

Original Article

High expression of ZBED1 affects proliferation and apoptosis in gastric cancer

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Abstract: Background: ZBED1 (zinc finger BED-type containing 1) is a transcription factor. However, its expression, role and clinical significance in cancer are unclear. The purpose of this study is to investigate the expression of ZBED1 and its effect on the proliferation of gastric cancer (GC). Methods: Quantitative PCR was used to detect the mRNA level of ZBED1 in GC tissues and normal gastric tissues. Proliferation and colony formation assays were conducted when ZBED1 was expressed ectopically or silenced by constructed vectors. Moreover, chemotherapy-drug induced apoptosis rates were examined by flow cytometry when ZBED1 was expressed ectopically or silenced. Results: The mRNA level of ZBED1 was significantly elevated in 10 out of 11 cases of GC tumor tissue specimens. Results of our analysis derived from a public clinical microarray database suggest that a high expression of ZBED1 predicts a poor outcome. ZBED1 promotes cell proliferation and colony formation. Moreover, ZBED1 decreased the chemosensitivity of GC cells. Conclusions: ZBED1 expression is up-regulated in GC cells. ZBED1 promotes proliferation and decreases the chemosensitivity of GC cells. ZBED1 may be a potential therapeutic target and predictive biomarker in gastric cancer.

Keywords: Gastric cancer, ZBED1, proliferation

Introduction

Gastric cancer (GC) is among the most prevalent malignancies in the world and remains the second leading cause of cancer-related death, which has posed a serious threat to human health and survival [1, 2]. The high mortality rate of GC mainly results from its delayed diagnosis due to the absence of early-stage clinical symptoms and prognostic and predictive biomarkers [3, 4]. GC is considered as a heterogeneous disease carrying various genetic mutations [5]. Therefore, it is necessary to investigate the biological characteristics of GC carcinogenesis and progression and to provide potential biomarkers to improve GC diagnosis, prognosis and treatment prediction.

ZBED1 (zinc finger BED-type containing 1), also known as DREF (DNA replication-related element binding factor), was first identified as a transcription factor in *Drosophila* [6]. It binds to box A and positively regulates a set of genes involved in DNA replication and cell proliferation, such as the proliferating cell nuclear antigen and DNA polymerase α and dE2F [7-9].

The underlying transcriptional regulation mechanism of various housekeeping genes by ZBED1 may be that ZBED1 possesses a small ubiquitin-like modifier (SUMO) ligase activity, specifically SUMOylate Mi2 α , that leads to the dissociation of Mi2 α from the gene loci [10]. Currently, the functions of ZBED1 in cancers, especially gastric cancer, are unclear.

In this study, we show that ZBED1 expresses higher in clinical GC tissues compared with adjacent non-cancerous tissues, and higher ZBED1 levels predict a poor outcome. We also show that ZBED1 could promote GC cell proliferation and inhibit chemotherapeutic agents induced apoptosis *in vitro*. Moreover, we provide the first evidence that ZBED1 may play an oncogene role in gastric cancer.

Materials and methods

Cell culture and sample collection

Six human GC cell lines (AGS, BGC-823, HGC27, MKN45, MGC-803, and SGC-7901) were cultured in Dulbecco's modified Eagle's medium

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(DMEM, Life Technologies) supplemented with 10% fetal bovine serum (Gibco). All cell lines were incubated in a humidified chamber with 5% CO₂ at 37°C. 11 fresh primary GC tissues and 11 paired normal gastric tissues were obtained at the time of diagnosis before any therapy from the Jiangxi Cancer Center (Nanchang, China). The clinical processes were approved by the ethics committees of Jiangxi Cancer Center and a signed informed consent was collected from each patient.

Construction of stable lines overexpressing ZBED1

Vector constructions were performed as described previously [11, 12]. Full-length human ZBED1 cDNA was cloned into pSin-puro vector, and ZBED1 was verified by DNA sequencing (Focus Bioscience Co., Ltd, Nanchang, China). The primers were as follows: 5'-CCCGGACG AATTCTTCGAAATGGAGAATAAAAGCCTGGAGAG-3' (forward), 5'-TGCGGATCACTAGTGCTAGCC TACAGGAAGCTGCTGTCCCTAATG-3' (reverse). pS-in-puro delivering ZBED1 or an empty vector were co-transfected with pMD.2G and psPAX2 into HEK-293T cells for 48 hours. The recombinant virus was collected and added to HGC27 and MKN45 cells cultured with 8 µg/ml polybrene for 24 hours, the stable lines were selected with 1 µg/ml of puromycin for two weeks (Focus Bioscience Co., Ltd, Nanchang, China).

Construction of stable lines silencing ZBED1

Vector constructions were performed as described previously [11, 12]. The vectors expressing either ZBED1 short hairpin RNAs (shRNAs) or a scrambled shRNA were generated using the plko.1-puro vector according to the manufacturer's instructions (Focus Bioscience Co., Ltd, Nanchang, China). The targets of human ZBED1 shRNA#1, and #2, are 5'-GTGG CCATGTACATGCTCTAT-3', and 5'-GCCTCCCGTC AAGAAGCTCAT-3', and respectively. ZBED1 shRNAs or scrambled shRNA were co-transfected with pMD.2G and psPAX2 into HEK-293T cells for 48 hours. The recombinant virus was collected and added to SGC7901 cells cultured with 8 µg/ml polybrene for 24 hours. The stable lines were selected with 1 µg/ml of puromycin for two weeks (Focus Bioscience Co., Ltd, Nanchang, China).

RNA extraction and quantitative RT-PCR (qPCR)

Quantitative RT-PCR was performed as described previously [12, 13]. The total RNA of the tissue specimens was isolated using Trizol (Life Technologies) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript® RT reagent Kit with gDNA Eraser. Quantitative PCR was performed for the detection of ZBED1 mRNA using Premix Ex Taq™ II (Takara). The sequences of primers were as follows: for ZBED1: 5' GTGTG GAAGTATTTTCGGCTTCG 3' (forward), 5' ATTCCTC GGGGTGGTTCTTCT 3' (reverse); for GAPDH: 5' ACAGTCAGCCGCATCTTCTT 3' (forward), 5' GAC AAGCTTCCCCTTCTCAG 3' (reverse). The PCR condition was: 95°C for 10 minutes, followed by 40 cycles of 95°C for 20 s, 60°C for 20 and 70°C for 30 s.

Cell proliferation and cell viability assays

In vitro cell proliferation was assessed using the CCK-8 assay, and the cells were seeded in 96-well plates at a density of 1,000 cells/well and incubated for 1, 2, 3, 4, or 5 days. Ten microliters of the CCK-8 reagent (Cell Counting Kit-8, Beyotime, China) was then added to each well, followed by incubation for 1.5 h. The absorbance value (OD) of each well was measured at 450 nm. For each experimental condition, 6 wells were used.

Colony formation assay

The cells were plated in the 6-well culture plates at 250 cells per well. Each group had 3 wells. After incubation for 15 days at 37°C, the cells were washed twice with PBS and stained with Giemsa solution. The number of colonies containing ≥50 cells was counted under a microscope.

Western blotting

As we described previously [11, 14, 15], the cells were collected and lysed by a RIPA buffer (150 mM NaCl, 0.5% EDTA, 50 mM Tris, 0.5% NP40) and centrifuged for 20 min at 12000 rpm at 4°C. Fifty micrograms of harvested total protein was loaded, separated in 8% sodium dodecyl sulfate-polyacrylamide gradient gels and transferred onto PVDF membranes followed by blocking with 5% non-fat milk for 2

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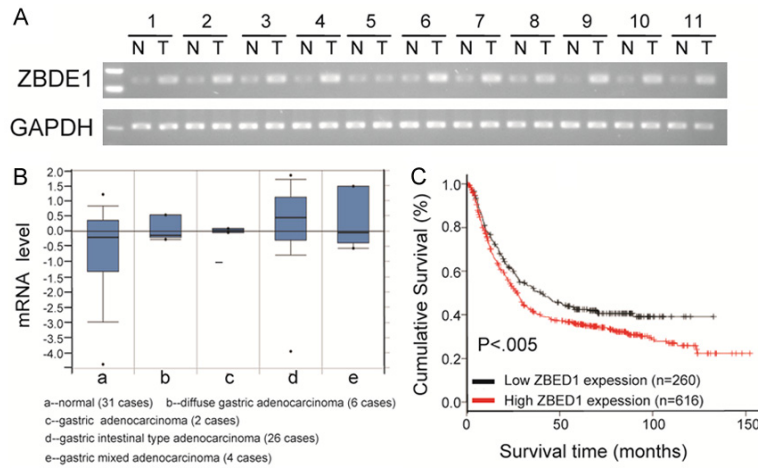


Figure 1. ZBED1 was up-regulated in primary human gastric cancer tissues. Compared to their non-tumor counterparts (N), the mRNA level of ZBED1 was examined in GC tumor tissues (T) by semi-quantitative RT-PCR (A). In addition, the mRNA levels of ZBED1 in gastric adenocarcinoma and non-tumor tissues were analyzed in online databases (B). Furthermore, an overall survival curve was generated based on the ZBED1 expression statuses from 876 GC tumor tissues in a large public clinical microarray database (C).

hours at room temperature. The membranes were incubated with a primary antibody and a horseradish peroxidase-conjugated secondary antibody, and then detected using the ECL chemiluminescence system (Pierce, Rockford, USA). The antibodies against CA IX, mTOR, and p-mTOR (Ser2448) were from Cell Signaling Technology. The antibody against Tubulin was from Bioworld Technology.

Flow cytometry

Briefly, the apoptosis analysis was conducted with an Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, China) according to the manufacturer's protocol. The percentage of apoptotic cells was determined using FACS flow cytometer equipped software (BECKMAN), as we described previously [14].

Clinical data set analysis

The correlations of ZBED1 and the clinical characteristics or survivals of GC patients were analyzed using the online KMplot database (<http://www.KMplot.com>). All statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS). All values from the in vitro assays are expressed as the mean \pm SD of at least three independent experiments or replicates. *P* values were calculated using the two-tailed Student's test. A *p* value <0.05 is considered statistically significant.

Results

ZBED1 expression is up-regulated in GC cells

To evaluate the expression level of ZBED1 in GC, we detected the mRNA level of ZBED1 in GC tissues and normal gastric tissues using quantitative PCR (qPCR). As shown in **Figure 1A**, the mRNA level of ZBED1 was significantly elevated in tumor tissue specimens versus the corresponding adjacent non-tumor tissue specimens from logically confirmed gastric cancer patients in 10 out of 11 cases. Furthermore, we analyzed its expression in GC and normal gastric tissues using online databases, as shown in **Figure**

1B, ZBED1 is significantly up-regulated in GC tissues compared with the normal tissues ($P < 0.01$), which is consistent with our results of detection.

To investigate whether ZBED1 expression can serve as a novel prognostic marker for GC patients, based on the ZBED1 expression levels reported in a large public clinical microarray database of GC, GC samples were subdivided into two groups, and the associated overall survival (OS) was analyzed. Individuals with high ZBED1 levels were observed to exhibit shorter OS than those with low levels (**Figure 1C**). Collectively, these results indicate that ZBED1 is up-regulated in GC and that its high expression predicts a poor outcome in GC.

Overexpression of ZBED1 promotes cell proliferation and colony formation in GC

Given that ZBED1 expression is up-regulated in GC, we carried out further research on the effects of ZBED1 on regulating the biological behaviors of GC cells. We detected the expression of ZBED1 in GC cell lines (AGS, BGC-823, HGC27, MKN45, MGC-803, SGC-7901) by quantitative real-time PCR (**Figure 2A**). Based on the expression level of ZBED1 in these GC cells, we constructed stable cell lines with ectopic expressions of ZBED1 in HGC27 and MKN-45 gastric carcinoma cells (**Figure 2B, 2C**). As shown in **Figure 2D, 2E**, colony formation

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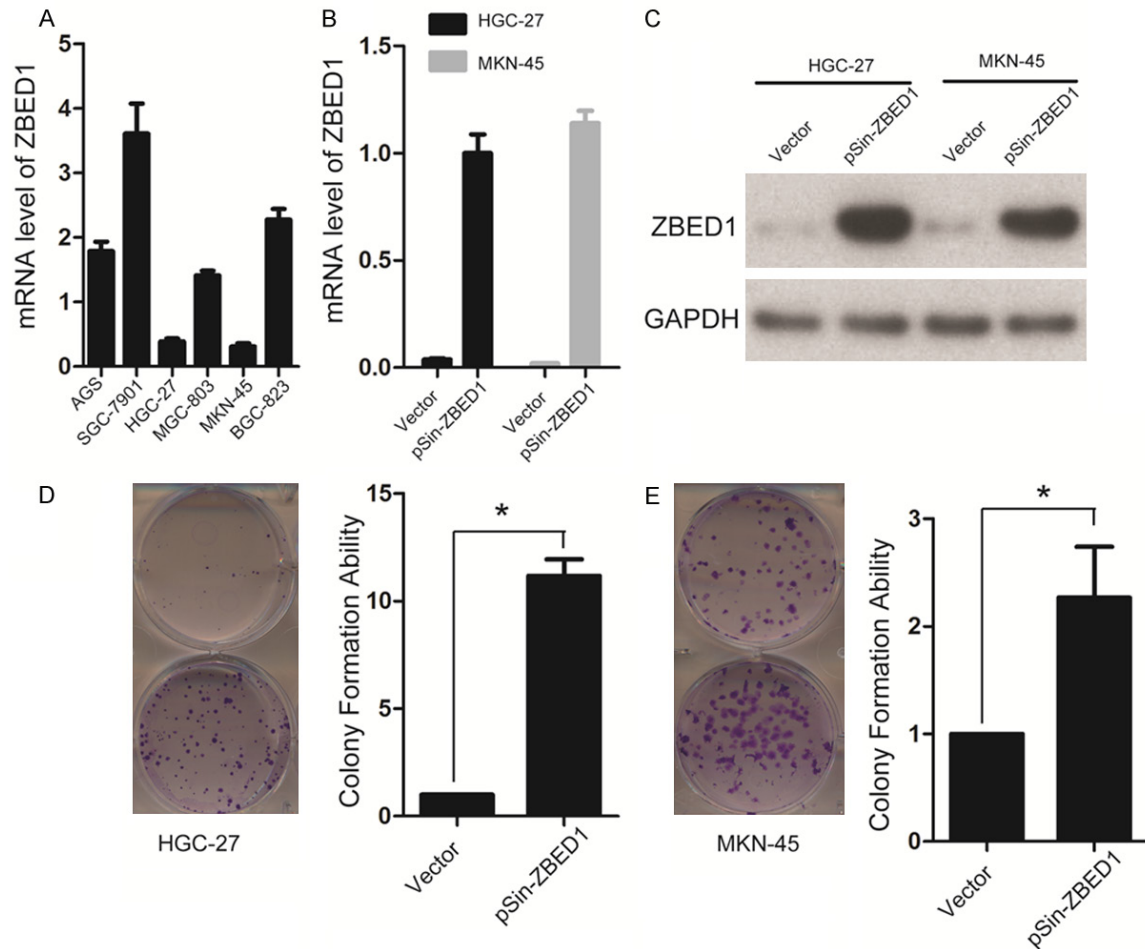


Figure 2. Ectopic expression of ZBED1 promotes proliferation and colony formation of GC cells. The mRNA level of ZBED1 was detected in six human GC cell lines including AGS, BGC-823, HGC27, MKN45, MGC-803, and SGC-7901 by semi-quantitative RT-PCR (A). The ectopic expression of ZBED1 was constructed in HGC27 and MKN45 GC cells, determined by semi-quantitative RT-PCR (B) and Western blotting (C). The colony formation ability was examined as indicated (D, E). The results are expressed as the mean \pm SD of three independent experiments, * $P < 0.05$, using an independent Student's *t* test.

assays indicated that the ectopic expression of ZBED1 significantly promoted cell proliferation and colony formation abilities.

Silencing ZBED1 suppresses cell proliferation and colony formation in GC

To further confirm the effect of ZBED1 on GC cells, two specific shRNAs targeting ZBED1 were used. As shown in **Figure 3A**, the expression of ZBED1 was dramatically decreased in the SGC7901 GC cell line when transfected with the two specific shRNAs. During cell culture we noticed that the knocked-down cells proliferated slower than the control counterparts, and the colony formation assay showed that silencing ZBED1 decreased colony formation ability (**Figure 3B**).

ZBED1 reduces the chemotherapy sensitivity of GC cells to 5-Fluorouracil

Because ZBED1 promotes GC cell proliferation, we next investigated whether ZBED1 affects the apoptosis of GC cells. The ectopic expression of ZBED1 was constructed in MKN-45 gastric cancer cells transfected with ZBED1-pSin or a vector. ZBED1 was knocked-down by two specific shRNAs in SGC-7901 gastric cancer cells. And then a Western blot was performed to examine the apoptosis-related protein marker c-PARP after the GC cells were subjected to 5-FU for 36 hours. The knockdown or overexpression of ZBED1 increased or decreased the cleavage of PARP (a well-known marker of cell apoptosis), respectively (**Figure 4A**). Moreover, a propidium iodide-annexin V assay was per-

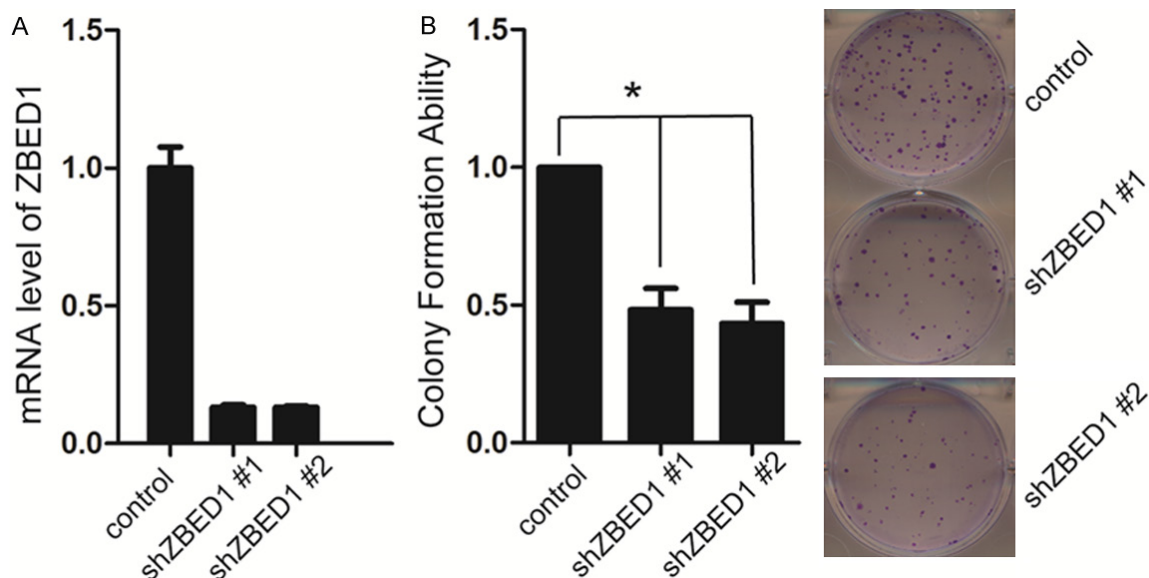


Figure 3. ZBED1 depletion suppresses the cell proliferation and colony formation of GC cells. Two specific shRNAs were used to silence ZBED1 in SGC-7901 gastric cancer cells (A). The colony formation ability was examined when ZBED1 was knocked-down (B). The results are expressed as the mean \pm SD of three independent experiments, * $P < 0.05$, using independent Student's t test.

formed to evaluate the effect of ZBED1 on the apoptosis ratios of GC cells after they were subjected to 5-FU for 36 hours. The forced expression of ZBED1 decreased the sensitivity of GC cells to 5-FU and vice versa, as shown in **Figure 4B, 4C**. Taken together, these findings suggest that ZBED1 inhibits the chemotherapy sensitivity of GC cells.

Discussion

Although it has been reported that ZBED1 regulates cell proliferation by positively regulating a set of human ribosomal protein genes [16], its expression and function in cancers have not been characterized. Our results revealed that ZBED1 was overexpressed in gastric cancer. High ZBED1 expression was shown to be associated with poor outcomes in patients with GC, indicating that a high level of ZBED1 was likely to present a poor prognostic value. Furthermore, we provided the first evidence that ZBED1 can promote GC cell colony formation and reduce the chemotherapy sensitivity by inhibiting GC cells apoptosis in vitro. Thus, we identified a pro-tumorigenic role of ZBED1 in gastric cancer.

Recent molecular profiling studies have pushed the traditional histological classification systems of GC forward molecularly, based on clas-

sification schemes. Gastric cancer is characteristic of DNA hypermethylation, amplification and mutations of tumor-associated genes including PTEN, SMADA, CDKN2A, ARIDA, JAK2, ERBB2, PD-L1 and PD-L, and so on [17, 18]. The oncogenic activation of tumor-associated signal pathways often occurs alongside pro-tumorigenic aberrations in other signaling networks [19]. The combination of these tumor-associated molecules may improve the effectiveness of early diagnosis, evaluation of chemotherapy response, therapeutic efficacy, and the minimizing of treatment-related toxicity in clinical practice. Our investigation provides the first evidence of ZBED1's role in gastric cancer. The crosstalk between ZBED1 and the tumor-associated signaling pathway needs further study.

In addition, the association between high ZBED1 expression and the poor outcome of gastric cancer patients indicates that ZBED1 may be a useful potential predictive biomarker for GC. Gastric cancer remains an aggressive and poorly understood malignancy with a heterogeneous presentation and tumor biology. GC harbors a diverse range of genetic mutations and tends to carry high mutational loads [5, 17, 20]. Until now, the complete surgical resection of localized GC with the removal of adjacent lymph nodes is the only known cure, and patients in

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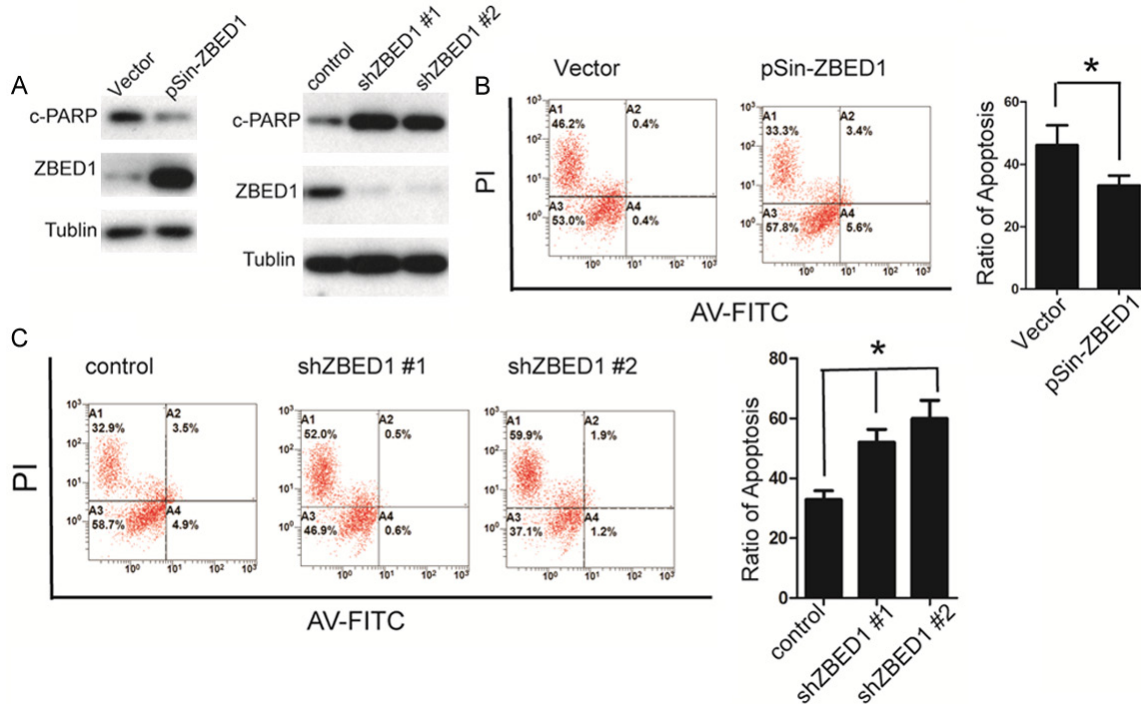


Figure 4. ZBED1 affects the chemotherapy-drug induced apoptosis of GC cells. The ectopic expression of ZBED1 was constructed in MKN-45 gastric cancer cells transfected with ZBED1-pSin or vector. In addition, ZBED1 was knocked-down by two specific shRNAs in SGC-7901 gastric cancer cells. And then a Western blot was performed to examine the apoptosis related protein marker c-PARP after being subjected to 5-FU for 36 hours (A). At the same time, the apoptotic ratios of MKN-45 (B) and SGC-7901 (C) were analyzed by flow cytometry after being stained and marked with Annexin V-FITC. The results are expressed as the mean \pm SD of three independent experiments, * $P < 0.05$, using independent Student's t test.

pathological stages II or III GC are recommended adjuvant therapy after surgery [21, 22]. For patients with advanced unresectable or metastatic disease, palliative systemic therapy and chemoradiation therapy are the standard treatment strategy [4]. Chemotherapeutic regimens currently being used for GC consist of anthracycline, fluoropyrimidine, taxane, and platinum-based agents. Fluoropyrimidine is an important part of the chemotherapy treatment for gastric cancer. Currently, there is no clinically available predictor of tumor response to the empiric use of these drug combinations. We urgently need biomarkers that are predictive of a chemotherapy response for GC in clinical practice [23]. Studies on the molecular characterization of GC may help identify the opportunity and challenge of developing strategies towards the goal of precision medicine in GC.

Collectively, our investigation may help to figure out the molecular characterization of GC and elaborate on the molecular profiling that can be

translated into therapeutic targets and predictive biomarkers for clinical use in the future.

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Disclosure of conflict of interest

None.

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