

RNAi-mediated downregulation of DNA binding protein A inhibits tumorigenesis in colorectal cancer

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Abstract. DNA binding protein A (dbpA) belongs to the Y-box binding protein family and has been reported to play an important role in carcinogenesis. Our previous study demonstrated that the knockdown of dbpA in gastric cancer cells inhibited cell proliferation by modulating the cell cycle. However, the role of dbpA in human colorectal cancer (CRC) remains unclear. In this study, immunohistochemical (IHC) staining and clinicopathological parameter analysis were employed to detect dbpA expression in 44 paired CRC samples and 7 CRC cell lines. Lentivirus-mediated short hairpin RNA (shRNA) was used to silence dbpA, and the effects of dbpA knockdown on cell proliferation were determined by MTT assay, colony formation assay and flow cytometry. Furthermore, a xenograft model was established to observe tumor growth *in vivo*. Functional analysis indicated that dbpA was overexpressed in the CRC tissues and cell lines, and a high dbpA expression was associated with the depth of invasion ($p < 0.001$), the degree of differentiation ($p < 0.001$), lymphatic metastasis ($p < 0.001$) and vessel invasion ($p < 0.001$). The suppression of dbpA expression resulted in decreased cell proliferation *in vitro* and tumor growth *in vivo*, and it induced cell cycle arrest and promoted the apoptosis of the CRC cells. As a whole, our findings illustrate the crucial role of dbpA in colorectal tumorigenesis. Thus, dbpA may be used as a novel and potent therapeutic target in CRC.

Introduction

Colorectal cancer (CRC) is among the most common malignancies and is becoming a leading cause of cancer-related mortality worldwide (1). Although the efficacy of multiple

drug treatments, surgical treatments and chemotherapy have extensively improved (2), CRC is still considered as a complex and difficult disease to deal with (3). In recent years, studies have focused on specific molecular targets, such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) to predict the progression of CRC (4,5). However, due to the heterogeneous characteristics of CRC, there is still controversy regarding the optimal treatment strategy for CRC. Thus, in order to develop more effective targeting agents against CRC, the identification of novel molecules is urgently required (6).

Human DNA binding protein A (dbpA), a member of the Y-box binding protein family, contains a highly conserved DNA binding domain, named the cold shock domain (CSD) (7,8). This family of proteins appears to play a critical role in cell proliferation and growth, transcriptional and translational regulation, DNA replication, drug resistance, the cell cycle and malignancy (9,10). dbpA can bind to EGFR, proliferating cell nuclear antigen, thymidine kinase and DNA polymerase (11,12) to participate in cellular activities. Previously, Tobita *et al* suggested that dbpA induced carcinogenesis by regulating the expression of cellular genes, such as insulin-like growth factor binding protein-1 (IGFBP-1) and carbonic anhydrase 3 (Car3) in dbpA-transgenic mice (13). Furthermore, dbpA has been reported as a prognostic marker for the advanced stages of and for the poor prognosis of hepatocellular carcinoma by enhancing cell proliferation and transformation (14,15). Our previous study demonstrated that dbpA played a crucial role in the development of gastric cancer by regulating the expression of E-cadherin, β -catenin, adenomatous polyposis coli (APC) and cyclin D1 (16). These findings indicate the significance of dbpA in the development of malignant diseases.

In the present study, to illustrate the role of dbpA in CRC, the expression of dbpA in CRC tissues and cell lines was examined. The effects of dbpA on CRC cells were investigated by lentivirus-mediated short hairpin RNA (shRNA) interference both *in vitro* and *in vivo*. Our findings indicate that dbpA is a vital driver of human CRC and that the knockdown of dbpA markedly reduces cell proliferation *in vitro* and decreases tumorigenesis *in vivo*. Our study may provide scientific evidence for the further development of reliable molecular biomarkers for CRC.

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Materials and methods

Clinical specimen collection. Fresh colorectal tumor and adjacent normal tissues were obtained from 44 patients who received surgery from May 2012 to July 2014 at the Department of General Surgery, the Third Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China. We only collected the clinical data of patients that were complete and from patients who had not received any radiotherapy or chemotherapy prior to surgery. All specimens were classified according to the TNM staging system enacted by International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC). The correlations between dbpA expression and clinicopathological parameters in CRC were analyzed by Pearson's Chi-square test. Written informed consent was obtained from all the patients, and this study was approved by the Human Ethics Committee of the Third Affiliated Hospital of Xi'an Jiaotong University and all experiments were performed in accordance with the 1964 Helsinki declaration and its later amendments.

Immunohistochemical (IHC) staining. The colorectal tissues obtained from patients were fixed immediately with 4% paraformaldehyde overnight at 4°C, and were then embedded in paraffin and sectioned (4- μ m-thick) onto slides. The sections were baked at 60°C for 2 h, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were heated in 20 mmol/l sodium citrate (pH 6.0) at 95°C for 15 min. The slides were treated then with 3% H₂O₂ to block endogenous peroxidase activity. Subsequently, the slides were incubated overnight at 4°C with the rabbit polyclonal anti-dbpA antibody (ab48952; Boster Biological Technology, Ltd., Wuhan, China) at a 1:500 dilutions. Subsequently, the sections were incubated with HRP polymer (1:500; ab6721; Boster Biological Technology, Ltd.) for 30 min at room temperature and DAB mix (Tiangen Biotechnology, Beijing, China) was applied for staining. Two independent pathologists blinded to the patient data evaluated the scores of the IHC results. The scoring criteria was based on the percentage of dbpA-positive cells in the tumor tissue as follows: - (<10%) was considered as no staining; + (11-40%) was considered weak staining; ++ (41-70%) was considered moderate staining; and +++ (71-100%) was considered strong staining. If a discrepancy was existed between the scores, the specimens would be re-examined by both researchers together.

Cell lines and cell culture. The human CRC cell lines, LoVo, SW480, RKO, HT-29, DLD-1, SW1463 and SW620, were obtained from the First Affiliated Hospital of the Medical College of Xi'an Jiaotong University. The normal colorectal mucosa cell line, FHC, and the 293T cell line were purchased from the Animal Center of the Fourth Military Medical University, Xi'an, China. All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂.

dbpA RNAi lentivirus design and packaging. The shRNA sequences were designed for dbpA as follows: sense, 5'-AGA CGUGGCUACUAUGGAATT-3' and antisense, 5'-UUCCAU AGUAGCCACGUCUGT-3' in accordance with our previous study (16). The negative control shRNA were randomly sequenced and homology with the dbpA sequence was avoided

using the Blast website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The shRNAs were then cloned into the pGV115 entry vector (Invitrogen Life Technologies, Carlsbad, CA, USA) and identified by PCR and DNA sequencing. Lentiviruses were produced and packaged using the 293T cells following co-transfection with pGV115 entry vector carrying dbpA-shRNA (shRNA-dbpA-Lv) or scrambled shRNA (shNC-dbpA-Lv) and pHelper plasmids, according to the lentivirus packaging protocol (Genechem Co., Ltd., Shanghai, China). The lentivirus contained the green fluorescent protein (GFP) and viral titers were then measured by GFP-positive cell counts under the observation of a fluorescence microscope (Olympus, Tokyo, Japan).

Infection of SW620 cells with shRNA-dbpA-Lv. The SW620 cells were seeded at 5x10⁵ cells/well in 6-well plates and incubated for 24 h at 37°C with 5% CO₂. The cells were infected with shRNA-dbpA-Lv (GenePharma, Shanghai, China) at a multiplicity of infection (MOI) of 10. The knockdown efficiency of dbpA was evaluated at 72 h by the percentage of GFP-positive cells (>50%). Cells were also infected with the shNC-dbpA-Lv plasmid as negative controls (NC). Cells transfected with empty vector were used as controls (CON). After infection at 120 h, the cells were harvested and prepared for reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis.

RNA extraction and RT-qPCR. RNA from the cell cultures was extracted using TRizol reagent (Gibco Life Technologies, Beijing, China) according to the manufacturer's instructions. First strand cDNA was synthesized from 5 μ g of total RNA using SuperScript II RT 200 U/ μ l (Invitrogen). dbpA mRNA expression was evaluated by qPCR on an ABI 7500 Real-Time PCR System (Applied Biosystems Life Technologies, Beijing, China) with SYBR-Green PCR core reagents. GAPDH was used as the input reference. The sequences of the primers used were as follows: dbpA sense, 5'-CGTCGCTCACGGGTCTTA-3' and antisense, 5'-CCTGA AGTTGTGCTCCCTCT-3'; GAPDH sense, 5'-TGACTTCAACAGCGACACCCA-3' and antisense, 5'-CACCTGTTGCTGTAGCCAAA-3'. RT-qPCR was performed in triplicate and the results are presented as the Ct values, defined as the threshold PCR cycle number at which an amplified product is first detected. The mean Ct value was calculated, and the Δ Ct value was determined as the mean Ct value for the target gene minus the mean Ct value for GAPDH.

Western blot analysis. The cells were collected, washed with PBS and then lysed in lysis buffer containing 100 mM Tris-HCl, pH 7.5, 0.5% NP-40 and protease inhibitor cocktail. The supernatant was collected after centrifugation (at 20,000 x g, for 15 min, at 4°C) and the protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The cell lysate samples (40 μ g) were separated by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride filter (Immobilon; Millipore, Bedford, MA, USA). After blocking with 5% milk, the filter was incubated overnight with a primary rabbit polyclonal anti-dbpA antibody (ab48952; Boster Biological Technology, Ltd.) at 1:500 dilutions for 1 h. The samples were then incubated with the HRP-conjugated secondary antibodies (ab6721) at 1:1,000, and the bands were detected by enhanced

chemiluminescence (both from Amersham Biosciences, Piscataway, NJ, USA), and then analyzed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The SW620 cells transfected with shRNA-dbpA-Lv or shNC-dbpA-Lv for 1 to 5 days, respectively, followed by diluting and seeding at a density of 2×10^3 cells/ml into a 96-well plate for 24 h. MTT solution was added to each well to a final concentration of 5 mg/ml and culture was continued for 4 h at 37°C. The supernatant mixed with MTT was removed and DMSO was added into each well. The OD data were analyzed once daily for 5 days using an ELISA reader (Bio-Rad Laboratories) at a wavelength of 490 nm.

Colony formation assay. The SW620 cells transfected with shRNA-dbpA-Lv or shNC-dbpA-Lv were plated into 6-well plates (2,000 cells/well) and incubated for 14 days at 37°C, with the medium replaced every 3 days. After cultivating for 14 days, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature and stained with Giemsa (Tiangen Biotechnology) for 15 min. The number of colonies containing >50 cells was counted under a microscope (CKX53; Olympus).

Cell cycle analysis. The effect of shRNA-dbpA-Lv on the cell cycle distribution was determined by flow cytometry. The cells were suspended at the concentration of 1×10^6 and centrifuged at 1,500 rpm for 5 min twice, then resuspended with 100 μ l PBS and fixed with 70% ice-cold ethanol at 4°C overnight. The cells were washed with PBS and resuspended in 1 ml PBS containing 50 μ g/ml PI and 100 μ g/ml RNase A for 1 h in the dark at 4°C. The cell cycle was analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) at 72 h after transduction. The proportions of cells in the G2/M, S, and G0/G1 phases were analyzed using special software FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Apoptosis analysis. SW620 cells transfected shRNA-dbpA-Lv or shNC-dbpA-Lv were collected and washed with PBS twice. Following centrifugation (at 100 x g, for 5 min, at 4°C), the cells were resuspended with 1X staining buffer at the concentration of 1×10^6 cells/ml, and the cells were then dyed with 5 μ l Annexin V-APC in the dark at room temperature for 15 min. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton-Dickinson) and analysis was performed with FlowJo software (Tree Star, Inc.).

Tumorigenicity assay. BALB/C nude mice (4 weeks old) were purchased from the Animal Center of the Fourth Military Medical University. Living SW620 cells were detected and harvested after mixing with 4% trypan blue, then washed and resuspended in PBS at 4°C. The animals were randomly divided into 3 groups (n=10) as follows: the mice subcutaneously injected with dbpA-shRNA (KD), those injected with negative-shRNA (NC) and those injected with normal cells (empty vector-transfected cells, CON). Each mouse was subcutaneously injected with 1×10^6 cells on the right side of axilla. The standard of tumorigenesis was based on a tumor diameter of ≥ 3 mm. Tumor sizes [volume (mm³) = width (mm²) / 2 x length (mm)]

Table I. Expression of dbpA in colorectal tumor and adjacent normal tissue samples.

	Total	DbpA (-)	DbpA (+)	P-value
Tumors	44	9 (20.5%)	35 (79.5%)	0.001 ^a
Normal tissues	44	40 (90.9%)	4 (9.1%)	

^aIndicates statistical significance. dbpA, DNA binding protein A.

were measured using calipers every 7 days. Furthermore, the physical conditions of the mice were monitored as follows: the thickness of subcutaneous fat of the mice was measured by calipers after pinching with fingers; the body weight of the mice was recorded using an electronic scale (Dongyi Biotechnology, Beijing, China). All mice were euthanized by cervical dislocation at 35 days post-inoculation, and the separated and complete tumors were collected and disposed for further analysis. The animal experiment was reviewed and approved by the Animal Care and Use Committee of Xi'an Jiaotong University.

Statistical analysis. Statistical analyses were performed using SPSS 12.0 software. Each experiment was repeated 3 times, unless otherwise indicated. All data are presented as the means \pm standard deviation (SD). Comparisons between groups were carried out using the unpaired Student's t-test. A p-value of <0.05 was considered to indicate a statistically significant difference for all analyses.

Results

Expression of dbpA is increased in CRC tumor samples and varies in CRC cell lines. To explore the role of dbpA in human colorectal tumors, we began by analyzing the expression of dbpA in human CRC tissues. Paired colorectal tumor samples were obtained from 44 patients and dbpA expression was examined by IHC staining. Approximately 79.5% (35/44; p<0.001) of the CRC samples exhibited a positive dbpA expression, while 20.5% (9/44) of the CRC samples were negative for dbpA expression; only 9.1% (4/44) of the adjacent normal tissue samples exhibited a positive dbpA expression (Table I). We also found that dbpA was predominantly expressed in the cytoplasm of the CRC cells rather than in the nucleus or the cytomembrane region (Fig. 1A, top panels). Furthermore, IHC analysis revealed that the level of dbpA expression correlated with the depth of invasion in CRC. With the progression of the depth of invasion of the CRC tumor tissues (T1 to T4), the level of dbpA expression gradually increased (Fig. 1B, bottom panels). Collectively, the dbpA expression levels were significantly higher in the CRC tissues than in the non-tumor tissues, which implies that dbpA may facilitate tumorigenesis in the colon.

In addition, the expression levels of dbpA were assessed in different CRC cell lines, namely in the RKO, SW480, LoVo, DLD-1, SW1463, HT-29, SW620 cells, and in the normal colorectal mucosa cell line, FHC. The results of RT-qPCR revealed that the mRNA expression levels of dbpA varied among the cell lines. In the CRC cell lines, the expression levels from lowest to highest were: LoVo, DLD-1, RKO, HT-29,

SW1463, SW480 and SW620 cells (Fig. 1B); however, no dbpA expression was detected in the normal colorectal mucosa cell line, FHC, which was consistent with the results of western blot analysis (Fig. 1C). The SW620 cells were selected for use in further experiments as they exhibited the highest expression of dbpA. Our findings indicated that dbpA is dominantly expressed in CRC tissues or cell lines rather than in normal colon tissues or cells, and the expression profile differs amongst CRC cell lines.

dbpA expression correlates with different clinicopathological parameters. Considering that the level of dbpA in CRC tissues was significantly higher than that in non-tumor tissues, the correlations between dbpA expression and clinicopathological parameters in CRC were analyzed by Pearson's Chi-square test. We subdivided the CRC-positive cases into 3 groups according to the criteria described in the Materials and methods. The percentage of dbpA weak expression (+) was 28.6% (10/35), moderate expression (++) was 31.4% (11/35), and strong expression was 40.0% (14/35). Correlation analysis demonstrated that the strong expression of dbpA was significantly associated with the degree of differentiation ($p < 0.001$), the depth of invasion ($p < 0.001$), lymphatic metastasis ($p < 0.001$), vessel invasion ($p < 0.001$) and the TNM stage ($p < 0.05$), but not with age, gender, tumor size, stage, type, distant metastasis, or surgical method (Table II). Therefore, we hypothesized that dbpA may be considered as a potential unfavorable prognostic biomarker for patients with CRC.

Effect of shRNA on dbpA expression in SW620 cells. Lentivirus-mediated shRNA interference was applied to suppress the expression of dbpA in the SW620 cells. The efficiency of shRNA-dbpA-Lv or shNC-dbpA-Lv was detected by fluorescent microscopy in the infected cells. The results revealed that a great proportion of cells was infected with the shRNA (Fig. 2A); the infected percentage was >90% (data not shown).

RT-qPCR and western blot analysis were performed to further examine the silencing efficiency of the shRNA against dbpA. At 72 h post-transfection, the mRNA level of dbpA in the shRNA-dbpA-Lv group (KD) was significantly decreased when compared to the shNC-dbpA-Lv (NC) or control (CON) group ($p < 0.01$; Fig. 2B). Western blot analysis also verified that the protein expression of dbpA was markedly decreased in the KD group in comparison to the CON or NC groups (Fig. 2C); no significant difference was observed between the NC and CON group. Therefore, our results indicated that lentivirus-mediated dbpA RNAi successfully and efficiently suppressed dbpA expression in the SW620 cells.

Silencing of dbpA suppresses the proliferation of SW620 cells. MTT assay was employed to assess the effects of the silencing of dbpA expression on the proliferation of the SW620 cells *in vitro*. Cell proliferation was analyzed by MTT assay once daily for 5 days. We found that the cells transfected with shRNA-dbpA-Lv proliferated more slowly than the cells in the NC and CON groups from 3 days onwards. In addition, on the 4th and 5th day, the proliferation rates in the KD group were significantly decreased ($p < 0.05$ and $p < 0.01$, respectively) when compared with the NC group (Fig. 3A).

Table II. Association between dbpA expression and clinicopathologic factors in patient with CRC.

Parameters	The level dbpA expression			P-value
	+	++	+++	
Age (years)				
>60	8	6	9	0.431
≤60	2	5	5	
Gender				
Male	7	7	8	0.708
Female	3	4	6	
Tumor size (cm)				
>4	6	5	4	0.528
≤4	4	6	10	
Degree of differentiation				
Well	7	2	0	<0.001 ^b
Moderate	3	9	5	
Poor	0	0	9	
Invasion depth				
T1	0	0	0	<0.001 ^b
T2	1	0	0	
T3	5	5	2	
T4	4	6	12	
Tumor site				
Colon	5	2	6	0.900
Rectum	5	9	8	
TNM stage				
I+II	5	4	0	<0.05 ^a
III+IV	5	7	14	
Lymphatic metastasis				
N0	7	5	1	<0.001 ^b
N1	3	4	10	
N2	0	2	3	
Distant metastasis				
M0	9	11	11	0.485
M1	1	0	3	
Tumor type				
Infiltrate type	1	0	2	0.788
Ulcerative type	9	9	12	
Protrude type	0	2	0	
Vessel invasion				
0	10	10	4	<0.001 ^b
1	0	1	10	
Surgical method				
Dixon	5	7	7	0.670
Mile's	0	2	7	
Hemicolectomy	5	2	0	

^{a,b}Indicates statistical significance and high statistical significance, respectively. CRC, colorectal cancer; dbpA, DNA binding protein A.

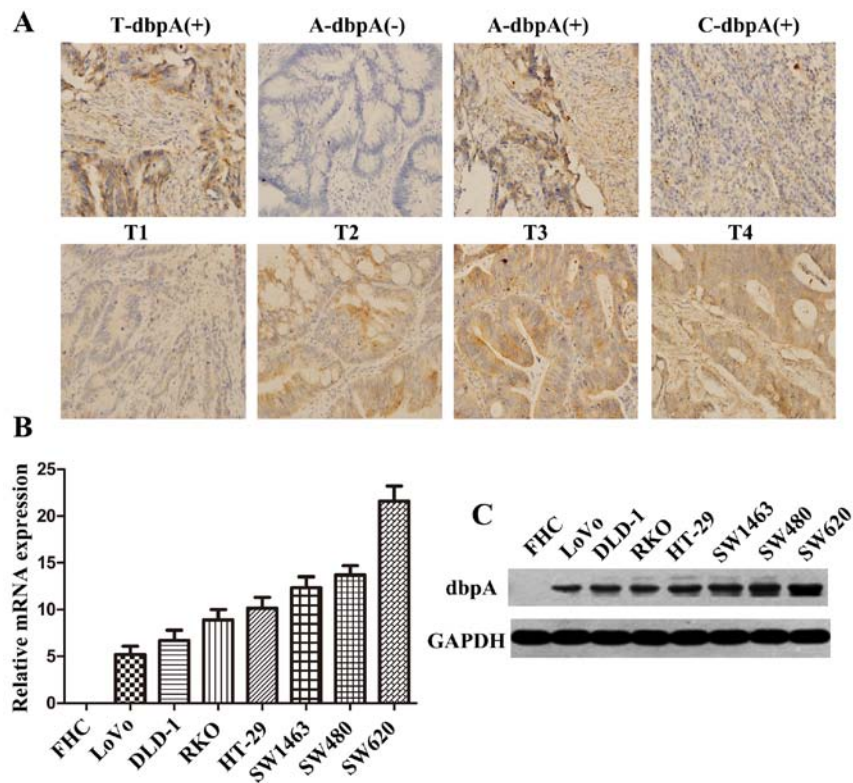


Figure 1. Expression of DNA binding protein A (dbpA) in tissues obtained from patients with colorectal cancer (CRC) and various CRC cell lines using immunohistochemistry (IHC). T, tumor tissues; A, adjacent normal tissues; C, cytoplasm; and T1-T4, invasion depth. (A) dbpA expression in tumors and adjacent normal tissues (x100 magnification). (B) The levels of dbpA expression in different invasion depths (x100 magnification). RT-qPCR analysis of the mRNA expression of the dbpA in various human CRC cell lines. (C) Western blot analysis of dbpA protein levels. GAPDH was used as an internal control. Data are shown as the means \pm standard deviation (SD).

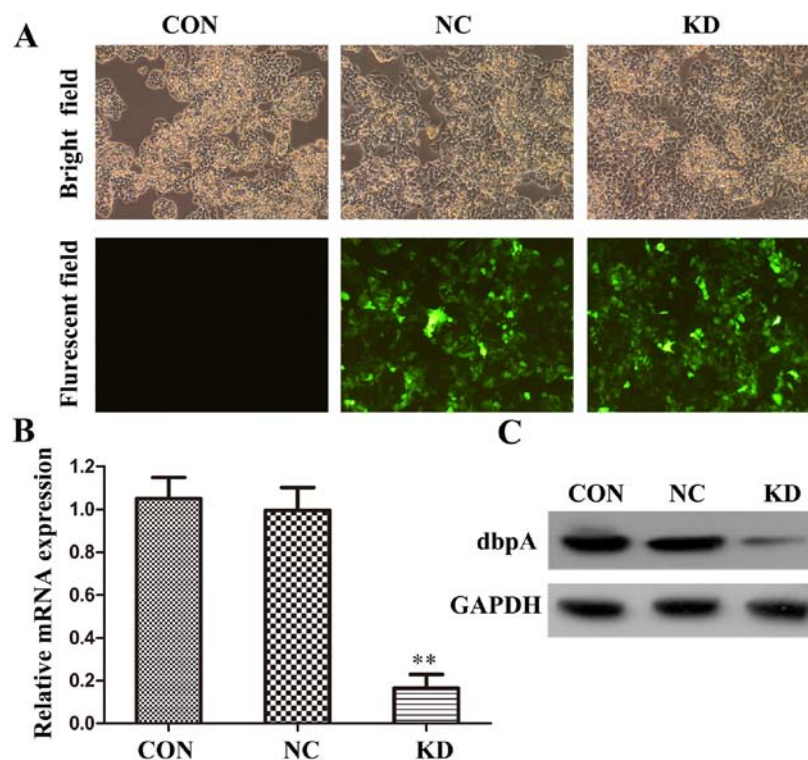


Figure 2. DNA binding protein A (dbpA) knockdown by lentiviral-mediated RNAi. (A) Representative images of SW620 cells transfected with shRNA-dbpA-Lv, shNC-dbpA-Lv, or untransfected cells (CON) for 72 h (x100 magnification). GFP expression indicated that the cells had been successfully infected with the shRNA. (B) RT-qPCR analysis of dbpA mRNA levels in cells treated as in (A). (C) Western blot analysis showing dbpA protein levels in cells treated as in (A). GAPDH was used as an internal control. Data are shown as the means \pm standard deviation (SD); ** $p < 0.01$ vs. NC.

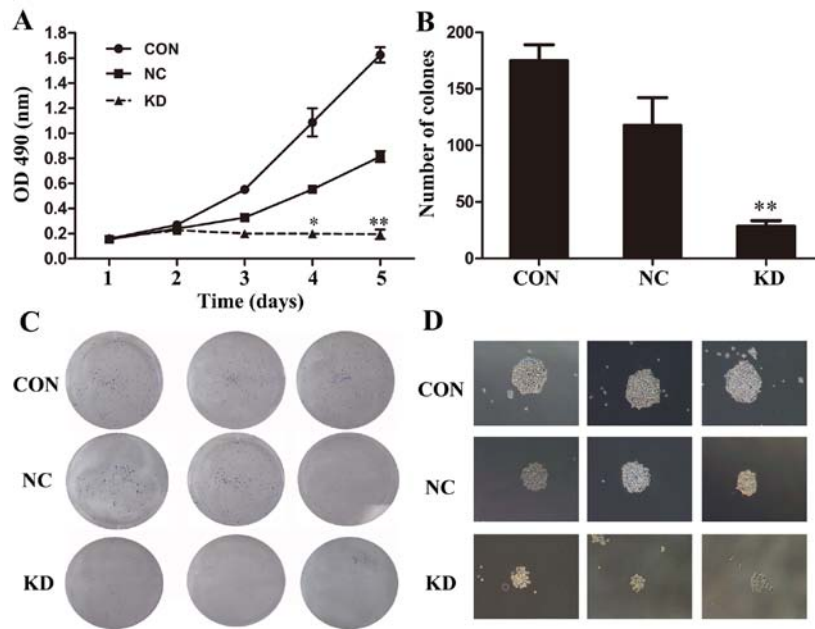


Figure 3. Effect of DNA binding protein A (dbpA) silencing on SW620 cell proliferation. (A) MTT assay measuring cell proliferation of lentivirus-transfected SW620 cells. (B) Quantification of clone number in SW620 cells transfected with shRNA-dbpA-Lv, shNC-dbpA-Lv, or empty vector-transfected cells (CON). (C) Representative images of colony formation assay for SW620 cells in a 6-well plate. (D) Representative images per colony for SW620 cells under a bright microscope. Data are shown as the means \pm standard deviation (SD); * $p < 0.05$, ** $p < 0.01$ vs. NC.

Downregulation of dbpA inhibits the colony-forming ability of SW620 cells. Furthermore, when the SW620 cells were transfected with dbpA-shRNA, negative-shRNA, or the empty vector-transfected cells were incubated for 14 days, the colony-forming capacity of the SW620 cells was determined. The number of colonies was counted following Giemsa staining. Our results revealed that the number cell colonies in the KD group declined significantly ($p < 0.01$) in comparison with the control group (Fig. 3B and C). In addition, the size of the colonies was markedly reduced in the KD group compared with the control group (Fig. 3D). On the whole, these findings indicated that the silencing of dbpA by lentivirus-mediated RNAi efficiently suppressed SW620 cell proliferation *in vitro*.

Silencing of dbpA expression induces SW620 cell cycle arrest. Flow cytometry was adopted to detect cell cycle progression in the dbpA-shRNA-transfected cells. The results revealed that compared with the NC group, the proportion of dbpA-shRNA-transfected cells in the S phase was significantly decreased from 43.92 to 25.19 ($p < 0.01$), while the ratio of cells in the G0/G1 phase was significantly increased from 35.24 to 54.45 ($p < 0.01$), indicating that dbpA-shRNA interfered with the distribution of the cell cycle, leading to cell cycle arrest at the G1 phase in the KD group (Fig. 4). However, no significant difference in the number of cells in the S phase or the G0/G1 phase was observed between the cells in the CON group and NC group. Thus, knocking down dbpA expression suppressed SW620 cell proliferation by leading to cell cycle arrest at the G0/G1 phase.

Knockdown of dbpA expression promotes the apoptosis of SW620 cells. The balance between the cell cycle and cell apoptosis is the key premise to maintain tumorigenesis in

patients (17). Hence, we considered it necessary to examine the effects of dbpA silencing on SW620 cell apoptosis in this study. Apoptosis was determined by FITC-labeled Annexin V/PI double staining and flow cytometric analysis. The results revealed that the silencing of the expression of dbpA in the KD group significantly increased the apoptotic rate when compared with the NC group ($p < 0.01$; Fig. 5A and B). These results indicated that the silencing of dbpA expression promoted cell apoptosis which correlated with the inhibitory effects on cell proliferation.

Silencing dbpA decreases tumorigenesis in CRC in vivo. Since the knocking down of dbpA exerted an inhibitory effect on tumorigenesis *in vitro*, we considered it crucial to assess its effects *in vivo*. A xenograft tumor model was established by subcutaneously injecting normal cells (empty vector-transfected cells), negative control- or dbpA-shRNA-transfected SW620 cells into nude mice. After 14 days, the average tumor volume in the mice in the KD group was markedly smaller than that in the NC or CON groups (Fig. 6A). Furthermore, through monitoring the physical conditions of the injected mice, at 30 days post-treatment, the mice in the NC and CON groups reserved less subcutaneous fat and exhibited more weight loss than the mice in the KD group. At the 35th day, during the process of tumor isolation, we found that in the NC and CON groups, the surfaces of tumors appeared uneven with ulcerations and bleeding, while in the KD group, the tumors appeared more complete and had smooth outer members (Fig. 6C). The average weight of the tumors in the KD group was significantly smaller than that in the NC group, with the tumor inhibition rate at 93.97% ($p < 0.05$) (Fig. 6B and C). However, no difference was observed either in tumor volume or tumor weight between the CON group and NC group. In addition, western

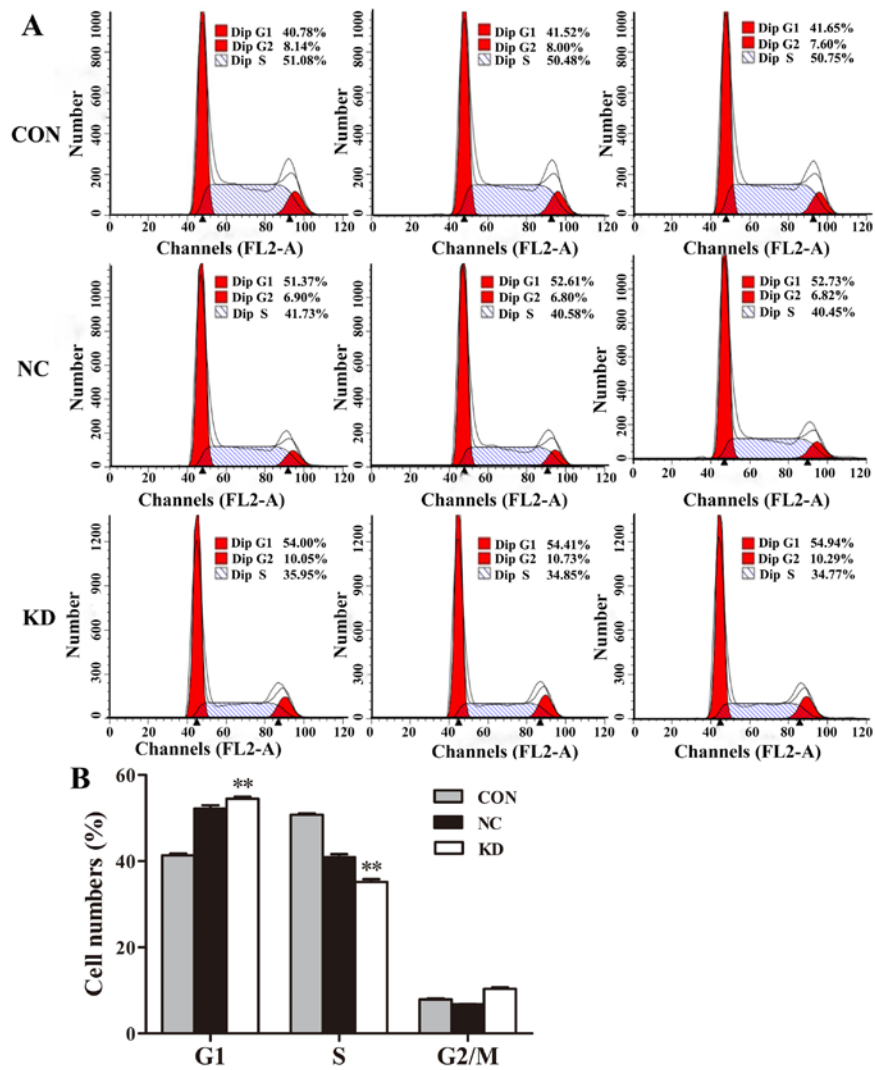


Figure 4. Effect of DNA binding protein A (dbpA) silencing on cell cycle progression in SW620 cells. (A) Representative images of cell cycle analysis at 72 h by flow cytometry. (B) Cell cycle distribution in SW620 cells transfected with shRNA-dbpA-Lv, shNC-dbpA-Lv, or empty vector. Data are shown as the means \pm standard deviation (SD); ** p <0.01 vs. NC.

blot analysis verified that the protein expression of dbpA was downregulated in the KD group in comparison to the NC or CON groups (Fig. 6D). Therefore, our results demonstrated that the suppression of the expression of dbpA in the SW620 cells significantly inhibited tumor growth *in vivo*.

Discussion

dbpA as a member of the Y-box protein family, has aroused great interest in recent years and has been reported to be involved in the development of malignant tumors, such as hepatocellular carcinoma (18) and gastric cancer (16). dbpA regulate the proliferation of epithelial cells, accelerates inflammation-induced hepatocarcinogenesis, and plays a vital role in the pathogenesis and development of gastric cancer (16). Although dbpA is considered as an oncogene in several tumors, its pathogenic mechanisms of action in CRC remain unknown. Hence, in this study, we examined dbpA expression in tumor samples from patients with CRC, and clarified that dbpA was overexpressed in CRC tumor tissues compared to paired adjacent normal tissues. To the best of our knowledge, we provided

the first evidence to verify that dbpA plays an important role in CRC tumorigenesis.

In this study, 35 CRC cases with various TNM stages (I-IV) were selected to investigate the association between the clinical characteristics of CRC tumors and dbpA expression levels. Our findings illustrated that dbpA expression positively correlated with the degree of differentiation, the depth of invasion, vessel invasion and an advanced TNM stage, which are all the key features to accelerate cancer development (19). Furthermore, IHC staining revealed that dbpA was mainly expressed in the cytoplasm and was associated with the progression of CRC in patients. Yasen *et al* had emphasized that both the cytoplasmic expression and the nuclear localization of dbpA, as a significant prognostic marker, was responsible for the advanced stages of hepatocellular carcinoma (14). However, during our study, no dbpA expression was found in the nucleus in our CRC cases. These exiting data indicate that the localization of dbpA may alternate, depending on advanced cancer stages or different cancer types. Further studies with larger sample sizes are warranted in order to deeply investigate the variation of dbpA localization in CRC progression.

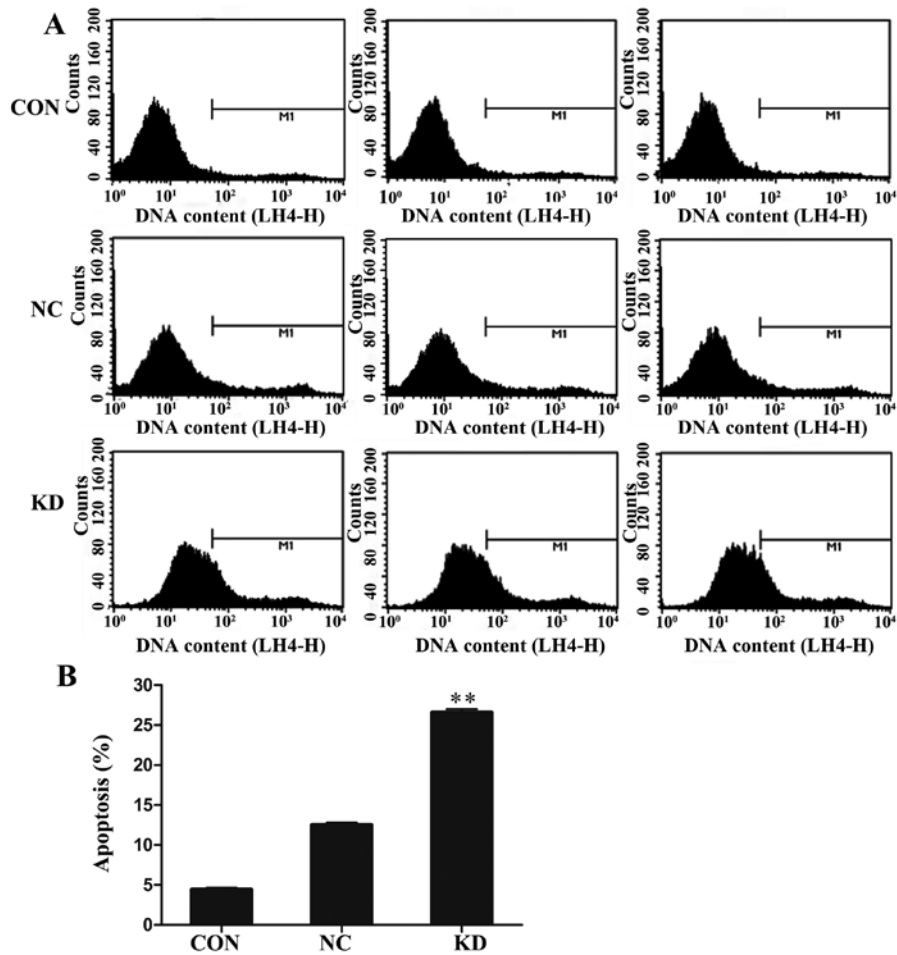


Figure 5. Apoptosis induction in SW620 cells transfected with short hairpin RNA (shRNA)-DNA binding protein A (dbpA)-Lv, shNC-dbpA-Lv, or empty vector. (A) Cell apoptosis was analyzed by flow cytometry. (B) Cell apoptosis in SW620 cells treated as in (A). Data are shown as the means \pm standard deviation (SD); ** $p < 0.01$ vs. NC.

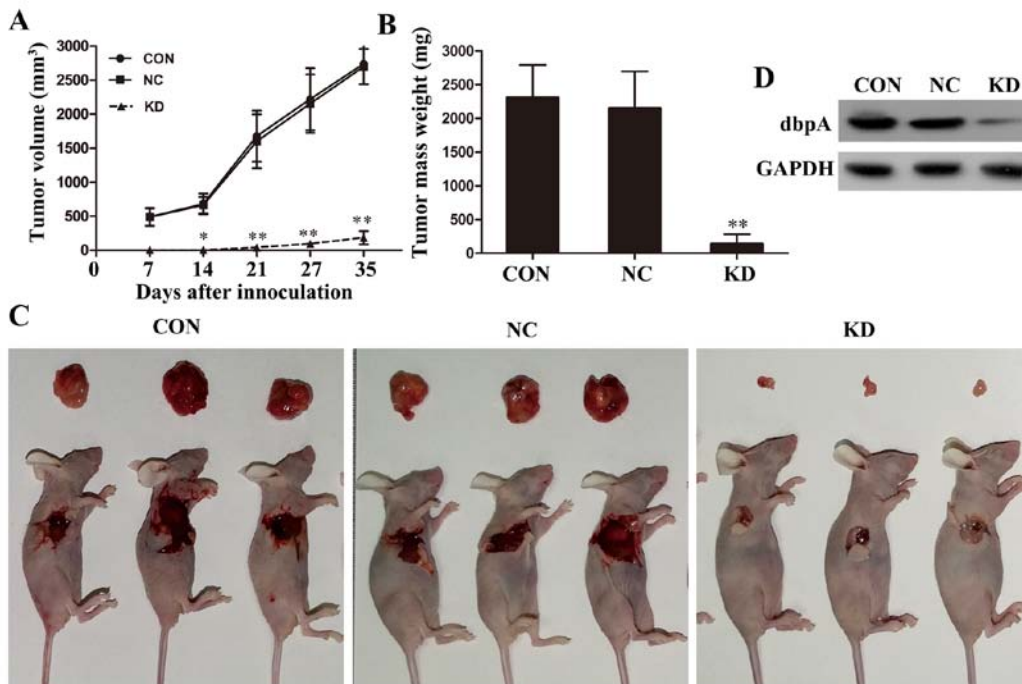


Figure 6. Effect of DNA binding protein A (dbpA) knockdown on tumorigenesis in nude mice. (A) Curve of tumor volume was assessed by caliper measurements. (B) Mass weight of tumor at the 35th day after inoculation. (C) Representative images of mice and tumors from each group. (D) dbpA expression was detected by western blot analysis from isolated tumors. GAPDH was used as an internal control. Data are shown as the means \pm standard deviation (SD); * $p < 0.05$, ** $p < 0.01$ vs. NC.

In the present study, we found that dbpA expression varied in different CRC cell lines. The SW620 cells derived from colorectal adenocarcinoma had the highest expression of dbpA. Thus, for this reason, we selected these cells for use in our subsequent experiments. Small interference RNA and lentiviral vector-mediated RNAi have been extensively used as efficient tools to investigate the specific genes involved in abnormal cell proliferation and are regarded as promising therapeutic methods to deal with malignant tumors (20,21). Lentivirus-mediated shRNA interference was conducted in this study to inhibit dbpA expression in the SW620 cells. As a result, the knockdown of dbpA suppressed SW620 cell proliferation by inducing cell cycle arrest in the G0/G1 phase *in vitro*. Furthermore, through establishing a xenograft model using nude mice, we found that dbpA silencing significantly inhibited tumor growth and tumorigenesis *in vivo*. Therefore, modulating dbpA expression resulted in changes in cell proliferation both *in vitro* and *in vivo*. Abnormal cell proliferation is considered as the key element in the progression of cancer (22). The occurrence of cell proliferation in physiological conditions is always regulated by specific molecular signaling pathways (23). For example, the duplicate progression of a cell is mediated by a group of proteins known as cyclins (24). Cyclins act in part with the cyclin-dependent kinases (CDKs) to phosphorylate key substrates that participate in each phase of the cell cycle (25). Researchers have reported that increased cyclin expression is frequently observed in human malignancies (26). For example, our previous study also proved that the silencing of dbpA suppressed the transcription of cyclin D1 and resulted in the inhibition of the proliferation of gastric cancer cells (16); this may also explain the similar results obtained in our present study.

Apoptosis is a natural way of removing aged cells from the living body (27), whereas under cancer conditions, the uncontrolled regulation of apoptotic signals assists cancer cells to escape from this programmed death and leads to abnormal proliferation (28). Various molecular signaling pathways have been found to be involved in this complex program, such as the B-cell lymphoma 2 (Bcl-2) signaling pathway (29), the heat shock protein signaling pathway (30) and the proteasome pathway (31). In this study, we confirmed that the silencing of dbpA significantly increased the apoptosis of SW620 cells, suggesting that dbpA may regulate apoptosis by activating or inactivating certain signaling pathways. The exact underlying molecular mechanisms responsible for the apoptosis observed by the silencing of dbpA in CRC requires further investigation.

In conclusion, our study confirmed that dbpA was overexpressed in CRC tissues and cell lines. The high expression level of dbpA closely correlated with certain clinicopathological parameters and tumor progression in CRC. Lentivirus-mediated RNAi of dbpA inhibited SW620 cell growth *in vitro* and tumorigenesis *in vivo*. Furthermore, the silencing of dbpA induced cell cycle arrest and promoted cell apoptosis. Hence, our findings illustrate the biological significance of dbpA in tumorigenesis in CRC and provide scientific evidence to develop a novel therapeutic target for the more effective treatment of patients with CRC. Moreover, further investigations are required in order to comprehensively reveal the intrinsic mechanisms of action of dbpA in CRC.

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