Expression of RUNX3 protein in human lung adenocarcinoma: Implications for tumor progression and prognosis

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Runt-related transcription factor 3 belongs to the runt domain family of transcription factors that play a pivotal role during normal tissue development and tumorigenesis in several organs. We directed our attention to the expression of RUNX3 protein in human lung AC and non-neoplastic lung tissues, comparing the results with clinicopathological profiles. We evaluated the expression of RUNX3 protein in 17 pairs of lung AC and non-neoplastic lung tissue. Furthermore, 98 lung AC were studied to examine the frequency of RUNX3-positive cells. Western blot analysis showed a single band at 45 kDa in all 17 AC and non-neoplastic tissues. Immunohistochemistry revealed immunoreactivity in alveolar type II pneumocytes or Clara cells. RUNX3 was expressed more frequently in the carcinomas with a BAC component than in those without ($P < 0.01$). Lower RUNX3 levels were associated with poorly differentiated types ($P = 0.049$). The five-year survival rate was significantly higher in the 50 patients with higher levels of RUNX3 expression than in the 48 patients with lower levels ($P = 0.027$). The expression of RUNX3 protein in lung AC might play a pivotal role in tumor progression and patients' survival. (*Cancer Sci* 2005; 96: 227–231)

Lung cancer is currently the leading cause of carcinoma-related deaths in most countries.[1] The long-term survival rate, even with complete clinical resections, remains unsatisfactory.[2,3] Among NSCLC, AC are increasing in frequency and account for almost half the number of lung carcinomas.[1] There is a great need to identify molecular markers that will eventually lead to improved survival in patients with lung AC.

The RUNX3 gene, which is located at 1p36.1, encodes a protein that belongs to the runt domain family of transcription factors that act as master regulators of gene expression in major developmental pathways.[4–7] Li *et al*. reported that the RUNX3 protein has essential functions in both cell proliferation and differentiation in gastric epithelium.[7] The gastric epithelium of RUNX3 knockout mice demonstrated hyperplasia and a reduced rate of apoptosis, accompanied by a reduced sensitivity to TGF-β.[7,8]

Recently, many studies have disclosed that the expression of RUNX3 is frequently deleted or silenced as a result of hypermethylation in a variety of human malignancies including lung carcinoma.[9–17] Cancer-specific hypermethylation of the RUNX3 gene was even frequently found in lung AC.[10] Therefore, the decreased expression of RUNX3 might be responsible for the occurrence and progression of lung AC.

In the present study, we examine the expression of RUNX3 protein in human lung AC and non-neoplastic tissue, comparing histological or clinicopathological profiles to clarify the precise pathological role of the expression.

Materials and Methods

Tissue samples. We obtained frozen tissue samples of 17 pairs of lung AC and corresponding non-neoplastic tissues that were removed in 2002, and 98 paraffin-embedded tumor tissues of lung AC resected in 1990–2001 at Yonago National Hospital. Paraffin blocks were sectioned in 3 μm slices and stained with hematoxylin and eosin.

The tumors were histologically diagnosed as 66 mixed subtypes with BAC components and 32 types without BAC (non-BAC) according to the WHO's classification,[1] and Noguchi's criteria,[18] (Table 1). All of the 66 AC with BAC were non-mucinous BAC type. The 17 lung AC included 10 with BAC and seven without.

Protein extraction from lung tissue samples. The frozen tissue samples were solubilized in a lysis buffer [150 mM NaCl; 20 mM Tris/HCl (pH 7.4); 0.1% SDS; 1% sodium deoxycholate; 1% Triton X-100, containing a mixture of proteinase inhibitors (5 μg/mL aprotinin and 1 μg/mL leupeptin)] on ice using a homogenizer. All lysates were centrifuged at 11 000 *g* for 5 min. The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratory, Richmond, CA, USA) with bovine serum albumin as a standard protein.

Western blot analysis. Fifty micrograms of protein was separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (12% gel). In addition, as a positive control for RUNX3, HeLa cells transfected with a RUNX3 expression plasmid vector were prepared as described in our previous report.[19] Then the proteins were electrotransferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corporation, MA, USA). After 1 h incubation in a blocking solution (5% non-fat dry milk in PBS-0.5% Tween 20), the membrane was blotted with the anti-RUNX3 polyclonal antibody AS251,[19] (1:5000) and an anti β-actin monoclonal antibody (1:1000; AC15, Sigma, St. Louis, MO, USA). The anti-RUNX3 antibody was generated in our laboratory and confirmed to react with RUNX3, but not with RUNX1 or 2.[19] The blots were developed with peroxidase-labeled secondary antibodies. After extensive washing, specific bands were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Immunohistochemistry The following antibodies were used for immunohistochemistry; anti-RUNX3 polyclonal antibody,[19]

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Abbreviations: RUNX3, runt-related transcription factor; NSCLC, non-small cell lung carcinoma; AC, adenocarcinoma; BAC, bronchioloalveolar carcinoma; WHO, World Health Organization; SP-A, surfactant apoprotein A; LI, labeling indices; TGF-β, transforming growth factor-β.

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Table 1. Relationship between RUNX3 expression and clinicopathological data in 98 lung adenocarcinoma patients

	No. cases	RUNX3 expression		*P* value‡
		Higher (=10.0%)†	Lower (<10.0%)†	
Case	98	50	48	
Age (years)				
=66	46	21	25	
>66	52	29	23	0.317
Sex				
Male	45	24	21	
Female	53	26	27	0.673
Differentiation				
Well	33	20	13	
Moderately	54	26	28	
Poorly	11	2	9	0.049
Stage				
IA	56	34	22	
IB	15	7	8	
IIA	5	3	2	
IIB	8	2	6	
IIIA	14	4	10	0.118

†Cut off = median; ‡chi-squared test.

(1:2000) and antihuman SP-A monoclonal antibody (1:100; DAKO Cytomation, Tokyo, Japan).

Endogenous peroxidase activity was blocked by immersing slides in 0.6% hydrogen peroxide in methanol for 30 min. No antigen retrieval method was performed in both immunohistochemistry procedures against RUNX3 and SP-A. The sections were reacted with primary monoclonal or polyclonal antibodies overnight at 4°C. The tissue sections were treated with secondary antibody and biotin-streptoavidin complex for 30 min each at 37°C. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with methyl-green, then dehydrated and mounted. To confirm the specificity of the immunostaining results, sections immunoreacted without the primary antibodies were used as negative controls.

Labeling indices of RUNX3 were determined by counting the number of RUNX3-positive cells per 1000 cells under a 200-fold magnification in BAC region, non-BAC region or non-neoplastic area, respectively, showing the most prominent within the section. Anti-SP-A antibody was used to identify type II pneumocytes or Clara cells in non-neoplastic tissues.[20]

Statistical analysis. Student's *t*-test was used to assess the correlation of RUNX3 expression between each location (i.e. alveolar epithelia, BAC, and non-BAC). The correlations between RUNX3 expression and several clinicopathological factors were assessed with the chi-squared test. The probability of overall survival for RUNX3 expression was determined by the Kaplan-Meier method. Different survival curves were compared using the log-rank test. All *P*-values were derived from two-sided tests and a probability less than 0.05 was considered statistically significant.

Results

RUNX3 expression in human lung tissues. Western blot analysis disclosed a RUNX3 protein of 45 kDa in the 17 paired carcinomas and non-neoplastic lung tissues and in the three non-cancerous tissues, as well as in the RUNX3-expressing HeLa cells used as a positive control (Fig. 1). There was no significant difference in the level of RUNX3 expression between the lung AC and non-neoplastic lung tissues. Similarly, there was no obvious difference in RUNX3 expression between those with BAC ($n = 10$; case no.s 1–10) and those without ($n = 7$; case no.s 11–17).

Localization of RUNX3 protein in human lung tissues. Immunohistochemistry using AS251 Ab was performed to examine the expression of RUNX3 protein and the distribution of RUNX3-positive cells.

Immunoreactivity against RUNX3 was detected mainly in the cytoplasm (Figs 2a,c) in alveolar epithelia of the non-neoplastic tissue. RUNX3-positive cells were mostly concordant with SP-A expressing cells, which were considered to be type II pneumocytes or Clara cells in non-neoplastic region adjacent carcinoma with inflammation mimicking interstitial pneumonia (Figs 2a,b). However, RUNX3 immunoreactive cells were extremely rare in normal lung without inflammation. RUNX3 was also expressed in a few inflammatory cells including lymphoid cells and

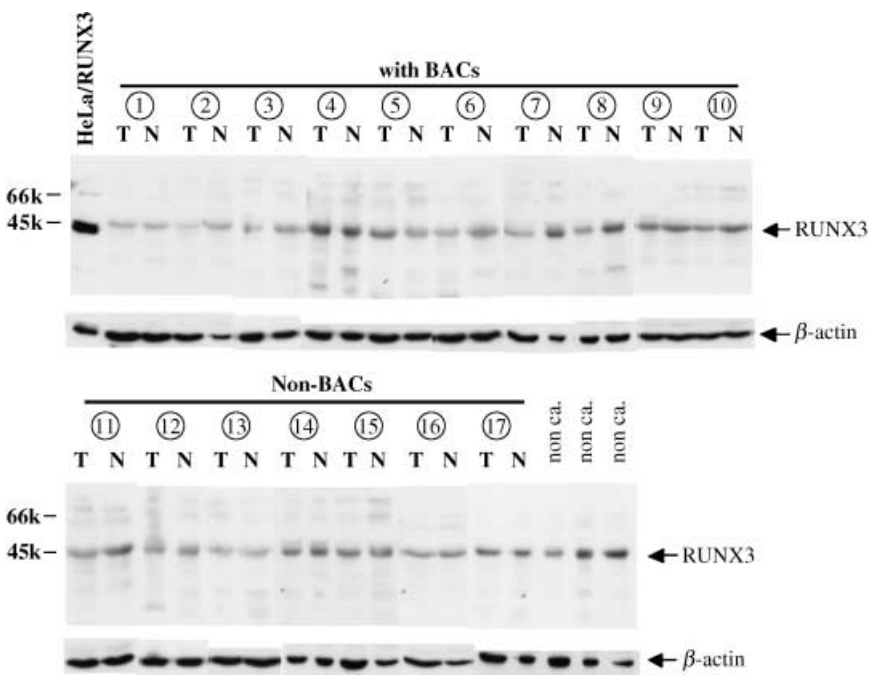

Fig. 1. Expression of RUNX3 protein in human lung AC analyzed by Western blotting. RUNX3 expression in tumor (T) and non-neoplastic tissue (N) in human lung AC tissues. RUNX3 is clearly expressed in (T) and (N) in all cases. In addition, three non-cancerous tissues (non-c) diagnosed as emphysematous bullae, fibrous nodule and atelectasis also show a single band at 45 kDa. HeLa cells transfected with the RUNX3 expression vector were used as a positive control. There is no obvious difference in the RUNX3 expression level between the tumor and the corresponding non-neoplastic tissue, or between the tumors with BAC ($n = 10$) and without ($n = 7$). Lower bands show the expression of β-actin as a quantitative control.

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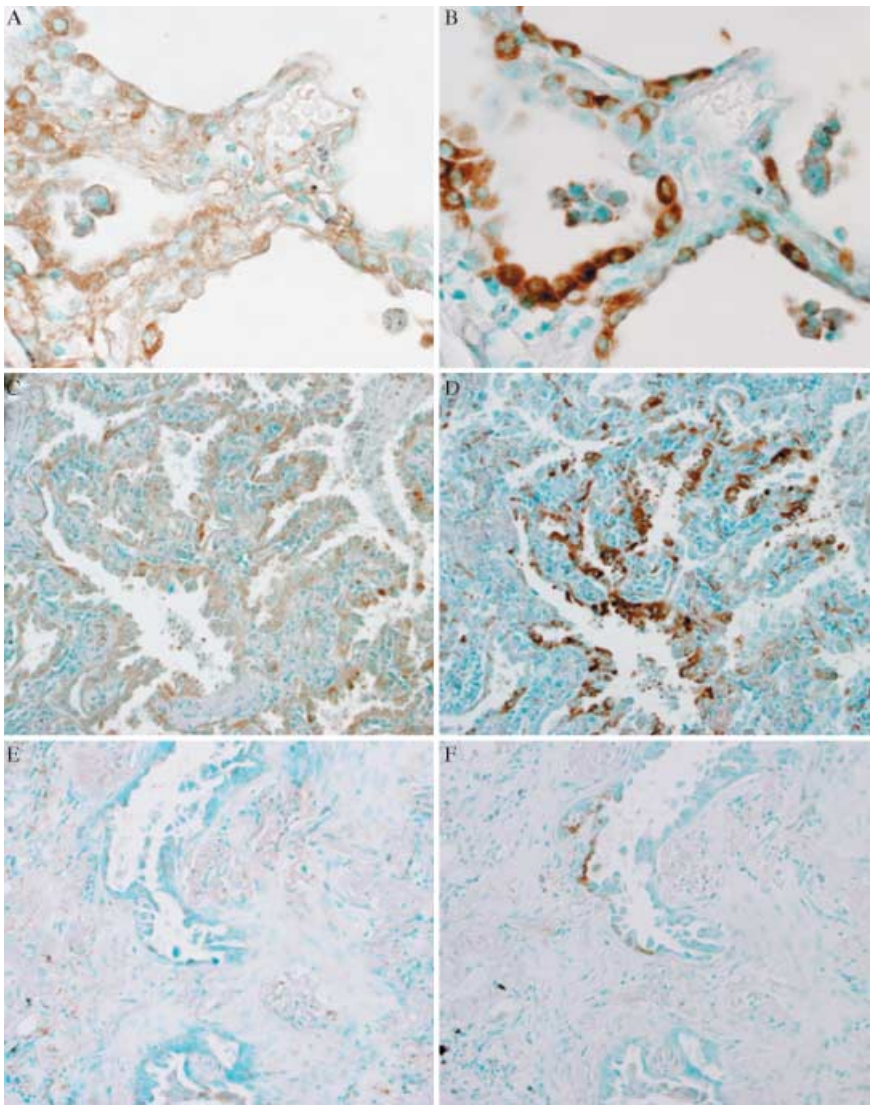

Fig. 2. Immunohistochemical analysis of RUNX3 expression in human lung tissues. RUNX3 is expressed in alveolar macrophages and several covering cells in alveolar epithelia (a). These alveolar cells are mostly consistent with SP-A-expressing cells shown in the semiserial sections (b), corresponding to type II pneumocytes or Clara cells. RUNX3 is also expressed in a few inflammatory cells including lymphoid cells and alveolar macrophages, and carcinoma cells showing BAC feature (c), which are also immunoreactive to SP-A (d). In contrast, RUNX3 expression is less frequent in invasive non-BAC cells with stromal formation (e). Only a few SP-A-expressing cells are noted in the invasive lesion (f).

alveolar macrophages (Figs 2a,c,e). RUNX3 was expressed in carcinoma cells showing BAC (Fig. 2c), most tumor cells being immunoreactive to SP-A (Fig. 2d). In contrast, the LI of RUNX3 was obviously lower in the invasive carcinoma cells with stromal formation (Fig. 2e), which were not immunoreactive to SP-A (Fig. 2f).

The RUNX3 LI was $20.02 \pm 16.92\%$ in the 66 cases with BAC and $9.41 \pm 11.01\%$ in the 32 without, the value being significantly higher in the former than in the latter ($P < 0.01$; Student's *t*-test). In contrast, the LI was $20.66 \pm 21.06\%$ in non-neoplastic alveolar epithelia.

The cut off level of the RUNX3 LI in carcinoma cells was fixed at 10%, which was the median for all cases. Higher levels of RUNX3 expression (≥10%) were noted in 50 cases, and lower (<10%) levels in 48 cases. RUNX3 expression was significantly correlated with the histological differentiation of the tumors ($P = 0.049$), with higher levels of expression noted in 20 well, 26 moderately, and two poorly differentiated carcinomas. In contrast, there was no significant correlation between RUNX3 expression and any of the other clinicopathological parameters including age, sex and histological stage (Table 1).

Survival analysis. Kaplan-Meier survival curves showed that the 5-year survival rate was 79.9% in the 50 patients with higher RUNX3 expression and 57.5% in the 48 patients with lower RUNX3 expression, the value being significantly different ($P = 0.027$) (Fig. 3).

Discussion

We confirmed the expression of RUNX3 protein in lung AC and non-neoplastic lung tissues by Western blot analysis, which showed a specific band at 45 kDa. The antibody, AS251, has been confirmed to react with RUNX3 specifically, but not with RUNX1 or 2. Therefore, the data showed that the AS251 antibody could recognize RUNX3 protein extracted from human lung tissue specimens.

Immunohistochemistry revealed that RUNX3 was frequently observed in cells expressing SP-A, corresponding to type II pneumocytes or Clara cells in clearly thickened alveolar septa or bronchiolar with inflammation mimicking interstitial pneumonia, but not in normal area. It might be considered that RUNX3 does not expressed in normal alveolar cells or the expression level is too low to detect by immunohistochemistry. In carcinoma lesion, RUNX3 was frequently expressed in carcinoma cells showing BAC components, which were also immunoreactive to SP-A. In the histologic classification of lung and pleural tumors by WHO,[1] lung AC have been devided into five histological subtypes; BAC, acinar, papillary, solid with mucin, and AC with mixed subtypes.

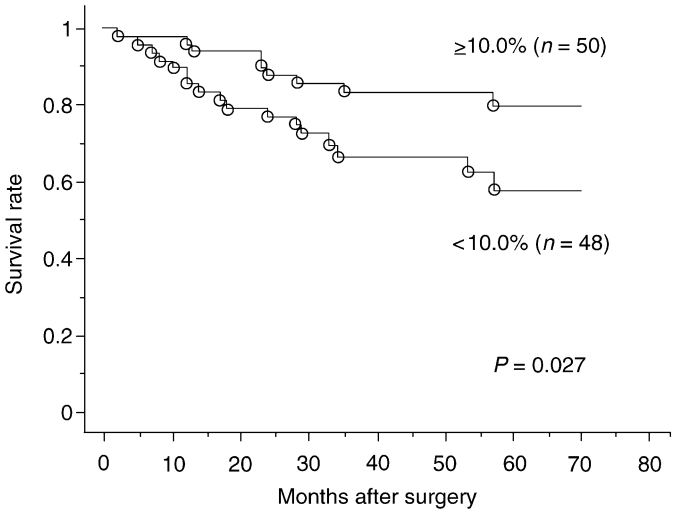

Fig. 3. Kaplan-Meier survival curves with respect to overall survival. The 5-year survival rate is 79.9% in the 50 patients with higher levels of RUNX3 expression (positive cells ≥10%), and 57.5% in the 48 patients with lower levels (<10%). The survival rate is significantly different. ($P = 0.027$; log-rank test).

BAC is thought to show cellular differentiation to type II pneumocytes or Clara cells.[21,22] BAC is the only subtype showing no invasive features, and an excellent prognosis can be expected for non-invasive BAC.[18,23,24] However, most lung AC have mixed subtypes,[23] which show invasive growth. Therefore, we divided the histological types showing invasive growth into two groups: those with BAC and those without (non-BAC), and evaluated the difference in RUNX3 expression between them. The RUNX3 LI was significantly lower in the non-BAC, which were not immunoreactive to SP-A. These findings suggest that RUNX3 expression correlates with cell differentiation to type II pneumocytes and Clara cells, and the lung AC lost the differentiation characteristics in the early stages of tumor progression. In fact, there was a relationship between the frequency of RUNX3 expression and the histological grade.

Western blot analysis showed no obvious difference in the RUNX3 expression level between the tumors with BAC and without. In contrast, the LI was significantly lower in the non-BAC. This discrepancy might be partly a result of the expression of RUNX3 in non-neoplastic cells including lymphoid cells and macrophages in the lung AC.

Finally, it is worthwhile touching upon the prognostic significance of the RUNX3 expression. Lower levels of RUNX3 in human lung AC were significantly associated with a worse patient's survival. RUNX3 appears to be an important component of the TGF-β-induced tumor suppressor pathway.[8] TGF-β itself was found to be an independent risk factor for metastasis in lung cancer,[25] and also a predictor of survival in patients with lung AC.[26] Moreover, Smad, activated by TGF-β,[27] followed by interaction with RUNX3,[28] has been found to be an independent predictor of survival in patients with various human carcinomas.[29,30] These two molecules play a critical role in the induction and integration of RUNX3 signaling.[31] Thus, RUNX3 also might be a candidate for prognostic factor and molecular target therapy.

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