CREB Overexpression: A Feature Associated with Negative Prognosis in Never-Smokers with NSCLC

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Abstract

Lung cancer is the leading cause of cancer deaths worldwide. Recent advances in targeted therapies hold promise for the development of new treatments for certain subsets of cancer patients by targeting specific signaling molecule. Based on the identification of the transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) as an important regulator of growth of several types of cancers and our recent findings of its importance in normal differentiation of bronchial epithelial cells, we hypothesized that CREB plays an important pathobiologic role in lung carcinogenesis. We conducted this initial study to determine whether the expression and activation status of CREB are altered in non-small cell lung cancer (NSCLC) and of any prognostic importance in NSCLC patients. We found that the expression levels of mRNA and protein of CREB and phosphorylated CREB (p-CREB) were significantly higher in most of the NSCLC cell lines and tumor specimens than in the normal human tracheobronchial epithelial (NHTBE) cells and adjacent normal lung tissue, respectively. Analysis of CREB mRNA expression and the *CREB* gene copy number showed that CREB overexpression occurred mainly at the transcriptional level. Immunohistochemical analysis of tissue microarray (TMA) slides containing sections of NSCLC specimens obtained from 310 patients showed that a decreased survival duration was significantly associated with overexpression of CREB or p-CREB in never-smokers but not in current or former smokers with NSCLC. These are the first reported results illustrating the potential of CREB as a molecular target for the prevention and treatment of NSCLC, especially in never-smokers.

Keywords

CREB overexpression; prognosis; NSCLC; adenocarcinoma; squamous cell carcinoma

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Introduction

Lung cancer is the leading cause of cancer deaths, and its incidence is rising (1). In the United States, 215,020 new cases and 161,840 deaths of lung and bronchial cancer are projected to occur in 2008 (2). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers and is subdivided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (3). Recent advances in targeted therapies directed against the epidermal growth factor receptor and vascular endothelial growth factor receptor pathways showed marked improvement in treatment in a subset of patients with lung cancer (4–6). Thus, identifying new molecular targets for treatment and/or prevention of NSCLC is warranted and urgently needed to improve the control of this deadly form of lung cancer.

Studies have shown that cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) plays important roles in cell differentiation (7), survival (8,9), proliferation (10), development (11,12), cell cycle progression (10), and glucose metabolism (13). CREB is activated by cAMP, growth factors, hormones, retinoids (14), cytokines (15), and prostaglandins (16) via multiple signaling pathways (13,17), including the cAMP/ protein kinase A, phosphatidylinositol 3-kinase/Akt, extracellular signal-regulated kinase/ p90 ribosomal S6 kinase, and p38/mitogen- and stress-activated protein kinase pathways. Once activated, CREB induces the expression of cAMP response element-containing target genes (13,18,19), which play important roles in differentiation (14), cell cycle progression (20), apoptosis suppression (21), proliferation (22), neovascularization (23), inflammation (24), and tumorigenesis (25).

Recently, we showed that CREB plays a physiologic role in mucous differentiation of normal human tracheobronchial epithelial (NHTBE) cells (14,26). Previous studies found that CREB has a pathobiologic role in the growth of breast cancer, melanoma, and hepatocellular carcinoma cells (7,27,28). Recent studies also showed that CREB acts as a proto-oncogene to regulate hematopoiesis and to contributes to the leukemia phenotype (29–31). However, whether CREB expression is altered in human NSCLC tumors and whether CREB/phosphorylated CREB (p-CREB) expression correlates with the survival rate in patients with NSCLC has not been previously shown.

Based on these previous findings, we hypothesized that CREB, which plays an important role in normal differentiation of bronchial epithelial cells, may also have an important pathobiologic role in lung carcinogenesis as a transcriptional regulatory factor. To test this hypothesis, we compared the CREB expression levels and activation statuses and the *CREB* gene copy numbers in 10 NSCLC cell lines, NHTBE cells, 6 frozen human NSCLC tissue specimens, and paired normal lung tissue specimens. We also analyzed CREB and p-CREB expression in 45 paraffin-embedded whole specimens of NSCLC tumor and adjacent normal bronchial or bronchiolar epithelial tissue specimens. Lastly, we assessed the levels of CREB and p-CREB expression in association with clinicopathologic parameters and overall survival duration of 310 NSCLC patients with banked NSCLC tissue specimens using tissue microarray (TMA) analysis. Although studies have consistently demonstrated that smoking is an important etiological factor for lung cancer, about 15% of men and 53% of women with this disease worldwide are never-smokers (32). Thus, we also analyzed the effects of CREB and p-CREB expression on overall survival duration in patients with NSCLC according to smoking status.

Materials and Methods

NSCLC tissue specimens and TMA construction

Six frozen tumor tissue specimens (three squamous cell carcinoma and three adenocarcinoma) and six adjacent normal lung tissue specimens surgically resected from patients who underwent lobectomies or pneumonectomies for primary NSCLC were obtained from the tissue bank of The University of Texas M. D. Anderson Cancer Center Specialized Program of Research Excellence in Lung Cancer. All of the tumors were histologically examined and classified using the 2004 World Health Organization International Classification of Lung Tumors (33). Also, specimens of tumor and adjacent normal lung tissue (including bronchial and bronchiolar epithelia) obtained from 45 patients with surgically resected NSCLC (26 adenocarcinoma, 19 squamous cell carcinoma) were randomly selected for assessment of immunohistochemical expression of CREB and p-CREB in whole histologic sections. After histologic examination of 310 NSCLC specimens (194 adenocarcinoma or bronchioloalveolar carcinoma and 116 squamous cell carcinoma) in the tissue bank of The University of Texas M. D. Anderson Cancer Center, tumor TMAs were constructed using three tissue cores per tumor that were 1 mm in diameter to obtain tissue from central, intermediate, and peripheral tumor areas. The M. D. Anderson Cancer Center Institutional Review Board approved the use of the archived clinical tissue specimens.

Smoking history

Patients who had smoked at least 100 cigarettes in their lifetime were defined as ever-smokers, and patients who quit smoking at least 12 months before their lung cancer diagnosis were defined as former smokers. Current smokers were defined as active smokers who had been smoking for at least 6 months. Subjects were asked whether they had ever smoked any tobacco products (nonfiltered or filtered cigarettes, cigars, or pipes) for at least 6 months and were classified as never-smokers if they had not, as current smokers if they smoked daily at the date of diagnosis (for patients) or interview (for controls), or as former smokers if they had stopped smoking daily before those dates.

Cell cultures

NHTBE cells obtained from Cambrex (East Rutherford, NJ) were organotypically cultured and maintained as described previously (34–37). Basically, NHTBE cells from passage 2 were seeded at a density of 1×10^5 per insert onto 24-mm, uncoated, semipermeable membranes (Transwell clear; Costar, Cambridge, MA) in a 1:1 mixture of Dulbecco's modified Eagle's medium (Invitrogen Co., Carlsbad, CA) and bronchial epithelial cell basal medium (Cambrex) supplemented with transferrin (10 ng/mL), epinephrine (0.5 μg/mL), insulin (5 μg/mL), triiodothyronine (6.5 ng/mL M), hydrocortisone (0.5 μg/mL), epidermal growth factor (0.5 ng/mL), bovine pituitary extract (1% w/v), bovine serum albumin (1.5 μg/mL), gentamicin (10 μg/mL), and retinoic acid (5×10^{-8} M). The cells were grown submerged in the media for the first 7 days, after which time an air-liquid interface was created. The cells were then cultured in the air-liquid interface for 3 weeks, with the medium changed every 24 h. Fully differentiated 28-day-old cultures developed mucociliary phenotypes similar to that of *in vivo* bronchial epithelium. Also, 10 NSCLC cell lines (H226, H292, H520, H2170, H1563, H1734, H1975, H2228, A549, H1703) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum and gentamicin (10 μg/mL).

Western blot analysis

Western blot analysis was performed as described previously (14) to measure the expression of CREB and p-CREB in whole cell extracts from the NHTBE cells and NSCLC cell lines and from the six archived NSCLC specimens and paired normal lung tissue specimens.

CREB mRNA expression and *CREB* gene copy number

Total RNA and genomic DNA from the NHTBE cells, NSCLC cells, and frozen NSCLC specimens were extracted using the QIAGEN RNeasy Mini Kit and Blood & Cell Culture DNA Mini Kit (QIAGEN, Valencia, CA) and subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR) and conventional polymerase chain reaction (PCR) analysis to determine CREB mRNA expression and *CREB* gene copy number, as described previously (14). The primer sequences used for detection of CREB mRNA in qRT-PCR were as follows: forward, 5′-ACTGTAACGGTGCCAACTCC-3′; reverse, 5′-GAATGGTAGTACCCGGCTGA-3′. The mRNA level of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected with VIC dye (Applied Biosystems, Foster City, CA) was used as endogenous control. The primer sequences used for determination of the *CREB* gene copy number in conventional PCR were as follows: forward, 5′-AAGAGGAGACTTCAGCACCTG-3′; reverse, 5′-GCAAAACTGAGAAGACTTGGC-3′. For endogenous control, the following β-actin primer sequences were used: forward, 5′-AGGTCATCACCATTGGCAAT-3′; reverse, 5′-AATGAGGGCAGGACTTAGCTT-3′.

Immunohistochemical analysis

Immunohistochemical analysis of the NSCLC and adjacent normal bronchial and bronchiolar epithelial tissue specimens and of the TMA NSCLC tissue specimens was performed using anti-CREB and anti-p-CREB antibodies (Upstate, Lake Placid, NY) at a dilution of 1:100 according to the manufacturer's instructions. Immunostaining was visualized using the Histostain-Bulk-SP kit and the AEC red substrate kit (Zymed Laboratories, South San Francisco, CA). Immunohistochemical staining without a primary antibody was performed as a negative control. Distinct nuclear immunostaining for CREB and p-CREB was quantified by an experienced lung cancer pathologist (I.I.W.) under a light microscope. The observer quantified immunohistochemistry expression in a blindly fashion regarding the clinical features of the cases (38,39). In each specimen, up to 1000 tumor and epithelial cells were examined using a $\times 20$ magnification objective. The intensity of CREB and p-CREB immunostaining was graded on a scale of 0 to 3, with 0 indicating no staining, 1 indicating weak staining, 2 indicating moderate staining, and 3 indicating strong staining. The extent of positive immunoreactivity for CREB and p-CREB (0 to 1; 1 = 100%) was calculated as the percentage of cells that had nuclear staining for CREB or p-CREB. Scoring of immunohistochemical staining in each specimen was determined as the product of positive immunostaining intensity (0 to 3) and positive immunoreactivity extent (0 to 1).

Statistical analysis

A mixed-effect general linear model was used to assess the differences in CREB and p-CREB expression in the normal lung and NSCLC tissue specimens. The Kruskal-Wallis test and Wilcoxon rank sum test were used to assess the relationships between CREB and p-CREB expression in the TMA specimens and patients' demographic and clinicopathologic characteristics. Cox proportional hazards regression models were used to assess the effect of the CREB and p-CREB immunostaining scores on overall survival duration (time from surgery to death of any cause). Survival curves were determined using the Kaplan-Meier product limit estimates, and differences in probability of survival between groups were assessed statistically using the log-rank test. The need for transformation of predictive variables in the Cox proportional hazards regression models was assessed using martingale

residual plots. Predictive variables with *P* values < 0.10 for the univariate Cox proportional hazards regression model were included in a multivariate model. In this multivariate model, backward elimination with a *P* value cutoff of 0.05 was used; any previously deleted variables were then allowed to reenter the final model if *P* < 0.05.

Results

Higher expression of CREB and p-CREB in NSCLC cell lines than in NHTBE cells

Western blot analysis showed that most of the NSCLC cell lines had higher levels of CREB (9 of 10 cell lines) and p-CREB (7 of 10 cell lines) protein expression than did the NHTBE cells (Fig. 1*A*). qRT-PCR analysis showed a similar pattern in CREB mRNA expression: about 2-fold to 12-fold higher levels in 6 of 10 NSCLC cell lines than in NHTBE cells (*P* < 0.05 in 4 cell lines; *P* < 0.01 in 2 cell lines) (Fig. 1*B*); these 6 cell lines also had the highest p-CREB protein expression levels. Unlike CREB protein and mRNA expression, PCR analysis showed that the *CREB* gene copy number was significantly increased in only 2 of the 10 NSCLC cell lines (*P* < 0.05 for both cell lines) (Fig. 1*C*). These data clearly show that CREB was overexpressed and highly activated in most of the NSCLC cell lines. Moreover, CREB overexpression in these cell lines mainly resulted from increased *CREB* gene transcription rather than an amplified *CREB* gene copy number.

Higher expression of CREB in frozen NSCLC specimens than in adjacent normal lung tissue specimens

CREB protein was overexpressed (by 50 to 92%) and more highly activated (by 18 to 119%) in frozen NSCLC tissue specimens than in adjacent normal tissue specimens according to our western blot analysis (Fig. 2*A*). Among the six frozen specimens, qRT-PCR analysis showed that CREB mRNA expression was significantly higher in all three squamous cell specimens (by 73 to 95%) and in two of the three adenocarcinoma specimens (by 65 and 73%) than in the adjacent normal tissue specimens (*P* < 0.05 for all five specimens) (Fig. 2*B*). PCR analysis revealed that only one squamous cell carcinoma tissue specimen and one adenocarcinoma tissue specimen had a significantly increased *CREB* gene copy number when compared with normal tissue specimens (*P* < 0.05 for both pairs of specimens) (Fig. 2*C*).

The overexpression of CREB and p-CREB in these frozen NSCLC tissue specimens was concordant with our results in the NSCLC cell lines. Taken together, our cell line and frozen tissue specimen results showed that CREB overexpression occurred primarily at the gene transcription level but in some cases could have resulted from an amplified *CREB* gene copy number.

Higher expression of CREB in paraffin-embedded NSCLC specimens than in adjacent normal lung tissue specimens

Immunohistochemical analysis of CREB and p-CREB expression in the 45 whole paraffin-embedded NSCLC specimens and adjacent normal bronchial and bronchiolar epithelial tissue specimens showed stronger nuclear staining for CREB and p-CREB in both adenocarcinoma and squamous cell carcinoma tissue than in normal tissue (Fig. 3*A*). The distributions of the staining scores for the tumor tissue and normal epithelium are shown in Fig 3*A*. Statistical analysis showed significantly higher immunostaining scores for both CREB (1.37 *versus* 0.73; *P* = 0.013) and p-CREB (1.96 *versus* 1.05; *P* = 0.0002) in tumor tissue than in normal tissue.

Association of CREB and p-CREB expression with histologic NSCLC subtype and patients' demographic and clinicopathologic characteristics

We performed immunohistochemical analysis of CREB and p-CREB expression in the 310 NSCLC TMAs to assess potential associations of CREB and p-CREB overexpression with the patients' demographic and clinicopathologic characteristics and histologic subtypes of NSCLC (listed in Supplementary Table 1). Representative images of the staining of CREB and p-CREB in the TMA specimens are shown in Fig. 3*B*. According to the Wilcoxon rank sum test, the CREB and p-CREB immunostaining scores were significantly higher in the squamous cell carcinoma specimens than in the adenocarcinoma and bronchioloalveolar carcinoma specimens (Fig. 3*B*, CREB: 0.61 *versus* 0.43, $P = 0.002$; p-CREB: 0.32 *versus* 0.25, $P = 0.008$). The levels of immunohistochemical staining of CREB and p-CREB were lower in the TMA specimens than in the paraffin-embedded whole NSCLC tissue specimens. This phenomenon probably resulted from larger tissue areas and stronger immunostaining in the whole tissue specimens than in the TMA specimens. We detected no significant differences in CREB or p-CREB expression between other demographic and clinicopathologic subpopulations (Table 1).

Effects of CREB and p-CREB overexpression on overall survival duration

The Kaplan-Meier survival curves shown in Fig. 4 demonstrate that overexpression of CREB or p-CREB was related to a lower probability of survival. Overexpression of CREB (immunostaining score > 0.9 (E/N = 25/56)) or p-CREB (immunostaining score > 0.7 (E/N = 23/45)) was significantly associated with decreased overall survival duration in patients with NSCLC (log-rank test; $P = 0.02$ and $P = 0.002$, respectively). We also used univariate Cox proportional hazards regression models to determine the effects of covariates on overall survival duration. Factors that significantly affected overall survival were age, histologic subtype, p-CREB expression, pathologic T classification, pathologic N classification, and pathologic TNM stage (Table 1). Multivariate Cox proportional hazards regression model analysis showed that the expression of both CREB and p-CREB was significantly associated with decreased overall survival after accounting for the effects of age and pathologic T and N classification (CREB: $P = 0.02$, p-CREB: $P = 0.01$). One-unit increases in the CREB and p-CREB immunostaining scores increased the risk of death by 51% and 80%, respectively (Table 1).

Effects of CREB and p-CREB overexpression on survival duration according to smoking status

The inverse relationship between the level of CREB and p-CREB expression and the survival duration was significantly dependent on smoking status in the 310 patients from whom the TMA NSCLC specimens were obtained. Overexpression of CREB and p-CREB significantly lowered the probability of survival in never-smokers (CREB immunostaining score > 0.5 (E/N = 16/38) (p-CREB immunostaining score > 0.5 (E/N = 10/17) (Fig. 4, right-hand panels) but not in former- or current- smokers (data not shown). Also, univariate Cox proportional hazards regression model analysis demonstrated that both CREB and p-CREB immunostaining scores were significantly inversely correlated with overall survival duration in never-smokers, increasing the risk of death by 73% ($P = 0.02$) and 169% ($P = 0.02$), respectively. We also observed this tendency in former-smokers, as both CREB and p-CREB immunostaining scores were inversely correlated with overall survival duration, increasing the risk of death by 28% ($P = 0.39$) and 113% ($P = 0.01$), respectively. In contrast, in current-smokers, neither CREB nor p-CREB immunostaining scores affected overall survival duration ($P = 0.41$ and $P = 0.96$ respectively; Table 2).

Discussion

Our study of the role of CREB in the development and pathogenesis of NSCLC, showed that CREB and p-CREB are overexpressed in patients with NSCLC and that this overexpression is associated with a negative prognosis in never-smokers with this disease. CREB expression levels were higher and CREB was more highly activated constitutively in NSCLC cell lines than in NHTBE cells. CREB and p-CREB were also expressed at higher levels in frozen NSCLC tumor specimens than in paired normal tissue specimens. This overexpression of CREB protein resulted primarily from transcriptional overexpression of CREB mRNA rather than from amplification of the *CREB* gene copy number. These results are consistent with a recent report showing that CREB expression is upregulated at both the protein and mRNA levels in primary acute myeloid leukemia cells compared with that in normal blood cells (9). However, the report indicated that CREB overexpression was associated mainly with an amplified *CREB* gene copy number (in three of four patients with acute myeloid leukemia), whereas we found that CREB overexpression occurred mainly at the gene transcription level (and possibly as a result of an increased *CREB* gene copy number in a few cases). This discrepancy implies mechanistic variation in CREB overexpression in different cancer types. Immunohistochemical analysis of slides containing whole sections of NSCLC and adjacent normal bronchial or bronchiolar epithelial tissue specimens confirmed that expression of both CREB and p-CREB was significantly higher in the tumor specimens than in the normal tissue specimens. Also, the results of our immunohistochemical study on NSCLC TMAs showed that CREB and p-CREB immunostaining scores were significantly higher in the squamous cell carcinoma specimens than in the adenocarcinoma specimens. Taken together, these results clearly indicate overexpression of CREB in NSCLC.

In addition, the Kaplan-Meier survival curves revealed that overexpression of both CREB and p-CREB was significantly associated with decreased overall survival durations in the 310 patients from whom the TMA NSCLC specimens were obtained. This observed effect of CREB and p-CREB overexpression on survival was mainly contributed by the never-smokers portion of patients. The survival durations in these patients were strongly influenced by the levels of CREB and p-CREB expression, whereas the durations in former and current smokers were less or not affected by them. These data suggest that the expression level of both CREB and p-CREB is a useful biomarker for predicting survival duration and therefore CREB could be a therapeutic target for never-smokers with NSCLC. Prognosis for and treatment outcome of NSCLC are often in favor of never-smokers (40,41). The reason why overexpression of CREB and p-CREB is a negative prognostic factor in never-smokers but not in former or current smokers is unclear. One possible explanation is that survival of cancer cells depends substantially on CREB activity in never-smokers, less so in former smokers, and not at all in current smokers. Alternatively, former and current smokers may have other confounding factors that predominate over CREB activity in affecting overall survival.

Cancers in ever-smokers may use multiple (proto)oncogenic pathways for growth and survival. While tobacco smoking is the leading cause of lung cancer, several studies have shown that the biology of lung cancer differs between never-smokers and ever-smokers (41–45) see Sun *et al.* for a comprehensive review (46). For examples, some well characterized mutations of EGFR are more frequently detected in a subset of lung cancer patients who are never smokers whereas mutations in KRas are more prevalent in smokers and such mutation statuses are mutually exclusive in each given set of lung cancers (42,46). CREB is known to be activated by two downstream pathways of EGFR signaling, namely Ras-Raf-ERK-RSK (26,47) and PI3K-Akt (48) pathways, but it is not clear whether these pathways differentially regulate CREB expression and activity. Further studies are required to

determine whether CREB plays a differential role in the pathogenesis of lung cancers between smokers and never smokers.

Of note is that CREB may also play a significant role in the progression of lung cancer at its early stage. Studies showed that the transcriptional activity of CREB mediated tobacco smoke-stimulated overexpression of amphiregulin (49), which has been shown to be associated with poor prognosis in patients with NSCLC indicating that CREB may play an important role in the early stage of lung carcinogenesis in smokers (50). Recent studies examining the role of CREB in lung cancer have shown an elevated expression of p-CREB in lung tumors generated in insulin-like growth factor II-overexpressing transgenic mice and that CREB played an important role in survival of lung cancer cell lines (51,52). Further studies are warranted to molecularly characterize the differential role of CREB in smokers and never-smokers with NSCLC tumors.

CREB overexpression may lead to upregulation of genes and activation of signaling pathways that support lung tumor growth and survival. To test this hypothesis, we have gathered data indicating that inactivation of CREB or reduction of CREB expression (via the expression of a dominant repressor of CREB or small interfering RNA against CREB) reduces the expression of antiapoptotic genes and consequently inhibits the growth and survival of NSCLC cells (53). The present study showed that increased expression of CREB and p-CREB correlates with decreased overall survival durations in lung cancer patients, suggesting that sustained overexpression of CREB in malignant cells supports the growth and survival of tumor cells.

In summary, the present study provided important initial insights into the role of CREB in the development and pathogenesis of NSCLC. First, CREB was overexpressed and highly active constitutively in NSCLC cell lines and banked NSCLC specimens. Second, CREB overexpression appeared to be more attributable to increased CREB mRNA transcription than *CREB* gene amplification, suggesting that CREB overexpression is a correctable therapeutic target for patients with NSCLC. Third, overexpression of both CREB and p-CREB was independently correlated with significantly decreased overall survival durations in never-smokers but not in current or former smokers with NSCLC. However, further extensive studies are required to determine whether CREB plays a differential role in the development of lung cancer in never- and ever-smokers. To the best of our knowledge, this study is the first to provide evidence that CREB overexpression and p-CREB overexpression are negative prognostic factors in never-smokers with NSCLC. Therefore, targeting CREB, such as with the use of CREB inhibitors, may be a preventive strategy for individuals at high risk for NSCLC and/or a targeted therapeutic strategy for this disease, especially among never-smokers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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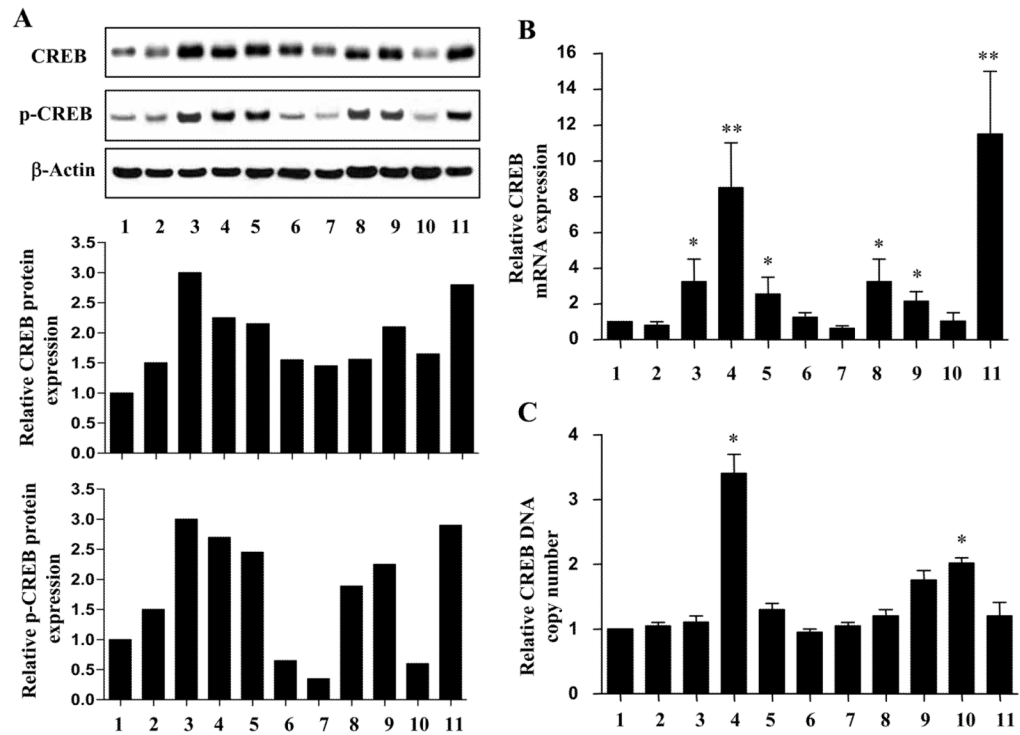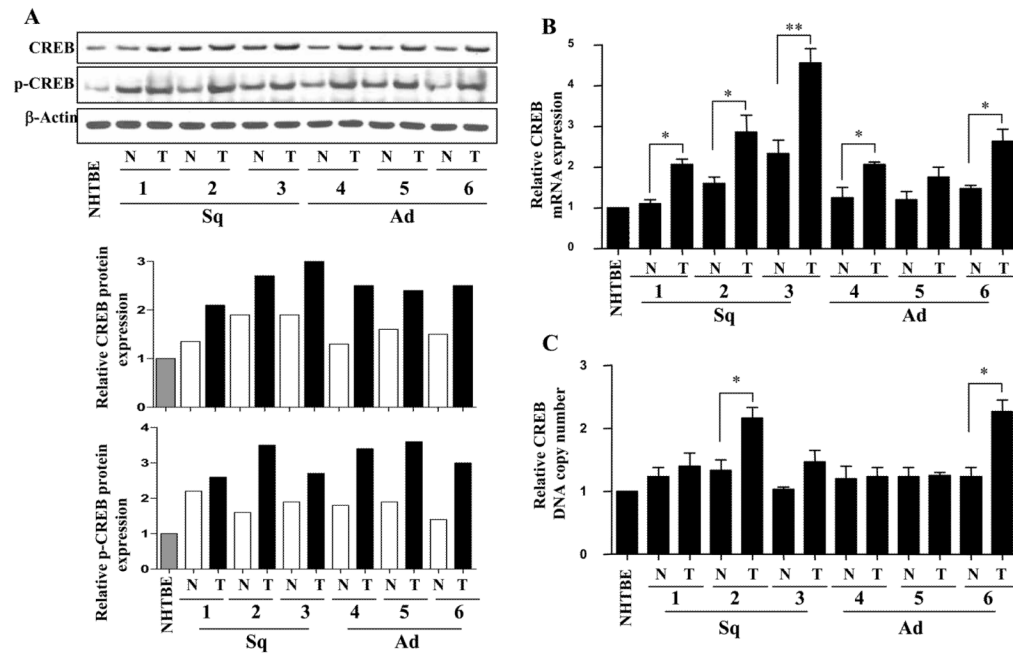
Figure 1.
Expression of CREB and p-CREB in NHTBE and NSCLC cells. *A*, Western blot analysis of
CREB and p-CREB expression. Whole-cell lysates were prepared from fully differentiated
NHTBE cells (*lane 1*) and the 10 NSCLC cell lines (*lane 2*, H226; *lane 3*, H292; *lane 4*,
H520; *lane 5*, H2170; *lane 6*, H1563; *lane 7*, H1734; *lane 8*, H1975; *lane 9*, H2228; *lane 10*,
A549; *lane 11*, H1703) grown in optimal medium up to confluence. Equal amounts of lysate
were subjected to SDS-PAGE and western blotting. The blots were probed with anti-CREB
and anti-p-CREB antibodies. Equal protein loading was confirmed by stripping the blots and
reprobing them with an anti-β-actin antibody. The expression levels of CREB and p-CREB
proteins for each cell line in relative to the NHTBE cells were quantitated and shown below.
B, qRT-PCR analysis of CREB mRNA expression. The CREB mRNA level in each cell line
was analyzed with qRT-PCR. The values shown are the ratios of the CREB mRNA
expressed in NSCLC cells to that expressed in NHTBE cells, with CREB mRNA levels
normalized against the GAPDH mRNA level. The results shown are from a representative
experiment performed twice, each run in triplicate. The data are expressed as the mean ±
standard error (S.E.). *: $P < 0.05$; **: $P < 0.01$ *versus* NHTBE cells (*lane 1*) with Student's
t-test. *C*, *CREB* gene copy number analysis with PCR. The genomic DNA obtained from
each cell line was subjected to PCR. The results shown are from a representative experiment
performed twice, each run in triplicate. The values are the ratios of the *CREB* DNA copy
number in NSCLC cells to that in NHTBE cells, with CREB DNA normalized against the β-
actin DNA level. The data are expressed as the mean ± S.E. *: $P < 0.05$; **: $P < 0.01$ *versus*
NHTBE cells (*lane 1*) with Student's *t*-test.

Figure 2.
Expression of CREB and p-CREB in frozen human NSCLC specimens and adjacent normal bronchial and bronchiolar epithelial tissue specimens. *A*, Western blot analysis of CREB and p-CREB expression. Soluble proteins obtained from three squamous cell carcinoma (Sq) and three adenocarcinoma (Ad) tissue specimens (T) and paired matching normal tissue specimens (N) were subjected to western blot analysis for the levels of total CREB and p-CREB expression. Equal protein loading was confirmed by stripping the blots and reprobing them with an anti-β-actin antibody. The expression levels of CREB and p-CREB proteins in tissue specimens in relative to that of the NHTBE cells (hatched bar) were quantitated and shown below. *B*, qRT-PCR analysis of CREB mRNA expression. Total mRNA from the NSCLC tissue specimens and paired normal tissue specimens described in *A* was subjected to qRT-PCR. The values shown are the ratios of the CREB mRNA expressed in tissue specimens to that expressed in NHTBE cells, with CREB mRNA levels normalized against the GAPDH mRNA level. The results are from a representative experiment performed twice, and samples were run in triplicate. The data are expressed as the mean ± S.E. *: $P < 0.05$; **: $P < 0.01$ *versus* matched normal tissue with Student's *t*-test. *C*, PCR analysis of *CREB* gene copy number. The genomic DNA extracted from the NSCLC tissue specimens and paired normal tissue specimens was subjected to qPCR. The values are the ratios of the *CREB* DNA copy numbers in normal and tumor tissues to the *CREB* DNA copy numbers in NHTBE cells, with *CREB* DNA normalized against the β-actin DNA level. The results are from a representative experiment performed twice, and samples were run in triplicate. The data are expressed as the mean ± S.E. *: $P < 0.05$; **: $P < 0.01$ *versus* matched normal tissue with Student's *t*-test.
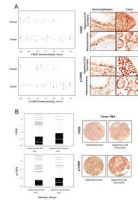
Figure 3.
Immunohistochemical analysis of CREB and p-CREB expression in normal and tumor lung tissues. *A*, Fixed NSCLC tissue specimens (26 adenocarcinoma, 19 squamous cell carcinoma) and adjacent normal bronchial and bronchiolar epithelial tissue specimens were subjected to immunohistochemical staining, and then scored according to the criteria mentioned in *Method and Material*. BLiP plots displaying the distribution of CREB and p-CREB immunostaining scores in normal and tumor tissues are shown (*right*). Average scores for CREB are 1.37 in tumor *versus* 0.73 in normal ($P = 0.013$) and for p-CREB 1.96 in tumor *versus* 1.05 in normal ($P = 0.0002$). Representative Images (*left*) were captured at a magnification of ×200. Arrows indicate nuclear CREB and p-CREB immunostaining in the basal layer of normal bronchial epithelial and tumor tissue specimens. *B*, CREB and p-CREB immunostaining of TMA specimens from a total of 310 patients with either adenocarcinoma (194) or squamous cell carcinoma (116) are included in the analysis. The distributions of the staining scores for the two histological types of NSCLC are presented in the box plots (*left*). The number of samples measured was indicated under each category. X marks and lines inside the quartile boxes are means and medians respectively (for CREB, 0.43 and 0.23 in adenocarcinoma/BAC versus 0.61 and 0.45 in squamous cell carcinoma; for p-CREB, 025 and 0.03 versus 0.32 and 0.2). $P = 0.002$ for CREB; $P = 0.008$ for p-CREB. Representative images of CREB and p-CREB immunostaining of TMA NSCLC specimens are shown in the right.(magnification, ×40).
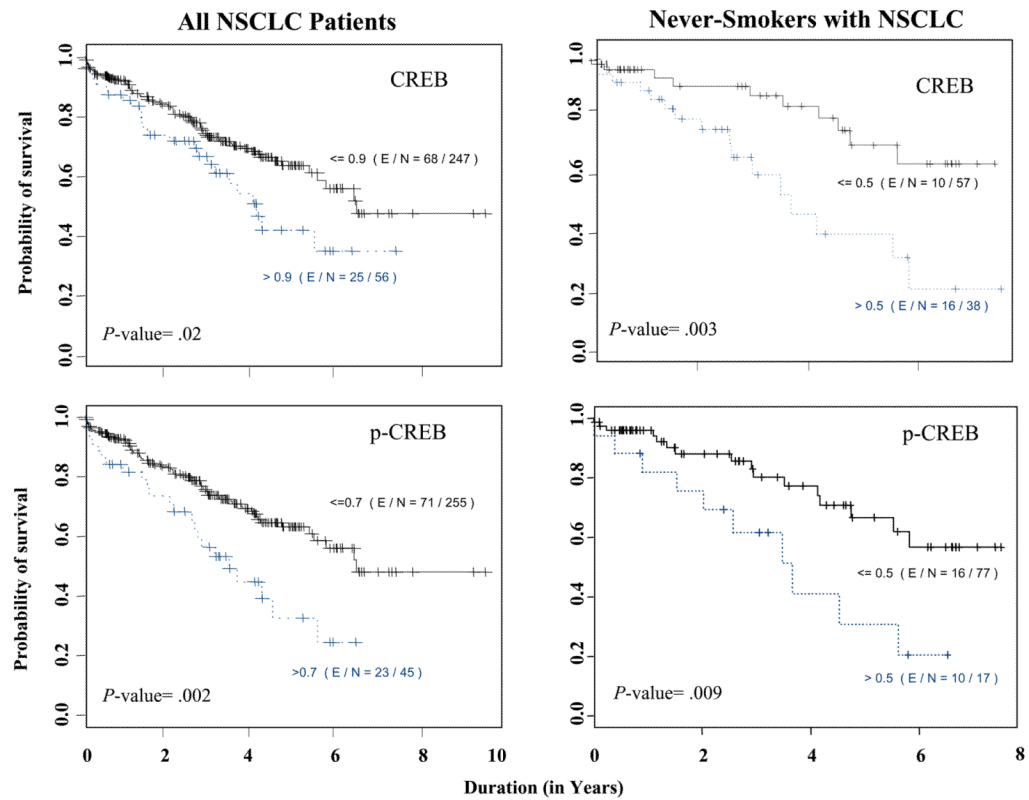
Figure 4.
Kaplan-Meier curves of overall survival duration stratified according to CREB (top) and p-CREB (bottom) expression in the entire cohort (left panel) and in never-smokers (right panel). A total of 310 patients had information available for the survival analysis, 94 of whom died. The median overall survival duration was 6.5 years, and the median follow-up duration was 3.2 years. The cut-off points for the CREB and p-CREB immunostaining scores were identified using martingale residual plots with respect to the overall survival duration. The curves are labeled with the corresponding immunostaining scores.

Table 1

Estimation of overall survival durations per demographic and clinicopathologic characteristics in NSCLC patients from whom tumor TMA specimens were obtained.

Univariate Cox proportional hazards regression models			
Characteristic	**Parameter estimate ±S.E.**	**P**	**Hazards ratio**
Age	0.04 ± 0.01	0.0002	1.04
Sex (male *versus* female)	0.34 ± 0.21	0.1000	1.40
Race (white *versus* other)	−0.31 ± 0.34	0.3500	0.73
Smoking status			
Former *versus* never	0.24 ± 0.25	0.3300	1.27
Current *versus* never	0.03 ± 0.29	0.9300	1.03
Histologic subtype (squamous cell carcinoma *versus* adenocarcinoma/BAC)	0.65 ± 0.21	0.0020	1.92
CREB expression	0.28 ± 0.17	0.1000	1.33
p-CREB expression	0.66 ± 0.23	0.0040	1.93
Pathologic T classification (T2 + T3 + T4 *versus* T1)	0.97 ± 0.27	0.0002	2.63
Pathologic N classification (N1 + N2 + N3 *versus* N0)	0.73 ± 0.23	0.0010	2.08
Pathologic M classification (M1 *versus* M0)	1.05 ± 0.72	0.1400	2.87
Pathologic TNM stage			
II *versus* I	0.65 ± 0.26	0.0100	1.92
III + IV *versus* I	0.86 ± 0.29	0.0030	2.37

Multivariate Cox proportional hazards regression models			
Characteristic	**Parameter estimate ±S.E.**	**P**	**Hazards ratio**
Model A: CREB			
Age	0.04 ± 0.01	0.0002	1.04
CREB expression	0.41 ± 0.18	0.0200	1.51
Pathologic T classification (T2 + T3 + T4 *versus* T1)	0.84 ± 0.28	0.0030	2.31
Pathologic N classification (N1 + N2 + N3 *versus* N0)	0.47 ± 0.24	0.0490	1.60
Model B: p-CREB			
Age	0.04 ± 0.01	0.0005	1.04
p-CREB expression	0.59 ± 0.23	0.0100	1.80
Pathologic T classification (T2 + T3 + T4 *versus* T1)	0.69 ± 0.28	0.0100	2.00
Pathologic N classification (N1 + N2 + N3 *versus* N0)	0.47 ± 0.24	0.0500	1.59

Table 2

Estimation of overall survival durations per smoking status in NSCLC patients from whom tumor TMA specimens were obtained using univariate Cox proportional hazards regression models

Smoking status	Parameter estimate	S.E.	*P*	Hazards ratio
Never				
CREB	0.55	0.24	0.02	1.73
p-CREB	0.99	0.43	0.02	2.69
Former				
CREB	0.24	0.28	0.39	1.28
p-CREB	0.75	0.30	0.01	2.13
Current				
CREB	−0.46	0.56	0.41	0.63
p-CREB	−0.03	0.60	0.96	0.97