

Cyclic adenosine monophosphate-responsive element-binding protein activation predicts an unfavorable prognosis in patients with hepatocellular carcinoma

Lingxiang Yu*
Xiaodong Guo*
Peirui Zhang
Ruizhao Qi
Zhiwei Li
Shaogeng Zhang

Department of Hepatobiliary Surgery,
302 Military Hospital of China,
Beijing, People's Republic of China

*These authors contributed equally
to this work

Aim: To investigate the clinical significance of cyclic adenosine monophosphate-responsive element-binding (CREB) and phosphorylated CREB (pCREB) expression in human hepatocellular carcinoma (HCC).

Materials and methods: Immunohistochemistry and Western blot analyses were performed to detect the expression and subcellular localizations of CREB and pCREB proteins in 130 pairs of HCC and adjacent nonneoplastic liver tissues.

Results: Both immunohistochemistry and Western blot analyses showed that the expression levels of CREB and pCREB proteins in HCC tissues were significantly higher than those in the adjacent nonneoplastic liver tissues (both $P < 0.001$). In addition, the combined upregulation of CREB and pCREB proteins (CREB-high/pCREB-high) was significantly associated with serum α -fetoprotein ($P = 0.02$), tumor stage ($P < 0.001$), and tumor grade ($P = 0.01$). Moreover, HCC patients with CREB-high/pCREB-high expression showed shortest 5-year disease-free survival and 5-year overall survival (both $P < 0.001$). Furthermore, the multivariate survival analysis found that the combined upregulation of CREB and pCREB proteins may be an independent unfavorable prognostic factor for both 5-year disease-free survival and 5-year overall survival (both $P = 0.01$) in HCC.

Conclusion: Our data indicate for the first time that the activation of the CREB protein may be associated with tumor progression in HCC, and may serve as a valuable marker of prognosis for patients with this malignancy.

Keywords: hepatocellular carcinoma, cyclic AMP-responsive element-binding protein, tumor progression, prognosis

Introduction

Hepatocellular carcinoma (HCC) represents the fifth-most frequent human malignancy in terms of its incidence rate and the third-leading cause of cancer-related deaths globally.¹ More than 700,000 new cases are diagnosed worldwide, and more than 600,000 deaths attributed to HCC annually.² HCC is especially prevalent in certain areas of Asia and Africa.³ As a highly aggressive solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor prognosis.⁴ Despite improvements in diagnostic and therapeutic approaches, the 5-year survival rate of this cancer is merely 7%, because of a high incidence of postoperative metastasis and recurrence, and the high resistance of HCC to chemotherapy.⁵ This disappointing outcome clearly suggests that the current knowledge regarding diagnosis, prevention,

Correspondence: Zhiwei Li; Shaogeng Zhang
302 Military Hospital of China,
100 West Fourth Ring Road, Fengtai,
Beijing 100039, People's Republic
of China
Tel/Fax +86 10 6693 3260
Email lizhiwei112013@163.com;
sgzhang2014@163.com

and treatment of this cancer is insufficient. Therefore, further investigations should focus on the identification of specific biomarkers with high sensitivity and specificity for HCC in order to screen patients with poor prognosis or at high risk of early recurrence and to serve as a basis for understanding the complex molecular mechanisms governing the progression and aggressiveness of this malignancy.

The cyclic adenosine monophosphate (cAMP)-response element-binding (CREB) protein, which belongs to a subfamily of the leucine zipper with basic domain family, is a 43 kDa basic/leucine-zipper transcription factor.⁶ It binds to the cAMP-responsive element (TGACGTCA) on the somatostatin promoter in the presence of cAMP, and activates transcription in response to a variety of extracellular signals, such as hormones, growth and neurotrophic factors, neurotransmitters, and membrane depolarization.⁷ CREB plays crucial roles in cell differentiation, proliferation, cell-cycle progression, survival, development, and glucose metabolism through being activated by cAMP, growth factors, hormones, retinoids, cytokines, and prostaglandins via multiple signaling pathways.⁸ Pathologically, the CREB protein has been demonstrated to be upregulated in several human malignancies and act as an important oncogene.⁹ In particular, a study by Abramovitch et al¹⁰ showed that CREB could promote the tumor progression of HCC by supporting angiogenesis and rendering HCC cells resistant to apoptosis. Kovach et al¹¹ also found that total CREB and phosphorylated CREB (pCREB) proteins were both significantly elevated in HCC versus normal liver. However, their clinical implications are still unclear. The aim of this study was to investigate the correlations of CREB and pCREB expression with clinicopathological characteristics and HCC patient survival.

Materials and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of the 302 Military Hospital of China (PLA30200198). Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to ethical and legal standards.

In this retrospective study, a total of 130 patients with primary HCC who underwent a curative liver resection at the 302 Military Hospital of China in Beijing were enrolled. A total of 130 HCC and adjacent nonneoplastic liver tissues obtained from these patients were retrieved from the tissue bank of the Department of Pathology in the hospital. These patients had been diagnosed with HCC between 2001 and 2006. None of the patients recruited in this study had chemotherapy or

radiotherapy before the surgery. HCC diagnosis was based on World Health Organization criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child–Pugh scoring system. Tumor staging was determined according to the sixth edition of the *TNM Classification of Malignant Tumors* of the International Union Against Cancer.¹² The clinicopathological features of 130 patients are summarized in Table 1.

The median follow-up period of all 130 patients with HCC was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 months.

Immunohistochemistry analysis

Immunohistochemistry was performed to detect the expression patterns and the subcellular localizations of CREB and pCREB

Table 1 Association of combined CREB and pCREB upregulation with clinicopathological features of 130 hepatocellular carcinoma patients

Clinicopathological features	Case	CREB-high/ pCREB-high (n, %)	P
Age (years)			
≤50	72	32 (44.44)	—
>50	58	28 (48.28)	
Sex			
Male	96	44 (45.83)	—
Female	34	16 (47.06)	
Serum AFP			
Positive (≥400 µg/L)	72	40 (55.56)	0.02*
Negative (<400 µg/L)	58	20 (34.48)	
Tumor stage			
T1	23	0	<0.001**
T2	40	10 (25.00)	
T3	52	35 (67.31)	
T4	15	15 (100.00)	
Tumor grade			
G1	31	8 (25.81)	0.01*
G2	76	34 (44.74)	
G3	23	18 (78.26)	
Growth pattern			
Trabecular	101	45 (44.55)	—
Nontrabecular	29	15 (51.72)	
Cirrhosis			
Yes	86	40 (46.51)	—
No	44	20 (45.45)	
Underlying liver disease			
Alcoholic	25	12 (48.00)	—
Hepatitis B	49	22 (44.90)	
Hepatitis C	35	16 (45.71)	
Unknown	21	10 (47.62)	

Notes: * $P<0.05$; ** $P<0.001$.

proteins in HCC and adjacent nonneoplastic liver tissues following the protocol of our previous studies.^{13–15} The primary antibodies were rabbit monoclonal anti-CREB (ab32515; Abcam, Cambridge, UK) and rabbit monoclonal anti-pCREB (phospho S133) (ab32096; Abcam). The secondary antibody for the detection of primary antibodies was anti-rabbit immunoglobulin G (sc-3739; Santa Cruz Biotechnology, Dallas, TX, USA). The specificities of anti-CREB and anti-pCREB antibodies (as shown in Figure 1) were validated by Western blot analysis using CREB Blocking Peptide (ab4963, 0.5 µg/mL, incubation at room temperature for 30 minutes; Abcam) and Phospho-CREB (Ser133) Blocking Peptide (1090, 0.5 µg/mL, incubation at room temperature for 30 minutes; Cell Signaling Technology, Danvers, MA, USA). The negative controls were processed in a similar manner with phosphate-buffered saline instead of primary antibody. The positive CREB and pCREB expressions confirmed by Western blotting were used as positive controls for immunostaining.

Evaluation of immunostaining results

Immunohistochemistry results were evaluated by two independent experienced pathologists who were blinded to the clinicopathological data and clinical outcomes of the patients. Their scores were compared, and any discrepant scores were reexamined by both pathologists to reach a consensus score. The number of positive-staining cells in ten representative microscopic fields was counted, and the percentage of positive cells was calculated. The percentage scoring of positive tumor cells was 0 (0), 1 (1%–10%), 2 (11%–50%), and 3 (>50%). The staining intensity was visually scored and stratified as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final score was obtained for each case by multiplying the percentage and

the intensity score. Therefore, tumors with a multiplied score less than the median of the total score for CREB (median = 5.56) or pCREB (median = 4.48) were deemed to be low expressions of CREB or pCREB; all other scores were considered to be high expressions of CREB or pCREB.

Western blot

Western blot analysis was carried out according to the protocol of our previous studies.^{13,14} Rabbit monoclonal anti-CREB antibody (ab32515; Abcam) and rabbit monoclonal anti-pCREB (phospho S133) antibody (ab32096; Abcam) were used, and GAPDH antibody (CW0266, dilution 1:1,000; CoWin Biotech, Beijing, People's Republic of China) was used as the internal control. The relative CREB- and pCREB-expression levels were both indicated after normalization to GAPDH protein (internal control).

Statistical analysis

Statistical analysis was performed by SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC, USA). The χ^2 test was used to show differences in categorical variables. Correlations between CREB and pCREB expression were calculated using Spearman's correlation. Differences in patient survival were determined by the Kaplan–Meier method and log-rank test. A Cox regression analysis (proportional hazards model) was performed for the multivariate analyses of prognostic factors. Differences were considered statistically significant when $P < 0.05$.

Results

Expression patterns and subcellular localizations of CREB and pCREB proteins in HCC

Expression patterns and subcellular localizations of CREB and pCREB proteins in 130 self-pairs of HCC and adjacent nonneoplastic liver tissues were observed by immunohistochemistry analysis. As shown in Figure 2, CREB and pCREB stainings were both primarily localized in the nucleus of both normal and tumor cells, and were higher in HCC tissues than those in adjacent nonneoplastic liver tissues. In addition, statistical analysis showed that the expression levels of CREB and pCREB proteins in HCC tissues were both significantly higher than those in the adjacent nonneoplastic liver tissues (both $P < 0.001$, Figure 2C and F). Of note, the expression levels of CREB and pCREB in a high tumor grade (G3; 6.62 ± 1.78 and 5.38 ± 1.02 , respectively) were both significantly higher than in lower grades (G1–G2; 3.37 ± 0.42 and 2.91 ± 0.33 , respectively; both $P = 0.02$) HCC. Moreover, based on the scoring system

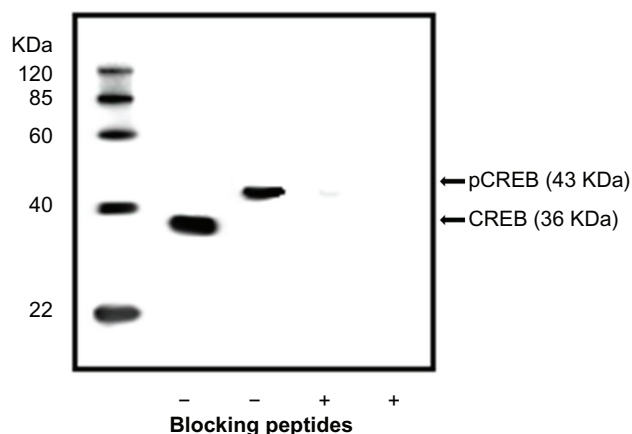


Figure 1 Specificities of anti-CREB and anti-pCREB antibodies. Western blot analysis of hepatocellular carcinoma tissues using CREB and pCREB antibodies with and without preincubation with respective blocking peptides.

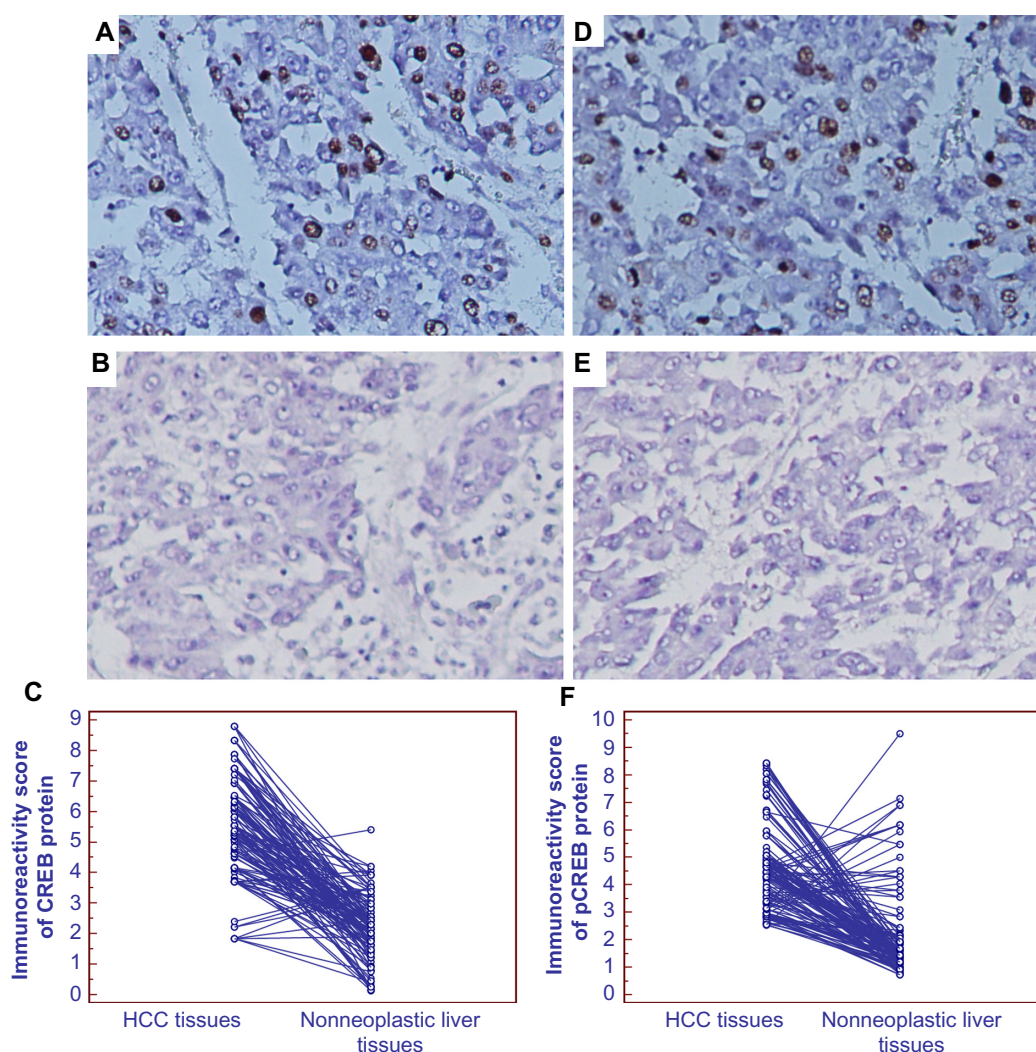


Figure 2 (A–F) Expression patterns and subcellular localizations of CREB and pCREB proteins in hepatocellular carcinoma (HCC) and adjacent nonneoplastic liver tissues. Representative immunohistochemistry images for CREB (**A** and **B**) and pCREB (**D** and **E**) proteins in HCC and adjacent nonneoplastic liver tissues, respectively (magnification $\times 200$). Statistical analysis showed that the expression levels of CREB and pCREB proteins in HCC tissues were both significantly higher than those in the adjacent nonneoplastic liver tissues (both $P < 0.001$ [**C** and **F**], respectively).

used in the present study, 60 (43.08%) cases showed high expression of both CREB and pCREB, 45 (40.00%) cases showed low expression of both CREB and pCREB, 12 (9.23%) cases were CREB-high and pCREB-low expression, and 13 (7.69%) cases were CREB-low and pCREB-high expression. As determined by Spearman's correlation, CREB expression was closely correlated with pCREB expression ($r=0.68$, $P=0.002$, Figure 3). Of note, the results of the immunohistochemistry analysis were further confirmed with the findings of the Western blot analysis, as shown in Figure 4.

Upregulation of CREB and pCREB proteins is associated with the aggressive clinicopathological features of HCC

We then correlated immunoreactive scores of CREB and pCREB proteins with clinicopathological features of

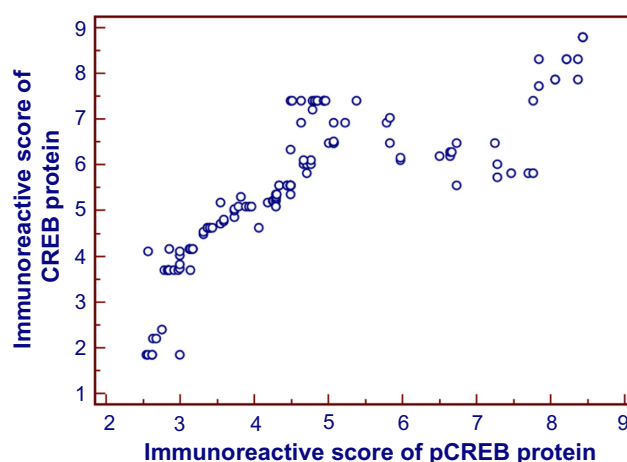


Figure 3 Scatter diagram of immunoreactive scores of CREB and pCREB proteins in hepatocellular carcinoma (HCC) tissues. As determined by Spearman's correlation,¹⁶ CREB expression was closely correlated with pCREB expression ($r=0.68$, $P=0.002$).

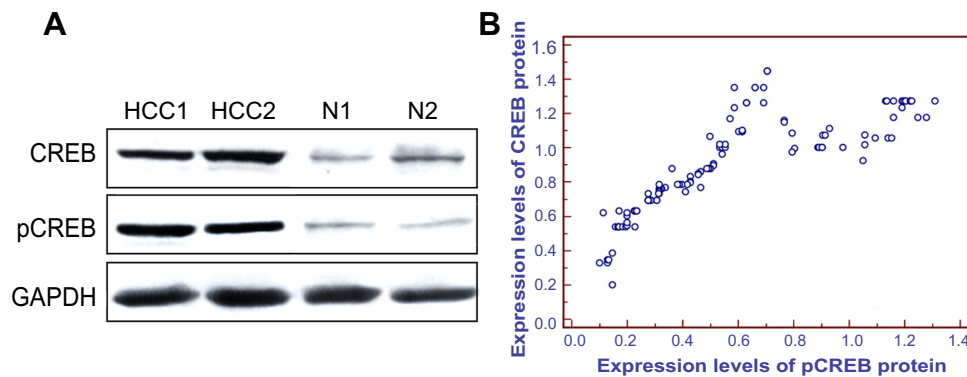


Figure 4 (A and B) Expression levels of CREB and pCREB proteins in hepatocellular carcinoma (HCC) and adjacent nonneoplastic liver tissues detected by Western blot. **(A)** Representative Western blotting of CREB and pCREB protein levels in HCC tissues and adjacent nonneoplastic liver tissues. **(B)** Scatter diagram of expression levels of CREB and pCREB proteins in HCC tissues detected by Western blot. Relative CREB- and pCREB-expression levels were both indicated after normalization to GAPDH protein (internal control). As determined by Spearman's correlation,¹⁶ CREB expression was closely correlated with pCREB expression ($r=0.56$, $P=0.008$).

HCC (Table 1). We found that the combined upregulation of CREB and pCREB proteins (CREB-high/pCREB-high) was significantly associated with serum α -fetoprotein (AFP; $P=0.02$, Table 1), tumor stage ($P<0.001$, Table 1), and tumor grade ($P=0.01$, Table 1).

Upregulation of CREB and pCREB proteins predicts poor prognosis in HCC

Five-year disease-free survival was observed in 30 (23.08%) patients, whereas in 100 (76.92%) patients, disease recurred, and 88 (67.69%) died during the 5-year follow-up period. The association between coexpression of CREB/pCREB and the survival rates was tested by the Kaplan–Meier method. The χ^2 value by log rank (Mantel–Cox) indicated a significant difference among different groups with regards to the conjoined expression status of CREB/pCREB (Figure 5A and B).

Results of pair-wise comparisons showed that statistically significant differences in disease-free survival and overall survival existed between CREB-high/pCREB-high patients and all of the other three groups (both $P<0.001$). In all four groups, CREB-high/pCREB-high patients had the poorest prognosis. More interestingly, disease-free survival and overall survival of patients with CREB-low/pCREB-high expression were both significantly shorter than those with CREB-high/pCREB-low (both $P=0.001$, Figure 5A and B), suggesting that high levels of the activated form pCREB may correlate with poor prognosis.

Furthermore, in a multivariate Cox model, including serum AFP, tumor stage, tumor grade, presence of cirrhosis, sex, age, and combined CREB/pCREB expression, we found that tumor stage ($P=0.009$ and 0.01 , Table 2) and combined CREB/pCREB expression (both $P=0.01$, Table 2) were

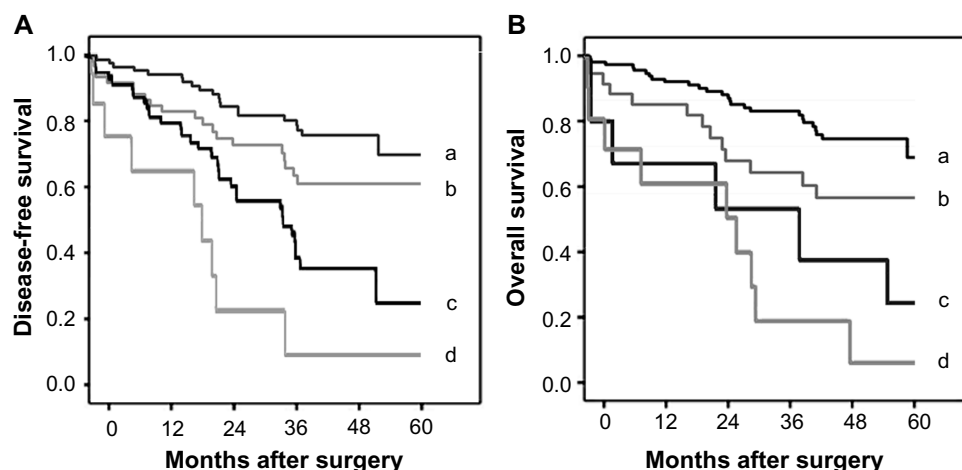


Figure 5 Disease-free survival and overall survival curves for four groups defined by the combined expression of CREB **(A)** and pCREB **(B)** in patients with hepatocellular carcinoma (HCC). CREB-low/pCREB-low (a); CREB-high/pCREB-low (b); CREB-low/pCREB-high (c); CREB-high/pCREB-high (d). Results by pair-wise comparisons showed that statistically significant differences of overall and disease-free survival existed between CREB-high/pCREB-high patients and all the other three groups (both $P<0.001$). In all four groups, CREB-high/pCREB-high patients had the poorest prognosis.

Table 2 Multivariate survival analysis of 5-year overall and disease-free survival in 130 patients with hepatocellular carcinoma

Features	5-year overall survival			5-year disease-free survival		
	HR	95% CI	P	HR	95% CI	P
Age	1.132	0.316–3.516	–	1.536	0.322–3.736	–
Sex	1.191	0.345–3.857	–	1.559	0.357–3.831	–
Serum AFP	1.931	0.685–4.056	–	1.953	0.615–4.273	–
Tumor stage	2.879	1.366–5.196	0.009**	2.686	1.386–6.009	0.01*
Tumor grade	1.563	0.609–4.088	–	1.551	0.607–4.466	–
Presence of cirrhosis	1.919	0.738–4.102	–	1.921	0.793–4.219	–
CREB/pCREB expression	5.296	1.296–11.661	0.01*	5.828	1.322–12.687	0.01*

Notes: *P<0.05; **P<0.01.

Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; HR, hazard ratio.

independent poor prognostic factors for both 5-year disease-free survival and 5-year overall survival in HCC.

Discussion

As a common malignancy, HCC incidence is still on the rise in developing countries. It is generally believed that early detection offers curative treatment for HCC patients. Therefore, it is extremely important to develop effective screening strategies for high-risk patients. In the current study, we observed the expression patterns and subcellular localizations of CREB and pCREB proteins in 130 HCC tissues and paired adjacent nonneoplastic liver tissues using immunohistochemistry and Western blot analyses. At first, we confirmed that the positive stainings of CREB and pCREB proteins mainly occurred in the nucleus of tumor cells in HCC tissues, and that the expression levels of the two proteins were significantly upregulated in HCC tissues compared with paired adjacent nonneoplastic tissues, suggesting the activation of the CREB protein in HCC. Since both the immunohistochemistry and Western blot results showed that CREB expression was closely correlated with pCREB expression, we further investigated the association of the combined expression of the two proteins with tumor progression of HCC. As a result, we found that the combined upregulation of CREB and pCREB proteins was significantly associated with aggressive clinicopathological features and poor prognosis in patients with HCC, indicating for the first time that the coexpression of CREB and pCREB proteins may be a useful diagnostic and prognostic marker for HCC patients. These findings may be important for the prevention and treatment of this malignancy.

CREB functions as a regulator of the expression of several genes in response to hormones, ion fluxes, growth factors, and stress signals.¹⁷ CREB-binding sites (CRE1 and CRE2) are essential for cAMP-induced promoter II activity, which requires phosphorylation by cAMP-dependent protein kinase at serine 119 and 133 for interaction with

deoxyribonucleic acid and ribonucleic acid polymerase II.¹⁸ Recent studies have identified several downstream genes of CREB, including the cell cycle-related genes cyclin A1, cyclin B1, and cyclin D2, the oncogene *Bcl-2*, activated transcription factor 3, NF- κ B, signal-transduction proteins, and other growth-related genes.^{19,20} Accumulating evidence shows that CREB is overexpressed in several neoplastic cancers, functions as an important oncogene that promotes tumor cell growth, and is involved in the proliferation, survival, and metastasis of tumor cells.^{21–24} For example, CREB is present at a high level in human glioma tissues,²¹ CREB is upregulated in malignant mesothelioma cells through an epidermal growth-factor receptor/protein-kinase A pathway,²² CREB is also found to be involved in the occurrence of breast cancer²³ and CREB overexpression may counteract the suppression of growth in gastric cancer cells.²⁴ Similarly to these previous studies, our data showed that CREB- and pCREB-expression levels were both significantly increased in HCC tissues versus nonneoplastic liver tissues. In addition, we observed that the combined upregulation of CREB and pCREB was significantly associated with advanced tumor stage, high tumor grade, high serum AFP levels, and unfavorable prognosis, suggesting that the activation of the CREB protein may serve as a molecular prognostic marker for HCC, and that CREB overexpression in combination with pCREB upregulation may be associated with even worse prognosis of HCC patients.

Taken together, these findings indicate for the first time that activation of the CREB protein may be associated with tumor progression in HCC, and may serve as a valuable marker of prognosis for patients with this malignancy. Although the role of CREB in human tumor pathology has been explored extensively, its molecular mechanisms in different tumor types have not been fully elucidated. Further studies are needed to investigate the precise mechanisms of CREB in the progression of HCC.

Disclosure

The authors report no conflicts of interest in this work.

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