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Original Article

Upregulation of human DNA binding protein A (dbpA) in gastric cancer cells

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Aim: To determine the effect of human DNA binding protein (dbpA) on the biology of gastric cancer cells.
Methods: DbpA expression was analyzed by Western blot analysis and immunofluorescence staining in gastric cancer tissues and cell lines. A dbpA-specific small interference (si) RNA was designed and synthesized. Suppressive effect of siRNA on dbpA expression was assessed by real-time RT-PCR. Transwell migration and colony formation assays were used to assess the inhibitory effects of dbpA siRNA on cell invasion and tumorigenesis *in vitro*. Drug-sensitivity was evaluated using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.
Results: The expression of dbpA was upregulated in gastric cancer tissues and cell lines as compared to adjacent normal tissues or gastric epithelial cells. siRNA treatment successfully silenced dbpA expression. Silencing of dbpA increased expression of E-cadherin, decreased expression of adenomatous polyposis coli (APC), β-catenin and cyclin D1, but had no effect on expression of NF-κB. Silencing of dbpA also suppressed cell invasion and colony formation of SGC7901 cells, and enhanced their chemosensitivity to 5-fluorouracil.
Conclusion: DbpA plays an important role in the pathogenesis and development of gastric cancer, and the process involves E-cadherin, APC, β-catenin and cyclin D1. Silencing of dbpA might be a novel therapeutic strategy for increasing chemosensitivity to 5-fluorouracil in gastric cancer.

Keywords: stomach neoplasms; CSDA protein, human; small interfering RNA; fluorouracil

Introduction

Gastric cancer is a common cancer, and it is the second most common cause of death in China[1]. This type of cancer is not sensitive to antitumor therapies such as chemotherapy. Therefore, it is crucial to understand the molecular mechanisms of gastric tumor development.

Human DNA binding protein A (dbpA), a member of the Y-box binding protein family, was first identified as the protein binding to the EGFR enhancer and c-erb-2 promoter[2, 3]. DbpA contains a highly conserved nucleic acid binding domain named cold-shock domain (CSD)[4, 5]. These domains have pleiotropic functions in the regulation of gene transcription and translation, DNA repair, RNA packaging, drug resis-

tance and cellular responses to environmental stimulation[6, 7]. DbpA has been shown to promote cell proliferation by regulating the expression of cyclin D1 and proliferating cell nuclear antigen *in vitro*[8]. Expression of dbpA mRNA is enhanced in transgenic mice by upregulated carcinogenesis-related genes, such as insulin-like growth factor binding protein 1[9]. In addition, studies have indicated that dbpA is positively regulated by E2F1 and is involved in hepatocarcinogenesis *in vitro*[10]. Furthermore, dbpA expression is correlated with the stage of hepatocellular carcinoma and is linked with poor prognosis in patients, these traits make dbpA a good prognostic marker for hepatocellular carcinoma[11].

These observations suggest that dbpA may play a role in the abnormal proliferation of cells and that it is involved in the pathogenesis and development of tumors. Therefore, we examined the role of dbpA in gastric tumor tissues and cell lines. We constructed small interference (si) RNAs to use as

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tools to suppress dbpA expression in SGC7901 gastric tumor cells. Our results indicate that silencing of dbpA can reduce cell invasion and tumorigenesis, and that it can enhance the cells' (chemo)sensitivity to 5-fluorouracil. The silencing effects of siRNAs likely involve gene activity of E-cadherin, adenomatous polyposis coli (APC), β-catenin, and cyclin D1.

Materials and methods
Tissue collection
Fresh gastric tumor and adjacent normal tissues were obtained from 18 patients who underwent surgery between 2007 and 2008 at the Department of General Surgery, First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, Xi'an, China. All gastric cancer cases were clinically and pathologically verified. Standard protocols established by the Hospital's Protection of Human Subjects Committee were followed in this study.

Cell lines and reagents
Preserved samples of gastric cancer cell lines SGC7901, MKN45, MKN28, and BGC823 were available for this study from Institute of Urology, First Affiliated Hospital of Medical College of Xi'an Jiaotong University (Xi'an, China). Samples of the immortalized gastric mucosal epithelial cell line GES-1 were purchased from the Laboratory Animal Research Centre of the Fourth Military Medical University at Xi'an, China. All cell lines except BGC823 were maintained in RPMI1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. BGC823 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA). The rabbit anti-human dbpA polyclonal antibody (COOH terminal) was a gift from Dr Kazunori KAJINO (Second Department of Pathology, Juntendo University, School of Medicine, Tokyo, Japan). Mouse anti-human GAPDH monoclonal antibodies, mouse anti-human β-catenin monoclonal antibodies (WB 1:600), rabbit anti-human E-cadherin polyclonal antibodies (WB 1:800), rabbit anti-human cyclin D1 polyclonal antibodies (WB 1:500), rabbit anti-human APC polyclonal antibodies (WB 1:1 000), and mouse anti-human NF-κB (p65) monoclonal antibodies (WB 1:500) were purchased from Santa Cruz Biotech Inc(Santa Cruz, CA, USA). 5-fluorouracil was purchased from Sigma (St, Louis, MO, USA).

siRNA design and preparation
Three pairs of siRNA oligonucleotides targeting human dbpA with the following sense and antisense sequences were used:
dbpA siRNA1: 5'-UGGAGAGGCUGAGAAUAAATT-3' (sense) and 5'-UUUAUCUUUCAGCCUCUCCATT-3' (antisense);
dbpA siRNA2: 5'-AGACGUGGCUACUAUGGAATT-3' (sense) and 5'-UUCCAUAGUAGCCACGUCUGT-3' (antisense);
dbpA siRNA3: 5'-AAAUCGAAAUGACACCAAATT-3' (sense) and 5'-UUUGGUGUCAUUUCTAUUUUAT-3' (anti-

sense).

All siRNAs were designed using the siRNA selection web server (http://jura.wi.mit.edu/bioc/siRNA), an online design tool for siRNA at WHITEHEAD. The negative control duplexes of siRNA (siRNA-NC) were random sequences and did not target any known mammalian gene according to Genbank searches. All of the siRNA duplexes were chemically synthesized and samples with an optical density of 1.0 of siRNA-NC were labeled with fluorophore FAM by Shanghai GenePharma (Shanghai, China). Each siRNA sample was freeze-dried and reconstituted with RNase-free water to prepare a 20 µM stock solution.

Cell culture and transfection of siRNAs
SGC7901 cells were plated at a density of 1×10^5 cells/mL in 6-well dishes. When cells reached 70%–80% confluence (at approximately 24 h of culture), the cells were treated with siRNAs in serum-free medium with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer.

Total RNA extraction and real-time RT-PCR
Total RNA was extracted from cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Three µg of total RNA were reverse-transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas LIFE SCIENCES, Vilnius, Lithuania) according to the manufacturer's instructions. Specific primers for dbpA were 5'-GCC GCC GCC TCT TTA GCC-3' (forward) and 5'-TTC CGT GGG TTA TTC TTG ATG G-30 (reverse). For β-actin, the oligoenucleotides were 5'-ATC GTG CGT GAC ATT AAG GAG AAG-3' (forward) and 5'-AGG AAG GAA GGC TGG AAG AGT G-3' (reverse). Real-time PCR was performed using the BioEasy SYBR Green I mix (Bioer, Hangzhou, China). Amplification and detection of dbpA mRNA were conducted using the Line-gene K System (Bioer) according to the manufacturer's specifications. The program was run with an initial predenaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. After amplification, melting curve analysis was performed for accurate identification of the PCR amplicons. A standard curve that correlated the cycle number with the amount of product formed was plotted for each sequence of interest. The quantity level of dbpA transcripts was then normalized to that of β-actin.

Western blotting
Western blotting was performed as described previously[12]. Briefly, a total protein extract (30 µg) isolated from tissues or cultured cells was separated by 12% SDS-PAGE, and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk in tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1.5 h at room temperature. The membrane was then incubated with rabbit anti-human dbpA polyclonal antibodies (COOH terminal) at a dilution of 1:800 overnight at 4 °C, and washed six

times, and then hybridized for 1 h with the secondary antibody at a dilution of 1:3000. Immunodetection was performed using the ECL detection system (Pierce, Rockford, IL, USA). Protein loading equivalence was assessed by the expression of GAPDH.

Immunofluorescence staining

Immunofluorescence staining was conducted with minor modifications as described previously[13]. Briefly, cell monolayers on cover slips were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.2% Triton X-100 for another 15 min, and then blocked with goat serum for 30 min. The cells were then incubated with a rabbit anti-human dbpA polyclonal antibody (COOH terminal) at a dilution of 1:150 overnight at 4 °C. After washing three times with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit antibody at 37 °C for 1 h. The fluorescence staining intensity and intercellular location were then examined using a fluorescence inverted microscope (Olympus IX50, Tokyo, Japan).

Soft agar clonogenic assay

After transfection with siRNA or control siRNA (siRNA-NC) for 24 h along with no treatment and mock cells were collected and dispersed into a suspension of single cells in growth medium. Cells were resuspended in complete medium containing 0.3% low melting temperature agarose (Promega, Madison, WI, USA) and were seeded at a concentration of 1000 cells/mL in 0.6% low melting temperature agarose in a 6-well dish. The dishes were incubated for 3 weeks at 37 °C in a 5% CO_2 atmosphere until colonies formed. Colonies larger than 100 μmol/L were counted. Descriptive statistics (mean± SD) on colony sizes were calculated.

Matrigel invasion assay

Cells were suspended in 0.2 mL of serum-free medium and were added to the upper chamber of a transwell plate (Costar, Corning, NY, USA) precoated with Matrigel (Invitrogen). Culture medium with 10% fetal bovine serum (FBS) was plated in the lower chamber. Cells in the invasion chambers were incubated for 24 h. The cells on the upper surface of the Matrigel filter were completely removed using cotton swabs. The cells that traversed the membrane pores and spread to the lower surface of the filters were stained with 5% Giemsa solution for visualization. The average number of invading cells was acquired in three representative fields using microscopy (100×magnification).

Drug sensitivity assay *in vitro*

Drug sensitivity was evaluated using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. SGC7901 cells were plated in 96-well plates with six replicates per dose at 3×10^3 cells/well and incubated for 24 h at 37 °C in their normal culture conditions. Cells were treated with siRNA or control siRNA for 4 h. The medium was removed and cells were exposed to 5-fluorouracil (1 mg/L).

After incubation for 24, 48, 72, and 96 h, followed by the addition of 20 μL 5 mg/mL MTT (Sigma) to each well, the cells were incubated for another 4 h. Next, 150 μL dimethylsulfoxide (DMSO) (Sigma) was added to lyse the cells, the mixture was placed on a shaker for 10 min. The absorbance at 490 nm was measured on a microplate reader (Bio-Rad).

Statistical analysis

All data are presented as mean±standard deviation (SD). The statistical analysis was performed using Student's *t*-test and $P<0.05$ indicates significant differences between the entities being compared.

Results

Overexpression of dbpA in gastric cancer tissues and cell lines

We tested dbpA expression in gastric cancer tissues and adjacent normal tissues using Western blot analysis. Sixteen out of 18 cases showed a significantly higher level of dbpA in the tumor tissue compared to adjacent normal tissues, in the other two cases, no difference was observed (Figure 1A). In addition, dbpA was detected in four gastric cancer cell lines SGC7901, MKN45, MKN28, and BGC823 and in one immortalized gastric mucosal epithelial cell line (GES-1). As shown in Figure 1B, dbpA expression in the four gastric cancer cell lines was much higher than in GES-1. Furthermore, dbpA in SGC7901 and MKN45 was more abundant than in BGC823 and MKN28.

siRNA silencing of dbpA at both the RNA and protein levels

The SGC7901 cell line was chosen for siRNA treatment because of its abundant dbpA expression and because it is easily cultured. Total RNA and protein were isolated and analyzed by real time RT-PCR and Western blot, respectively, 48 h after transfection. Compare to control, the expression of dbpA was obviously suppressed by all of 3 pairs of siRNA oligonucleotides at both protein (Figure 2A) and mRNA levels (Figure 2B). We used immunofluorescence analysis to determine the location of dbpA in SGC7901 cells, dbpA was located exclusively in the cytoplasm of gastric cells (Figure 2C). Signal intensity of dbpA in SGC7901 cells was stronger than that in cells treated with dbpA specific siRNA. Among the different siRNA species, siRNA2 was the most effective in decreasing signal intensity of dbpA and was therefore selected for further investigations.

Silencing of dbpA alters the expression of downstream genes

To explore the mechanism of dbpA in the pathogenesis and development of gastric cancer, we used siRNA2 to transfect SGC7901 cells, 48 h later we isolated total protein and detected several potential downstream proteins (β-catenin, E-cadherin, APC, cyclin D1, and NF-κB) using Western blot analysis. Protein expression of β-catenin, APC and cyclin D1 decreased after treatment with dbpA siRNA, whereas protein expression of E-cadherin was induced. Thus, we suggest that the the effect of dbpA on gastric cancer is mediated by these proteins in contrast, NF-κB expression was not affected, indicating that
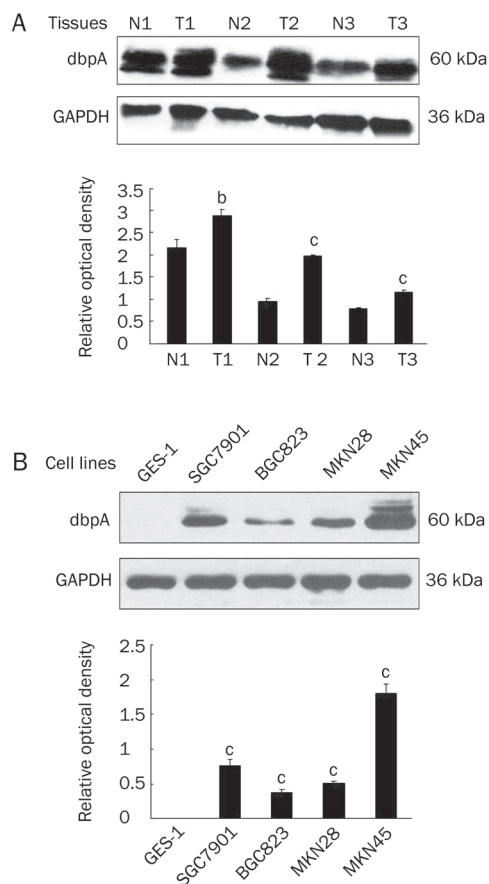
Figure 1. DbpA protein in human gastric cancer tissues and cell lines. (A) Protein isolated from gastric cancer and adjacent non-tumorous tissues from 18 patients was separated by SDS-PAGE and subjected to Western blot analysis using an anti-human dbpA polyclonal antibody and mouse anti-human GAPDH monoclonal antibodies. Upper panel: three representative pairs of gastric cancer tissues (T) and adjacent normal gastric mucosa (N) are presented. Lower panel: Semi-quantification of densitometry. The relative dbpA protein expression level was normalized against GAPDH. $^bP<0.05$, $^cP<0.01$, for the comparison between N and T. Values are the mean±SD of three independent experiments. (B) Upper panel: Western blot analysis of four human gastric cancer cell lines (SGC7901, BGC823, MKN45, MKN28) and the non-cancer cell line GES-1 using the same antibodies as in panel A. GAPDH was used as an internal control. Lower panel: Semi-quantification of densitometry. The relative dbpA protein expression level was normalized against GAPDH. $^cP<0.01$ for the comparison between gastric cancer cell line and GES-1. Values are the mean±SD of five independent experiments.

the molecule is outside of the dbpA pathway (Figure 3).

Silencing of dbpA suppresses cell invasion and colony formation of SGC7901 cells

To determine the role of dbpA in the development of cancer, the effect of siRNA knock-down in SGC7901 tumor cells was analyzed using cell invasion assays. As shown in Figure 4A, there was no significant difference at 24 h in the number of migrated cells between siRNA treated cells and control cells.
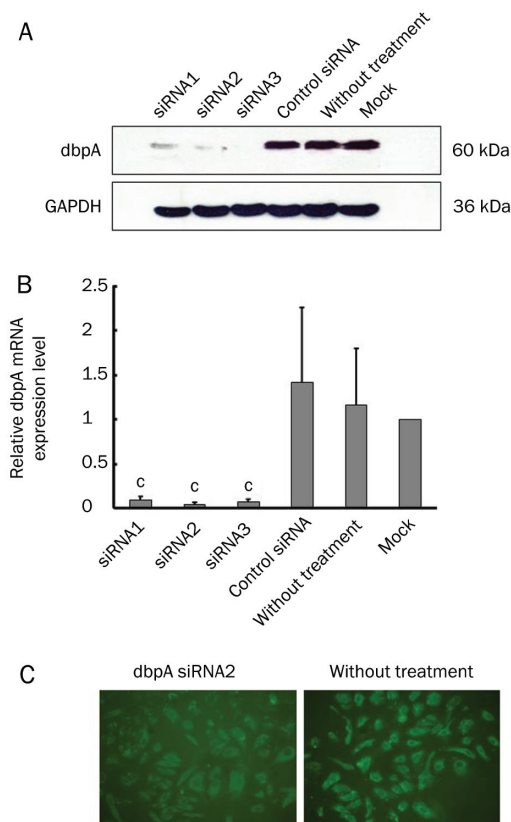

Figure 2. Levels of dbpA protein and mRNA in the SGC7901 cell line treated with different siRNAs. (A) Protein isolated from SGC7901 cells treated with siRNA (1–3) or control siRNA were separated by SDS-PAGE and subjected to Western blot analysis using an dbpA-antibody and GAPDH-antibodies. One representative of six separate experiments is shown. (B) The amount of transcripts was determined by real time RT-PCR. Values represent the mean±SD of six separate experiments. $^cP<0.01$ for the comparison of between siRNA (1–3) and control siRNA (C) Immunofluorescent staining of dbpA in SGC7901 cells treated with/without siRNA.

However, significant differences were observed after 48 h (Figure 4A, 4B). This result indicated that dbpA silencing suppressed the invasion activity of SGC7901 cells. We also performed colony formation assays with SGC7901 cells. Colony formation was inhibited after treatment with dbpA siRNA2 (Figure 5).

Silencing of dbpA increases chemosensitivity to 5-fluorouracil

SGC7901 cells were seeded in 96-well plates and then treated with siRNA or control siRNA. The cells were then exposed to 5-fluorouracil (1 mg/L) for 24, 48, 72, and 96 h. At each time point the growth rates of cells were assessed by MTT assays. The growth of cells treated only with siRNA or treated with 5-fluorouracil alone were inhibited, although not significantly when compared to non-treated cells (Figure 6). However, the combination of siRNA2 with 5-fluorouracil (1 mg/L) significantly inhibited growth at both 72 h and 96 h. We conclude that the chemosensitivity to 5-fluorouracil was enhanced by
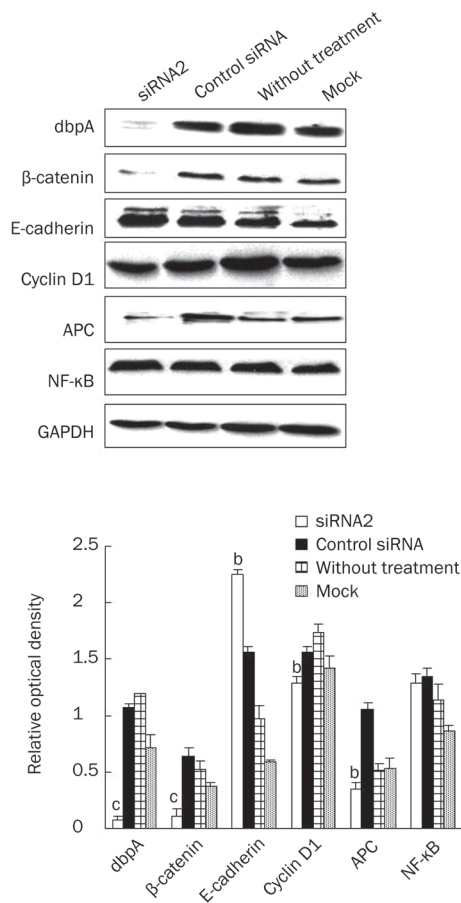
Figure 3. Effect of dbpA on the expression of target genes. Upper panel: Cells were treated with dbpA-siRNA2 for 48 h. The amount of protein was analyzed by Western blot using corresponding antibodies. Lower panel: Semi-quantification of densitometry. The relative dbpA protein expression level was normalized against GAPDH. $^{b}P<0.05$, $^{c}P<0.01$ for the comparison between siRNA2 and control siRNA. Values are the mean±SD of three independent experiments.
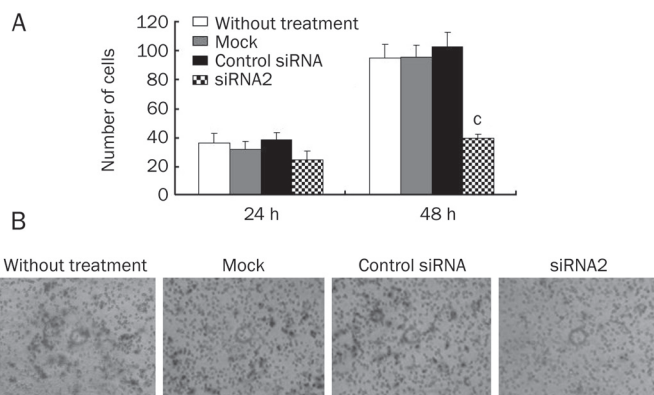

Figure 4. Silencing of dbpA reduces the invasion of SGC-7901 cells. (A) Quantification of invasion activity of cells treated with siRNA2 at 48 h compared to 24 h. (B) Visualization of cell invasion at 48 h. Values represent the mean±SD of three separate experiments. $^{c}P<0.01$ for the comparison between siRNA2 and control siRNA.
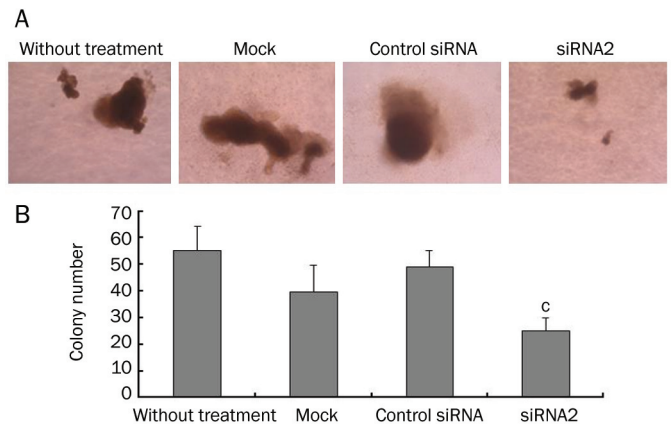

Figure 5. Silencing of dbpA inhibits colony formation of SGC7901 cells. (A) Visualization of colony formation of cells with different treatments. Cells were placed in media in six-well plates containing soft agar and incubated for 20 days, after which the number of foci >100 μm was counted. (B) Quantification of colony formation assays. Data represent mean±SD of three independent experiments. $^{c}P<0.01$ for the comparison between siRNA2 and control siRNA.
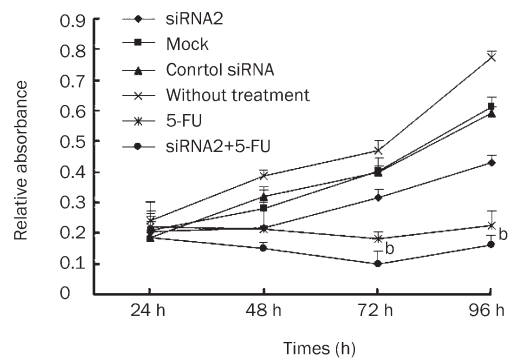

Figure 6. Silencing of dbpA affects chemosensitivity of SGC7901 cells to 5-fluorouracil. Cells were placed in 96-well plates with media, treated with dbpA siRNA2, and then treated with 5-fluorouracil (1 mg/L). Values represent the mean±SD of at least three separate experiments. 5-FU: 5-fluorouracil. $^{b}P<0.05$ for the comparison between siRNA2 with 5-fluorouracil and siRNA2 or 5-fluorouracil only.

dbpA siRNA 2 in SGC7901 cells.

Discussion

Our results show that dbpA protein is much higher in gastric cancer tissues than in adjacent normal tissues. The protein is also expressed at a much higher level in the gastric cancer cell lines SGC7901, MKN45, MKN28, and BGC823 compared to its expression in GES-1, an immortalized gastric mucosal epithelial cell line. Treatment of SGC7901 cells with dbpA siRNA suppressed the expression of dbpA at both the mRNA and protein level. Silencing of dbpA with siRNA upregulated the expression of E-cadherin, downregulated the expression of APC, β-catenin and cyclin D1, and had no effect on NF-κB.

Phenotypically, silencing of dbpA suppressed cell invasion and colony formation of SGC7901 cells and increased their sensitivity to 5-fluorouracil, indicating an important role of dbpA in the biology of gastric cells.

Previous studies indicated that dbpA can regulate cell proliferation in epithelial cells[9]. Our results suggest that dbpA might be involved in abnormal proliferation, not only in gastric cells, but also in gastric cancer tissues. Because abnormal expression of adhesion molecules is a frequent event in gastric cancer, and because dysfunction of E-cadherin, APC and β-catenin frequently occur in gastric cancer[14, 15], we hypothesized that dbpA might have an effect on cell proliferation by regulating adhesion molecule protein (E-cadherin, APC, and β-catenin), and cell cycle related protein cyclin D1 in SGC7901 cells. Our results on siRNA silencing of dbpA provide initial evidence for this function. As a transmembrane protein, E-cadherin plays a key role in the maintenance of intercellular adhesion junctions[16]. E-cadherin interacts with β-catenin or γ-catinen to form a complex with α-catinen, APC, and β-catinen. The complex degrades β-catinen in the cytoplasm[17, 18]. If β-catinen fails to be degraded, it subseqeuntly is translocated to the nucleus, which leads to cyclin D1 gene transcription[18]. Previous studies indicated that lymphoid-enhancing factor (LEF) could bind and regulate the cyclin D1 gene, a key regulator of cell proliferation by promoting the cell cycle[19]. Our results showed that silencing of dbpA causes upregulation of E-cadherin, which can form a stable E-cadherin-catinen complex, resulting in strengthened cell-cell adhesion. Silencing of dbpA might promote the combination of APC and β-catinen, causing downregulation of APC and β-catinen, and thus maintaining a low cytoplasmic level of β-catinen to inhibit its nuclear translocation. An increase in E-cadherin has a similar effect[17]. The suppression of cyclin D1 transcription can result in inhibition of cell proliferation. We did not find a close link between dbpA and NF-κB, indicating that the function of dbpA might be independent of NF-κB.

Transwell migration and colony formation assays using SGC7901 cells demonstrated the inhibitory effects of dbpA siRNA on cell invasion and tumorigenesis *in vitro*. This, combined with the above results, suggests that dbpA may positively regulate initiation and metastasis in gastric cancer cells.

The YB-1 protein, the prototype of the Y-box binding protein, is 52% identical to dbpA, has been reported to be associated with drug resistance in both cancer cell lines and human tumor tissues[20]. Antisense RNAs against YB-1 increases sensitivity to cisplatin in MCF7 cells[21]. This finding indicates that a decreases in YB-1 protein expression may contribute to the sensitivity of cells to chemotherapy in malignant disease. Therefore, we used a low dose of 5-fluorouracil to treat the SGC7901 cells to determine if dbpA also has an effect on chemosensitivity. Our studies showed that dbpA siRNA combined with a low dose of 5-fluorouracil affected the inhibition of cell proliferation. Silencing of dbpA using siRNA increased the sensitivity of gastric cancer cells to 5-fluorouracil. Like YB-1, dbpA might be also associated with drug resistance in gastric cell lines, and might be a potential target gene for anti-resistance treatment.

In conclusion, we demonstrated that dbpA plays an important role in the pathogenesis and development of gastric cancer. Silencing of dbpA reduced cell invasion and tumorigenesis but enhanced the chemosencsitivity of cells to 5-fluorouracil, these findings may provide a novel strategy for the enhancement of chemosensitivity of gastric cancer. The effect of dbpA in gastric cancer might be exerted via E-cadherin, APC, β-catinen, and cyclin D1.

Author contribution
Guo-rong WANG conceived and designed the basic experiments, acquired data, and analyzed and interpreted data, and helped drafting the paper; Yan ZHENG conceived and designed the clinical experiment, collected data from patient, analyzed and interpreted data, and helped drafting the paper. Xiang-ming CHE designed the basic experiment, analyzed data, critically evaluated the manuscript for important intellectual content, and corrected the manuscript. Xin-yang WANG, Jia-hui ZHAO, Kai-jie WU, and Jin ZENG designed the clinical experiment, analyzed data, critically evaluated the manuscript for its important intellectual content. Chen-en PAN and Da-lin HE designed the clinical experiment, analyzed data, critically evaluated the manuscript for its important intellectual content, and corrected the manuscript.

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