Regulation of Proliferation and Epithelial-to-Mesenchymal Transition (EMT) of Gastric Cancer by ZEB1 via Modulating Wnt5a and Related Mechanisms

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Background: As a member of the zinc-finger E-box binding protein (ZEB) family, ZEB1 can modulate onset and progression of various tumors, but its regulatory effect or mechanism in GC has not been defined.

Material/Methods: GC tumor tissues and adjacent tissues were collected from GC patients across different TNM stages. Real-time PCR was used to measure ZEB1 expression to analyze its correlation with pathological features of tumors. Cultured GC cell lines SGC-7901 and MGC-803 were randomly assigned into control group, scramble group, and ZEB1 siRNA group. Real-time PCR was employed to analyze ZEB1 expression, and MTT approach was used to measure cell proliferation. Cell apoptosis was evaluated by flow cytometry. Wound healing assay was used to detect its effect on cell migration. Expression of E-cadherin and Vimentin involved in epithelial-to-mesenchymal transition (EMT) was measured by Western blot analysis, along with Wnt5a proteins.

Results: GC tissues had upregulation of ZEB1 (P<0.05 compared to adjacent tissues), whose expression level was correlated with differentiation grade, lymph node metastasis, and tumor pathological stage (P<0.05). Transfection of ZEB1 siRNA into SGC-7901 or MGC-803 cells can suppress ZEB1 expression, inhibit tumor cell proliferation, enhance apoptosis, and inhibit cell migration. Transfected GC cells had higher E-cadherin expression and decreased Vimentin expression or Wnt5a expression (P<0.05 compared to the control group).

Conclusions: ZEB1 expression is increased in GC tumor tissues and is associated with pathological features. The downregulation of ZEB1 can facilitate cell apoptosis via mediating Wnt5a, further suppressing GC cell proliferation and migration, and reducing EMT occurrence.

MeSH Keywords: Apoptosis • Cell Proliferation • Epithelial-Mesenchymal Transition • Stomach Neoplasms
Background

Gastric carcinoma (GC) has high incidence and unfavorable prognosis, and GC patients have shorter life span and severely reduced quality of life [1]. GC usually derives from gastric epithelium, and is a common malignant tumor in the digestive tract [2]. GC has geographic patterns, and, in general, Asia, Africa, and Latin America are epidemic regions. Due to unique environmental factors and dietary habits, China has relatively higher GC occurrence, especially in eastern and northwestern regions [3,4]. According to WHO statistics, GC is currently the second most common cancer in China, and its morality is the third highest, accounting for almost 50% of GC patients worldwide [5]. GC most commonly occurs in middle-aged males. A recent study showed a younger trend of newly diagnosed GC patients, and this trend is increasing [6]. Due to an incomplete screening system for GC in China, many patients are already at terminal stage at the time of primary diagnosis, causing heavy burdens for the family and society, making it a major health and economic issue worldwide [7]. GC has a complicated pathogenesis mechanism and involves multiple factors, including environment, diet, and Helicobacter pylori (Hp) infection [8]. To date, the pathogenesis mechanism of GC has not been fully defined, causing major difficulties for disease treatment.

Zinc-finger transcriptional factor is part of the zinc-finger E-box binding protein (ZEB) family, which has become the focus of recent studies [9]. The ZEB family mainly consists of ZEB1 and ZEB2 proteins [10]. A previous study showed that ZEB1 participates in various transcriptional activity modulations. ZEB1 participates in embryogenesis and formation, and its gene mutation can lead to severe deformation [11]. A recent study showed the involvement of ZEB1 in onset and progression of multiple tumors [12,13], whose metastasis and invasion are closely related with EMT [14,15]. However, the expression profile of ZEB1 in GC tissues and its regulatory mechanism for tumors have not been defined.

Material and Methods

Patients

We enrolled 48 patients diagnosed with GC and receiving surgical resection in the First Affiliated Hospital of Bengbu Medical College (Bengbu, Anhui, China) from January 2017 to December 2017. There were 31 males and 17 females, ages 40–75 years old, with an average age of 58±7 years. Tissue samples were collected during surgery for pathological examination, staging, and sub-typing. Inclusion criteria were: primary GC diagnosed by pathological examination, and had not received other treatment such as surgery, chemotherapy or radiotherapy. Exclusive criteria were: recurrent GC patients, received surgery, chem-, or radiotherapy, and complicated with other diseases [6]. Clinical staging of GC patients followed the TNM guideline stipulated by UICC in 2003 [7], including 8 patients at stage I, 13 at stage II, 11 at stage III, and 16 at stage IV. A further examination for tumor differentiation showed 10, 9, and 12 cases of high-, moderate-, and low-differentiation adenoma, plus 7 cases of undifferentiated tumors. In examining peri-gastric lymph node metastasis, we found 28 patients presented lymph node lesions, while 20 patients did not develop lymph node metastasis. Both GC tumor tissues and adjacent tissues (with larger than 5 cm distance toward cancer lesion) were resected during the surgery and stored at -80°C. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (Bengbu, Anhui, China) and all participants signed informed consent.

Major materials and equipment

GC cell line SGC-7901 (CRL-1740™) was purchased from the ATCC Cell Bank (USA). The MGC-803 cell line was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai). DMEM medium, fetal bovine serum (FBS), and penicillin-streptomycin were bought from Hyclone (USA). ZEB1 siRNA sequence and si-NC sequence were synthesized by Toyobo Bio (China). DMSO and MTT reagent were purchased from Gibco (USA). Trypsin-EDTA digestion buffer was purchased from Sigma (USA). PVDF membrane was purchased from Pall Life Sciences (USA). EDTA was bought from Hyclone (USA). Western blot reagents were purchased from Beyotime Biotech (China). ECL reagent was purchased from Amersham Biosciences (USA). Rabbit anti-human E-cadherin, Vimentin, rabbit anti-human Wnt5a monoclonal antibody, and horseradish peroxidase (HRP) conjugated-mouse anti-rabbit IgG secondary antibody were purchased from Cell Signaling (USA). RNA extraction kits and reverse transcription kits were purchased from Axygen (USA). TaqMan microRNA reverse transcription kits were purchased from Thermo (USA). The LabSystem version 1.3.1 microplate reader was bought from Bio-Rad (USA). An ABI 7700 Fast quantitative fluorescent PCR cycler was purchased from ABI (USA). An Ultrapure workstation was purchased from Sutai Purification Engineer (China). A CO2 cell incubator was purchased from Thermo (USA). The DNA amplifier AMP PCR system 2400 was purchased from PE (USA).

GC cell line SGC-7901 culture and grouping

SGC-7901 and MGC-803 cell lines preserved in liquid nitrogen were resuscitated for incubation. SGC-7901 cells at 2nd to 8th generation of log-growth phase were used for experiments in 3 groups: the Control group received normal cell culture...
condition; the Scramble group, in which ZEB1 siRNA NC was used to transfect SGC-7901 cells; and the ZEB1 siRNA group that received ZEB1 siRNA transfection into SGC-7901 cells.

Transfection of ZEB1 siRNA into SGC-7901 cells

ZEB1 siRNA NC and ZEB1 siRNA were transfected into SGC-7901 or MGC-803 cells. ZEB1 siRNA sequence was 5’-GCU UAU CGC AUG CAT AUA T-3’ and 5’-UAU ACU AGG GAA UGC GAG AUG-3’. Cells were cultured in 6-well plates until reaching a confluence of 70~80%. ZEB1 siRNA NC or ZEB1 siRNA liposome was added into 200 μl serum-free DMEM medium for mixture, followed by 15-min incubation at room temperature. Lipofectamine 2000 mixture was incubated for 30 min at room temperature. Serum was removed from the culture medium and cells were rinsed gently in PBS, then 1.6 ml serum-free DMEM medium was added into all systems for 6-h incubation at 37°C in a 5% CO₂. Serum-containing DMEM medium was switched for 48-h continuous incubation in further assays.

Real-time PCR analysis of ZEB1 mRNA expression

Total RNA was isolated from GC tumor tissues and adjacent tissues using Trizol reagent. Following the instructions of the test kit, DNA was synthesized by reverse transcription. Primers were designed using Primer Premier 6.0 and synthesized by Invitrogen Biotech (China). The primer sequence for ZEB1 was: Forward-5’-TCTTCTCTAGACCTG-3’ and Reverse-5’-TGGTGTATAGATGGTGTT-3’. GAPDH was: Forward-5’-AGTACCATGCTTGCTGG-3’ and Reverse-5’-TAAATAGGCGGATGCTGTT-3’. Real-time PCR was performed using the following conditions: 56°C 1 min, followed by 35 cycles each consisting of 92°C 30 s, 58°C 45 s, and 72°C 35 s. Data were analyzed and quantified with GAPDH as the internal reference using 2⁻ΔΔCT method.

Western blot analysis of the protein expression of E-cadherin, Vimentin, and Wnt5a

Total proteins were isolated from GC tumors and adjacent tissues, and from SGC-7901 cells. RIPA lysis buffer was mixed with proteinase inhibitor for 15~30 min lysis on ice. Cells were ruptured (5 s, 4 times) and were centrifuged at 4°C at 10 000 g for 15 min. The supernatant was saved, measured by Bradford method, and stored at –20°C. Proteins were separated in 10% SDS-PAGE, followed by transfer to PVDF membranes. Then, 5% defatted milk powder was added into the membrane for blocking the non-specific background. Primary antibody including E-cadherin (1: 1000 dilution), Vimentin monoclonal antibody (1: 1000), and Wnt5a monoclonal antibody (1: 2000) was added and incubated at 4°C overnight. After that, the membrane was washed with PBST. Goat anti-rabbit secondary antibody (1: 2000) was added for 30 min at room temperature for incubation. After PBST rinsing, the membrane was developed for 1 min, followed by X-ray film exposure. The protein band density was measured by Quantity One software.

Analysis of cell proliferation by MTT assay

SGC-7901 and MGC-803 cells at log-growth phase were inoculated into 96-well plates using DMEM medium containing 10% FBS. After 24-h incubation, the supernatant was removed and cells were randomly divided into 3 groups: control, Scramble, and ZEB1 siRNA group, which were treated as detailed above. After 48-h incubation, 20 μl MTT reagent was added into each well. After 4-h incubation, the supernatant was completely removed and 150 μl DMSO was added into each well for 10-min vortexing. After complete resolve of crystal violet, the absorbance (A) values at a wavelength of 570 nm were measured by a microplate reader. All experiments were repeated at least 3 times.

Cell scratching assay

Cell scratching assay was used to measure the change in cell migration. In brief, 5×10⁴ cells were inoculated into 6-well plates and parallel lines were drawn with a marker pen at 0.5~1 cm intervals. The scratch line was made using pipette tips following the pre-drawn lines. Cells were rinsed in PBS 3 times, and detached cells were removed. Serum-free medium was added for culture, and cell migration was observed in all groups after 48 h.

Cell apoptosis assay

Cell culture medium was discarded from all groups. Cells were digested after rinsing in cold PBS, and were collected for transferring into a centrifuge tube for 1000 g centrifugation for 5 min. The supernatant was discarded and cells were re-suspended in 1× binding buffer. We mixed 100 μl cells with 5 μl Annexin V-FITC and 5 μl PI staining buffer, followed by gentle mixing and 15-min room temperature incubation in the dark. We added 300 μl 1× binding buffer for mixture and flow cytometry assay.

Statistical processing

All data were analyzed by SPSS 11.5 software and are shown as mean ± standard deviation (SD). The t test was performed for comparison of differences between 2 groups, and analysis of variance (ANOVA) was used for comparing differences among multiple groups. Enumeration data were tested by χ² test. Pearson analysis was used for correlation analysis. P<0.05 indicated statistical significance.
Results

ZEB1 expression in GC

Real-time PCR was performed to analyze the expression of ZEB1 in GC. Results showed significantly elevated ZEB1 levels in GC tumor tissues (P<0.05 compared to tumor-adjacent tissues, Figure 1A).

Relationship between ZEB1 expression and clinical features and prognosis of GC

ZEB1 expression level was not associated with sex or age of GC patients, but was correlated with tumor differentiation grade, lymph node metastasis, and TNM pathological staging of tumors (P<0.05, Table 1).

To evaluate the relationship between ZEB1 expression and prognosis of GC, we analyzed whether ZEB1 expression affected...
the overall survival of GC patients. GC patients were divided into a low ZEB1 expression and a high ZEB1 expression group based on the median level of ZEB1 expression, and we found a significantly different overall survival in these 2 groups of patients, with longer overall survival observed in patients with low ZEB1 expression (P<0.05, Figure 1B).

Effects of ZEB1 siRNA on ZEB1 expression in GC cells

Real-time PCR was performed to analyze changes in expression of ZEB1 in GC cell lines SGC-7901 and MGC-803 after transfecting ZEB1 siRNA. Results showed that ZEB1 mRNA was significantly inhibited in GC cells (P<0.05 compared to the control group). Transfection of ZEB1 siRNA-negative control (NC) into GC cells did not significantly change ZEB1 mRNA expression compared to the control group (Figure 2A).

The effect of ZEB1 on GC cell proliferation

MTT assay was conducted to assess the influence of siRNA interference of ZEB1 on the proliferation of GC cell lines SGC-7901 and MGC-803 and revealed that transfection of ZEB1 siRNA into SGC-7901 and MGC-803 cells decreased ZEB1 expression, and significantly suppressed the proliferation of SGC-7901 and MGC-803 cells (P<0.05 compared to the control group, Figure 2B).

Influence of ZEB1 on GC cell migration

Cell scratching assay was performed to evaluate the influence of siRNA interference of ZEB1 on the migration of GC cell lines SGC-7901 and MGC-803. Results demonstrated that after transfecting ZEB1 siRNA, the migration of SGC-7901 and MGC-803 was remarkably inhibited (P<0.05 compared to control group, Figure 3).
The effect of ZEB1 on GC cell apoptosis

The effect of ZEB1 siRNA interference on the apoptosis of GC cell line SGC-7901 was analyzed by flow cytometry and revealed that transfecting ZEB1 siRNA into SGC-7901 and MGC-803 cells significantly facilitated apoptosis of SGC-7901 and MGC-803 cells (P<0.05 compared to control group, Figure 4).

Effect of ZEB1 on EMT of GC cells

Western blot analysis was conducted to analyze the effect of ZEB1 siRNA interference on EMT of GC cell lines SGC-7901 and MGC-803. The transfection of ZEB1 siRNA into SGC-7901 and MGC-803 cells increased E-cadherin and decreased Vimentin expression (P<0.05 compared to control group, Figure 5).

As Wnt5a has been demonstrated to be a key regulator of the epithelial-mesenchymal transition and cancer stem cell properties in human gastric carcinoma cells [16], we also measured the effect of ZEB1 siRNA interference on the expression of Wnt5a in SGC-7901 and MGC-803 GC cells and showed that transfection of ZEB1 siRNA into SGC-7901 and MGC-803 cells inhibited Wnt5a expression (P<0.05 compared to control group, Figure 5).
Figure 5. Effects of ZEB1 regulation on EMT of GC cells. (A) Western blot for the effect of siRNA interference of ZEB1 on the expression of Vimentin, E-cadherin, and Wnt5 in GC cell lines SGC-7901 and MGC-803. (B, C) the expression of Vimentin, E-cadherin, and Wnt5. * P<0.05 compared to control group. # P<0.05 compared to scramble group.

Discussion

GC has a complicated pathogenesis mechanism involving genetics, environment, and diet [7]. Due to the atypical symptom of GC at early stage, patients usually cannot obtain timely diagnosis and some patients are already at terminal stage, causing tremendous difficulty for clinical treatment [17]. The onset, progression, and metastasis of GC is a dynamic process involving several factors. Under the control of multiple genes and factors, EMT plays important roles in GC migration and metastasis [18,19]. Therefore, the identification of GC-related molecular targets can benefit the management of GC onset and progression. This study analyzed the expression of ZEB1 in GC tumor tissue and adjacent tissues, and confirmed upregulation of ZEB1 in GC tissues. Further analysis for its correlation with clinical pathology of GC confirmed that ZEB1 expression level was correlated with differentiation grade, lymph node metastasis, and tumor pathology stage of GC, indicating the important role of ZEB1 in onset and progression of GC. Further studies on effects on GC cells showed that ZEB1 downregulation inhibits GC cell proliferation, facilitates cell apoptosis, and inhibits migration.

Due to strong correlation between ZEB1 and EMT-derived malignant tumors [20], ZEB1 may affect E-cadherin expression through specifically binding to E-cadherin, leading to the morphological and identity change of epithelial cells that are originally interconnected by polarity to transform into mesenchymal cells. Those cells further move freely among the cellular matrix to facilitate EMT formation [21,22]. Our study demonstrated that downregulation of ZEB1 can facilitate E-cadherin expression of GC cells and decrease Vimentin expression, indicating the involvement of ZEB1 in mediating EMT onset. The Wnt5a signal pathway is closely related with tumor migration and EMT occurrence, and can facilitate tumor progression via modulating polarity and directional migration of cells [23,24]. This study demonstrated that downregulation of ZEB1 can inhibit Wnt5a expression in GC cells, thus inhibiting EMT onset. However, the functional mechanism of ZEB1 in vivo remains unclear and requires further investigations to identify novel targets of GC treatment.
Conclusions

GC tissues have increased ZEB1 expression, which is correlated with clinical pathology of GC. Downregulation of ZEB1 expression can inhibit GC cell proliferation and migration via modulating Wnt5a to facilitate apoptosis, thus impeding EMT occurrence.

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Conflict of interest

None.

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