

Original Article

CREB5 promotes cell proliferation and correlates with poor prognosis in hepatocellular carcinoma

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Abstract: CAMP responsive element binding protein 5 (CREB5) has been reported to be overexpressed in several types of human cancers and has crucial roles in regulating cell growth, proliferation, differentiation, and the cell cycle. However, the expression and function of CREB5 in hepatocellular carcinoma (HCC) remains unclear. The purpose of this study was to investigate the role of CREB5 in HCC, and its prognostic significance. We measured the expression of CREB5 in 91 specimens of paraffin-embedded HCC tissue by immunohistochemistry and performed a clinicopathological analysis. Gain of function and loss of function assays were used to evaluate the effect of CREB5 on cell proliferation *in vitro*. We found that up-regulation of CREB5 was associated with a poor prognosis, and CREB5 status was an independent prognostic factor. The overexpression of CREB5 increased the proliferation of SMMC-7721 cells, but the knockdown of CREB5 had the opposite effect. The results indicate that CREB5 may be useful when determining a treatment strategy for patients with HCC.

Keywords: CREB5, HCC, proliferation, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and represents the third leading cause of cancer mortality worldwide with a 30% to 40% 5-year postoperative survival rate [1-3]. The high mortality of HCC is mainly related to HCC metastasis, which is a major cause of tumor recurrence after tumor resection [4-7]. However, the molecular mechanism of HCC metastasis remains largely unknown. Therefore, it is critical to identify the mechanisms underlying HCC migration and invasion in order to develop novel therapeutic strategies.

CAMP responsive element binding protein 5 (CREB5) belongs to the CRE (cAMP response element)-binding protein family. The protein specifically binds to CRE as a homodimer or heterodimer with c-Jun or CRE-BP1 to function as a CRE-dependent transactivator [8, 9]. Previous studies suggested that CREB5 might play a key role in the tumorigenesis of colorec-

tal cancer, lung cancer, and ovarian cancer [10-12]. However, the expression and function of CREB5 in HCC remains unknown. Thus, the purpose of this study was to examine the expression of CREB5 in HCC and determine its prognostic significance.

Materials and methods

HCC patient samples

Ninety-one patients with a median follow-up time of 18 months with HCC who underwent hepatectomy at the First Affiliated Hospital of Sun Yat-sen University were included in the study. All patients were diagnosed with primary liver cancer, and none had received preoperative radiotherapy or chemotherapy. The study was carried out under the approval of the Committees for Ethical Review of Research involving Human Subjects of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). All patients, or their guardians or caregivers, provided written informed consent for the use of clinical specimens in medical

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Table 1. Relationship of CREB5 expression with pathological parameters of HCC

Variables	Low CREB5 Group	High CREB5 Group	P
Age (yrs)			
≤ 50	35	12	0.625
> 50	35	9	
Sex			
Male	60	20	.446
Female	10	1	
HBsAg			
Positive	58	19	0.508
Negative	12	2	
Tumor size (cm)			
≤ 10 cm	57	12	0.039
> 10 cm	13	9	
Tumor number			
Solitary	57	14	0.227
Multiple	13	7	
Vascular invasion			
Yes	9	4	0.488
No	61	17	
Encapsulation			
Intact	46	15	0.793
Non-intact	24	6	
Edmonson			
I-II	51	17	0.573
III-IV	19	4	
AFP (μg/l)			
≤ 400	40	13	0.803
> 400	30	8	
TNM stage			
I-II	59	10	0.001
III	11	11	

research. Of the 91 patients (80 men and 11 women; mean age, 51 years; range, 21 to 73 years), 70 were serum hepatitis B virus (HBV)-positive, and 53 had an elevated alpha-fetoprotein level (AFP; ≥ 200 ng/mL) (**Table 1**).

Immunocytochemistry

Paraffin-embedded sections of HCC tissue or adjacent non-cancerous liver tissue were cut and dehydrated in xylene, followed by microwave antigen retrieval. The sections were incubated with an anti-ELMO3 antibody and then analyzed using the Evison/HRP Kit (Dako, CA, USA). The degree of immunostaining was reviewed and scored by two observers accord-

ing to the proportion of positively stained tumor cells and the intensity of staining. Tumor cell proportion was scored as follows: 0 (no positive tumor cells), 1 (< 35% positive tumor cells), 2 (35-70% positive tumor cells), and 3 (> 70% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index was calculated as the product of the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated CREB5 expression by determining the staining index, with scores of 0, 1, 2, 3, 4, 6, or 9. An optimal cutoff value was identified: a staining index score of > 5 was used to define tumors with high CREB5 expression, and a staining index score of < 5 was used to define low CREB5 expression.

HCC cell line

The HCC cell line SMMC-7721 was obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Shanghai, China). The cells were maintained in high-glucose Dulbecco's modified Eagle's media (DMEM) (Gibco, Australia) supplemented with 10% fetal bovine serum (Gibco, Australia).

Plasmid constructs and siRNA transfection

Full-length CREB5 cDNA was amplified and cloned into the pReciever M68 expression vector (FulenGen, Guangzhou, China). The expression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Oligonucleotide siRNA duplex was synthesized by Shanghai Gene Pharma (Shanghai, China). The siRNA sequence of CREB5 was: 5'-GG-UGGAAAUGGACUGGCUTT-3'. The transfection of siRNA in the HCC cells was carried out with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Cell cycle analysis

To evaluate the cell cycle, 1×10⁶ cells were harvested and washed in PBS, then fixed in 75% alcohol for 30 min at 4°C. After washing in cold PBS three times, the cells were resuspended in

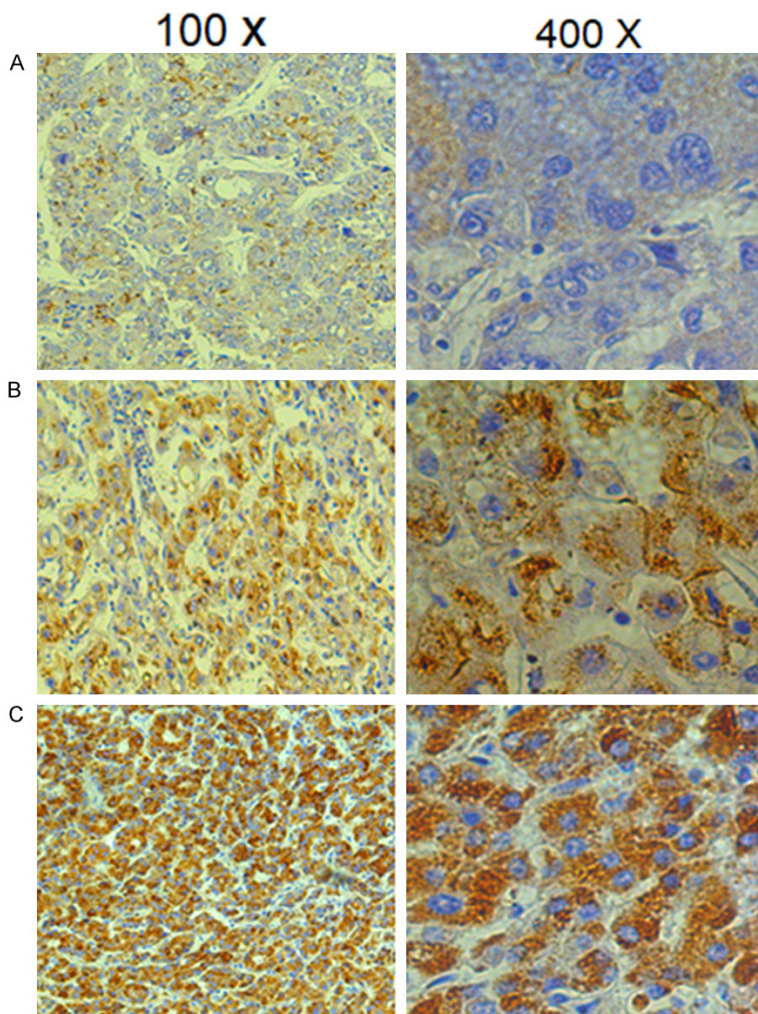


Figure 1. Representative immunohistochemical staining of CREB5 in tumor tissues. The micrographs showed weak (A), medium (B), and strong (C) expression of CREB5 in liver cancer tissues. (Magnification: left panel 100×, right panel 400×).

1 ml of PBS solution with 40 ug of propidium iodide (Sigma) and 100 ug of RNase A (Sigma) for 30 min at 37°C. Samples were then analyzed for their DNA content by FACSCalibur (Becton Dickinson, Mountain View, CA). The proliferative index (Plx), a measure of the number of cells in a tumor that are dividing (proliferating), was calculated with the following formula: $Plx = (S+G2M)/(G0/G1+S+G2M) \times 100\%$.

Colony formation assays

For colony formation assays, 500 cells were plated onto 6-well plates and incubated at 37°C. When the cells had grown to form visible colonies, the colonies were washed once with PBS and fixed with 4% paraformaldehyde for 20

min. The cells were stained with crystal violet, and images were collected by a video camera.

Western blot analysis

Tissues or harvested cultured cells were homogenized in a lysis buffer (50 mmol/L Hepes pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10 mmol/L NaF, 10 mmol/L Na₄P₂O₇, 1 mmol/L Na₃VO₅, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 20 µg/ml aprotinin). Protein was quantified by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). After blocking in 5% skim milk for 1 h at room temperature, membranes were incubated with a primary antibody at 4°C overnight, followed by a horseradish peroxidase-conjugated secondary antibody, and analysis by a chemiluminescence assay (Amersham Biosciences, Piscataway, NJ). Quantification of the Western blot data were

performed by measuring the intensity of the hybridization signals using an image analysis program (Fluor-Chem™ 8900, Alpha Inotech).

Statistical analysis

The differences between the two groups were examined using Student's t test. A value of $P < 0.05$ was considered statistically significant. Data were expressed as the mean ± standard deviation from at least three experiments. Comparisons between groups were performed with two-tailed paired Student's t test. The chi-square test was used to analyze the relationship between the CREB5 expression and the clinicopathological features. Bivariate correlations between variables were determined by

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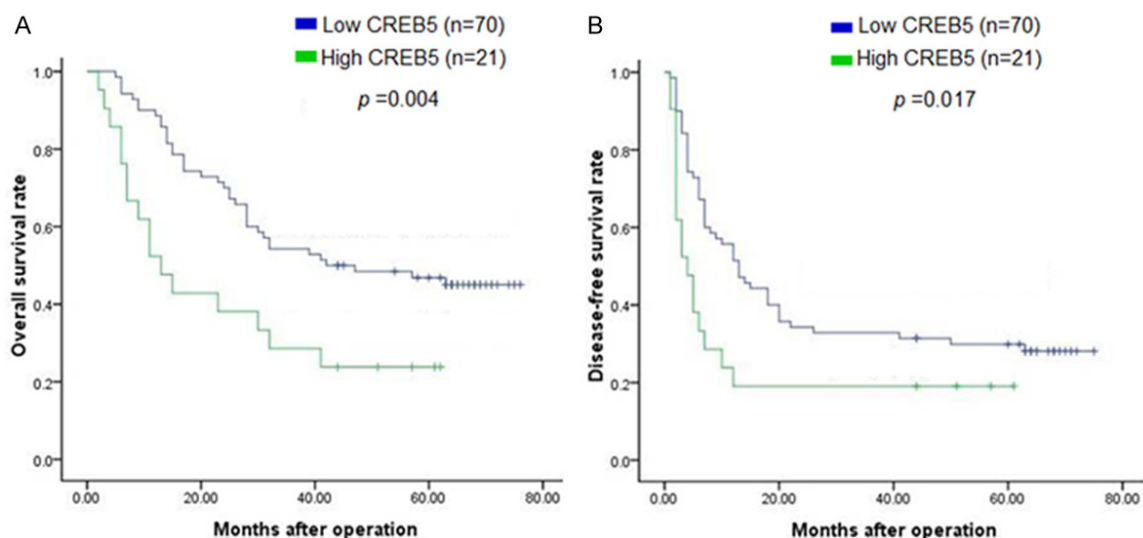


Figure 2. Kaplan-Meier survival curves of hepatocellular carcinoma (HCC) patients based on CREB5 expression. HCC patients with high CREB5 expression had a significantly shorter (A) overall survival ($P = 0.004$) and (B) disease-free survival ($P = 0.017$) than those with low CREB5 expression.

Table 2. Univariate analysis of different prognostic factors in 91 HCC patients by cox regression analysis

Variables	n	DFS				P	OS			
		1-yr	3-yr	5-yr	P		1-yr	3-yr	5-yr	P
Age (yrs)										
≤ 50	47	44%	30%	26%	0.622	79%	49%	41%	0.524	
> 50	44	49%	25%	24%		76%	45%	36%		
HBsAg										
Positive	77	45%	29%	24%	0.484	78%	47%	38%	0.726	
Negative	14	61%	30%	30%		78%	48%	39%		
Tumor size (cm)										
≤ 10 cm	69	25%	9%	7%	0.000	85%	55%	47%	0.000	
> 10 cm	22	55%	35%	32%		59%	25%	15%		
Tumor number										
Solitary	71	56%	34%	30%	0.001	83%	55%	47%	0.000	
Multiple	20	27%	12%	6%		67%	29%	18%		
Vascular invasion										
Yes	13	10%	3%	3%	0.000	86%	55%	46%	0.000	
No	78	56%	35%	30%		40%	13%	7%		
Encapsulation										
Intact	61	61%	36%	32%	0.000	86%	55%	45%	0.001	
Non-intact	30	24%	17%	13%		63%	34%	27%		
Edmonson										
I-II	68	53%	31%	28%	0.026	79%	50%	43%	0.036	
III-IV	23	30%	16%	14%		73%	38%	24%		
AFP (μg/l)										
≤ 400	53	56%	34%	32%	0.012	84%	50%	44%	0.108	
> 400	38	37%	23%	17%		71%	44%	32%		
TNM stage										
I-II	69	59%	36%	33%	0.000	87%	57%	49%	0.000	
III-IV	22	30%	16%	14%		73%	38%	24%		
CREB5 expression										
High	21	56%	33%	30%	0.017	89%	54%	47%	0.004	
Low	70	19%	19%	19%		52%	29%	24%		

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Table 3. Multivariate analysis of different prognostic factors in 91 HCC patients using a Cox regression analysis

Variables	DFS			OS		
	HR	95% CI	P	HR	95% CI	P
Tumor size	1.014	0.456-2.253	0.973	1.050	0.467-2.363	0.906
Tumor number	0.882	0.375-2.075	0.774	0.933	0.358-2.435	0.888
Vascular invasion	1.666	0.744-3.726	0.214	1.672	0.602-4.638	0.324
Encapsulation	0.767	0.424-1.387	0.380	0.916	0.491-1.709	0.783
Edmonson	1.772	1.008-3.116	0.047	1.358	0.738-2.499	0.325
AFP	1.262	0.729-2.185	0.405			
TNM stage	1.786	0.592-5.384	0.303	1.795	0.503-6.399	0.367
CREB5 expression	1.865	1.017-3.422	0.044	1.962	1.008-3.817	0.047

HR, hazard ratio; CI, confidence interval; DFS, Disease-free survival; OS, overall survival; HCC, hepatocellular carcinoma.

Spearman's correlation coefficients. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. All statistical analyses were performed using SPSS 11.0 software.

Results

High CREB5 expression is associated with a poor prognosis of patients with HCC

We detected the expression of the CREB5 protein by immunohistochemistry in 91 pairs of HCC tissue and matched adjacent non-cancerous tissue samples after liver resection (**Figure 1**). Cancer tissue from 63 of the 91 (69.2%) patients exhibited higher CREB5 protein expression as compared to the expression of CREB5 in the adjacent non-cancerous tissues. The expression of CREB5 was significantly elevated (a staining index score > 5) in tumor tissue samples compared with the matched non-tumor tissues ($P < 0.001$). In the 91 cases examined, high CREB5 expression was detected in 21 (23.08%) HCC specimens, but only 10 (11.00%) of the non-tumor specimens (**Table 1**).

A clinical association analysis using the Pearson chi-square test revealed that CREB5 expression in the HCC tumors was significantly associated with a poor prognosis. All 91 patients were included in the overall survival (OS) analysis. A Kaplan-Meier analysis revealed the association of CREB5 expression with short OS times ($P = 0.004$) (**Figure 2A**). The 1-, 3-, and 5-year survival rates decreased from 89%, 54%, and 47%,

respectively, in the low CREB5 expression group to 52%, 29%, and 24%, respectively, in the high CREB5 expression group. The Kaplan-Meier analysis also revealed an association of CREB5 expression and disease-free survival (DFS) ($P = 0.017$) (**Figure 2B**). The 1-, 3-, and 5-year DFS rates decreased from 56%, 33%, and 30%, respectively, in the low CREB5 expression group to 19%, 19%, and 19%, respectively, in the high CREB5 expression group.

Univariate Cox regression analyses revealed that tumor size, tumor number, vascular invasion, encapsulation, Edmonson grade, TNM stage, and CREB5 expression were all predictors of worse OS and DFS of HCC patients (**Table 2**). In addition, multivariate Cox regression analysis revealed that a higher level of CREB5 expression and a higher Edmonson grade were independent prognostic factors for DFS, whereas only CREB5 expression was an independent prognostic factors for OS (**Table 3**).

A Spearman correlation analysis showed significant associations between CREB5 expression and tumor size and TNM stage.

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After identifying the significant association between CREB5 expression and tumor size, we overexpressed CREB5 in SMMC-7721 cells to better understand the role of CREB5 in HCC. Stable CREB5 and control transfectants were generated and examined for CREB5 protein levels (**Figure 3C**). We analyzed cell cycle kinetics in SMMC7721 cells transfected with either

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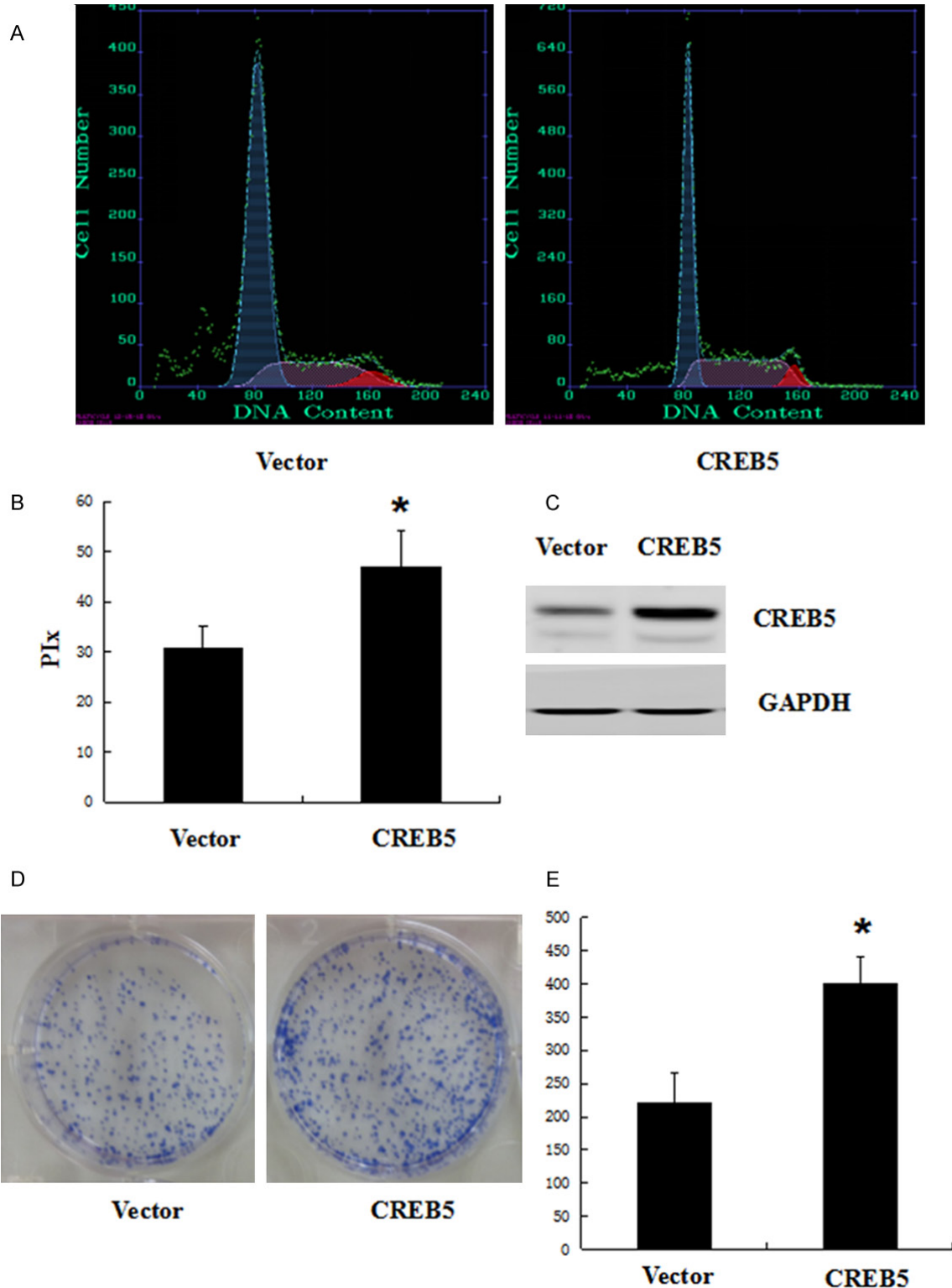


Figure 3. Overexpression of CREB5 promoted SMMC-7721 cell cycle progression and colony formation. SMMC-7721 cells were transfected with an empty vector or Ad-CMV-HA-CREB5. At 48 h after transfection, the cell cycle was analyzed by FACSCalibur (A). (B) The proliferative index (PIx) of CREB5-overexpressed cells was greater than that of the control cells ($P < 0.05$). The amount of CREB5 was determined by antibodies specific for CREB5 (C). For colony formation assays, we transfected the CREB5 overexpression construct into SMMC-7721 cells. Overexpressed CREB5 promoted colony formation in SMMC-7721 cells (D). Quantitative results of the colony-formation assay are also shown; * $P < 0.05$ (E).

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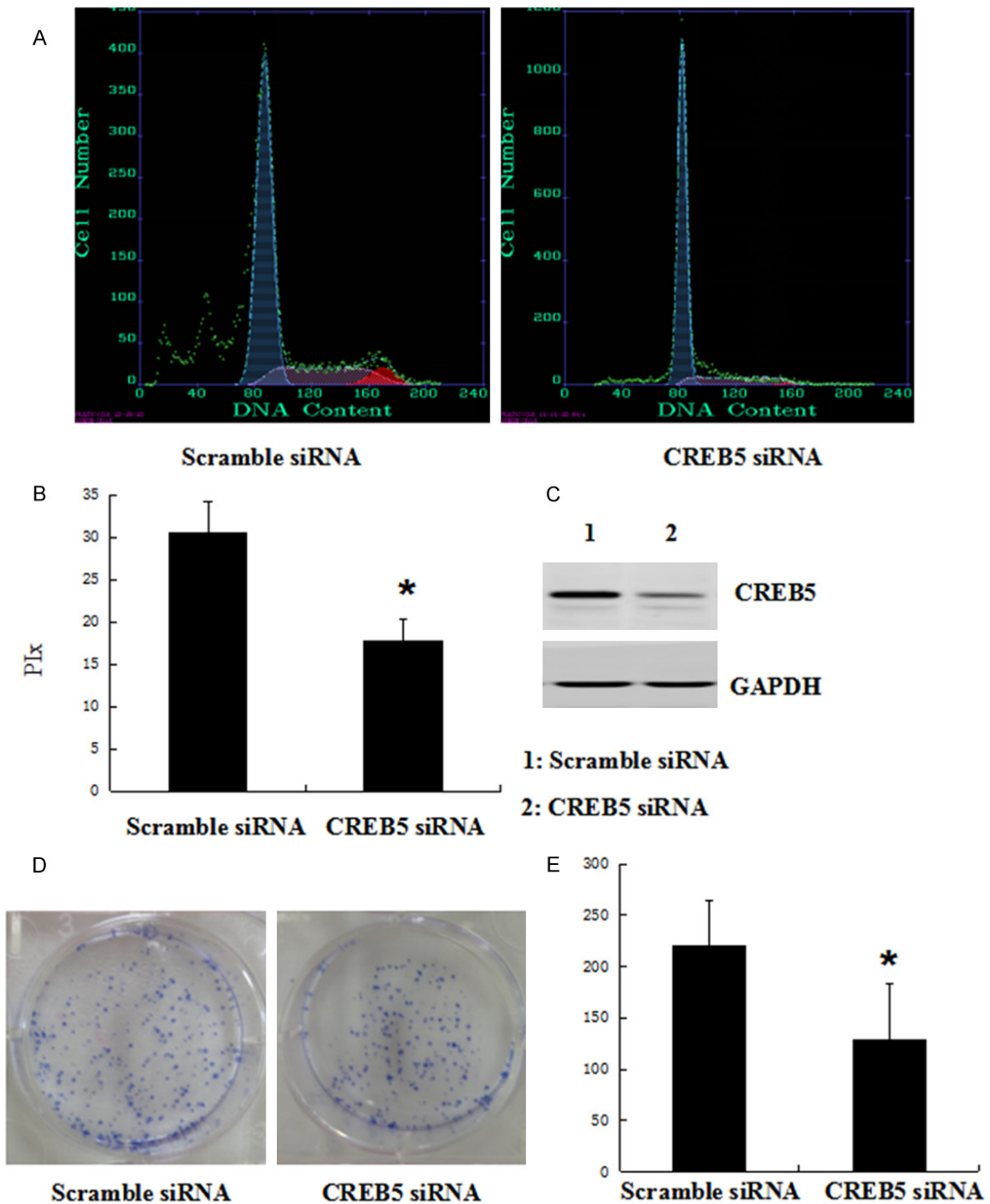


Figure 4. Knockdown of CREB5 inhibited SMMC-7721 cell cycle progression and colony formation. The SMMC-7721 cells were transfected with scramble siRNA or CREB5 siRNA. At 48 h after transfection, the amount of CREB5 and p21 was determined by antibodies specific for CREB5 (C). (A) The cell cycle was analyzed by FACSCalibur. (B) The proliferative index (PIx) was decreased compared to the control group ($P < 0.05$). (D) The knockdown of CREB5 in SMMC-7721 cells inhibited colony formation. Quantitative results of the colony-formation assay are shown; * $P < 0.05$ (E).

CREB5 or control. We further analyzed cell proliferation with a clone formation assay. The CREB5-overexpressing cells showed a statisti-

cally significant increase in proliferation when compared to the control cells (Figure 3A, 3B). In order to confirm these findings, we next

transfected specific-stranded RNA oligonucleotides against CREB5 to knockdown the CREB5 levels in the SMMC-7721 cells. When CREB5-specific oligonucleotides were used, a rapid down-regulation of CREB5 was detected (**Figure 4C**). The decrease in the CREB5 level induced G0/G1 cell cycle arrest in the SMMC7721 cells and a concomitant decrease in proliferation (**Figure 4A, 4B**). Using the clone formation assay, we found that the over-expression of CREB5 increased the CREB5er of the colonies as compared with the control (**Figure 3D**). The knockdown of CREB5 decreased the CREB5er of the colonies as compared with the control (**Figure 4D**).

Discussion

CREB5 belongs to the CRE-binding protein family. The protein specifically binds to CRE as a homodimer or heterodimer with c-Jun or CREBP1 to function as a CRE-dependent transactivator. CREB regulates a number of genes with diverse functions, including proliferation, survival, memory, and learning [8, 13-20]. Deregulation or aberrant expression of some of these genes has been associated with cancer. Seo et al. reported that CREB expression levels in non-small cell lung cancer cells were highly dependent on elevated CREB activity for their survival and cell growth, and the inhibition of CREB effectively suppressed the growth of non-small cell lung cancer cells [12]. He et al. reported that CREB5 was significantly overexpressed in epithelial ovarian cancer and was significantly correlated with aggressive features and an unfavorable prognosis [10].

In this study, the expression of CREB5 was detected in 91 specimens of paraffin-embedded HCC tissues. The immunohistochemistry results showed that the positive staining of CREB5 was observed in the cytoplasm of the hepatocytes. However, we did not perform corresponding HE staining of IHC, which should be addressed in a later study. Then, we studied the clinicopathological significance of CREB5 in HCC. We found tumor size, tumor number, vascular invasion, encapsulation, Edmonson grade, TNM stage, and CREB5 expression to be predictors of poorer OS and DFS in HCC patients. Besides CREB5 expression, all of the other factors are recognized indicators of HCC progression. Furthermore, a Spearman correla-

tion analysis showed that CREB5 expression was significantly correlated with tumor size and TNM stage. These data indicate that CREB5 expression is an independent prognostic indicator for survival in patients with HCC.

In addition, we further explored the possible role of CREB5 in the occurrence and development of HCC. We found that forced expression of CREB5 in SMMC-7721 cells promoted the progression of the cell cycle *in vitro*. The depletion of CREB5 in the SMMC-7721 cells had the opposite effect. The results of the clone formation assay indicated that CREB5 expression is positively correlated with cell proliferation in SMMC-7721 cells.

In conclusion, this is the first study to assess the role of CREB5 in HCC. The results suggest that CREB5 expression may be a useful prognostic indicator in patients with HCC. However, further study of the mechanisms by which CREB5 affects cell proliferation of HCC is required.

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Disclosure of conflict of interest

None.

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