Targeting the overexpressed CREB inhibits esophageal squamous cell carcinoma cell growth

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Abstract. Although several studies highlight the important role of cAMP-responsive element binding protein (CREB) in tumor progression, little is known concerning the expression and function of CREB in esophageal cancer. In the present study, the expression of CREB was evaluated using a human esophageal squamous cell carcinoma tissue array by immunohistochemical analysis, which was confirmed by western blot analysis of tissues from esophageal cancer, and adjacent esophageal tissue. The role of CREB on esophageal cancer cell growth was analyzed *in vitro* and *in vivo*. Results showed that CREB was overexpressed in esophageal squamous cell carcinomas tissues, which was positively correlated with lymph node metastasis and tumor-node-metastasis (TNM) stage of esophageal cancer patients. Downregulating the expression of CREB effectively reduced esophageal cell growth *in vitro* and *in vivo*, induced S phase cell cycle arrest, triggered apoptosis and inhibited cell migration and invasion. These findings suggested CREB as an attractive drug target for esophageal cancer.

Introduction

Esophageal cancer is a refractory disease and the sixth leading cause of cancer-related deaths worldwide (1,2). Esophageal squamous cell carcinoma (ESCC) is the major histologic subtype in Asia (1,2). Lack of effective diagnosis and prognosis marker account for the patients diagnosed at late stage and poor prognosis (3). It is important to identify new molecular target and develop novel therapeutic regiment for esophageal cancer.

Transcription factor has now been explored as attractive anticancer target (4). Previous studies showed that cAMP-responsive element binding protein (CREB) play an important role in tumor progression. For example, CREB was overexpressed in non-small cell lung cancer (NSCLC) and significantly associated with decreased survival duration in never smokers with NSCLC (5). CREB promoted abnormal proliferation and survival of myeloid cells *in vitro* and *in vivo* (6). Overexpressed CREB was detected in acute myeloid leukemia (6), acute lymphoblastic leukemia (7) and associated with relapse disease or a lower overall survival. Downregulation of CREB inhibited cancer cell growth (8-10), migration and invasion (11-14). However, Liu *et al* reported that the expression of CREB from Juvenile myelomonocytic leukemia patients was significantly lower than that from normal adults (15). Targeting CREB promoted cell proliferation in Hodgkin lymphoma (16). These results implied that CREB play an important role in tumor progression in a tumor-specific manner. However, the expression and role of CREB in ESCC remains elusive.

Here we found that CREB was overexpressed in esophageal squamous cell carcinoma tissues, which was positively associated with lymph node metastasis and tumor-node-metastasis (TNM) stage of ESCC patients. Knockdown of CREB reduced cell growth *in vitro* and *in vivo*, induced S phase arrest, triggered apoptosis, inhibited cell migration and invasion. These results imply that CREB may be an attractive anticancer target in ESCC.

Materials and methods

Cell lines. The human esophageal squamous cell carcinoma cell lines (EC1, EC9706, EC109, TE1, TE13, Kyse140 and Kyse450) were grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% FBS [Biological Industries (BI), Inc., Cromwell, CT, USA] at 37˚C with 5% CO_2. Human immortalized normal esophageal epithelial cell line (Het-1A) was a kind gift from Professor R. Liu (Southeast China University) and was cultured in Bronchial Epithelial Cell Medium (BEGM; BulleKit).

Immunohistochemistry (IHC) staining of human esophageal cancer tissue array. Human esophageal squamous cell carcinoma tissue array was purchased from Xi'an Alenabio Biotech Co. Ltd. (Xi'an, China). IHC staining was carried

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out with specific CREB antibody (Abcam Trading Co. Ltd., Shanghai, China). Briefly, the tissue array sections (5 μm) were dehydrated and peroxidase blocked. Primary antibodies were added and incubated at 4˚C overnight, followed by staining with a Histostain-SAP kit (ZSGB-BIO, Beijing, China). The slides were counterstained with hematoxylin. The stained slides were observed by microscopy, and images were acquired. Based on staining intensity, samples were classified into five groups from the lowest density (-) to the highest (++++) as previously described (17,18).

Collection of esophageal cancer tissues. Esophageal cancer and adjacent esophageal tissues were collected from esophageal squamous cell carcinoma patients undergoing resection at the Linzhou Cancer Hospital (Linzhou, Henan, China) from July 2012 to September 2014. Histological diagnosis and tumor-node-metastasis (TNM) stages of cancers were determined in accordance with the American Joint Committee on Cancer (AJCC) manual criteria for esophageal cancer.

Gene silencing using small interfering RNA (siRNA). Knockdown of CREB was carried out using siRNA oligonucleotides. The sequences of the siRNA are as follows: siCREB-1, CCAAGUUGUUGUUCAAGCU; siCREB-2, GAGAGAGGU CCGUCUAAUG; siControl, UUCUCCGAACGUGUCACGU.

Western blotting. Kyse450 and EC1 cells were transfected with siControl or siCREB, and then proteins were collected for western blot analysis, using antibodies against CREB (Abcam Trading Co. Ltd.), P27, WEE1, cleaved caspase-3, cleaved poly(ADP)-ribose polymerase (PARP) (Cell Signaling, Boston, MA, USA). CDC2, CDK2, cyclin B, cyclin A1, cyclin D, cyclin E (Abgent Biotech Co., Ltd., Suzhou, China). Tubulin (Shanghai Likun Trade Co. Ltd., Shanghai, China) was used as a control. Densitometric analysis for the quantification relative to tubulin was performed using the ImageJ software.

Cell viability and clonogenic assay. The cell viability was determined by Cell Counting Kit-8 (CCK-8; Beyotime Biotech Co., Haimen, China). EC1 and Kyse450 cells were transfected with siControl or siCREB for 24 h, and then seeded into 96-well plates ($2.5x10^3$ cells/well) for 72 or 96 h. Subsequently, CCK-8 solution was added to each well and incubated at 37˚C for 2 h. The absorbance at 450 nm was measured.

For the clonogenic assay, cells were transfected with siControl or siCREB, seeded into 6-well plates with 500 cells/well in triplicate, and then incubated for 12 days. The colonies were fixed by 4% paraformaldehyde, stained using crystal violet solution, and then the colonies with >50 cells were counted.

Cell cycle analysis. EC1 and Kyse450 cells were transfected with siControl or siCREB for 72 h, and then cells were harvested, fixed in 70% ethanol at -20˚C, stained with propidium iodide (PI; 50 μg/ml) containing RNase A (30 μg/ml) (both from Sigma, St. Louis, MO, USA) at 37˚C for 30 min, and analyzed for cell cycle profile by flow cytometry (FACScan; Becton-Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA). The expression of cell cycle-related proteins was detected using indicated antibodies.

Apoptosis assay and detection of the caspase-3 activity. Cells were transfected with siControl or siCREB for 96 h. Apoptosis was determined using the Annexin V-FITC/PI apoptosis kit (BioVision, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The activities of caspase-3 were measured using the CaspGLOW assay kit (BioVision, Inc.) according to the manufacturer's instructions. Cell proteins after transfection were collected, and then cleaved PARP and caspase-3 were detected.

Invasion assay. The invasion assay was carried out in Matrigel (Becton-Dickinson)-coated Transwell inserts with a pore size of 8-μm, as previously described (17). Briefly, the inserts were pre-coated with Matrigel. EC1 cells ($3x10^4$) transfected with siRNA for 48 h were seeded in serum-free medium in the upper chamber, whereas medium with 10% FBS was added to the lower well. After incubating for 24 h, the cells in the upper chamber were carefully removed with a cotton swab. The inserts were fixed in methanol, stained with 0.4% crystal violet, observed under microscopy, and images were acquired. Then, the dye was eluted by 33% acetic acid and detected at OD 570 nm.

Wound healing assay. For wound-healing assay, cells were seeded on 6-well plates and the confluent monolayer was scratched by a plastic pipette, then cells were washed three times with PBS. Images were captured at 0 or 36 h after wounding.

In vivo assay. The stable EC1 cell line with lentivirus targeting CREB was established as previously described (19,20). BALB/c nude female mice were subcutaneously injected with $5x10^6$ EC1 cells stably expressing lenti-shCREB or lenti-shControl, respectively. Tumor growth was observed and tumor area was recorded twice a week with a FluorVivo Model-300 imaging system (INDEC BioSystems, Santa Clara, CA, USA) (19). At the time of sacrifice, tumor tissues were harvested, photographed and weighed. Animal experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

Statistical analysis. The statistical significance of differences between groups was assessed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The t-test was used for the comparison of parameters between groups. For all tests, two levels of significance ($^*P<0.05$; $^{**}P<0.01$) were applied.

Results

CREB is overexpressed in esophageal squamous cell carcinoma tissues. To investigate whether CREB served as anti-esophageal cancer target, we firstly examined the expression levels of CREB by immunochemistry (IHC) staining of the tissue array derived from human ESCC. Results showed that CREB was overexpressed in ESCC tissues (Fig. 1A), which was confirmed by western blot assay using the esophageal cancer and adjacent esophageal tissues from esophageal squamous cell carcinoma patients (Fig. 1B). Furthermore, the overexpressed CREB was positively correlated with lymph

Table I. Correlation between the expression of CREB and clinical characteristics of ESCC patients.

Characteristics	Total n	CREB no.		P-value
		Negative n (%)	Positive n (%)	
Overall	114	18 (16)	96 (84)	-
Sex				0.804
Male	85	13 (15)	72 (85)	
Female	29	5 (17)	24 (83)	
Age (years)				0.725
<60	55	8 (15)	47 (85)	
≥60	59	10 (17)	49 (83)	
Lymph node metastasis (N)				0.029
N0	38	10 (26)	28 (74)	
N1-2	76	8 (11)	68 (89)	
TNM stage				0.018
I	14	6 (43)	8 (57)	
II	62	6 (10)	56 (90)	
III	35	6 (17)	29 (83)	
IV	3	0 (0)	3 (100)	
Tumor invasion				0.849
T1	14	2 (14)	12 (86)	
T2	50	9 (18)	41 (82)	
T3	50	7 (14)	43 (86)	
Differentiation				0.577
Well	21	3 (14)	18 (86)	
Moderate	49	7 (14)	42 (86)	
Poor	44	8 (18)	36 (82)	

[a]P<0.05 was defined as significant, Fisher's exact test. CREB, cAMP-responsive element binding protein; ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis.

node metastasis and TNM stage of ESCC patients (Table I). However, CREB was expressed higher in ESCC cell lines than human immortalized normal esophageal epithelial cell line (Het-1A) (Fig. 1C). These results implied that CREB may be an attractive anti-ESCC target.

Targeting CREB inhibits cell growth of ESCC. According to the above results, we next examined the effect of knockdown CREB on cell growth. Results showed that the expression of CREB was effectively downregulated using specific siRNA (Fig. 2A). Silencing of CREB inhibited cell growth by cell proliferation (Fig. 2B) and colony formation assay (Fig. 2C).

Knockdown of CREB induces S cell cycle arrest in esophageal cancer cells. To elucidate the growth suppression mechanism by CREB silencing, the cell cycle profile was examined after knockdown of CREB. As shown in Fig. 3, knockdown of CREB induced S cell cycle arrest (Fig. 3A) and downregulated the expression of cyclin A1 and D (Fig. 3B).

Silencing of CREB triggers apoptosis in esophageal cancer cells. We next examined whether apoptosis was also responsible for the growth inhibition effect of CREB silencing. Results showed that knockdown of CREB-induced apoptosis, as evident by increased Annexin V-positive cells (Fig. 4A), improved caspase-3 activity (Fig. 4B) and enhanced expression level of cleaved caspase-3 and cleaved PARP (Fig. 4C).

Targeting CREB inhibits cell invasion and metastasis of ESCC. Statistical analysis results showed that the overexpressed CREB was correlated with lymph node metastasis (Table I). So the expression of CREB was downregulated by siRNA and the effect on cell invasion and metastasis was examined. Results showed that silencing CREB inhibited ESCC cell invasion and metastasis by Transwell (Fig. 5A) and wound healing assay (Fig. 5B).

Targeting CREB inhibits ESCC cell growth in vivo. To further investigated the growth suppressive effect of CREB knockdown *in vivo*, BALB/c nude female mice were subcutaneously injected with $5x10^6$ EC1 cells stably expressing lenti-shCREB (marked as shCREB) or lenti-shControl (marked as shControl), respectively. Tumor growth was observed and tumor area was recorded twice a week. Results showed that CREB silencing

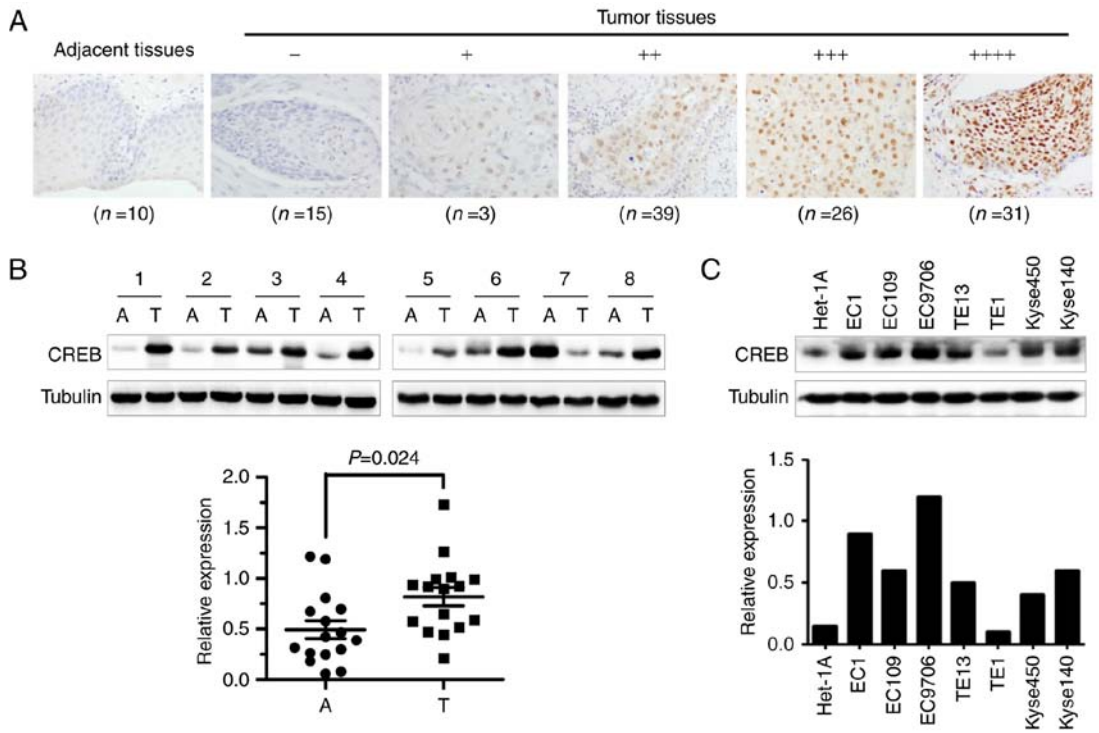

Figure 1. CREB is overexpressed in esophageal squamous cell carcinoma tissues. (A) The expression of CREB in human esophageal squamous cell carcinoma tissues was detected by immunohistochemistry (IHC) staining. According to staining intensity, samples were classified into five groups with increasing staining intensity from the weakest (-) to the strongest (++++). Representative images are shown. The number of each group is marked below the image. (B) Western blot analysis to determine the expression of CREB in ESCC and adjacent esophageal tissues. Representative results are shown in the upper panel and analysis of western blotting is shown in the lower panel. A, adjacent tissues, T, tumor tissues. (C) Expression of CREB in esophageal squamous cell carcinoma cell lines and human immortalized normal esophageal epithelial cell line (Het-1A). Results of western blotting are shown in the upper panel and analysis is shown in the lower panel.

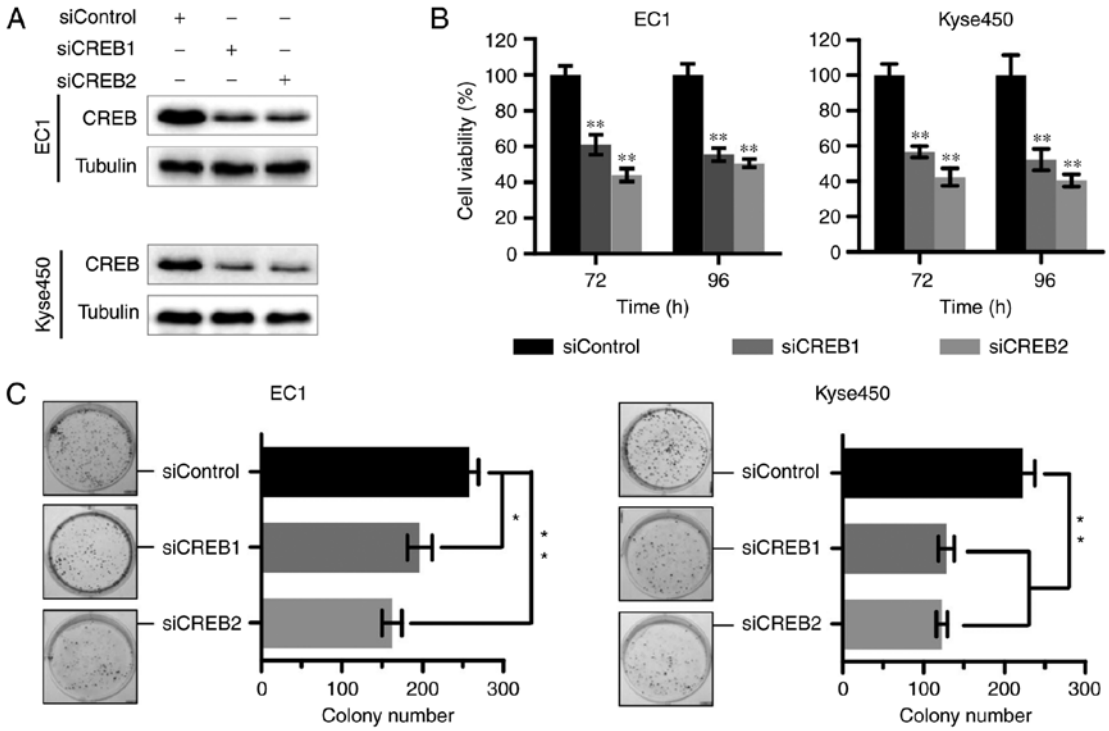

Figure 2. Knockdown of CREB inhibits cell proliferation of human esophageal cancer. (A) Detection of siRNA efficiency targeting CREB. Cells were transfected with siRNA for 96 h, and then proteins were collected and knockdown efficiency was determined by western blotting. (B) Effect of silencing CREB on the viability of esophageal squamous cell carcinoma cells EC1 and Kyse450. Cells were transfected with siRNA for 72 or 96 h and viability was assessed using the CCK-8 assay. (C) Effect of silencing CREB on clonogenic survival of esophageal squamous cell carcinoma cells EC1 and Kyse450. Representative images are shown in the left panel and colony count in the right panel. The statistical significance of differences between groups was assessed according to Materials and methods (*P<0.05; **P<0.01).

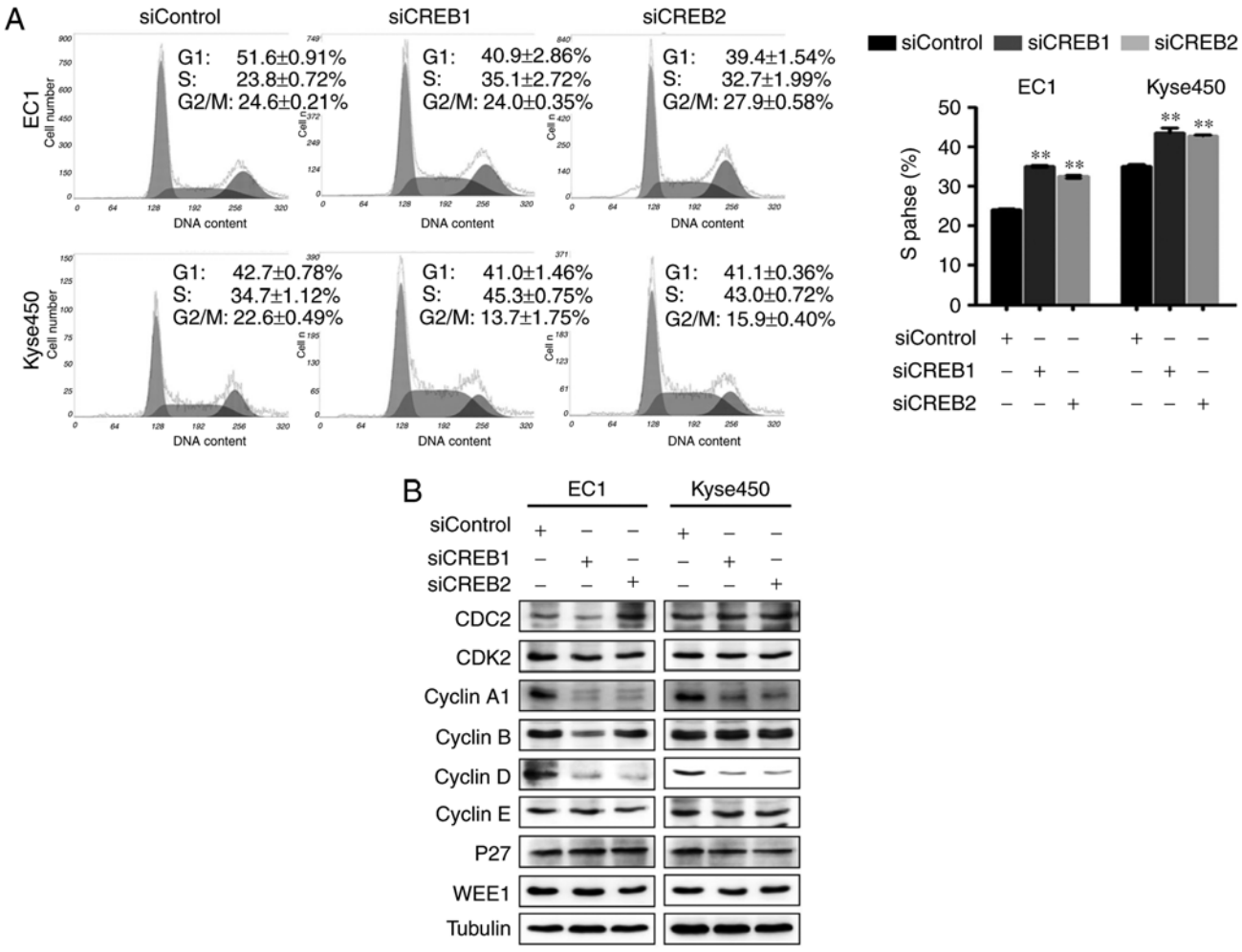

Figure 3. Knockdown of CREB induces S cell cycle arrest of human esophageal cancer cells. (A) EC1 and Kyse450 cells were transfected with siRNA, and then stained with PI staining. DNA contents were analyzed by fluorescence-activated cell sorting (FACS) analysis. Representative images are shown (left panel). The statistical significance of differences between groups was assessed using the GraphPad Prism 5 software (**P<0.01) were applied (right panel). (B) Effect of silencing CREB on the expression of cell cycle-related proteins was detected using indicated antibodies.

suppressed tumor growth over time while control tumors grew rapidly, as revealed by real-time images of tumors (Fig. 6A), tumor growth curve (Fig. 6B; P<0.05), tumor size (Fig. 6C) and tumor weight analysis (Fig. 6D; P<0.05).

Discussion

Despite the improvement in the surgical and non-surgical therapy for ESCC (2), the general outcome remains very poor for overall 5-year survival rates (~10%) and 5-year post-esophagectomy survival rates (~15-40%) (21). Diagnosis at advanced stage and resistance to chemotherapy still affect the refractory disease. Therefore, it is urgent to find new therapeutic targets.

CREB belongs to basic/leucine zipper (bZIP) transcription factor family (22) and is described as a proto-oncogene (6,23). CREB play an important role at early stage of papilloma formation (24), promoted abnormal proliferation of myeloid cells *in vitro* and *in vivo* and was implicated in myeloid cell transformation (6). However, CREB was overexpressed in ovarian adenocarcinoma (10), non-small cell lung (9,25) and breast cancer (12), leukemia (26), and highly associated with disease stage or poor clinical outcomes of the patients. In different conditions, CREB participated in tumorigenesis and influenced melanoma (14,27), T cell and myeloid leukemia (6) and hepatocellular carcinoma (28). Overexpressed CREB promoted tumor progression by regulating cell proliferation, cell cycle, apoptosis, angiogenesis or metastasis. These findings highlight a pivotal role of CREB in carcinogenesis. However, the expression and role of CREB in ESCC remains to be elucidated. In the present study, we reported that CREB was overexpressed in esophageal squamous cell carcinomas tissues, which was positively correlated with lymph node metastasis and TNM stage of ESCC patients. Moreover, knockdown of CREB significantly inhibited cell proliferation of ESCC cells *in vitro* and *in vivo*. These results indicated that CREB may be involved in ESCC cell growth.

Previous studies suggested CREB as a promising target for cancer therapy. Downregulating the expression of CREB by ectopic expression of dominant repressor CREB or siRNA against CREB suppressed the growth and survival of NSCLC cells and induced apoptotic cell death (9). Ectopic expression of dominant-repressor CREB inhibited acute myeloid leukemia cell proliferation *in vitro* and *in vivo* (6,22), sensitized melanoma

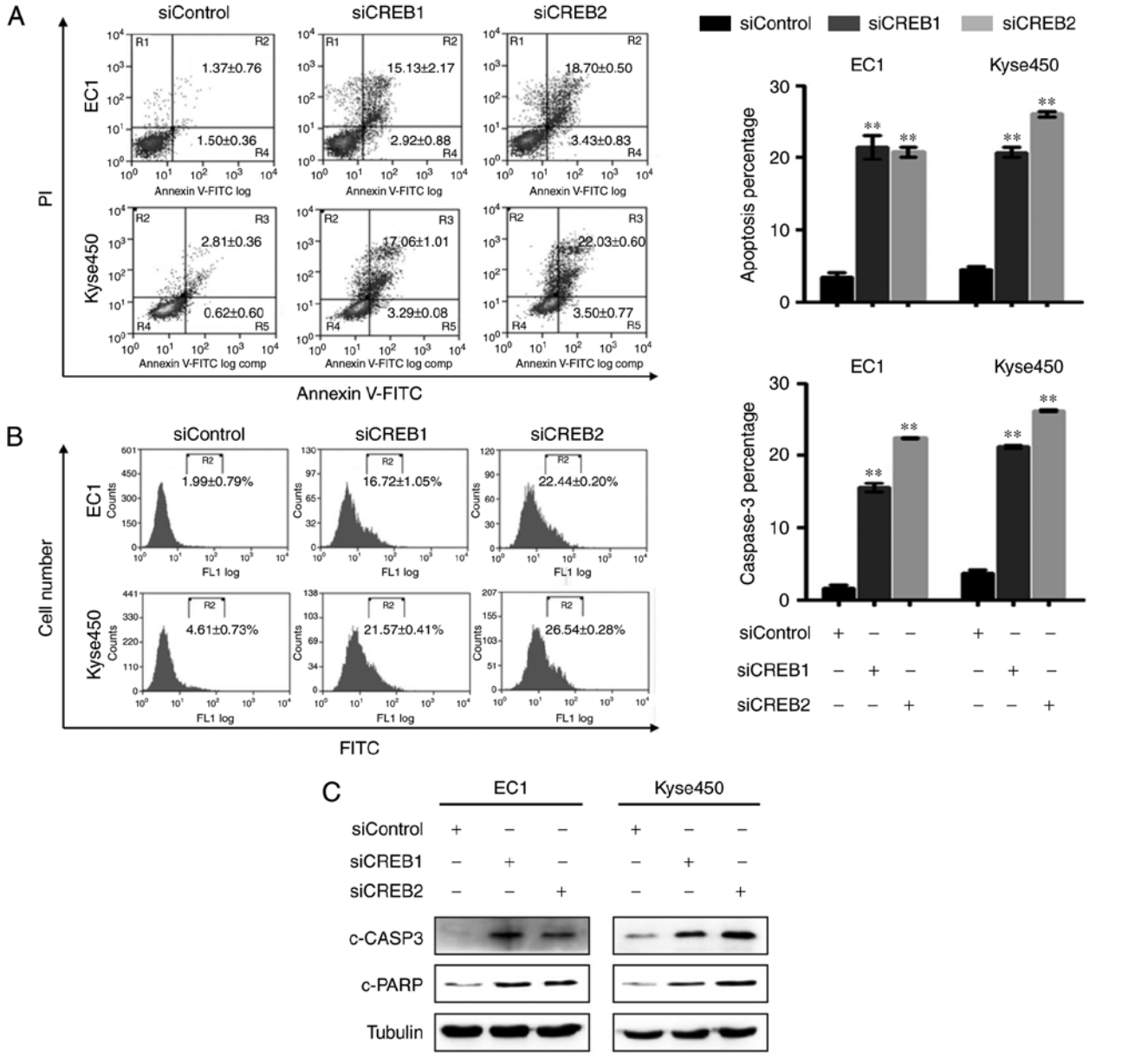

Figure 4. Knockdown of CREB triggers apoptosis of human esophageal cancer cells. EC1 and Kyse450 cells were transfected with siRNA for 96 h. Apoptosis was determined by Annexin V-FITC/PI double-staining analysis (A) Caspase-3 activity was analyzed by FACS (B) Cleaved PARP and caspase-3 were detected by western blot analysis (C). In the panel A and B, representative images are shown (left panel). The statistical significance of differences between groups was assessed using the GraphPad Prism5 software ($^{**}P<0.01$) (right panel).

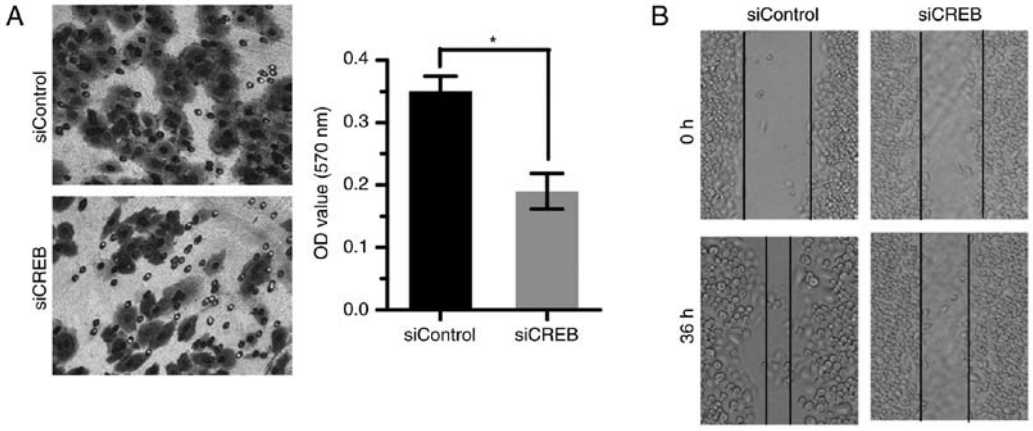

Figure 5. Knockdown of CREB inhibites cell migration and invasion in human esophageal cancer cells. EC1 cells were transfected with siControl or mixture of siCREB1 and siCREB2 for 48 h. (A) Cell invasion was determined by Transwell assay. (B) Cell migration was detected by wound healing assay.

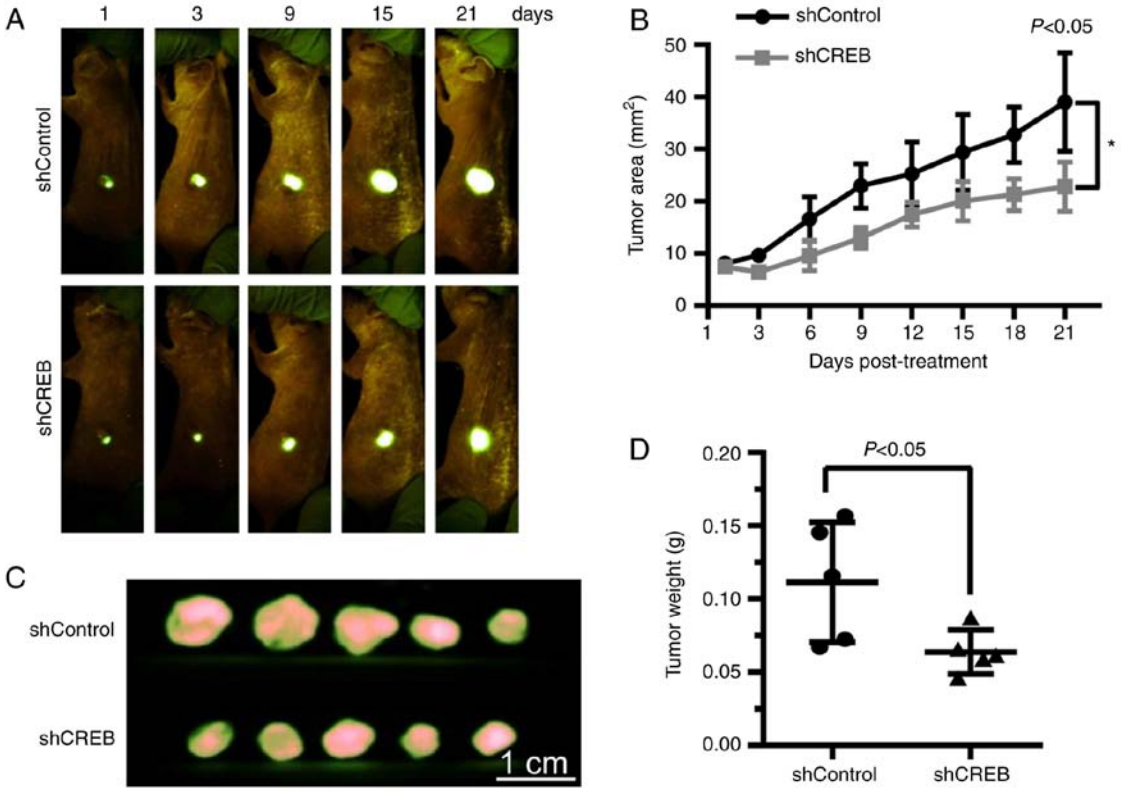

Figure 6. Knockdown of CREB suppresses esophageal tumor growth *in vivo*. Whole-body images of tumor model were captured (A). The data were converted to tumor growth curves (B). Mice were sacrificed and tumor tissues were harvested, photographed (C), and weighed (D) at the end of the study.

cells to apoptosis and downregulated their tumorigenicity and metastatic potential in nude mice (14,27,29). CREB knockdown inhibited human pre-B acute lymphoblastic leukemia cell growth and induced cell apoptosis (8). Furthermore, several small compounds were reported to target CREB or inhibit its transcriptional activity, exhibited efficient anticancer effect and showed little to no toxicity to normal epithelial cells, fibroblasts or hematopoietic cells (30-34); 666-15, a CREB inhibitor, showed promising potency against breast cancer *in vitro* and in a mouse model. Moreover, the mice treated with 666-15 showed no evidence of changes in body weight, complete blood count, blood chemistry profile, cardiac contractility and tissue histology from liver, kidney and heart (34). In contrast, CREB was involved in cisplatin/gemcitabine resistance (35) or radio sensitivity (36,37). These results implied potential of CREB-target therapy. Here, our results showed that CREB silencing suppressed esophageal tumor cell growth in a mouse model. Whether knockdown of CREB effectively sensitized esophageal cells to chemoherapy/radiotherapy still need to be further investigated.

Mechanistically, CREB was reported to be involved in the regulation of cell cycle machinery, including cyclin A1 and D1 (22,28,38-40). Desdouets *et al* reported that CREB was involved in regulation the expression of cyclin A, a pivotal regulatory protein which was involved in the S phase of the cell cycle (40,41). However, Linnerth *et al* reported that knockdown CREB using siRNA significantly reduced ovarian tumor cell proliferation, while there was no effect on apoptosis in these cells (10). Lu *et al* indicated that downregulation of CREB promoted cell proliferation by mediating G1/S phase transition in Hodgkin lymphoma (16). Inhibitor of the CREB signaling pathway Ro-31-8220 inhibited CREB activation and arrested the cell cycle at the G2-M phase (9). Here we found that knockdown of CREB downregulated the expression of cyclin A1 and D, induced S phase cell cycle arrest and apoptosis. These results implied that the anticancer mechanism of targeting CREB was in a tumor-specific manner.

Invasion and metastasis are the important characteristics of malignant tumor (42). Previous reports showed that CREB promoted cancer metastasis (12,14,43,44). CREB regulated vascular endothelial growth factor expression and was involved in human prostate cancer bone metastasis (45). In melanoma, CREB mediated tumorigenesis and metastatic potential (45,46). Downregulated CREB using a dominant-negative form of CREB and CREB silencing inhibited cell growth and metastasis (45). In accordance with these results, here we found that CREB was overexpressed in esophageal squamous cell carcinomas tissues, positively correlating with lymph node metastasis. Moreover, knockdown of CREB inhibited cell migration and invasion using wound healing and Transwell assay.

In summary, this is the first study to investigate the expression and clinicopathological significance of CREB in ESCC. Results demonstrated that CREB was hyperexpressed in human ESCC tissues and positively correlated with lymph node metastasis and TNM stage of esophageal cancer patients. In addition, knockdown of CREB effectively inhibited cell growth *in vitro* and *in vivo*. These findings expanded our knowledge of CREB in ESCC progression

and suggested CREB as a novel drug target for esophageal cancer.

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