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ORIGINAL RESEARCH

TGIF1 Knockdown Inhibits the Proliferation and Invasion of Gastric Cancer via AKT Signaling Pathway

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Introduction: Gastric cancer is a kind of cancer with high mortality. TGIF1, as a transcription inhibitor, can inhibit the transcription of specific genes. The purpose of this study was to investigate the role of TGIF1 in gastric cancer by knocking down TGIF1.

Methods: The expression of TGIF1 was detected by qPCR and Western blotting; CCK8 assay, colony formation assay, transwell, and wound-healing assay were used to evaluate the proliferation, migration, and invasion of gastric cancer cells; cell apoptosis was analyzed by flow cytometry and Hoechst-PI double staining; cell cycle was detected by flow cytometry. Gelatinase experiment was performed to detect the expression level of MMP-2; apoptosis related proteins and AKT singling pathway were assessed by Western blotting.

Results: Knockdown of TGIF1 inhibited the proliferation, migration, and invasion of gastric cancer cells and promoted apoptosis. TGIF1 knockdown down-regulated the expression levels of MMP-2, Bcl2, CyclinD1, and p-Akt, and up-regulated the expression levels of Bax and Caspase3. These data suggested that knockdown of TGIF1 inhibited the development of gastric cancer via AKT signaling pathway.

Conclusion: TGIF1 knockdown inhibited the proliferation, migration, and invasion and promoted apoptosis of gastric cancer cells via the AKT signaling pathway, suggesting that TGIF1 is considered a potential inhibitor in gastric cancer.

Keywords: TGIF1, gastric cancer, AKT pathway, apoptosis, proliferation

Introduction

Gastric cancer ranks fifth in the global incidence of malignant tumors and third in the mortality of malignant tumors.[1] At present, the treatment of gastric cancer mainly comprises surgery assisted radiotherapy and chemotherapy, but the treatment effect is not ideal. Moreover, due to the high recurrence and metastasis rate of gastric cancer, about 40% – 70% of patients have tumor recurrence,[2] and the 5-year survival rate is less than 30%.[3]

The occurrence and development of gastric cancer is a complex process, involving the inactivation of many tumor suppressor genes or the activation of cancer genes. It is of great significance to study the pathogenesis and mechanism of gastric cancer, for its diagnosis and treatment.[4]

TG-interacting factor 1 (TGIF1) is a highly conserved transcription factor in different types of vertebrates, which plays an important role in regulating fetal head and face, brain development, and differentiation of various cells.[5–8] TGIF1 is expressed in hematopoietic stem cells and participates in the regulation of many

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physiological processes. There has been a lot of research on its mechanism. Early studies have shown that TGIF1 protein is a transcriptional inhibitor, which can inhibit the transcription of specific genes.[9,10] The abnormal functioning of TGIF1 is related to many diseases. Several studies have proven that TGIF1 is closely related to the human genetic disease HPE which occurs in the fetal development stage.[11,12] On the other hand, the change of expression level and functional activity of TGIF1 is related to the occurrence and development of various cancers such as urothelial cancer,[7,13] liver cancer,[14] and lung cancer.[15] In addition, the expression level of TGIF1 is also significantly increased in lung cancer cells, indicating that TGIF1 may be related to the occurrence or development of lung cancer.[15] However, the effect and mechanism of TGIF1 on gastric cancer still remain unclear. In this study, the expression of TGIF1 gene was silenced by RNA interference to explore its effect and mechanism on the proliferation and apoptosis of gastric cancer cells, in order to provide a theoretical basis for the diagnosis and treatment of the disease.

Materials and Methods

Agents

Primary antibodies, including anti-TGIF1 (Cat # ab52955), Bcl-2 (Cat # ab32124), Active-Caspase3 (Cat # ab32042), Bax (Cat # ab182733), AKT (Cat # ab8805), p-AKT (Cat # ab38449), Cyclin D1 (Cat # ab134175), and anti-tubulin (Cat # ab210797) were purchased from Abcam (Cambridge, UK). HRP sheep anti-rabbit/mouse secondary antibodies were obtained from PTG Company.

Cell Culture and Transfection

Human gastric cancer cell lines AGS and MKN45 were obtained from the cell bank of Shanghai Chinese Academy of Sciences and cultured in DMEM medium (HyClone, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a 37°C humidified incubator containing 5% CO_2. The culture medium was changed 24 hours later, and then changed every 2–3 days according to the growth state of cells. The cells were transfected with TGIF1-specific siRNA (si-TGIF1) according to the instructions of Lipofectamine 2000 transfection kit (Invitrogen; Thermo Fisher Scientific, Inc.) at logarithmic stage. Non-specific siRNA was used as the control. The interfere sequences used were the following:

TGIF1-siNC: 5′-UUCUCCGAACGUGUCACGUTT-3′

TGIF1-siRNA1: 5′-GGCUGUAUGAGCACCGUUATT-3′

TGIF1-siRNA2: 5′-GCGUUGCUGUCCCAGCAAATT-3′

TGIF1-siRNA3: 5′-AGUGGAUGUUGCACUCAAATT-3′

TGIF1-siRNA4: 5′-CAGUGGAUUUCAGCUUCUATT-3′

Fluorescence Quantitative PCR

The total RNA of cells was extracted by RNA extraction kit. After reverse transcription, some of them were used for downstream experiments, and the rest were frozen in a refrigerator at - 80°C. SYBR method was used to detect the mRNA level of each sample and the data were analyzed by $2^{-\Delta CT}$ method. The primers (GENEWIZ, Inc) were as follows:

TGIF1-qPCR-up: 5′-TGAGCACCGTTACAATGCCT-3′

TGIF1-qPCR-down: 5′-GAAGTCCTGGTTGAGGTCCG-3′

human-β-actin-up: 5′-CCCGAGCCGTGTTTCCT-3′

human-β-actin-down: 5′-GTCCCAGTTGGTGACGATGC-3′

Cell Proliferation and Growth Ability Detection

Cell Counting Kit-8 (CCK8) assay was performed to detect cell proliferation. When the fusion rate reached 90%, cells were washed with PBS, digested with trypsin, and the cell suspension was prepared and counted. About 1000 cells were seeded into each well of a 96-well-plate. The cell viability was detected every 24 hours. Before detection, 10 μL CCK8 reagent was added to each hole and incubated in a 37°C incubator for 1.5 hours. The OD value was detected with 450 nm excitation light by enzyme labeling instrument and the proliferation curve was drawn.

Flat plate clone formation test was used to evaluate the ability of cell cloning. The cells of each group in logarithmic growth period were digested with trypsin, and 500 cells were inoculated into a 35 mm cell culture dish for 2–3 weeks. When there were visible clones in the dish, the culture was terminated and fixed with 4% POM for 20 minutes, stained with crystal violet for 30 minutes, then counted under a microscope. Finally, the clone formation rate was calculated.

Clone formation rate = (number of clones/number of inoculated cells) × 100%.

Cell Invasion and Migration Ability Detection

Transwell assay was used to assess the invasion and migration ability of AGS and MKN45 cells. For invasion assay, 100 μL prepared Matrigel (1:6 diluted by serum-free medium) was added to the upper chamber of transwell chamber (Millipore, Billerica, MA, USA), placed in a carbon dioxide incubator for 4–6 hours at 37°C to form the glue, then 500 μL of serum-free culture solution was added and it was left for half an hour to hydrate the basement membrane. 100 μL cell suspension (1×10^5 cells) was added to the upper chamber and 500 μL complete culture medium was added to the lower chamber. After being left overnight, the cells were moved from the upper chamber to lower chamber, and then fixed with 4% paraformaldehyde for 30 minutes after PBS cleaning, stained with 0.1% crystal violet for 20 minutes, observed and counted under the microscope. The procedure of migration experiment is similar to that of invasion experiment. Except the transwell chamber did not need to be treated with glue, and the cell number was 5000.

The migration ability was also detected by wound-healing assay. When the cells were 95% fused, a 10 μL pipette tip was used to make an artificial wound, and they were cultured with serum-free medium in a 5% CO_2 incubator at 37°C. The micrographs were taken at 0, 24, and 48 hours respectively and the migration area of the cells was analyzed to evaluate the migration ability of AGS and MKN45 cells.

Gelatinase Experiment

After 24 hours of transfection, the cells were washed with serum free medium and cultured in serum free medium. After 24 hours, the supernatant was collected and centrifuged to remove the cells. The supernatant of the diluted buffer solution without mercapto ethanol was added to each hole of the vertical electrophoresis bath and the samples were separated by SDS-page (0.5 mg/mL gelatin). Image scanner (Amersham) and Image Quant TL v2003 software were used to analyze the activity of MMPs.

Cell Apoptosis Detection

Cell apoptosis rate was analyzed with flow cytometry. After 24 hours of transfection, the cells were resuspended with 1 × buffer and the cell density was adjusted to 1–5 × 10^6 cells/mL. 100 μL cell suspension and 5 μL annexin V/FITC were added to 5 mL flow tube, then incubated at room temperature in the dark for 5 min, followed by adding 10 μL 20 μg/mL of double staining PI. Cell samples were analyzed using flow cytometric analysis and Flow Jo software.

Hoechst-PI fluorescence double staining experiment was performed to detect cell apoptosis. After 24 hours of transfection, Hoechst 33342 and PI staining solution were added to the culture solution. The fluorescence was observed by different excitation of fluorescence microscope. Hoechst staining was observed by blue light excited by ultraviolet light, and PI staining was observed by red light excited by green light.

Western Blot

After 24 hours of transfection, the protein was extracted with RIPA lysate. 20 μL protein samples were added into each pore of the vertical electrophoresis tank and separated by SDS-PAGE. Then, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skim milk for 1 hour, the PVDF membrane was probed with primary antibodies (1:1,000) at 4°C overnight and secondary (1:5,000) antibodies at room temperature for 1 hour. Then, immunoblotting was performed with ECL.

Cell Cycle Analysis

Flow cytometry was used to analyze cell cycle. After 24 hours of transfection, 70% ethanol was added to fix the cells at 4°C overnight. 500 μL PI was added and maintained in the dark for 30 min. Flowjo software was used to analyze the flow results.

Statistical Analyses

SPSS 18.0 software was used for statistical analysis. The gray values of Western blot positive bands were measured by Image J analysis software, and the internal reference bands were used as protein quantitative reference. Compared with *t*-test, $P < 0.05$ was statistically significant.

Results

Knockdown of TGIF1 Inhibits the Growth Capacity of Gastric Cancer Cells

To study the role of TGIF1 in gastric cancer, AGS and MKN45 cell lines were selected. The expression level of

TGIF1 was detected by qPCR. The results were as follows (Figure 1A), and si-RNA3 was selected as the interference plasmid to knock down TGIF1. In addition, the interference effect of other siRNAs was not significant, which may be due to the unreasonable sequence design. The increase in TGIF1 level after transfection of siRNA1 and siRNA4 in MKN45 cells may be due to the change of other genes' expression. Then, AGS and MKN45 cells were transfected with si-RNA3 to construct TGIF1 knockdown cell lines (si-TGIF1) with negative interference sequence as negative control group (NC). RNA and protein of NC and si-TGIF1 groups were collected. The results of qPCR showed that the mRNA level of TGIF1 in si-TGIF1 group was significantly lower than that in the NC group (Figure 1B). The protein level was evaluated with Western blotting. As shown in Figure 1C, the expression level of TGIF1 in si-TGIF1 group was decreased significantly compared with that in the NC group ($P<0.05$).

CCK8 assay was performed to detect cell proliferation. The results showed that TGIF1 knockdown inhibited the proliferation of AGS and MKN45 cells (Figure 1D and E). In addition, the colony forming ability of si-TGIF1 cells was decreased significantly compared with the NC group ($P<0.05$, Figure 1F).

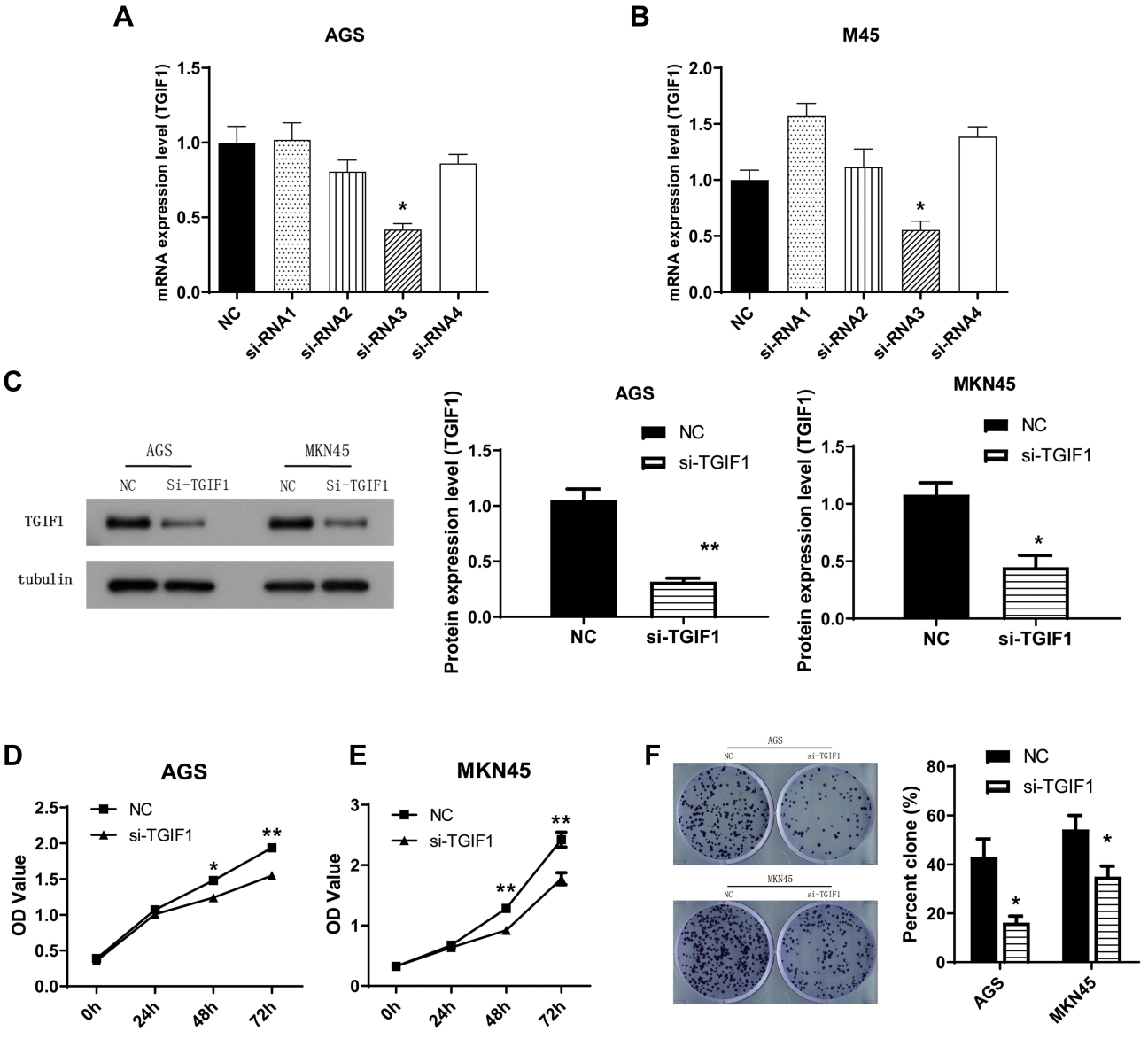

Figure 1 Knockdown of TGIF1 inhibited growth capacity of gastric cancer cells.

Notes: (**A** and **B**) Four TGIF1 siRNAs (si-RNA1, si-RNA2, si-RNA3 and si-RNA4) were transfected into gastric cancer cells respectively. TGIF1 mRNA expression level was detected with qPCR. (**C**) TGIF1 si-RNA3 was transfected into AGS and MKN45 cells to generate TGIF1 knockdown cell model (si-TGIF1). TGIF1 protein expression level was evaluated by Western blotting. (**D** and **E**) CCK8 assay was performed to assess the cell proliferation. (**F**) Plate clone formation test. *P<0.05, **P<0.01. Results are from three repeated experiments.

Taken together, knockdown of TGIF1 significantly inhibited the growth capacity of gastric cancer cells in vitro.

TGIF1 Knockdown Inhibits the Invasion and Migration of Gastric Cancer Cells

Transwell assay was performed to assess the effect of TGIF1 on the invasion and migration in gastric cancer cells. After transfection of si-RNA, the number of migrating cells and invading cells decreased significantly (Figure 2A and B). In parallel, from the results of wound-healing assay, TGIF1 knockdown inhibited the migration of both AGS and MKN45 cells (Figure 2C). Furthermore, the results of gelatinase spectrum showed that the expression level of MMP-2 protein in si-TGIF1 group was decreased significantly compared with the NC group (Figure 2D). These results suggested that TGIF1 knockdown inhibited the invasion and migration of gastric cancer cells in vitro.

TGIF1 Knockdown Promotes the Apoptosis of Gastric Cancer Cells

The apoptosis of AGS and MKN45 cells was detected using cell flow-cytometry. After 48 hours of transfection, cells were double stained by Annexin V and PI. As shown in Figure 3A, the apoptosis rates of AGS and MKN45 cells in si-TGIF1 group were increased significantly compared with the NC group. Moreover, the results of Hoechst-PI double staining showed that the apoptotic ratio of si-TGIF1 group was significantly higher than that of NC group both in AGS and MKN45 cells (Figure 3B). To further investigate the mechanism of apoptosis, Western blot analysis was adopted to determine the effect of si-TGIF1 on the expression levels of apoptosis-related proteins. The results indicated that TGIF1 knockdown down-regulated the expression of anti-apoptotic Bcl-2, but up-regulated the expression of pro-apoptotic Bax and

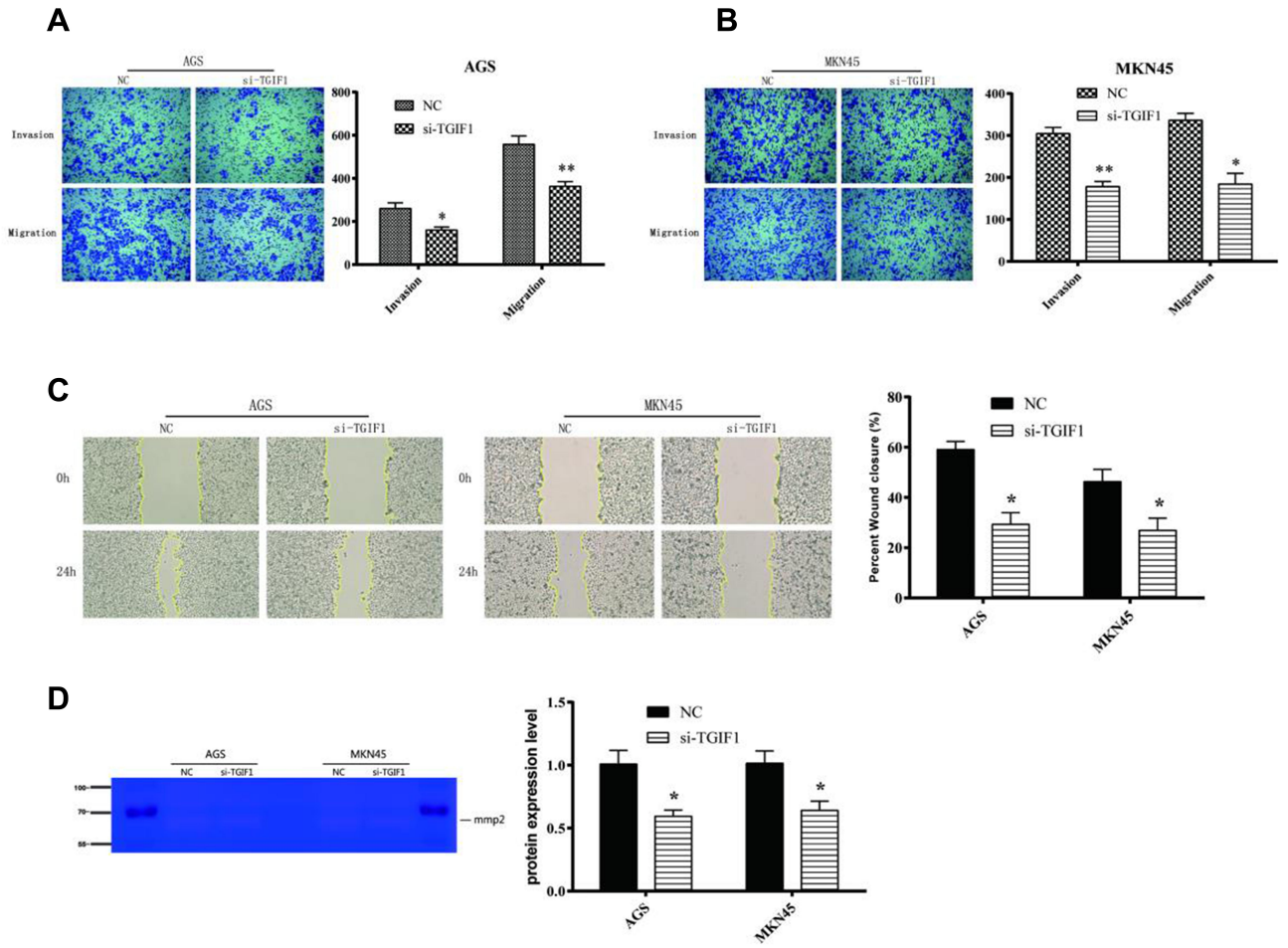

Figure 2 TGIF1 knockdown inhibited the invasion and migration of gastric cancer cells.

Notes: (**A** and **B**) Transwell assay was used to evaluated the invasion and migration of AGS and MKN45 cells. (**C**) Wound-healing assay was performed to detect the migration of cells. (**D**) Gelatinase experiment for MMP-2. *P<0.05, **P<0.01. Results are from three repeated experiments.

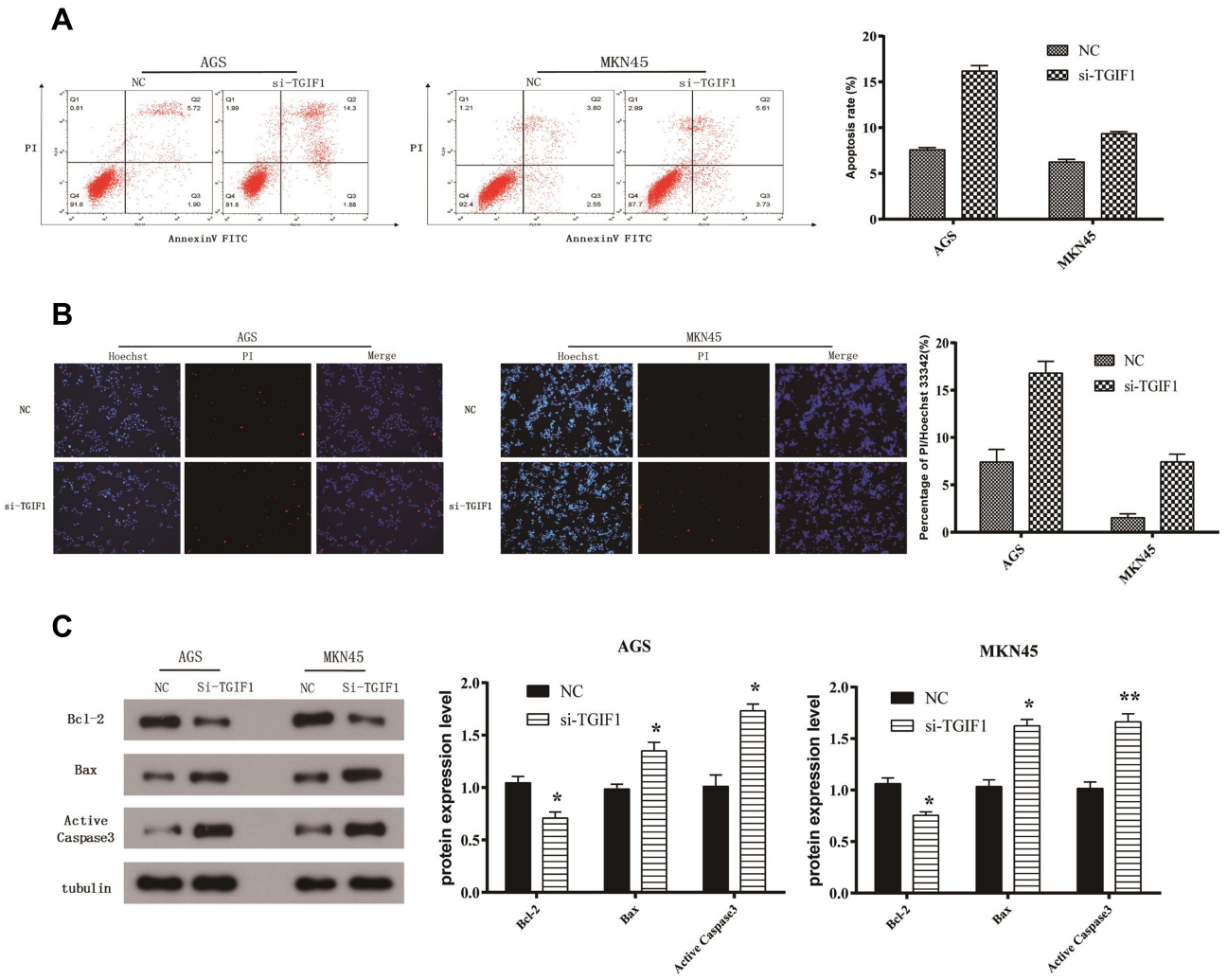

Figure 3 TGIF1 knockdown promoted the apoptosis of gastric cancer cells.
Notes: (**A**) Cell apoptosis was evaluated using flow cytometry assay. (**B**) Hoechst-PI double staining of apoptosis. (**C**) The expression of apoptosis-related proteins (Bcl-2, Bax, and active caspase3) was analyzed with Western blotting. *P<0.05, **P<0.01. Results are from three repeated experiments.

Caspase3 (Figure 3C). Taken together, knockdown of TGIF1 promoted the apoptosis of gastric cancer cells by regulating the expression of apoptosis-related proteins.

TGIF1 Knockdown Blocks Cell Cycle of Gastric Cancer Cells

Then, we detected the effect of TGIF1 on cell cycle in AGS cells using flow cytometry. As shown in Figure 4A, the cells in G1 phase of TGIF1 knockdown increased significantly compared with the control cells; moreover, the cells in S phase of TGIF1 knockdown increased significantly compared with the control cells. These results indicated that TGIF1 knockdown blocked the transformation progression of AGS cells from G1 phase to S phase.

TGIF1 Affects the AKT Signaling Pathway in Gastric Cancer Cells

To further investigate the mechanism of TGIF1 in gastric cancer cells, the activity of PI3K/AKT signaling pathway was detected using Western blotting. AKT pathway, as a key pathway of cell proliferation and apoptosis, is involved in tumor progression and metastasis. AKT pathway is often activated in gastric cancer.[16] Western blot analysis showed that there was no significant difference in total AKT expression between si-TGIF1 group and NC group, but TGIF1 knockdown reduced the level of the phosphorylated form p-Akt and its downstream protein Cyclin D1 both in AGS and MKN45 cells (Figure 4). Therefore, the activation of AKT signaling pathway was suppressed

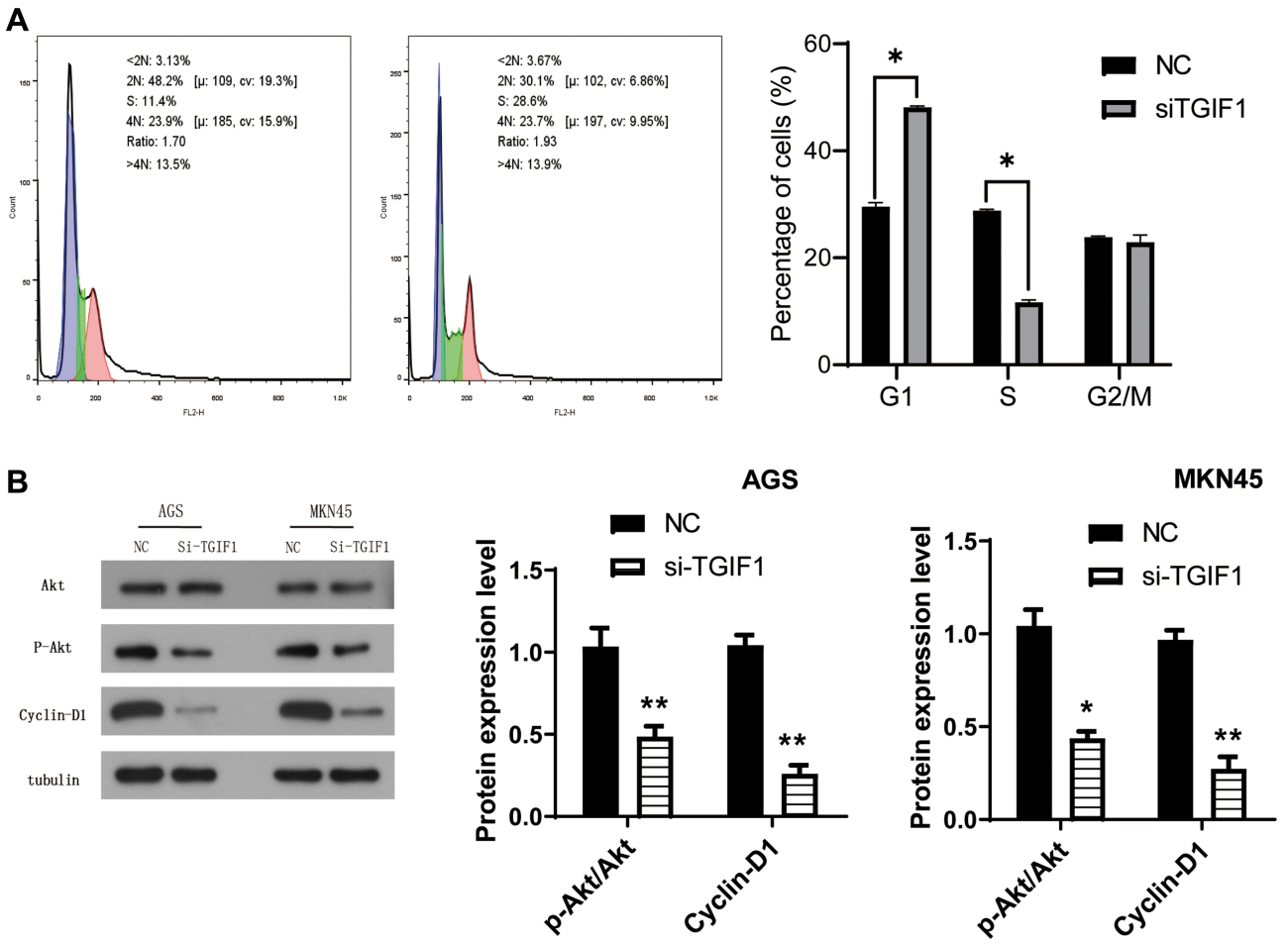

Figure 4 TGIF1 affected the AKT signaling pathway in gastric cancer cells.
Notes: (**A**) Cell cycle was evaluated using flow cytometry assay. (**B**) Western blot assay was used to analyze the effect of TGIF1 on AKT signaling pathway. *P<0.05, **P<0.01. Results are from three repeated experiments.

by TGIF1 knockdown. These results suggested that TGIF1 affects the development of gastric cancer cells by regulating the AKT signaling pathway.

The Expression of TGIF1 Was Significantly Correlated with M and T Stages of Gastric Cancer

Finally, we analyzed the clinical significance of TGIF1 in stomach adenocarcinoma (STAD) using LinkedOmics database on the TCGA dataset (www.linkedomics.org/).[17] As shown in Table 1, TGIF1 was significantly correlated with histological type of gastric cancer patients. Importantly, TGIF1 was also associated with the M stage and T stage of gastric cancer patients, suggesting that TGIF1 might be involved in the metastasis of gastric cancer. However, TGIF1 was not associated with the prognosis of gastric cancer patients.

Discussion

Due to the lack of early specific symptoms, gastric cancer is difficult to diagnose, so the mortality rate remains high. The discovery of targets in the diagnosis and treatment of gastric cancer provides great help for the diagnosis and treatment of gastric cancer.[18,19] TGIF1 (TG-interacting Factor 1) is a member of the TALE (three-amino-acid-loop-extension superclass) superfamily, named for its conserved DNA binding sequence and its specific binding with TGFP. It has been shown that TGIF1 plays a major role in transcriptional inhibition,[20] and its specific binding sequence is CTGTCAA. In the latest study, TGIF1 was found to be involved in the progression of gastric carcinoma as a downstream target of XTP8 stimulates migration and invasion through interacting with TGIF1.[21] In this study, si-TGIF1 was transfected to reduce the expression of TGIF1 in gastric cancer cells. The proliferation, invasion, migration, and apoptosis of gastric cancer cells were

Table 1 Clinical Significance of TGIF1 Was Obtained Using LinkedOmics Based Analysis

Attribute	Statistic	P-value	FDR (BH)
Histological type (Kruskal–Wallis Test)	26.9700	0.0001	0.0018
Pathology M stage (Wilcox Test)	−0.0298	0.0155	0.0930
Pathology T stage (Kruskal–Wallis Test)	7.8240	0.0498	0.1461
Number of lymph nodes (Spearman Correlation)	−0.1047	0.0553	0.1461
Residual tumor (Kruskal–Wallis Test)	5.3840	0.0678	0.1461
Years to birth (Spearman Correlation)	0.0934	0.0730	0.1461
Pathologic stage (Kruskal–Wallis Test)	5.0150	0.1707	0.2926
Pathology N stage (Kruskal–Wallis Test)	4.3790	0.2234	0.3350
Overall survival (Cox Regression Test)	−0.1393	0.3025	0.3682
Race (Kruskal–Wallis Test)	2.3630	0.3069	0.3682
Ethnicity (Wilcox Test)	0.0356	0.3444	0.3757
Radiation therapy (Wilcox Test)	−0.0037	0.9855	0.9855

detected; the results showed that inhibition of TGIF1 expression can significantly reduce the growth activity and promote apoptosis of gastric cancer cells.

In order to study the mechanism of the inhibitory effect of si-TGIF1 on gastric cancer cells, we analyzed the expression of MMP-2, a cell migration marker, by gelatinase. The high expression of MMP-2 plays an important role in tumor invasion and metastasis.[22] In breast cancer, up-regulating the expression of MMP-2 can significantly promote the invasion and metastasis of breast cancer;[23] in lung cancer, the expression of MMP2 in high metastatic potential lung cancer cells was significantly higher than that in low metastatic potential lung cancer cells. Interference with the expression of MMP2 can significantly inhibit the metastasis of lung cancer.[24] Our results showed that knockdown of TGIF1 can down-regulate the expression of MMP2.

Bcl2 and Bax are the two key factors in apoptosis, which are the regulatory genes that inhibit and promote apoptosis.[25] The ratio of Bax/Bcl2 can be used to determine whether the cells are in apoptosis or not.[26] Among most of the factors involved in the complex process of apoptosis, Caspase enzyme cascade reaction is the core, and Caspase 3 usually exists in the form of inactive proenzyme. Caspase 3, when activated, can catalyze the cleavage of a series of key intracellular proteases downstream, leading to the damage of cell structure and function.[27] So we detected the expression of apoptosis-related proteins Bax, Bcl2, and Caspase3. The results of Western blot showed that TGIF1 knockdown increased the expression of Bax and Caspase3, and decreased the expression of Bcl2. Furthermore, we evaluated the AKT signaling pathway, the results indicated that the levels of the phosphorylated form of AKT and Cyclin D1 were significantly decreased by si-TGIF1. Akt is a key factor in PI3K/Akt pathway. It promotes the growth, metastasis and inhibits apoptosis of tumor cells by phosphorylating a variety of substrates, such as mTOR, P21Cipl/WAF1, GSK3, TSC2 and FOXO family. These data prove that TGIF1 can affect the development of gastric cancer cells via the AKT signaling pathway, suggesting that TGIF1 is a potential target for gastric cancer treatment. In addition, we predict that TGIF1 affects the AKT pathway through specific downstream targets, which will be the focus of our future research.

Finally, we found that the expression of TGIF1 was significantly correlated with histological type, M and T stages, but not OS of gastric cancer through LinkedOmics based analysis. It has been found that the expression of TGIF1 is increased in oral squamous cell carcinoma (OSCC), and is related to cell differentiation, vascular invasion, pathological stage, lymphatic invasion, and overall survival.[28,29] In addition, TGIF1 overexpression is associated with poor prognosis of upper urinary tract urothelial carcinoma and acute myeloid leukemia.[7,13] The clinical significance of TGIF1 in gastric cancer needs to be further verified by clinical samples.

Conclusion

TGIF1 knockdown inhibited the proliferation, migration, and invasion and promoted apoptosis of gastric cancer cells via the AKT signaling pathway. Our results enriched our understanding of the mechanism of gastric cancer progression and revealed that siRNA of TGIF1 can be considered potential gastric cancer inhibitors in therapy.

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Disclosure

The authors report no conflicts of interest in this work.

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