European Review for Medical and Pharmacological Sciences 2018; 22: 6809-6815

GLI-1 facilitates the EMT induced by TGF-β1 in gastric cancer

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Abstract. – OBJECTIVE: To explore how GLI-1 affects the EMT induced by TGF-β1 in gastric cancer.

MATERIALS AND METHODS: Following 24 hours of culture of SGC-7901 cells in presence of TGF-β1, we observed the changes in morphology as well as mRNA and protein expressions of GLI-1, E-cadherin and Vimentin by RT-PCR and Western blot. Transwell assay was conducted to evaluate the changes in invasion ability of SGC-7901 cells. Then, SGC-7901 cells were co-treated with TGF-β1 and GANT 61, and changes of the above indexes were also detected using the corresponding methods.

RESULTS: In presence of TGF-β1, EMT was initiated in SGC-7901 cells EMT with increased cell invasion ability, and the mRNA and protein expressions of E-cadherin were downregulated, while those of the GLI-1 and Vimentin were upregulated. Conversely, the co-treatment of TGF-β1 and GANT 61 suppressed the increased cell invasion ability induced only by TGF-β1, and the changes in mRNA and protein expressions of these factors were abolished.

CONCLUSIONS: We found that GLI-1 facilitates the EMT induced by TGF-β1 in SGC-7901 cells, which may serve as a potential target in developing the clinical treatment of gastric cancer.

Key Words:
Gastric cancer, Transforming growth factor-beta1, Glioma associated oncogene homolog 1, Epithelial mesenchymal transformation, Invasion.

Introduction

Gastric cancer is one of the most frequent malignancies in the digestive system[1,2], and ranks first for incidence rate among all malignancies worldwide. In China, gastric cancer has evolved into a kind of malignancy with a high morbidity rate and mortality rate only secondary to the lung cancer[3-5]. Gastric cancer, with insignificant clinical symptoms in an early stage, is usually diagnosed only at the advanced stage with tumor-adjacent or distant metastasis, poor prognosis and a 5-year survival rate of lower than 15%[6-9]. Epithelial-mesenchymal transformation (EMT) refers to a process, in which the epithelial cells, under a series of stimuli, are transformed into the cells with mesenchymal phenotype with an enhancement in the migration ability of cells, which is closely associated with the distant invasion and metastasis of malignancy. Previous investigations have shown that abnormal activation of the Hedgehog (Hh) signal pathway is involved in the invasion and metastasis of gastric cancer in advanced stage. However, GLI-1, as a key factor regulating the transcription in Hh signal pathway, is up-regulated in a variety of malignant tumors, and also modulates the expressions of downstream targeted genes, thereby affecting the invasion and metastasis of tumor cells. EMT is activated by modulation of multiple signal pathways, in which TGF-β is the most classical pathway, and TGF-β1 has been proved as the activator of EMT in many malignancies[10]. Nevertheless, involvement of Hh signal pathway in the TGF-β1-induced EMT in gastric cancer remains unknown. Thus, we investigated the role of GLI-1 in the TGF-β-induced EMT, the cellular invasion, and its mechanism, so as to provide new evidence for the molecular-targeted therapy of gastric cancer in an early stage.

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Material and Methods

Materials

Human SGC-7901 cells (Shanghai Ruilu Biotechnology Co., Ltd.); recombinant human TGF-β1 (Sigma-Aldrich, St. Louis, MO, USA); GANT 61, the antagonist of GLI-1 (Selleck, USA); TRIzol kit (Dalian, China); Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South-Logan, UT, USA); fetal calf serum (Hao Yang Biological Manufacture Co., Ltd., Tianjin, China); penicillin-streptomycin mixture (Beyotime Biotechnology Co., Ltd, Shanghai, China); cDNA reverse transcription kit and SYBR Real-time polymerase chain reaction kit (RT-PCR) (TaKaRa, Otsu, Shiga, Japan); PCR primers (synthesized by Tianyi Huiyuan Co., Ltd, Beijing, China); horseradish peroxidase(HRP)-conjugated goat anti-rabbit anti-IgG (Solarbio Biotechnology Co., Ltd., Beijing, China); Matrigel, Matrix and 8 μL Transwell chambers (Corning Costar, Corning, NY, USA); protein markers (Thermo Fisher, Waltham, MA, USA); enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA); fetal bovine serum (FBS) (Gibco, Rockville, MD, USA); radio-immunoprecipitation assay kit and bicinchoninic acid (BCA) kit (Beyotime Biotechnology Institute, Shanghai, China); 24-well Transwell chambers (8 μm) (Corning Costar, Corning, NY, USA); Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Culture

SGC-7901 cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2. After confluence reached 70-80%, cells were starved using the serum-free medium, followed by treatment with TGF-β1, and then grouped in accordance with the experiment requirement.

Cell Morphology Observation

SGC-7901 cells were digested and passaged, followed by inoculation on a 6-well plate. After cells adhered to the wall, they were starved using the serum-free medium, and on the next day they were treated with TGF-β1 for 24 h, followed by observation of the morphological changes in cells under the phase-contrast microscope and photographing.

RNA Extraction and Real-Time PCR Detection

After treatment, SGC-7901 cells were used for extraction of total RNA using the TRIzol kit following the instruction of kit, followed by the cDNA preparation through reverse transcription in accordance with the instructions of manufacturer. In this study, primers for PCR were synthesized by Tianyi Huiyuan Co., Ltd., (Beijing, China). The sequences are shown as follows: GAPDH, upstream 5'-AGGTCGGTGTGAACGGATTTG-3', downstream 5'-GGGGTCGTTGATGGCAACA-3'; GLI-1, upstream 5'-CCCAATCACAAGTCAGGTTCCT-3', downstream 5'-CCTATGTGAAGCCCTATTTGCC-3'; E-cadherin upstream 5'-TGATTCTGCTGCTCTTGCTGTT-3', downstream 5'-CAAAGTCCTGGTCCTCTTCTCC-3'; Vimentin upstream 5'-GTACTTGTAATGACACATCTC-3', downstream 5'-TGCCAGTITCTGCATCTGC-3'. Amplification was performed as follows: 95°C for 5 min; 95°C for 10 s, 60°C for 35 s, and 40 cycles. Melting curve was prepared to evaluate the reliability of PCR results, and mRNA expressions were quantified using $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

SGC-7901 cells in logarithmic phase were collected and treated for 24 h, followed by three washes using phosphate-buffered saline (PBS). Then, total protein was extracted using radioimmunoprecipitation assay (RIPA) reagent, and the concentration was determined using BCA method. After 20 to 100 μg proteins were loaded in the wells, 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate the proteins which were later transferred regularly on the polyvinylidene difluoride (PVDF) membrane. Following 1.5 h of blocking in 5% skimmed milk at 37°C, rabbit anti-human anti-GLI-1, E-cadherin, Vimentin and β-actin were used to probe the proteins on the membrane at 4°C overnight; next, proteins were incubated with HRP-conjugated secondary antibody diluted at 1:3500 for 1.5 h at room temperature. Blot bands were exposed in a dark room, and grey value analysis was carried out for targeted bands using Image-Pro Plus 6.0 software, with β-actin as internal reference.

Cell Invasion Experiment

Matrigel was melted at 4°C prior to this experiment. On the membrane surface of each well, 60 μL diluted Matrigel were spread and solidified in an incubator for 4 hours for later use. SGC-7901 cells in logarithmic phase were collected and cultured in serum-free RPMI-1640 medium, followed by digestion using 0.25% EDTA-trypsin

and resuspending in the serum-free RPMI-1640 medium for single-cell suspension at a density of 2×10^5/mL. In the upper chamber, 200 μL cell suspension without serum were added, and cells were grouped in accordance with the experiment requirement, with 3 replicate wells. In the lower chamber, RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) was added (600 μL/well), followed by culture in an incubator for 48 h. After that, chambers were removed and washed three times using icy PBS to remove the residual medium. Cells failing to pass through the membrane were scrapped using a wet cotton swab, fixed using 4% paraformaldehyde (PFA) for 20 min, and dried at room temperature. Cells were stained using the crystal violet for 20 min and washed three times using the icy PBS. In PBS, chambers were placed on the inverted microscope to observe the cells that passed through the membrane; cells in the central and surrounding fields were counted at magnification (200×) with the average as the results.

Statistical Analysis

All data were presented as (s), and one-way analysis of variance was performed using SPSS 19.0 (IBM, Armonk, NY, USA). Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA as post-hoc test to find means that were significantly different from each other. $p<0.05$ suggested that the difference had statistical significance.

Results

TGF-Ð1 Induces EMT in SGC-7901 Cells

Following 24 h of treatment with 10 ng/mL TGF-β1 for SGC-7901 cells, they were placed under the inverted microscope to observe the morphological changes of cells; we found that in the control group, SGC-7901 in padstone shape was in alignment, while in the TGF-β1 group cells were in long-fusiform without close connection. Cells were independent from each other, manifesting the typical features of mesenchymal cells. RT-PCR and Western blot assays showed that after 24 hours of treatment using 10 ng/mL TGF-β1, in comparison with the control group, significant decreases were found in mRNA and protein expressions of E-cadherin in the TGF-β1 group with significant increases in the expressions of mRNA and protein of Vimentin (Figure 1).

TGF--Ð1 Enhances the In-Vitro Invasion Ability of SGC-7901 Cells

Transwell invasion experiment showed that compared to the control group, SGC-7901 gained an enhanced invasion ability after 24 hours of TGF-β1 (10 ng/mL) (Figure 2).

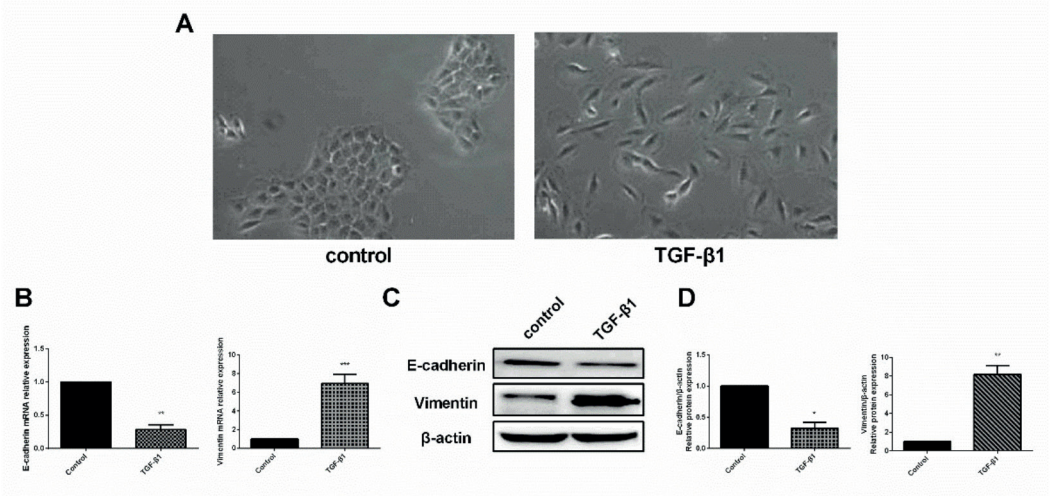

Figure 1. TGF-β1 induces lung cancer cell line SGC-7901 EMT. ***A,*** Morphological change of SGC-7901 cells treated with or without 10 ng /mL TGF-β1; ***B,*** The mRNA levels of E-cadherin and Vimentin in SGC-7901 cells induced with or without TGF-β1; ***C,*** The protein levels of E-cadherin and Vimentin in SGC-7901 cells induced with or without TGF-β1; ***D,*** Quantification of the protein level of E-cadherin and Vimentin.

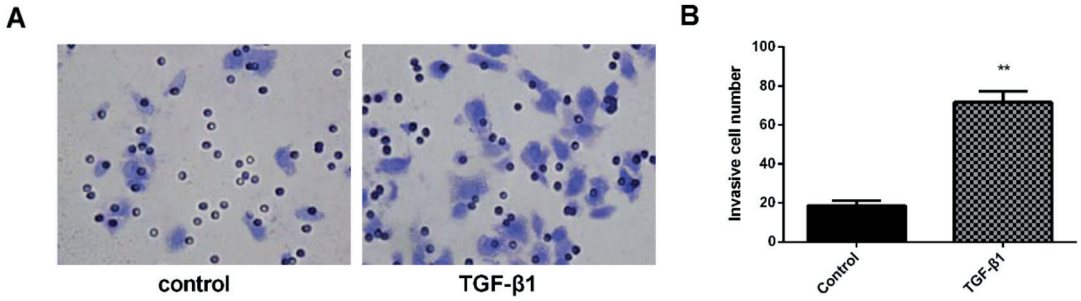

Figure 2. TGF-β1 promotes invasion of SGC-7901 cells (Coomassie brilliant blue staining × 200). A: The Matrigel invasion chamber assay was performed to detect the invasive capability of SGC-7901 cells; B: Quantification of the number of invaded cells.

TGF-β1 Up-regulates the mRNA and Protein Expressions of GLI-1 in SGC-7901 Cells

After 24 hours of treatment with TGF-β1 (10 ng/mL), RT-PCR results indicated that in presence of TGF-β1, GLI-1 mRNA and protein expression in SGC-7901 were significantly elevated (Figure 3).

Blocking GLI-1 Signal Pathway Inhibits the TGF-Ð1-Induced EMT

To further validate the role of GLI-1 signal pathway in the regulation of TGF-β1-mediated EMT, we used the GANT61, antagonist specific to GLI-1, to block the expression of GLI-1. RT-PCR and Western blot assays showed that GANT61 could significantly inhibit TGF-β1-induced GLI-1 expression, while facilitating the mRNA and protein expressions of E-cadherin, and suppressing those of Vimentin. These results indicated that targeted silence of GLI-1 signal pathway can block the TGF-β1-induced EMT in SGC-7901 cells (Figure 4).

Blocking GLI-1 Signal Pathway Attenuates the TGF-β1-Induced Invasion Ability

After 24 hours of treatment with TGF-β1 (10 ng/mL), transwell migration experiment indicated that compared to the control group, the migration ability of Tca8113 cells in the TGF-β1 group were significantly enhanced (Figure 5).

Discussion

Gastric cancer is a common kind of malignancy in digestive system originated from the gastric mucosa epithelium with a high morbidity rate and mortality rate ranking 2nd among all malignancies in China. Development and progression of gastric cancer are modulated by multiple genes and factors, which hinder the understanding on the pathogenesis. At the time of diagnosis, most of the patients have evolved into the advanced stage, and, due to the lack of effective treatment methods and susceptibility

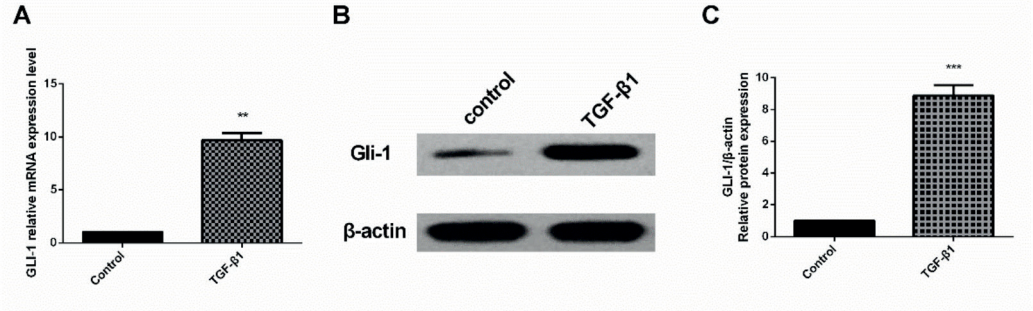

Figure 3. TGF-β1 upregulates mRNA and protein expression of GLI-1 in SGC-7901 cells. ***A,*** The mRNA levels of GLI-1 in SGC-7901 cells induced with or without TGF-β1; ***B,*** The protein levels of GLI-1 in SGC-7901 cells induced with or without TGF-β1; ***D,*** Quantification of the protein level of GLI-1.

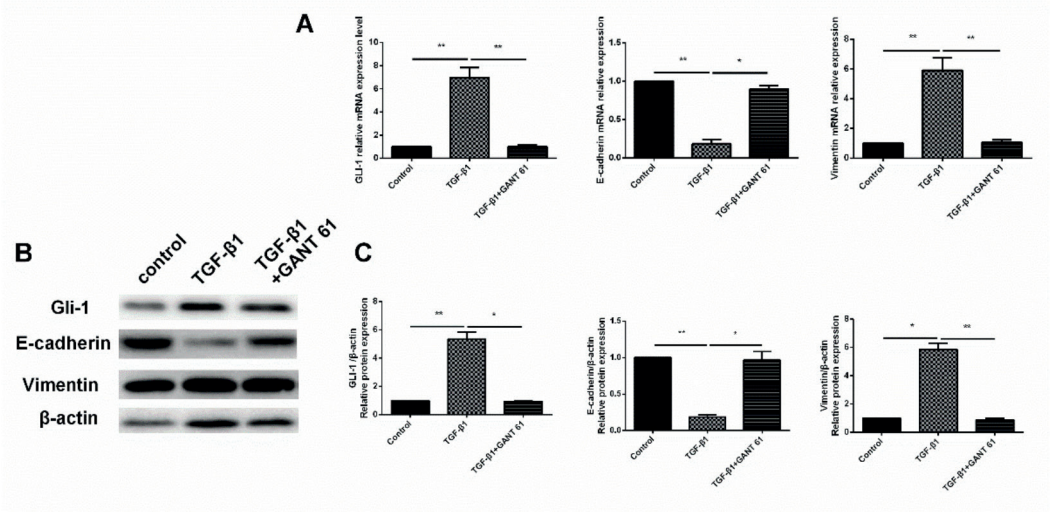

Figure 4. Blocking GLI-1 inhibits the TGF-β1-induced EMT. ***A,*** The mRNA levels of GLI-1, E-cadherin and Vimentin in SGC-7901 cells; ***B,*** The protein levels of GLI-1, E-cadherin and Vimentin in SGC-7901 cells; ***C,*** Quantification of the protein level of GLI-1, E-cadherin and Vimentin.

to distant invasion and metastasis, patients suffer from the multi-organ involvement, contributing to the poor prognosis and a low 5-year survival rate below 25%. Recent evidence has shown that EMT is critical to the infiltration, invasion and distant lesion-implantation in many malignancies, and additionally involved in the modulation of proliferation and apoptosis of tumor cells. EMT procedure, as a trigger, not only initiates the invasion and metastasis of tumor, but also acts as a key marker reflecting the distant invasion ability of tumor cells. Some scholars believed that EMT status and phenotypic changes in proteins can be used as the independent factors in evaluation of prognosis of gastric cancer patients[11]. Thus, research on EMT modulation in gastric cancer is conducive to ameliorating the prognosis of gastric cancer patients. Various endogenous and exogenous stimulators can initiate the EMT procedure, and TGF-β1, as a widely-recognized potent inducer of EMT, plays a key role in the development and progression of EMT. Previous studies have indicated the inducing effect of TGF-β1 in the development of EMT in gastric cancer cells, but the specific mechanism remains to be investigated in further studies[12]. Hedgehog (Hh) signal pathway has been proved to be involved in the formation and development of multiple tissues and organs in human beings, including brain, gastrointestinal tract, etc.. Major molecules in Hh signal pathway include Shh, Ptched, Smo, GLI and downstream genes; generally, Hh signal pathway is abnormally activated in the embryonic development and deactivated in mature body. GLI, the downstream transcrip-

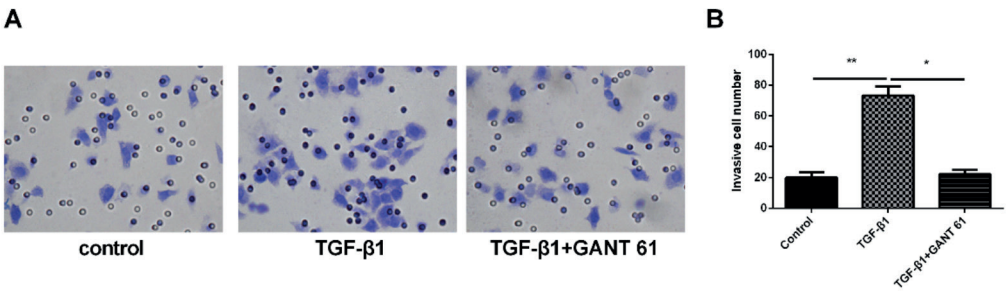

Figure 5. Inhibition of GLI-1 suppressed TGF-β1 induced EMT (Coomassie brilliant blue staining × 200) ***A,*** The Matrigel invasion chamber assay was performed to detect the invasive capability of SGC-7901 cells; ***B,*** Quantification of the number of invaded cells.

tion regulator in Hh signal pathway, is mainly responsible for the initiation of transcription of target genes in Hh signal pathway. So far, there are a total of three GLI transcription factors, i.e. GLI-1, GLI-2 and GLI-3, with highly-conservative DNA-binding region. GLI-1, as a key role, can activate the Hh signal pathway, which is considered as a marker of activation of Hh signal pathway[13]. It has been found that in gastric cancer samples, GLI-1 is abnormal upregulated, which is negatively correlated with the prognosis. In addition, emerging evidence has shown the promoting effect of GLI-1 on EMT of tumor cells through decreasing the intercellular adhesive force and enhancing the migration ability, contributing to the invasion and diffusion of tumors[14]. Thus, based on the results of these studies, we inferred that TGF-β1 can induce the initiation of EMT in SGC-7901 cells, thus facilitating the invasion and metastasis, and the distant metastasis of tumors. In this study, after 24 hours of treatment with 10 μg/L TGF-β1 for SGC-7901 cells, we found the EMT in these cells, and under the microscope, we observed the transition of cell morphology from the padstone shape to the thin, long-spindle or fusiform shapes with loosened intercellular connection. Meanwhile, mRNA and protein expressions of E-cadherin, the marker of epithelial cells, were decreased concomitant with increases in the levels of Vimentin, the marker of mesenchymal cells. Transwell experiment showed that SGC-7901 cells with EMT had a significantly enhanced *in-vitro* invasion ability. To further investigate the molecular mechanisms underlying in TGF-β1-induced EMT, RT-PCR and Western blot assays were adopted to detect the expressions of GLI-1; we found that compared to the control group, in SGC-7901 cells after treatment with 10 μg/L TGF-β1 for 24 h, GLI-1 gene was significantly activated, suggestive of the potential involvement in TGF-β1-mediated EMT process. To clarify the role of GLI-1 in EMT, we blocked the expression of GLI-1 using its antagonist, GANT 61, and further explored the effect on EMT initiation and invasion ability of SGC-7901 cells. The results showed that after the expression of GLI-1 was blocked, TGF-β1-mediated EMT process in SGC-7901 cells was blocked with a significant reduction in invasion ability of cells, which confirmed the important role of GLI-1 in this event. Thus, we inferred that TGF-β1 can trigger the EMT process in SGC-7901 through acting on GLI-1, a critical gene in Hh signal pathway, thus enhancing the invasion ability of gastric cancer cells and promoting the progression of gastric cancer.

Conclusions

We found that GLI-1 mediated the TGF-β1-induced EMT and invasion of SGC-7901 cells. Thus, with promising clinical significance and prospects, in-depth studies on the roles of GLI-1 in the development and progression of EMT in gastric cancer, reversing the EMT process through interfering on the expression of GLI-1, and decreasing the invasion and metastasis of tumor cells, can provide new suggestions and evidence for specific treatment of gastric cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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