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Expression of prothymosin α in lung cancer is associated with squamous cell carcinoma and smoking

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Abstract. Prothymosin α (ProTα) is a nuclear protein that serves a role in oncogenesis, by promoting proliferation and inhibiting apoptosis in various malignancies. The present study was designed to investigate ProTα expression in resected human non-small cell lung cancer to define the clinicopathological associations of ProTα-positive lung cancer. Immunohistochemical staining of ProTα was performed using tumor sample slides from 149 patients with non-small cell lung cancer, who underwent surgical resection. Association between the expression of ProTα and the following clinicopathological parameters was accessed: Age, sex, stage, lymph node involvement, pathological subtype, recurrence and cigarette smoking. A total of 85 tumors (57%) were classified as ProTα-positive lung cancer by staining intensity and 73 tumors (49%) were regarded as ProTα-positive by scoring index. The majority of patients with ProTα-positive tumors were younger (P=0.05) and had squamous cell carcinoma (P<0.01) compared with older and adenocarcinoma. Positive expression of ProTα by staining intensity was associated with a higher incidence rate of cancer recurrence (P=0.05) compared with negative ProTα expression. ProTα was also associated with cigarette smoking, particularly in the group with squamous cell carcinoma. Therefore, the present data suggested that ProTα-positive non-small cell lung cancer was associated with younger patients, squamous cell carcinoma, cigarette smoking and a higher incidence recurrence rate, subsequently indicating a subtype consisting of patients with smoking-associated inferior outcomes.

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Introduction

Prothymosin α (ProTα) is a 12.5 kDa acidic nuclear protein, initially isolated from rat thymus as the putative precursor of thymosin α1, and is regarded as a thymic immunoregulatory hormone (1). The biological function of ProTα contributes to cell cycle regulation, transcription, proliferation and apoptosis (2-4). The ProTα gene is upregulated by MYC proto-oncogene, BHLH transcription factor (c-Myc), E2F transcription factor 1 and the human papilloma virus type 16 E6 oncogene, whereas ProTα is downregulated by the p53 tumor suppressor (5). In addition, ProTα is present only in cells that are in the proliferative cycle, and therefore, is not expressed in non-proliferative cells (5). In colon cancer cells, ProTα mRNA expression has been reported to be positively correlated with *c-myc*, and its expression level was higher in the tumor tissue compared with the adjacent normal tissue (6). Overexpression of ProTα has been associated with a poor prognostic outcome in urinary tract transitional cell carcinoma, head and neck cancer, hepatocellular carcinoma and colon cancer (7-10). However, to the best of our knowledge, studies on the association between ProTα and lung carcinogenesis are limited. Previous study has indicated that the secreted thymosin-α1 in plasma from patients with lung cancer was higher compared with healthy individuals, but was not associated with age or pathological subtype of lung cancer in the first human lung

cancer study (11). In a urethane injection carcinogenesis A/J mouse model, daily administration of thymosin-α1 significantly reduced lung adenoma multiplicity, providing a different biological perspective on ProTα (12). A study of 20 lung cancer tissues reported that overexpression of ProTα mRNA was associated with poor prognosis (13).

Our previous research focused on the contribution of ProTα to the acetylation of histone and nuclear factor-κB, and particularly on smoke exposure (14). ProTα transgenic mice are prone to develop emphysema when exposed to cigarette smoke extract (14). However, the association of lung cancer with ProTα, in terms of cigarette exposure and pathological subtypes, has not been well defined (14). The aim of the present study was to investigate the impact of ProTα on pathological subtypes and clinical parameters in patients with lung cancer.

Materials and methods

Patient characteristics. A total of 149 patients (mean, 66; range, 28-90 years), including 87 male and 62 female patients, with a pathological diagnosis of lung carcinoma were included in the present study. Lung metastasis from other primary site was excluded. The lung cancer tissues were harvested between 1997 and 2008 by surgical resection at Chi-Mei Medical Center (Yong Kang, Taiwan). Data on parameters including age, sex, operative procedure, recurrence, disease-free survival, pathological subtypes of lung carcinoma and history of cigarette smoking were collected from the patients' medical records (Table I).

Table I. Clinicopathological parameters of the present study population.

Parameter	n=149
Median age (range), years	66 (28-90)
Sex (%)	
Male	87 (58)
Female	62 (42)
Pathological subtype (%)	
Squamous cell carcinoma	30 (20)
Adenocarcinoma	119 (80)
Tumor stage (%) (TNM system) (17)	
I	79 (53)
II	35 (24)
III	32 (21)
IV	3 (2)
Cigarette smoking (%)	
Yes	23 (15)
No	126 (85)
Intensity of ProTα expression (%)	
Negative	22 (15)
Weak	63 (42)
Moderate	33 (22)
Strong	31 (21)
ProTα score (%)	
≤50	73 (49)
>50	76 (51)

ProTα, prothymosin α.

Immunohistochemistry stain. Immunohistochemistry staining of 5 μm thick paraffin-embedded sections was carried out using the 2-step protocol Novolink Polymer Detection System (Leica Microsystems. Ltd., Milton Keynes, UK), according to the manufacturer's protocols. In brief, the sections were first deparaffinized in xylene two times for 5 min to remove paraffin and subsequently rehydrated through a gradient of ethanol for 3 min in each concentration, 100, 100, 95, 70 and 50%, followed by de-ionized water. Following microwave 10 mM sodium citrate buffer (pH 6.0) boiled for 10 min, slides were washed for 5 min x 2 in PBS. Endogenous peroxidase was neutralized using a peroxidase block (3.5% hydrogen peroxide) for 5 min. Following incubation for 1 h at room temperature, the sections were washed three times in PBS for 5 min each. Subsequently, the slides were treated with 1% skimmed milk in PBS for 30 min at room temperature, and non-specific background staining was minimized further by incubation in 0.3% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 0.1 M Tris-buffered saline for 1 h at room temperature. Sections were incubated with antibody diluent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 h at room temperature and washed again in PBS in triplicate for 5 min each. The primary monoclonal antibody used was anti-human-prothymosin α antibody (4f4 clone; culture supernatant generated from Professor Chao-Liang Wu's lab according to references) (15,16). Following serial incubation with the primary antibody overnight at 4˚C, the sections were washed in triplicate with PBS for 5 min each, and incubated with goat anti-mouse IgG-HRP (115-035-003, dilution, 1:300; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. Following incubation, the slides were washed five times in PBS for 5 min each. Negative controls included sections stained with mouse universal negative control with the same concentration of primary antibodies (Dako; Agilent Technologies, Inc.) overnight at 4˚C. Reactivity was visualized with DAB Quanto (Thermo Fisher Scientific, Inc.) and counterstained with hematoxylin (MUTO, 5X dilution) for 10 min at room temperature. The sections were washed in di-H_2O for 10 min prior to dehydration, clearing and mounting. Slide scorings were based on intensity of stain as follows: 0, negative; 1, weak; 2, moderate; 3, strong Fig. 1) and percentage of area stained (0-100%), with both scores multiplied to yield the total score. The definition of a high ProTα score was >50. The results were interpreted by light microscope under the power of x100.

Statistical analysis. All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc., San Jose, CA, USA). The unpaired t-test and χ^2 test were used to evaluate the differences in discrete variables and continuous variables between the expression of ProTα and the clinicopathological parameters. Values are presented as the mean ± standard deviation. For disease-free survival, the Kaplan-Meier method was adapted to generate survival curves, and the log-rank test

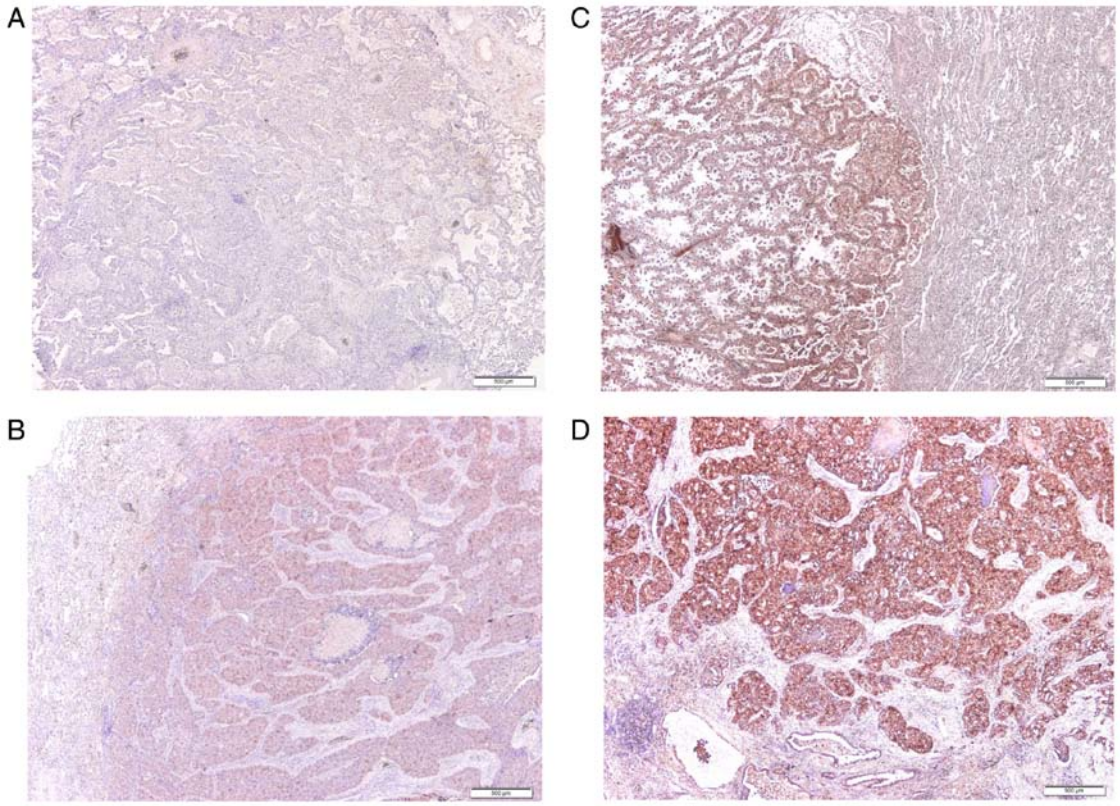

Figure 1. Expression of Prothymosin α by immunohistochemistry stain in patients with non-small cell lung cancer observed by light microscope under the power of x100 magnification. (A) Negative stain. (B) Weak stain. (C) Moderate stain and (D) strong stain.

was used to estimate the differences. All tests were two-tailed, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient demography. A total of 149 patients with resected lung cancer were enrolled for the present study between September 1998 and September 2008. Participating patients did not receive adjuvant chemotherapy, since adjuvant chemotherapy was not the recommended treatment at the time of diagnosis (1998-2008) or in that physical condition of poor performance status or significant organ dysfunction. Patients, who had undergone peri-operative radiotherapy were excluded. The median age of these patients was 66 years (range, 28-90); there were 87 male and 62 female patients. Regarding pathological subtypes, 30 cases were squamous cell carcinoma and 119 cases were adenocarcinoma. A total of 79 cases were stage 1, 35 cases were stage 2, 32 cases were stage 3 and 3 cases were stage 4 by TNM system (based on 6th edition of cancer staging manual, American Joint Committee on Cancer) (17). The 3 patients with stage 4 underwent operation for primary lung tumor and distant metastasis, due to solitary metastasis. Primary lung cancer resection with metastasectomy was suggested in the aforementioned conditions, based on the decision of the physicians at Chi-Mei Medical Center. The majority of the cases, 126, had no history of cigarette smoking, while 23 cases presented with a smoking history. A total of two methods were used to measure the expression of ProTα: staining intensity and the percentage of area stained. The results of staining intensity indicated that the expression of ProTα was negative in 22 cases, weak in 63 cases, moderate in 33 cases and strong in 31 cases. Using the scoring system described above for the percentage of area stained, 76 cases had a high ProTα score (score >50; Table I). Nuclear and nucleo-cytoplasmic staining of ProTα were regarded as positive for ProTα expression. However, in the present study, sole nuclear stain of ProTα was rare.

ProTα expression and clinicopathological parameters. In order to verify the association between clinicopathological characteristics and the expression of ProTα, the following parameters were assessed: Age, sex, pathological subtype, stage, disease recurrence and cigarette smoking. Using the ProTα scoring system, squamous cell carcinoma and cigarette smoking were the only 2 parameters that were significantly associated with a high ProTα score (Fig. 2). Patients with recurrence of lung cancer tended to have a higher ProTα score, however, the result was not statistical significant. Although cigarette smoking was associated with a high ProTα score in the analysis of these 149 patients, only 20% (30 cases) had squamous cell carcinoma and 18% cigarette exposure (23 cases), which may render difficult an accurate interpretation of the contribution of ProTα relative to cigarette smoking and pathological subtypes. The results of the association between clinicopathological parameters and ProTα expression are presented in Table II. Further, the association of ProTα expression with cigarette smoking was evaluated in patients with squamous cell carcinoma or adenocarcinoma. It was indicated that the ProTα score was higher among patients with exposure to cigarettes compared among patients without

Table II. Association between clinicopathological parameters and ProTα expression.

Parameter (number)	Positive ProTα expression by intensity (%)	P-value	High ProTα score (>50) (%)	P-value
Age, years		0.05		0.04
≤65 (67)	34 (51)		40 (60)	
>65 (82)	30 (37)		35 (43)	
Sex		0.90		0.69
Male (87)	37 (43)		42 (48)	
Female (62)	27 (44)		34 (55)	
Stage (TNM system)		0.49		0.22
I (79)	36 (46)		44 (56)	
II/III/IV (70)	28 (40)		32 (46)	
Lymph node involvement		0.58		0.49
Negative (94)	42 (45)		50 (53)	
Positive (55)	22 (40)		26 (47)	
Pathological subtype		<0.01		<0.01
Squamous cell carcinoma (30)	23 (77)		24 (80)	
Adenocarcinoma (119)	41 (34)		52 (44)	
Recurrence		0.05		0.42
Negative (112)	43 (38)		55 (49)	
Positive (37)	21 (57)		21 (57)	
Cigarette smoking		0.61		0.90
Negative (126)	53 (42)		64 (51)	
Positive (23)	11 (48)		12 (52)	

ProTα, prothymosin α.

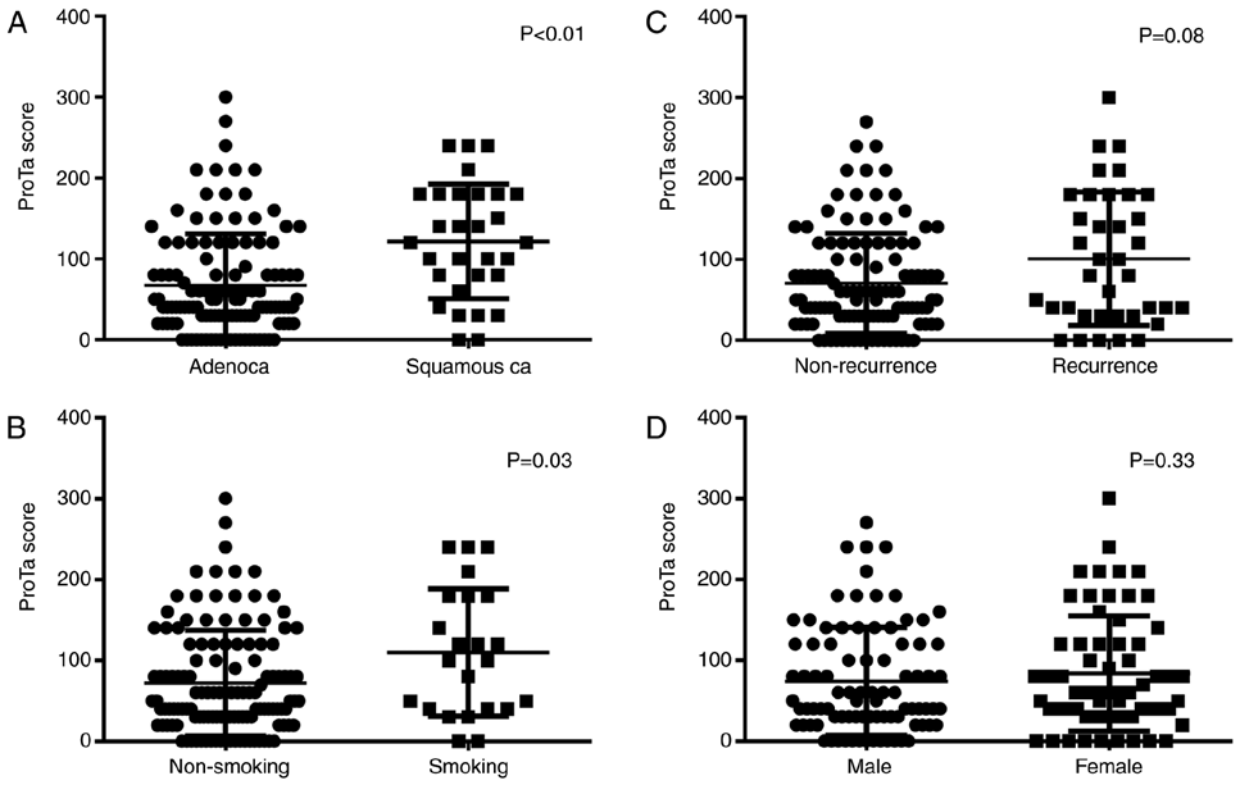

Figure 2. Expression of ProTα score by clinicopathological parameters. Expression of ProTα score in (A) adenocarcinoma and squamous cell carcinoma, (B) non-smoking and smoking (C) non-recurrence and recurrence, and (D) male and female. ProTα, prothymosin α.

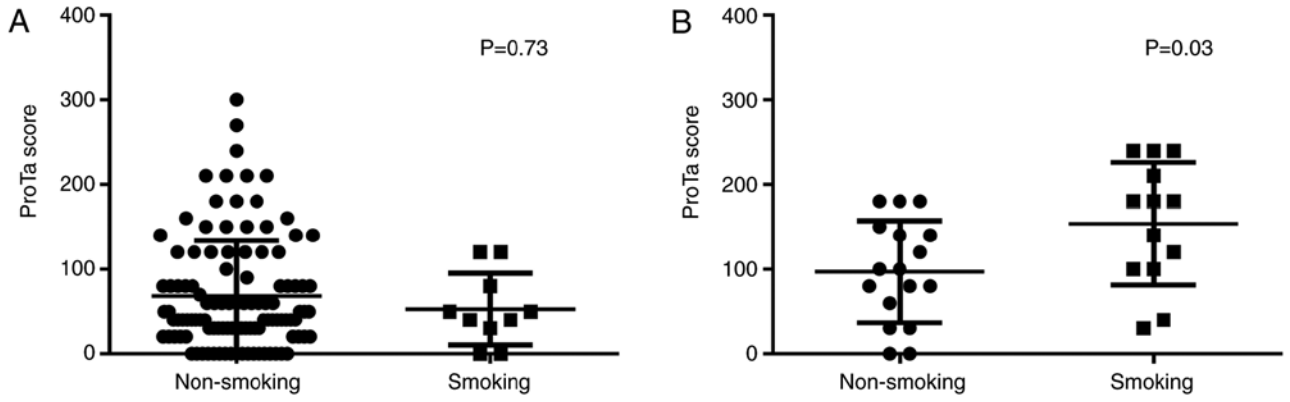

Figure 3. Expression of ProTα score categorized by pathological type for non-smoking and smoking status. ProTα score categorized by non-smoking and smoking in (A) adenocarcinoma and (B) squamous cell carcinoma. prothymosin α.

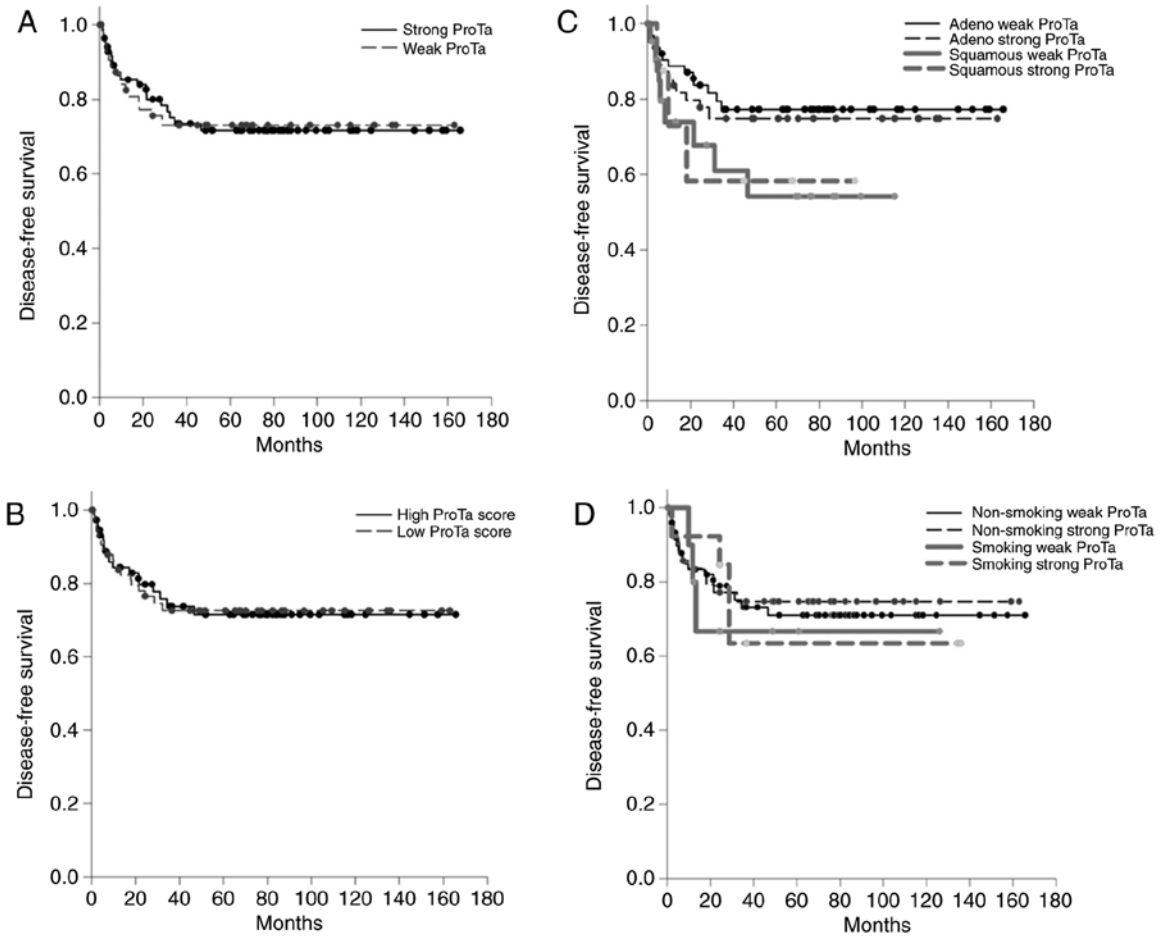

Figure 4. Disease-free survival categorized by ProTα expression and parameters. Kaplan- Meier survival plots for (A) positive and negative ProTα expression according to staining intensity, (B) high and low by ProTα score with a cut-off value of 50, (C) pathological subtypes and staining intensity of ProTα expression and (D) smoking and staining intensity of ProTα expression. Adeno, adenocarcinoma; Squamous, squamous cell carcinoma; ProTα, prothymosin α.

exposure to cigarettes in the squamous cell carcinoma group (P=0.03), however, this was not the case in the adenocarcinoma group (P=0.73) (Fig. 3). These results indicate that ProTα may serve a role in cigarette smoking-mediated carcinogenesis.

Survival analysis of patients with lung cancer. In a previous study with 20 cases of lung cancer, it was suggested that the presence of ProTα may be a poor prognostic factor for lung cancer (13). In the present study, involving 149 patients with lung cancer with operable disease, neither ProTα expression intensity nor ProTα expression score was associated with disease-free survival (Fig. 4A and B). Following categorization by pathological subtypes of adenocarcinoma and squamous cell carcinoma, squamous cell carcinoma was indicated to be associated with poor disease-free survival compared with cases of adenocarcinoma. However, patients who smoked and

exhibited strong ProTα expression tended to have the poorest disease-free survival rate. However, the difference between the disease-free survival rate of patients with different ProTα expressions and cigarette exposure statuses was not statistically significant (Fig. 4C and D).

Discussion

To the best of our knowledge, there have been no previous studies that focus on the protein expression of ProTα in human lung cancer. The mRNA expression of a small group of patients with lung cancer has been investigated, however, the ProTα mRNA levels were not associated with stage or pathological subtype (13). Our previous findings suggested that ProTα was positively correlated with the severity of emphysema in ProTα transgenic mice and patients with emphysema (14). ProTα transgenic mice were susceptible to cigarette smoking extract-induced emphysema mainly due to the inhibition of histone deacetylases and the promotion of matrix metalloproteinase 2 and matrix metalloproteinase 9 (14). As a result, the association between ProTα and cigarette smoking requires further attention.

Cigarette smoking has been reported to have a stronger association with squamous cell carcinoma compared with adenocarcinoma (18). Aside from lung cancer, ProTα has been used to distinguish oral pre-malignant lesions from histologically normal oral tissues by tissue proteomic analysis (19). Overexpression of ProTα, as detected by immunohistochemistry, has been reported to have a positive correlation with nuclear staining of tumor at an advanced stage, nodal involvement and inferior disease-free survival in patients with squamous cell carcinoma of the head and neck undergoing curative cancer surgery (8). ProTα was regarded as a poor prognostic factor in primary breast cancer, hepatocellular carcinoma, gastric cancer and upper urinary tract cancer, as well as in prostate cancer (7,20-23). In the present study the association of ProTα with squamous cell carcinoma and cigarette smoking was defined in a small sample. However, the underlying mechanism beyond this association requires further examination.

In our previous report on ProTα transgenic mice, increased Smad family member 7 and reduced tissue inhibitor of matrix metalloproteinase-3 were indicated in mice with cigarette smoke extract-induced emphysema (24). A proteomic profile using ProTα as 1 out of 5 biomarkers was valid in predicting the disease-free survival of patients with oral squamous cell carcinoma undergoing curative surgery in India and Canada (25). However, the present study did not examine the association between ProTα and cigarette smoking in oral squamous cell carcinoma (25). ProTα has been revealed to protect cells against apoptosis and oxidative stress (3). Caspase-9 activation negatively regulated by ProTα can inhibit apoptosome formation (26). Elimination of ProTα expression by suppression of RNA has been reported to sensitize cells to ultraviolet irradiation-induced apoptosis (3). In human lung adenocarcinoma A549 cells, human PNAS4 had the ability to induce apoptosis through downregulation of annexin A1 and ProTα. However, no detailed information on the role of ProTα in lung adenocarcinoma was provided in the aforementioned study (26).

In conclusion, the data of the present study indicated that ProTα expression was higher in squamous cell carcinoma of the lung compared with adenocarcinoma. Patients with squamous cell carcinoma and who smoked had higher ProTα scores compared with patients with squamous cell carcinoma and who did not smoke. However, cigarette smoking did not contribute to a difference in ProTα expression in adenocarcinoma of the lung. These results indicate a potential association between ProTα and cigarette smoking in squamous cell carcinoma. However, this result is limited to reflect only the clinical implications of ProTα at present. Therefore, comparing the expression of ProTα in lung cancer and adjacent normal control tissue samples of smoking and non-smoking patients is required to investigate smoking-associated carcinogenesis of squamous cell carcinoma. Further investigations of the clinical impact of ProTα in lung cancer, including a larger sample size of patients with lung cancer, particularly patients with squamous cell carcinoma, are required.

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Availability of data and materials

The datasets used during the current study are available from corresponding author on reasonable request.

Author's contributions

YHK and YHF were major contributors in writing the manuscript and analyzing the patient data. CLT, YCS and CFL performed the histological examination. ALS, PW, BHS, CJT and CLW made substantial contributions to study design, data analysis and interpretation, and manuscript organization. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the Chi Mei Medical Center (approval no. 10308-002). The condition of inform consent was a waiver documentation of consent, based on the protection of patient identifiable information.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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