AP-4 predicts poor prognosis in non-small cell lung cancer

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Abstract. The basic helix-loop-helix transcription factor activating enhancer-binding protein (AP)-4 has been found to be involved in tumor biology. However, the role of AP-4 in non-small cell lung cancer (NSCLC) has yet to be elucidated. The present study aimed to investigate the role of AP-4 expression in NSCLC. AP-4 expression as analyzed using quantitative polymerase chain reaction and western blot analyses of 42 fresh NSCLC samples and matched adjacent noncancerous tissues. Immunohistochemistry was performed to assess the clinical significance of AP-4 expression in tumor tissues of NSCLC patients (n=240) and matched adjacent noncancerous tissues. The correlation between AP-4 expression, clinicopathological features and clinical outcome were investigated. AP-4 expression was found to be increased in the NSCLC samples at the gene and protein levels compared with the matched adjacent noncancerous tissues. Immunohistochemistry revealed that the positive expression rates of AP-4 in the 240 NSCLC samples and the matched adjacent noncancerous tissues were 48.3 and 5.8%, respectively. Positive AP-4 expression was found to be significantly associated with the tumor, nodes and metastasis stage and nodal status. Furthermore, patients with NSCLC tumors expressing AP-4 were observed to have a poorer prognosis than those without AP-4 expression. Multivariate analysis revealed that AP-4 expression was an independent prognostic marker (hazard ratio, 2.543; 95% confidence interval, 1.18-5.016; P=0.016) in NSCLC. Thus, positive AP-4 expression may be a potential prognostic marker for NSCLC.

Introduction

Lung cancer is among the most lethal types of cancer for both males and females worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~80% of all lung cancer cases and is the most prevalent type of lung cancer, with 1.2 million new cases reported annually worldwide (2). At present, the prognosis for patients with NSCLC is poor, with the five-year overall survival rate being <15% (3). Thus, the identification of potential molecular markers of NSCLC is required for the prediction of survival and the development of novel therapeutic targets.

Transcription factors belong to the basic helix-loop-helix (bHLH) family and are key regulators of cell proliferation, differentiation and cell lineage determination, as well as other essential processes (4). As a member of the bHLH leucine-zipper (LZ) subgroup of bHLH proteins (5), activating enhancer-binding protein (AP)-4 has been reported to have a role in tumor biology. The activation of AP-4 has been reported to induce epithelial-mesenchymal transition and enhance migration and invasion in colorectal cancer cells. Moreover, the downregulation of AP-4 has been found to cause mesenchymal-epithelial transition and inhibit migration and invasion, suggesting that AP-4 may be a novel regulator in cancer (6). It has also been reported that high expression of AP-4 predicts poor prognosis in hepatocellular carcinoma following curative hepatectomy (7). However, the role of AP-4 in NSCLC has yet to be elucidated.

The present study investigated the role of AP-4 expression in NSCLC by analyzing AP-4 expression in NSCLC and the correlation between AP-4 expression and clinicopathological characteristics and prognosis by using quantitative polymerase chain reaction (qPCR) analysis, western blot analysis and immunohistochemical staining.

Materials and methods

Patients. Patients with NSCLC who underwent radical resection of their primary cancer at the Department of Radiation Oncology, Jinan Military General Hospital (Jinan, China) were used in the present study. Patients were excluded from the study if they had previously undergone radiotherapy or chemotherapy for cancer treatment. Two groups were included in the present study. The first group included 42 fresh NSCLC tumor samples, which were immediately frozen and stored in liquid nitrogen for protein and RNA extraction following surgical resection. In addition to the first group, 240 NSCLC tissue specimens, including 139 adenocarcinomas and 101 squamous cell carcinomas, obtained from the Department of Radiation Oncology, Jinan Military General Hospital between January 2005 and January 2008, were used. All specimens were histologically analyzed and classified using the World Health Organization classification system. Detailed

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clinical, pathological and survival data were available. Written informed consent was obtained from all patients for the use of their tissues. Furthermore, the present study was approved by the Institutional Review Board at Jinan Military General Hospital. Patient follow-up was performed at three-month intervals. The median follow-up period was 48 months (range, 9-66 months) for all patients. Overall survival was defined as the period from the time of surgery to mortality.

qPCR analysis. The total RNA from frozen fresh samples was extracted using a TRIzol® extraction kit (Invitrogen Life Technologies, Carslbad, CA, USA) and reverse transcribed in a 25 μl reaction volume using Taqman® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The complementary (c)DNA was diluted and quantified using qPCR analysis using SYBR® Green I. The primer sequences used for the qPCR analysis were as follows: AP-4 forward, 5'-GAGGGCTCTGTAGCCTTGC-3' and reverse, 5'-GAATCCCGCGTTGATGCTCT-3'; GAPDH forward, 5'-ACAACTTTGGTATCGTGG-3' and reverse,5'-GCCATCACGCCACAGTTTC-3'. Data were analyzed using the ΔΔCt method and normalized using GAPDH expression.

Western blot analysis. Frozen tumor tissues were prepared by washing twice in cold phosphate-buffered saline (PBS). Approximately 20 mg tissue from each fresh sample was homogenized in 0.5 ml ice-cold cell lysis buffer (Roche Applied Science, Penzberg, Germany) containing fresh protease and phosphate inhibitors. Lysates were then centrifuged at 12,000 x g in a microcentrifuge at 4˚C for 20 min and the resulting supernatants were used as tissue extracts. The extracted proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked using Tris-buffered saline (TBS) containing 5% non-fat dried milk and then probed with primary antibodies in PBS containing 5% bovine serum. The following primary antibodies were used: Rabbit anti-AP-4 (Millipore Corp., Billerica, MA, USA) and mouse anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA). Immunoreactive bands were detected and quantified using an imaging system (Invitrogen Life Tecnologies).

Immunohistochemical analysis. The antibodies used for western blot analysis were also used for immunohistochemical staining. Formalin-fixed and paraffin-embedded tissue sections (5-μm thick) were deparaffinized, hydrated and heated in a steamer for 10 min for antigen retrieval. Peroxidase activity was blocked using 3% H_2O_2 in methanol at room temperature for 10 min, followed by incubation in 10% bovine serum albumin in TBS-Tween 20 for 30 min. Slides were then incubated with primary antibodies against AP-4 at a 1:100 dilution for 60 min at room temperature. Subsequent to washing with PBS, the slides were incubated with biotin-labeled secondary antibodies for 30 min. The samples were then incubated with streptavidin-peroxidase at a 1:40 dilution for 30 min. Samples were stained with 0.05% 3',3-diaminobenzidine tetrahydrochloride prepared in 0.05 mol/l TBS (pH 7.6) containing 0.02% H_2O_2, then counterstained with hematoxylin. Formalin-fixed and paraffin-embedded lung tissues with normal bronchial epithelia were used as a positive control. Tissue samples which were not incubated with the primary antibodies were used as a negative control. Immunohistochemical staining was quantified by two independent pathologists.

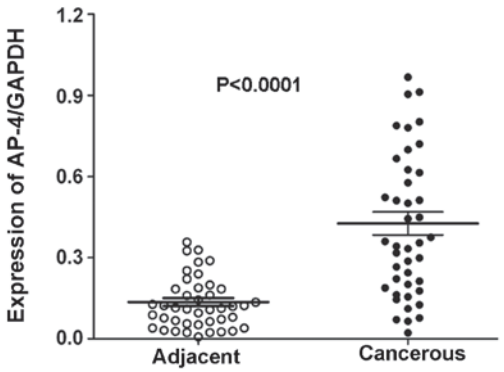

Figure 1. AP-4 mRNA expression is significantly increased in fresh non-small cell lung cancer tissues compared with their matched adjacent non-cancerous tissues (n=42), detected using quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard error of the mean. AP, activating enhancer-binding protein.

Assessment of immunohistochemical staining. Staining was quantified using a scoring method based on the intensity and proportion of the immunohistochemically stained cells. The proportion of positively stained tumor cells was determined semi-quantitatively and each sample was scored as follows: 0, <1%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The staining intensity of the positively stained tumor cells was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The immunoreactive score of each tumor was calculated by the sum of the two parameters. The immunohistochemical staining was ultimately graded as either negative (total score, 0-1) or positive (total score, 2-7). All stained sections were assessed by two independent pathologists without knowledge of the clinicopathological features.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Independent sample Student's t-tests and χ^2 tests were used to analyze the continuous and categorical variables, respectively. Survival probability was assessed using the Kaplan-Meier estimator. The log-rank test was used for the comparison of patient survival. The Cox proportional hazards model was used to calculate the effect of AP-4 expression on patient survival, with adjustments made for clinical and histopathological parameters, including age, gender and smoking status. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AP-4 mRNA and protein expression are significantly increased in fresh NSCLC tissue. AP-4 expression was assessed using qPCR analysis in 42 fresh NSCLC samples and matched adjacent non-cancerous lung tissues. AP-4 mRNA expression was found to be significantly higher in the NSCLC samples compared with the adjacent noncancerous tissues ($P<0.0001$; Fig. 1). Furthermore, western blot analysis revealed that AP-4

Table I. AP-4 expression and clinicopathological features in 240 patients with non-small cell lung cancer.

Parameter	Cases (n)	AP-4 expression		P-value
		Negative	Positive	
Gender				0.51
Male	154	80	74	
Female	86	44	42	
Age				0.44
<60 years	113	63	50	
≥60 years	127	61	66	
TNM stage				0.028
I	50	29	21	
II	69	33	36	
III-IV	121	62	59	
Histology				0.43
Squamous cell	101	49	52	
Adenocarcinoma	139	75	64	

AP, activating enhancer-binding protein.

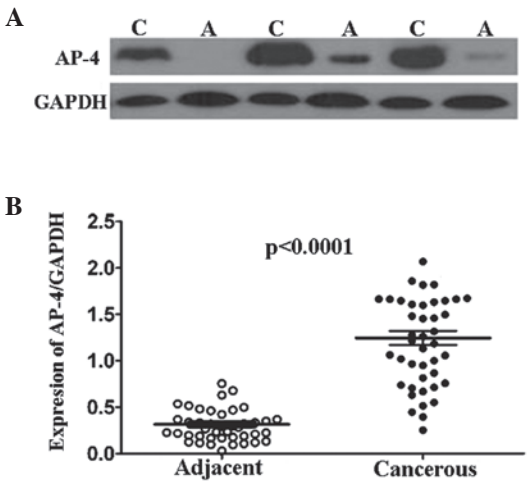

Figure 2. AP-4 protein expression is significantly increased in NSCLC tissues compared with matched adjacent non-cancerous tissues, detected using western blot analysis. (A) Western blot of AP-4 protein expression in the NSCLC tissues and matched adjacent non-cancerous tissues of three patients with NSCLC. (B) Quantification of AP-4 protein expression in the NSCLC tissues and matched adjacent noncancerous tissues of 42 patients with NSCLC. Data are presented as the mean ± standard error of the mean. NSCLC, non-small cell lung cancer; AP, activating enhancer-binding protein; C, cancerous tissue; A, adjacent non-cancerous tissue.

protein expression was significantly increased in the 42 fresh NSCLC samples compared with the matched adjacent noncancerous tissues, as quantified using densitometry (P<0.0001; Fig. 2).

Correlation between AP-4 expression and clinicopathological parameters. Immunohistochemical staining revealed that AP-4 was expressed in the nuclei of the cells in the NSCLC tissues. The rate of AP-4 expression was significantly higher in the NSCLC tissue samples (48.3%; 116/240) compared with the adjacent noncancerous lung tissues (5.8%; 14/240; P<0.01; Fig. 3). The correlation between AP-4 expression and clinicopathological features is shown in Table I. AP-4 expression was found to be significantly associated with the tumor, nodes and metastasis (TNM) stage.

AP-4 expression is associated with prognosis in patients with NSCLC. Kaplan-Meier survival estimates revealed that overall survival was significantly lower in patients with positive AP-4 expression than in those with negative AP-4 expression (P=0.0026; Fig. 4). Furthermore, Cox proportional hazard multivariate analysis was used to analyze the correlation between AP-4 expression in NSCLC tissues and other features, including patient gender and smoking history, as well as tumor histology, size, differentiation, metastasis status and TNM stage. Positive AP-4 expression (hazard ratio, 2.543; 95% confidence interval, 1.18-5.016; P=0.016) was found to be an independent prognostic indicator in patients with NSCLC, in addition to lymph node status and distant metastasis (Table II).

Discussion

NSCLC is one of the leading causes of mortality associated with cancer worldwide; therefore, improvements in the diagnosis and treatment of NSCLC are urgently required (8). The identification of novel biomarkers may help to guide the diagnosis and treatment of NSCLC. In the present study, AP-4 expression was found to be increased at the transcriptional and translational levels in fresh NSCLC samples. Moreover, the present study analyzed the correlation between AP-4 expression and clinical outcome and clinicopathological parameters. Positive AP-4 expression was identified in 48.3% (116/240) of NSCLC cases. By contrast, positive AP-4 expression was only observed in 5.8% (14/240) of the matched adjacent lung tissues. Of note, a significant correlation was identified between AP-4 expression and poor prognosis, independent of other clinicopathological parameters. These findings support the role for AP-4 as an oncogene and a novel prognostic marker in NSCLC.

Previous studies have demonstrated that AP-4 is involved in tumor biology. It was reported that AP-4 expression was significantly correlated with the progression of colorectal cancer and lymph node metastasis (9). AP-4 expression was also found to be associated with the expression of matrix metalloproteinase-9 and vascular endothelial growth factor in advanced colorectal cancer (9). A recent study has shown that AP-4 expression is associated with clinicopathological parameters in gastric cancer, including differentiation, lymph node metastasis, depth of invasion, TNM stage and poor prognosis (10). Furthermore, high AP-4 expression has been found to predict poor prognosis in hepatocellular carcinoma following curative hepatectomy (7). Thus, AP-4 may be a molecular marker to predict the progression and prognosis of the various types of tumors. However, the expression and clinical significance of AP-4 in NSCLC has yet to be elucidated. Therefore, the present study aimed to analyze the clinical significance of AP-4 expression in NSCLC.

The present study investigated AP-4 mRNA and protein expression in fresh NSCLC samples using qPCR and western

Table II. Mutivariate analysis of clinical features and prognosis in patients with non-small cell lung cancer.

Parameter	B	SE	Wald	P	HR	95% CI for HR	
						Lower	Upper
Histology	-0.02	0.17	1.48	0.345	0.74	0.49	1.26
Gender	0.596	0.31	3.31	0.081	4.91	0.83	3.29
Smoking	0.445	0.28	2.69	0.231	1.72	0.81	2.91
Tumor size	0.082	0.22	0.09	0.816	1.46	0.59	1.67
Position	-0.32	0.20	0.009	0.574	0.87	0.60	1.73
Differentiation	-0.03	0.23	0.04	0.892	0.98	0.57	1.60
TNM stage	0.16	0.19	0.40	0.432	1.21	0.77	1.63
Lymph node status	0.58	0.22	5.59	0.013	1.79	1.09	2.90
Distant metastasis	1.29	0.26	19.76	0.001	3.91	2.04	7.05
AP-4 expression	1.75	0.49	11.46	0.016	2.54	1.18	5.01

B, partial regression coefficient; SE, partial regression coefficient standard error; Wald, $(B/SE)^2$; HR, hazard ratio; CI, confidence interval; AP, activating enhancer-binding protein.

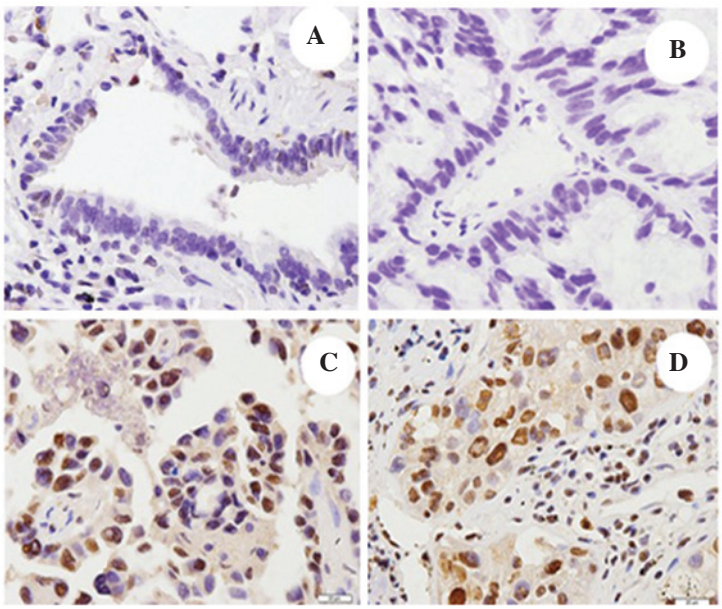

Figure 3. Immunohistochemistry of AP-4 in non-small cell lung cancer. (A) Negative AP-4 staining in the normal bronchial epithelium of the lung tissue. (B) Negative control using rabbit immunoglobulin G. (C) Positive AP-4 staining in lung adenocarcinoma. (D) Positive AP-4 staining in squamous cell carcinoma (magnification, x100). AP, activating enhancer-binding protein.

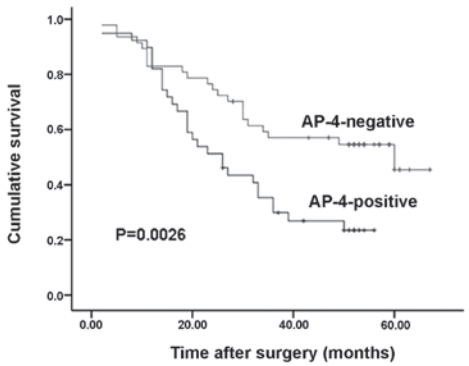

Figure 4. Kaplan-Meier analysis of overall survival in patients with non-small cell lung cancer (n=240). Overall survival in the AP-4-positive group (n=116) was significantly lower than that in the AP-4-negative group (n=124). P<0.01. AP, activating enhancer-binding protein.

blot analyses. AP-4 mRNA and protein expression were observed to be significantly increased in the tumor tissue samples compared with the adjacent non-tumor tissue samples. Moreover, in a relatively large number of NSCLC patients (n=240), high expression of AP-4 was found to be significantly correlated with the TNM stage of NSCLC, suggesting that an increase in AP-4 expression may promote tumor growth and invasion. These findings suggested that AP-4 may have an important role in the tumorigenesis or progression of NSCLC.

Kaplan-Meier survival analysis revealed that patients with positive AP-4 expression had a significantly lower overall survival than those with negative AP-4 expression. Multivariate analysis demonstrated that AP-4 expression was an independent prognostic factor in patients with NSCLC. These findings suggested that AP-4 may serve as a valuable prognostic biomarker for patients with NSCLC.

In conclusion, the present study revealed that positive AP-4 expression in NSCLC was correlated with a more malignant phenotype and poor prognosis in a large number of clinical samples. Thus, AP-4 may be utilized as a valuable prognostic biomarker for NSCLC. Translational studies of AP-4 as a therapeutic target in NSCLC are required.

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