expression levels of TGF β 1 and its receptors were entered into the database, and Kaplan-Meier survival curves were generated for the OS time of patients with GC. The patients were split into low- and high-expression groups according to the expression levels of TGF β 1 and its receptors with auto select best cutoff. The log rank P-value and the hazard ratio (HR) with a 95% confidence interval (CI) was calculated.

Cell culture and transfection. The AGS and MKN45 human gastric cancer cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and incubated in a 5% CO₂ incubator at 37°C for 48 h. Cells in the exponential growth phase were harvested and transfected with TGF β 1, TGF β R1, TGF β R2- or TGF β R3-specific siRNA (3 μ g) using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc) according to the manufacturer's protocol. The cells were incubated for 48 h prior to further experimentation. Following siRNAs were used (Ruibo; ribobio.com/): TGF β 1, 5'-GCCCATCTAGGT TATTTCCGTGG-3'; TGF β R1, 5'-AGGGTACTACGTTGA AAGACTTA-3'; TGF β R2, 5'-ACGATAATGTTTGTTAGT ATTCA-3'; TGF β R3, 5'-AACTTAAGATAGCAAGAAATA TC-3'; negative control siRNA (a scrambled siRNA control, siC) 5'-UUCUCCGAACGUGUCACGUTT-3'. Untreated AGS and MKN45 cells were used as the blank control, and cells treated with the scrambled siRNA control were used as the negative control.

Cell Counting Kit-8 (CCK-8) assay. After transfection, cells (1x10³ cells/well) were seeded into a 96-well plate, cultured at 37°C in a 5% CO₂ incubator. The proliferation of cells was measured every 24 h. Fresh DMEM containing 10 μ l CCK-8 solution (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well to detect cell proliferation according to the manufacturer's protocol. After incubation for 2 h at 37°C, cell proliferation was determined by measuring the optical

density (OD) value at a wavelength of 450 nm. The CCK-8 assay was performed in triplicate.

Transwell migration and invasion assays. Following transfection, cells (1x10³ cells/well) in serum-free medium were seeded into the upper chambers of transwell inserts, while medium supplemented with 10% FBS was added into the lower chambers. After incubation for 48 h, the cells that had migrated into the lower chamber were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 5 min. Images were captured using a light microscope at x100 magnification. For the invasion assay, the upper chambers were coated with Matrigel prior at 37°C for 4 h to the addition of the cells.

Reverse transcription fluorescence quantitative PCR (RTq-PCR). An Ultrapure RNA kit (CWBio) was used of the extraction of total RNA from AGS and MKN45 cells after the transfection for 24 h. A HiFiScript cDNA Synthesis kit (CWBio) was used for reverse transcription. The following thermocycling conditions were used for reverse transcription: Incubation at 42°C for 15 min and at 85°C for 5 min. Then, qPCR was performed using MagicSYBR Mixture (CWBio). The following primers was used: TGF β 1 forward, 5'-CCCCTACATTTG GAGCCTGG-3' and reverse, 5'-GCACGATCATGTTGGACA GC-3'; TGF β R1 forward, 5'-ACCGCACTGTCATTACCAT-3' and reverse, 5'-CTGAGCCAGAACCTGACGTT-3'; TGF β R2 forward, 5'-GCTCTGGTGCTCTGGGAAAT-3' and reverse, 5'-CCAGCACTCAGTCAACGTCT-3'; TGF β R3 forward, 5'-GCCCTGATGAGCTCCTGTTT-3' and reverse, 5'-GGC ACAGCCTGACAAAACAG-3'; β -actin forward, 5'-CCCGAG CCGTGTTTCCT-3' and reverse, 5'-GTCCAGTTGGTGAC GATGC-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 30 sec; 95°C for 5 sec, 60°C for 30 sec, with a total of 40 cycles. The relative expression levels of genes were analyzed using 2^{- $\Delta\Delta$ C_q} method (30).

Western blotting. Total protein was extracted from the AGS and MKN45 cells after the transfection for 48 h using a RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein determination was detected using a BCA Protein Assay kit (CWBio). A total of 20 μ g protein of each group was loaded on a 10% gel, resolved using SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 h. The protein was incubated with primary antibodies for at 4°C overnight and secondary antibodies at room temperature for 1 h. The following antibodies were used in this research: Anti-TGF β 1 antibody (1:500; ab92486; Abcam), anti-TGF β R1 antibody (1:500; ab31013; Abcam), anti-TGF β R2 antibody (1:500; ab186838; Abcam), anti-TGF β R3 antibody (1:200; ab97459; Abcam) and goat anti-rabbit secondary antibody (1:5000; ab6721; Abcam). An Enhanced ECL Chemiluminescent Substrate kit (Shanghai Maokang; maokangbio.com/index.action) was used for visualization. Protein level was analyzed using ImageJ version 1.41 (National Institutes of Health).

Statistical analysis. SPSS 20.0 (IBM Corp.) was used for the statistical analysis. All data in the present study are presented as the mean \pm SD. The data were analyzed from three separate experiments. Statistical significance was determined using

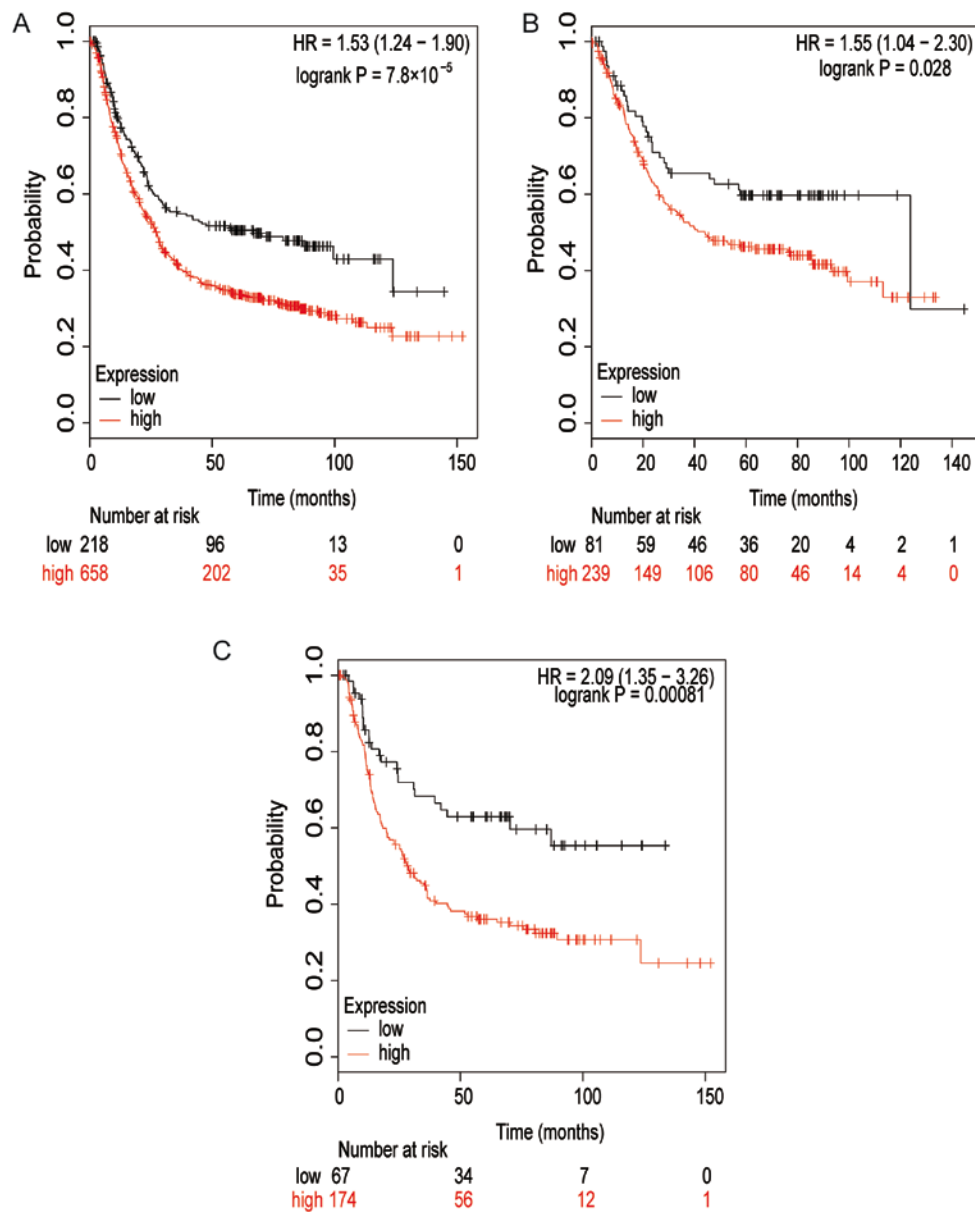


Figure 1. Determination of the prognostic value of TGF β 1 mRNA expression using the K-M plotter online tool. The desired Affymetrix ID is valid: 203084_at (TGF β 1). Survival curves representing overall survival times for (A) total patients with GC (n=876), (B) patients with intestinal type GC (n=320) and (C) patients with diffuse type GC (n=241). Patients were divided according to low- and high-TGF β 1 mRNA expression prior to survival curves being plotted. HR, hazard ratio; GC, gastric cancer; TGF β 1, transforming growth factor β 1.

one-way ANOVA followed by the Bonferroni's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Low expression of TGF β 1 and its receptors is associated with improved prognosis in patients with GC. The prognostic values of the mRNA expression of TGF β 1 and its receptors was determined using the online K-M plotter tool. The Affymetrix IDs of TGF β 1, TGF β R1, TGF β R2 and TGF β R3 are 203084_at, 206943_at, 207334_s_at and 204731_at respectively. Survival curves were generated for all patients with GC (n=876), patients with intestinal type GC (n=320) and patients with diffuse type GC (n=241). In 876 cases, only the above patients have clear pathological

classification information, therefore only these patient data were analyzed.

Firstly, the prognostic value of TGF β 1 mRNA expression was determined (Fig. 1). Low mRNA expression levels of TGF β 1 was associated with higher OS time and therefore, improved prognosis in patients with GC (HR, 1.53; 95% CI, 1.24-1.90; $P < 0.0001$; Fig. 1A). Low TGF β 1 mRNA expression was also observed to be associated with a higher OS time in patients with intestinal type GC (HR, 1.55; 95% CI, 1.04-2.30; $P = 0.028$; Fig. 1B), and patients with diffuse type GC (HR, 2.09; 95% CI, 1.35-3.26; $P = 0.00081$; Fig. 1C).

Next, the prognostic value of TGF β R1 mRNA expression was analyzed. Low TGF β R1 mRNA expression in patients with GC was associated with higher OS time (HR, 1.54; 95% CI, 1.30-1.83; $P < 0.0001$; Fig. 2A). Low TGF β R1 mRNA expression was also found to be associ-

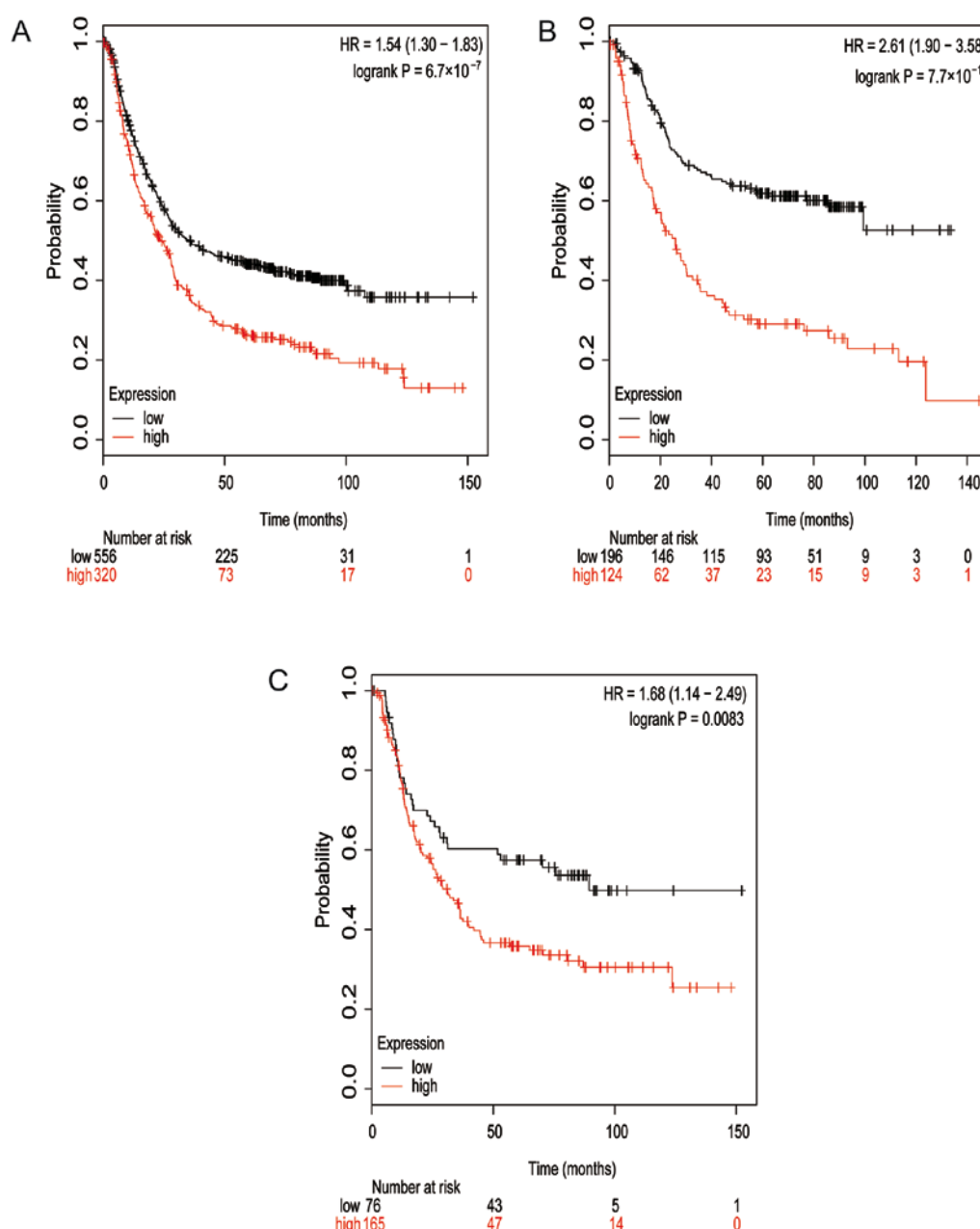


Figure 2. Determination of the prognostic value of TGF β 1 mRNA expression using the K-M plotter online tool. The desired Affymetrix ID is valid: 206943_at (TGF β 1). Survival curves representing overall survival times for (A) total patients with GC (n=876), (B) patients with intestinal type GC (n=320) and (C) patients with diffuse type GC (n=241). Patients were divided according to low- and high-TGF β 1 mRNA expression prior to survival curves being plotted. HR, hazard ratio; GC, gastric cancer; TGF β 1, transforming growth factor receptor β 1.

ated with higher OS time in patients with intestinal type GC (HR, 2.61; 95% CI, 1.90-3.58; $P < 0.0001$; Fig. 2B) and patients with diffuse type GC (HR, 1.68; 95% CI, 1.14-2.49; $P = 0.0083$; Fig. 2C).

The survival curves associated with TGF β 2 mRNA expression are represented in Fig. 3. Low expression levels of TGF β 2 mRNA were associated with an improved prognosis in patients with GC (HR, 1.25; 95% CI, 1.05-1.49; $P = 0.012$; Fig. 3A) and in patients with intestinal type GC (HR=1.82; 95% CI, 1.32-2.50; $P = 0.012$; Fig. 3B). TGF β 2 was also associated with a modest improvement in the prognosis of patients with diffuse type GC; however, this increase was not statistically significant (HR, 1.33; 95% CI, 0.94-1.89; $P = 0.11$; Fig. 3C).

The survival curves of TGF β 3 mRNA expression for all patient groups investigated are represented in Fig. 4. Low mRNA expression level of TGF β 3 was associated with improved prognosis in patients with GC (HR, 1.22; 95% CI, 1.03-1.45; $P = 0.021$; Fig. 4A). Low mRNA expression of TGF β 3 was also associated with improved prognosis in patients with diffuse type GC (HR, 2.14; 95% CI, 1.52-3.01; $P < 0.0001$; Fig. 4C). TGF β 3 was also associated with a modest improvement in the prognosis of patients with intestinal type GC; however, this increase was not statistically significant (HR, 1.33; 95% CI, 0.92-1.91; $P = 0.13$; Fig. 4B). According to the results of the present study, low mRNA expression levels of TGF β 1, TGF β 1, TGF β 2 and TGF β 3 were all associated with a higher OS time in patients with GC.

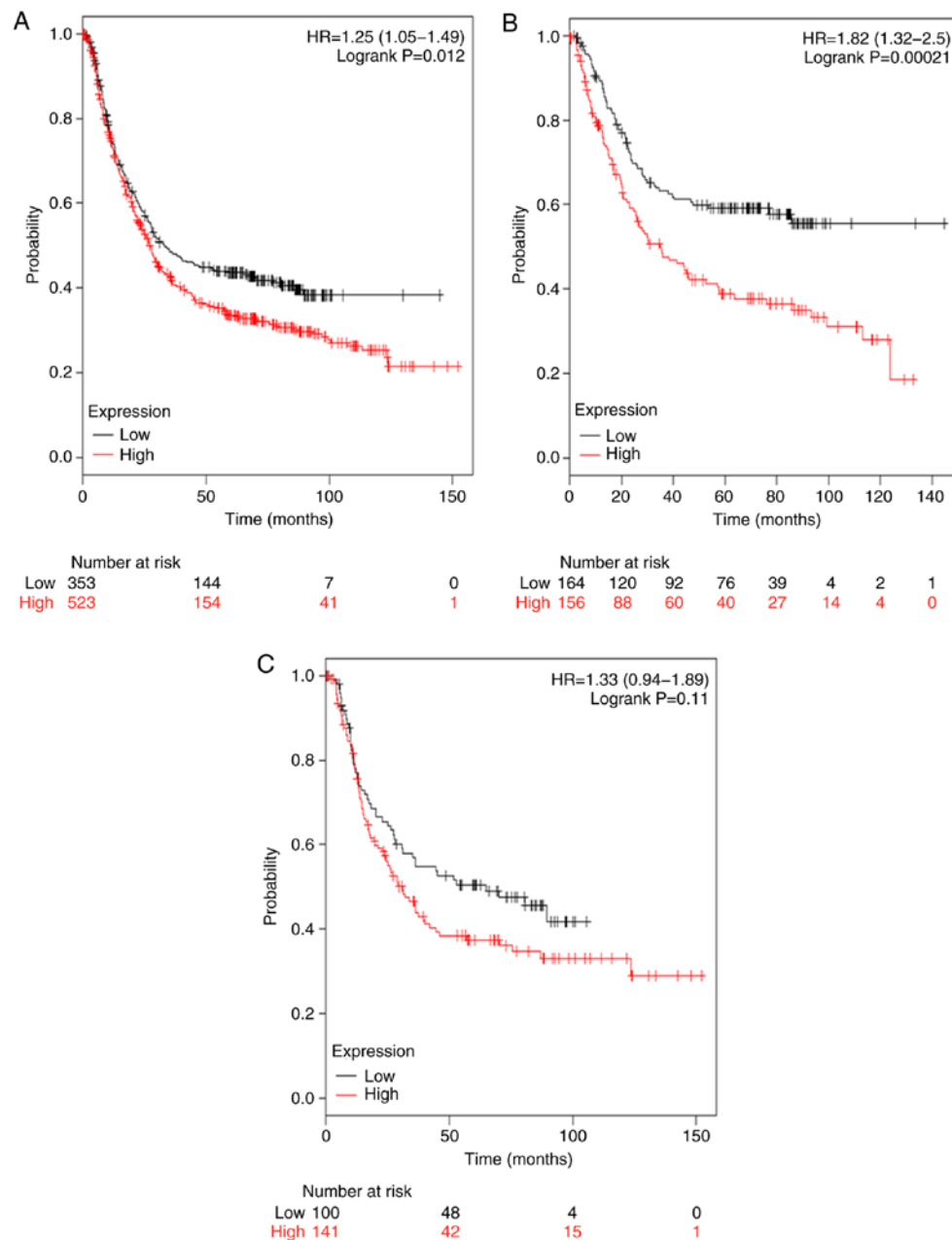


Figure 3. Determination of the prognostic value of TGFβ1 mRNA expression using the K-M plotter online tool. The desired Affymetrix IDs is valid: 207334_s_at (TGFβ2). Survival curves representing overall survival times for (A) total patients with GC (n=876), (B) patients with intestinal type GC (n=320) and (C) patients with diffuse type GC (n=241). Patients were divided according to low- and high-TGFβ1 mRNA expression prior to survival curves being plotted. HR, hazard ratio; GC, gastric cancer; TGFβ2, transforming growth factor receptor β2.

Furthermore, the association between TGFβ signaling and prognosis in patients with GC with different clinicopathological features, including clinical stages (Table I), HER2 status (Table II), pathological grades (Table III) and different treatment methods (Table IV) was analyzed. As presented in Table I, low TGFβ1 mRNA expression was associated with an improved prognosis at clinical stages 2 of GC (HR, 2.61; 95% CI, 1.16–5.86; P=0.016). Low mRNA expression of TGFβ1 was associated with a better prognosis at clinical stages 2 (HR, 3.39; 95% CI, 1.86–6.61; P<0.0001; Table I) and 3 (HR, 1.9; 95% CI, 1.42–2.55; P<0.0001; Table I) in patients with GC. Low mRNA expression of TGFβ2 was also associated with a more favorable prognosis at clinical stages 1 (HR, 9.1; 95% CI, 1.19–69.50; P=0.0099; Table I), 2 (HR,

2.32; 95% CI, 1.27–4.25; P= 0.0051; Table I) and 4 (HR, 1.76; 95% CI, 1.13–2.67; P= 0.012; Table I). Low mRNA expression of TGFβ3 was also found to be associated with better prognosis in clinical stages 2 (HR, 2.79; 95% CI, 1.53–5.08; P=0.00048; Table I), 3 (HR, 1.36; 95% CI, 1.02–1.8; P=0.035; Table I) and 4 (HR, 2; 95% CI, 1.33–3; P=0.00063; Table I) patients with GC.

Low mRNA expression levels of TGFβ1 (HR, 1.66; 95% CI, 1.27–2.15; P=0.00014; Table II) and TGFβ2 (HR, 1.33; 95% CI, 1.05–1.67; P=0.016; Table II) were associated with a better prognosis in HER2⁺ patients with GC. Low mRNA expression of TGFβ1 [(HER2⁺: HR, 1.67; 95% CI, 1.33–2.09; P<0.0001; Table II) (HER2⁺: HR, 1.48; 95% CI, 1.14–1.92; P=0.0028; Table II)] and TGFβ3 [(HER2⁺: HR,

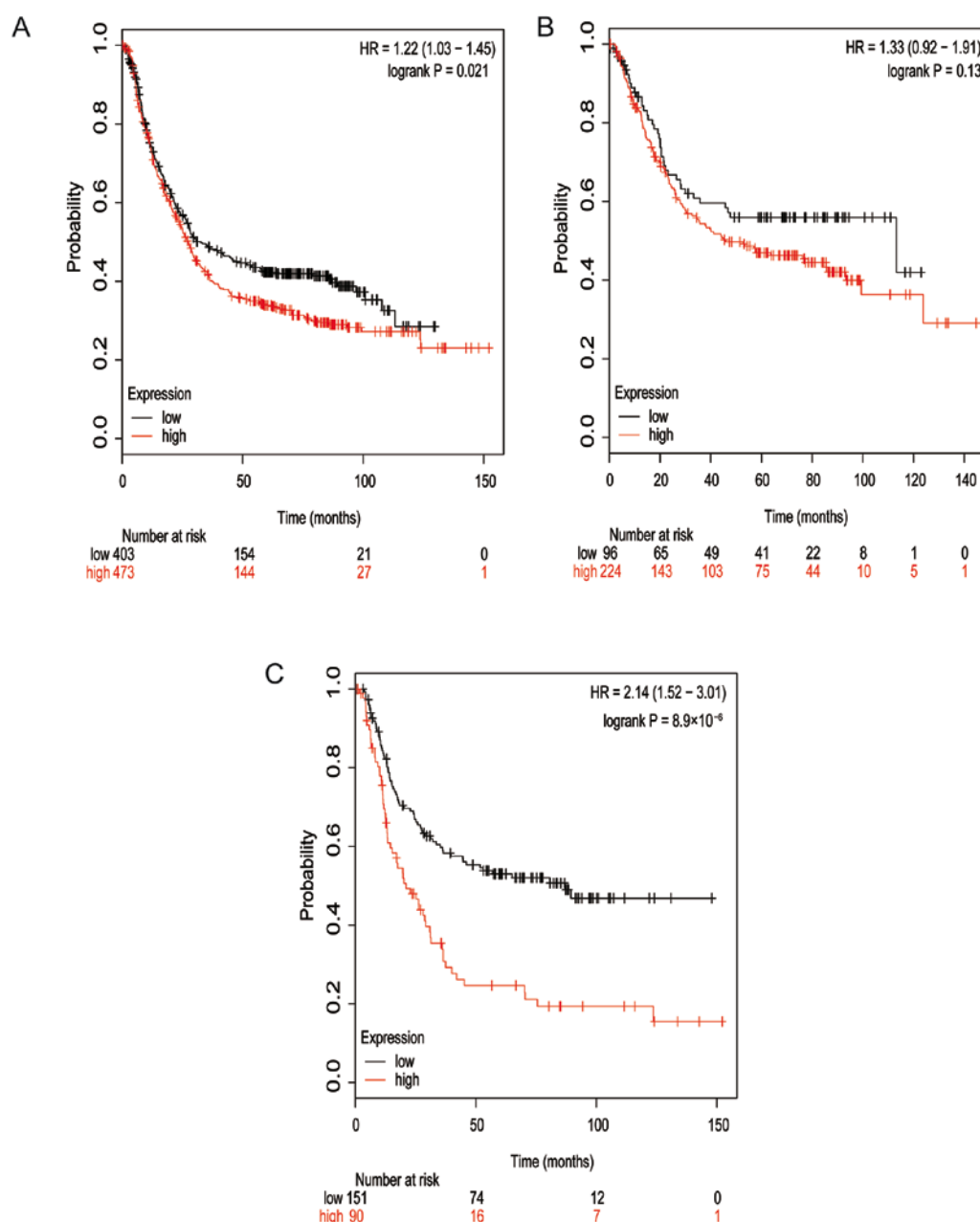


Figure 4. Determination of the prognostic value of TGF β 1 mRNA expression using the K-M plotter online tool. The desired Affymetrix IDs is valid: 204731_at (TGF β R3). Survival curves representing overall survival time for (A) total patients with GC (n=876), (B) patients with intestinal type GC (n=320) and (C) patients with diffuse type GC (n=241). Patients were divided according to low- and high-TGF β 1 mRNA expression prior to survival curves being plotted. HR, hazard ratio; GC, gastric cancer; TGF β R3, transforming growth factor receptor β 3.

1.48; 95% CI, 1.16-1.88; P=0.0013; Table II) (HER2⁺: HR, 1.32; 95% CI, 1.01-1.72; P=0.041, Table II)] were associated with a better prognosis in both HER2⁺ and HER2⁻ patients with GC.

Low mRNA expression of TGF β 1 (HR, 0.60; 95% CI, 0.37-0.99; P=0.042; Table III) and TGF β R2 (HR, 0.57; 95% CI, 0.37-0.89; P=0.012; Table III) was associated with higher OS time in grade I patients with GC. Additionally, TGF β R1 low mRNA expression was associated with higher OS time in grade II patients with GC (HR, 2.62; 95% CI, 1.32-5.20; P=0.0041; Table III). Low expression of TGF β R3 was associated with higher OS time in pathological grades II (HR, 0.29; 95% CI, 0.08-1.03; P=0.043; Table III) and III (HR, 4.48; 95% CI, 1.04-19.34; P=0.028; Table III) patients with GC.

Finally, as represented in Table IV, low mRNA expression of TGF β 1 was associated with higher OS times in patients with GC who had been treated with surgery alone (HR, 2.19; 95% CI, 1.47-3.25; P<0.0001). Concurrently, low mRNA expression levels of TGF β R1 were associated with higher OS times in patients with GC with the same method of treatment (HR, 1.53; 95% CI, 1.14-2.04; P=0.004; Table IV) and patients with GC who had fluorouracil (5-FU)-based adjuvant treatment (HR, 0.66; 95% CI, 0.47-0.94; P=0.02; Table IV). Low mRNA expression of TGF β R2 was associated with higher OS times in patients with GC who had received surgery alone (HR, 1.37; 95% CI, 1.03-1.84; P=0.031; Table IV) and patients with GC who had received 5-FU-based adjuvant treatment (HR, 0.42; 95% CI, 0.29-0.61; P<0.0001; Table IV). Low mRNA

Table I. Association between mRNA expression of TGFβ1 and its receptors and clinical stage in patients with gastric cancer.

Gene	Clinical stages	Cases, n	HR	95% CI	P-value
TGFβ1	1	69	3.92	0.89-17.33	0.052
	2	145	2.61	1.16-5.86	0.016 ^a
	3	319	0.77	0.58-1.03	0.074
	4	152	0.84	0.57-1.24	0.380
TGFβR1	1	69	1.95	0.61-6.19	0.250
	2	145	3.39	1.86-6.61	<0.001 ^c
	3	319	1.90	1.42-2.55	<0.001 ^c
	4	152	1.39	0.94-2.07	0.100
TGFβR2	1	69	9.10	1.19-69.51	0.010 ^a
	2	145	2.32	1.27-4.25	0.005 ^b
	3	319	1.28	0.96-1.71	0.090
	4	152	1.76	1.13-2.67	0.012 ^a
TGFβR3	1	69	1.63	0.60-4.41	0.330
	2	145	2.79	1.53-5.08	<0.001 ^c
	3	319	1.36	1.02-1.80	0.035 ^a
	4	152	2.00	1.33-3.00	<0.001 ^c

^aP<0.05, ^bP<0.01, ^cP<0.001. HR, hazard ratio; CI, confidence interval; TGF, transforming growth factor; TGFβR1, transforming growth factor receptor β1; TGFβR2, transforming growth factor receptor β2; TGFβR3, transforming growth factor receptor β3.

Table II. Association between mRNA expression of TGFβ1 and its receptors and HER 2 status of patients with gastric cancer.

Gene	HER status	Cases, n	HR	95% CI	P-value
TGFβ1	-	532	1.66	1.27-2.15	<0.001 ^c
	+	344	1.26	0.96-1.65	0.090
TGFβR1	-	532	1.67	1.33-2.09	<0.001 ^c
	+	344	1.48	1.14-1.92	0.003 ^b
TGFβR2	-	532	1.33	1.05-1.67	0.016 ^a
	+	344	0.76	0.57-1.03	0.072
TGFβR3	-	532	1.48	1.16-1.88	0.001
	+	344	1.32	1.01-1.72	0.041 ^a

^aP<0.05, ^bP<0.01, ^cP<0.001. HR, hazard ratio; CI, confidence interval; TGF, transforming growth factor; TGFβR1, transforming growth factor receptor β1; TGFβR2, transforming growth factor receptor β2; TGFβR3, transforming growth factor receptor β3.

expression of TGFβR3 was associated with higher OS times in patients with GC who were surgically treated (HR, 1.52; 95% CI, 1.12-2.07; P=0.0075; Table IV) and patients with GC who were treated with a 5-FU-based adjuvant (HR, 0.60; 95% CI 0.40-0.89; P=0.01; Table IV). The low expression levels of TGFβ1 (HR, 2.72; 95% CI, 1.12-6.58; P=0.021) and TGFβR3 (HR, 3.27; 95% CI, 1.26-8.52; P=0.01) (Table IV) were associated with higher OS times in patients receiving other adjuvant treatments.

Knockdown of TGFβ1 and its receptors inhibits the proliferation of human GC cells. Since a high mRNA expression level of TGFβ1 and its receptors is predictive of a poor prognosis in patients with GC, their direct effects on GC cells were subsequently investigated. In order to evaluate the role of

TGFβ1 and its receptors in AGS and MKN45 cells, specific siRNAs were transfected into cells and expression was quantified by RT-qPCR and western blotting. As presented in Fig. 5, the expression of TGFβ1 and its receptors was significantly suppressed in transfected GC cells. The proliferation of AGS and MKN45 cells was then determined using a CCK8 assay. Based on these results, it was determined that the knockdown of TGFβ1 and its receptors (with the exception of TGFβR3) inhibited the proliferation of GC cells (Fig. 6A and B).

Knockdown of TGFβ1 and its receptors inhibits the migration and invasion of human GC cells. Next, transwell assays were performed to explore the effects of TGFβ1 and its receptors on the migration and invasion of GC cells (Fig. 6C). With the exception of TGFβR3, TGFβ1 and

Table III. Association between mRNA expression of TGF β 1 and its receptors and pathological grades of patients with gastric cancer.

Gene	Pathological grades	Cases, n	HR	95% CI	P-value
TGF β 1	I	166	0.60	0.37-0.99	0.042 ^a
	II	67	0.57	0.30-1.09	0.087
	III	32	0.74	0.30-1.78	0.500
TGF β R1	I	166	1.52	0.93-2.50	0.094
	II	67	2.62	1.32-5.20	0.004 ^b
	III	32	2.23	0.68-7.90	0.170
TGF β R2	I	166	0.57	0.37-0.89	0.012 ^a
	II	67	2.16	0.90-5.19	0.078
	III	32	0.67	0.27-1.63	0.370
TGF β R3	I	166	1.20	0.79-1.83	0.390
	II	67	0.29	0.08-1.03	0.043 ^a
	III	32	4.48	1.04-19.34	0.028 ^a

^aP<0.05, ^bP<0.01, ^cP<0.001. HR, hazard ratio; CI, confidence interval; TGF, transforming growth factor; TGF β R1, transforming growth factor receptor β 1; TGF β R2, transforming growth factor receptor β 2; TGF β R3, transforming growth factor receptor β 3.

Table IV. Association between mRNA expression of TGF β 1 and its receptors and different treatment methods of patients with gastric cancer.

Gene	Treatment:	Cases, n	HR	95% CI	P-value
TGF β 1	Surgery only	393	2.19	1.47-3.25	<0.001 ^c
	5 FU based adjuvant	158	0.84	0.58-1.22	<0.001 ^c
	Other adjuvant	80	2.72	1.12-6.58	0.021 ^a
TGF β R1	Surgery only	393	1.53	1.14-2.04	0.004 ^b
	5 FU based adjuvant	158	0.66	0.47-0.94	0.020 ^a
	Other adjuvant	80	1.69	0.70-4.09	0.240
TGF β R2	Surgery only	393	1.37	1.03-1.84	0.031 ^a
	5 FU based adjuvant	158	0.42	0.29-0.61	<0.001 ^c
	Other adjuvant	80	1.66	0.69-4.00	0.260
TGF β R3	Surgery only	393	1.52	1.12-2.07	0.008 ^b
	5 FU based adjuvant	158	0.60	0.40-0.89	0.010 ^a
	Other adjuvant	80	3.27	1.26-8.52	0.010 ^a

^aP<0.05, ^bP<0.01, ^cP<0.001. HR, hazard ratio; CI, confidence interval; TGF β R1, transforming growth factor receptor β 1; TGF β R2, transforming growth factor receptor β 2; TGF β R3, transforming growth factor receptor β 3.

its receptors significantly inhibited the migration of AGS and MKN45 cells (Fig. 6C-E). Moreover, the results of the transwell assay for cell invasion demonstrated that except for TGF β 1, knockdown of TGF β 1 and its receptors suppressed cell invasiveness (Fig. 6C-E). Cumulatively, the data confirm that knockdown of TGF β 1, TGF β R1 and TGF β R2 inhibit the progression of human GC.

Discussion

The TGF β superfamily is a large class of cytokines that perform various biological activities. This superfamily is mainly comprised of TGF β , activin and bone morphogenetic

protein. These molecules are important in the regulation of cell growth, adhesion, migration, differentiation and apoptosis. In mammals, three subtypes of TGF β have been discovered: TGF β 1; TGF β 2; and TGF β 3 (31). TGF β 1 is the most commonly expressed form of TGF β in human tissues, and plays an important role in the regulation of cell growth, apoptosis, differentiation and the maintenance of normal immune homeostasis (32-34). TGF β signaling is a double-edged sword in the process of tumor formation and development (35). In tumor formation, the TGF β signaling pathway regulates downstream target genes, such as p21 cyclin dependent kinase (CDKN)1A and p15CDKN2B, to arrest cells in the G₁ phase of the cell cycle, and inhibit the proliferation of tumor cells (35). In

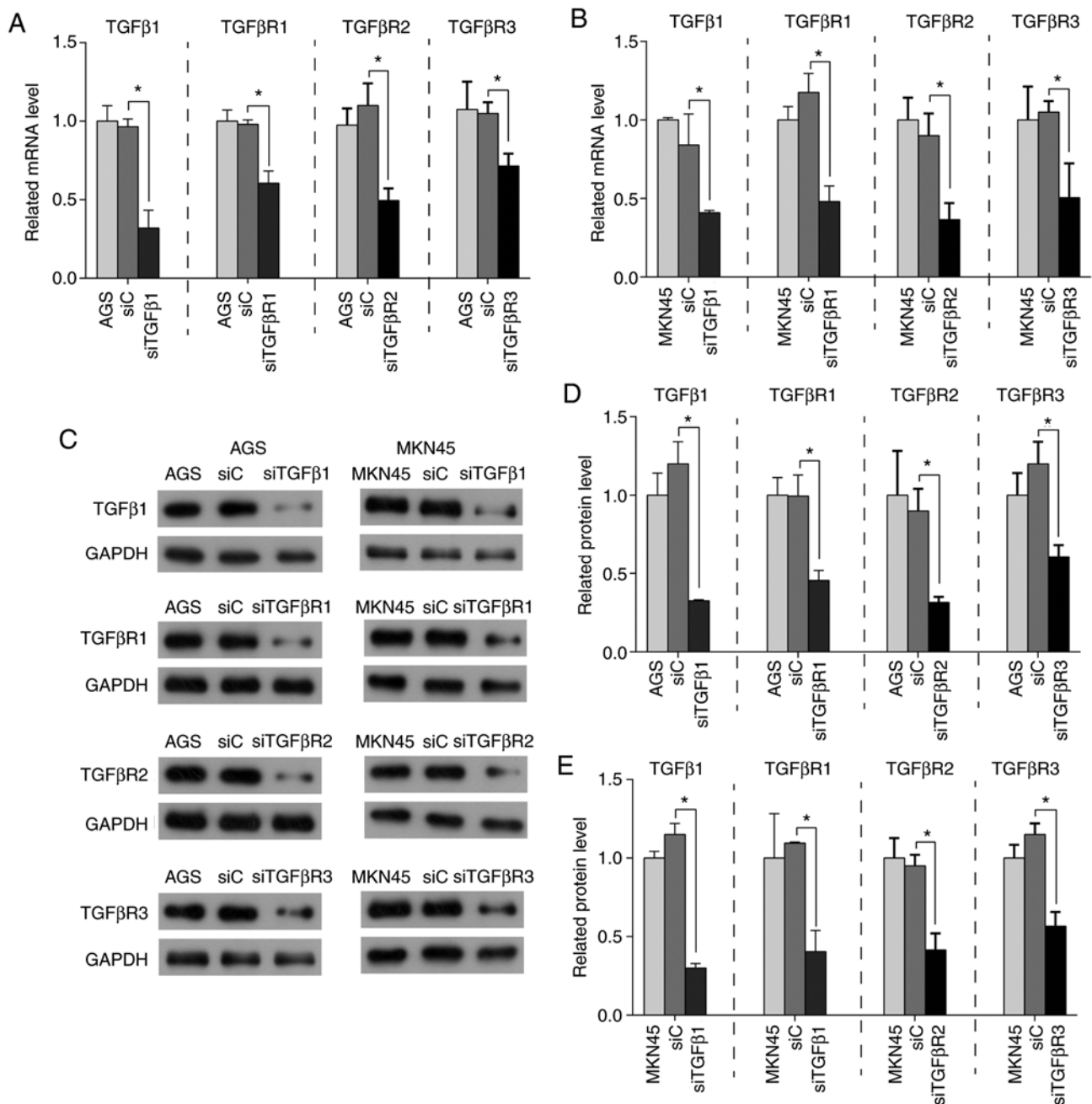


Figure 5. Inhibition of the expression of TGFβ1 and its receptors by specific siRNAs. mRNA levels of TGFβ1 and its receptors were detected using reverse transcription-quantitative PCR in (A) AGS and (B) MKN45 cells. (C) Protein expression level of TGFβ1 and its receptors was detected by western blot. Protein levels of TGFβ1 and its receptors in (D) AGS and (E) MKN45 cells as analyzed by ImageJ software. *P<0.05. TGFβ1, transforming growth factor β1; TGFβR1, transforming growth factor receptor β1; TGFβR2, transforming growth factor receptor β2; TGFβR3, transforming growth factor receptor β3.

tumor progression, TGFβ can promote invasion and metastasis through a variety of mechanisms, including immune suppression or escape, angiogenesis and by increasing the interaction between tumor cells and the extracellular matrix (35).

In previous years, numerous studies have demonstrated that TGFβ1 is associated with tumor occurrence and development, and is highly expressed in a variety of malignant tumor types, including prostate, breast gastric and colorectal cancer (36,37). Docea *et al* (38) noticed that the highest level of TGFβ was exhibited in GC compared with normal tissue and the expression of TGFβ progressively increased in the epithelium-intestinal metaplasia-dysplasia-carcinoma sequence. In intestinal variants, TGFβ immunoreactivity was significantly associated

with the degree of tumor differentiation and proliferative activity (38). According to another report, TGFβ1 mRNA levels were higher in tumor cells and were positively associated with Smad2 and Smad7 mRNA levels (39). Serum TGFβ1 levels have been demonstrated to be significantly higher in patients at both early and advanced cancer stages, compared with controls (39). TGFβ1 is closely linked to the initiation of the epithelial-mesenchymal transition (EMT) in the development and progression of carcinomas (40,41). In GC cells, TGFβ1 can induce the mRNA and protein expression of Krüppel-like factor 8 expression (42). It can also contribute to EMT via the downregulation of E-cadherin, and the upregulation of vimentin expression (43-45). TGFβ1 can interact with a variety

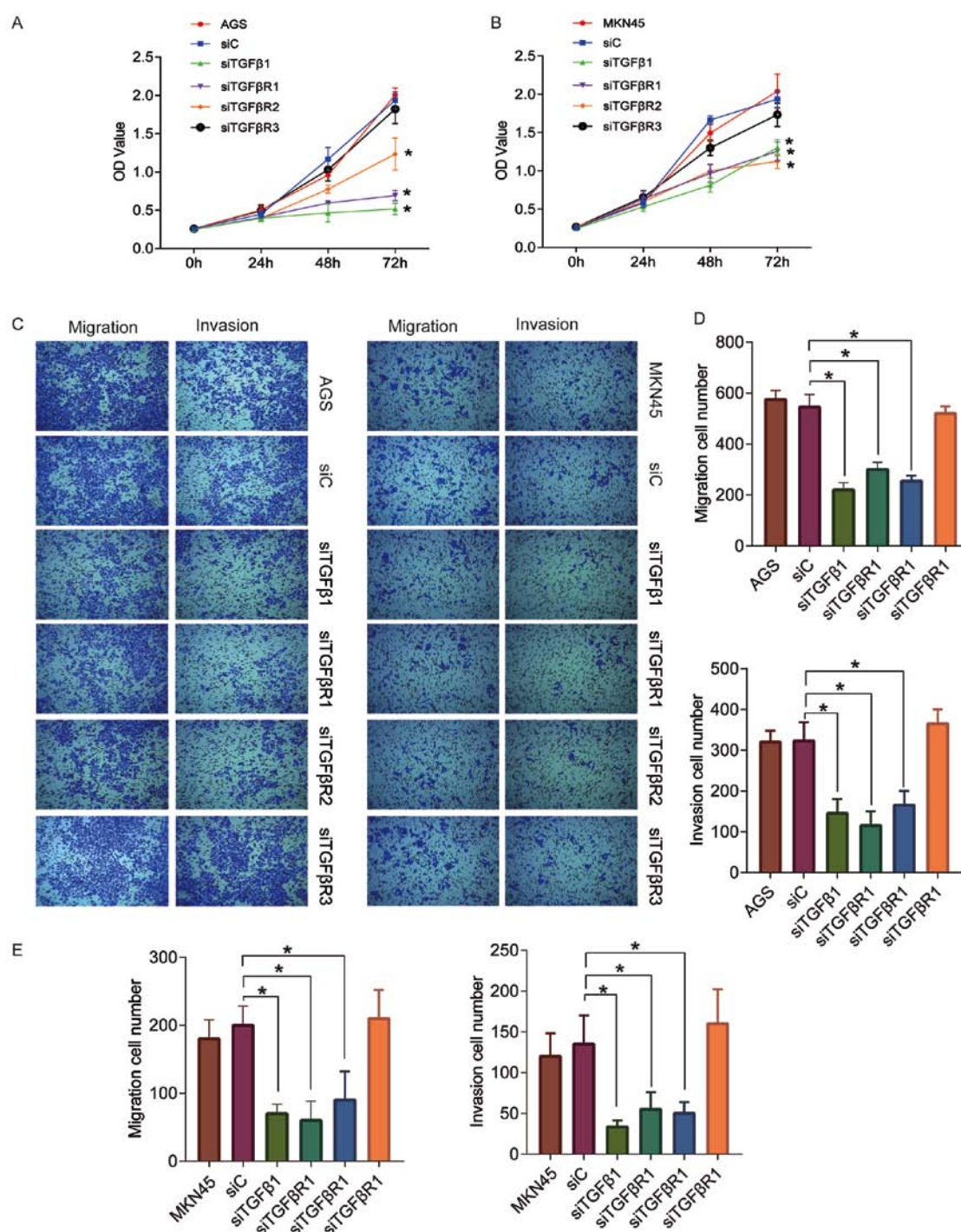


Figure 6. Inhibition of proliferation and induction of apoptosis by TGF β 1 and its receptors in human gastric cancer cell lines. The Cell Counting Kit-8 assay was performed to detect the proliferation of (A) AGS and (B) MKN45 cells. (C) Transwell assays were performed to analyze the migration and invasion of AGS and MKN45 cells. Column diagram showed the statistical analysis of migration and invasion cell number of (D) AGS and (E) MKN45 cells. * $P < 0.05$. siC, small interfering control; si, small interfering RNA; TGF β R1, transforming growth factor receptor β 1; TGF β R2, transforming growth factor receptor β 2; TGF β R3, transforming growth factor receptor β 3.

of tumor-related genes and proteins in TGF β -induced EMT in GC, such as SAM-domain and SH3-domain containing 1, microRNA-21 and Grainy head like 2 (43-45). In the present study, low mRNA expression of TGF β 1 was associated with an improved prognosis in patients with GC, including the intestinal and diffuse subtypes of GC. In addition, TGF β 1 can be associated with patient prognosis in GC, based on certain clinical features, including HER2 status, pathological grade I and different treatment methods. These results suggest that

TGF β 1 has potential as a new prognostic indicator of GC, including the intestinal and diffuse types.

The TGF β R includes three subtypes: TGF β R1, TGF β R2 and TGF β R3. TGF β R1 and 2 are categorized as type I transmembrane glycoproteins with serine/threonine kinase activity and collectively participate in the TGF β /Smad signaling pathway. Initially, TGF β binds to TGF β R2, and then activates TGF β R3 through phosphorylation. Together they form the TGF β R1-TGF β 1-TGF β R2 heterotetramer for transduction of

cell signaling. TGF β R3 can enhance the binding of ligand to TGF β R1 and 2, functioning as an accessory receptor (14). Wild-type TGF β R2 expression in GC cell lines can result in reduced proliferation compared with control cells (46). A case-control study was performed to evaluate the possible association of polymorphisms in TGF- β receptors with susceptibility to developing GC (47).

Polymorphisms of TGF β R1 and 2 may be associated with the risk of GC in the population of North China (48,49). However, TGF β R3 has not yet been studied in the context of GC. In the present study, it was revealed that low mRNA expression of TGF β R1, TGF β R2 and TGF β R3 was associated with a more favorable prognosis in patients with GC. While TGF β R2 was associated with OS time in patients with intestinal type GC, this was not observed for patients with diffuse type GC. Pak *et al* (50) determined that the expression of T β R2 was higher in patients with intestinal type GC compared with those with diffuse type GC. In addition, TGF β R was also associated with prognosis based on different clinical features in the aforementioned study (50). While TGF β 1 blockade has been proposed as an anti-cancer therapy, it is imperative to understand the best method of administration, and which specific pathological features will be most improved by this therapy (51).

Finally, the present study investigated the specific roles of TGF β 1 and its receptors on GC cells. The results of the present study demonstrated that knockdown of TGF β 1, TGF β R1 and TGF β R2 could significantly suppress the proliferation, migration and invasion of human GC cells. These results are consistent with previous studies (48,52-54). While TGF β R2 has been widely studied in different types of cancer (54,55), studies investigating TGF β R1 and TGF β R3 are limited. The current study confirms the inhibitory effect of TGF β R1 on the proliferation of GC cells, suggesting the involvement of TGF β 1 and TGF β R2, but also TGF β R1 in the progression of GC. However, the downstream targets and regulatory mechanism of TGF β R1 remain unclear, and cells cultured *in vitro* cannot precisely simulate the tumor microenvironment. The results of the present study need to be verified by further *in vivo* experiments.

In conclusion, the present study showed that TGF β 1 and its receptors were all associated with the prognosis of patients with GC. Consistently, low mRNA expression levels of TGF β 1 and TGF β R indicated a better OS time. Furthermore, knockdown of TGF β 1, TGF β R1 and TGF β R2 inhibited cell proliferation in GC. This suggests that TGF β 1 and TGF β R play important roles in the development of GC and may be investigated as therapeutic targets. These findings provide novel insights and approaches for the treatment of GC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FL and HW mainly performed the experiments and analyzed the data. FL performed the online analysis and wrote the paper. MZ carried out the experiment design and manuscript drafting. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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