

Thyroid Transcription Factor-1 (TTF-1) Expression in Human Lung Carcinomas: Its Prognostic Implication and Relationship with Expressions of p53 and Ki-67 Proteins

This study was aimed to evaluate the prevalence and prognostic implication of thyroid transcription factor-1 (TTF-1) immunoreactivity in 81 human lung carcinomas, including 65 cases of non-small cell lung carcinoma (NSCLC) and 16 cases of small cell lung carcinoma (SCLC); and also to investigate its relationship with the cell proliferation and regulation by immunostaining of Ki-67 and p53 proteins, respectively. The immunohistochemical staining for TTF-1 (clone 8G7G3/1) was performed and several clinicopathologic variables and the follow-up data were obtained. The immunostaining results for TTF-1 were semiquantitatively interpreted as negative and positive. Of NSCLCs, TTF-1 is highly expressed in adenocarcinomas (76%), whereas squamous cell carcinomas revealed no immunoreactivity (0%). SCLCs showed strong TTF-1 expression (88%). In NSCLC, TTF-1 expression was inversely correlated with Ki-67 proliferative activity and independent of p53 overexpression. TTF-1 (+) group tended to show better survival than TTF-1 (-) group in NSCLC. Conclusively, these observations suggest that TTF-1 is a sensitive and specific diagnostic marker for pulmonary adenocarcinomas and SCLCs; that TTF-1 might have a good prognostic implication based on its inverse correlation with Ki-67 proliferative activity and tendency for better survival in NSCLC; that this cell lineage marker may play a role in the molecular pathogenesis of lung cancers at the level of transcription.

Key Words : Transcription Factors; Thyroid Transcription factor-1 (TTF-1) Expression; Human; Lung Neoplasm; Ki-67 antigen; Protein p53; Pathogenesis

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INTRODUCTION

Thyroid transcription factor-1 (TTF-1) is a 38-kDa homeodomain containing DNA-binding protein of the *Nkx-2* gene family, which is expressed in the thyroid, lungs, and some restricted areas of diencephalon (1). During human development, it appears in early stages and thus could play an important role in cell differentiation and morphogenesis of both the thyroid and lung (1). TTF-1 activates transcription of thyroglobulin and thyroperoxidase genes in the follicular thyroid cells, but not in lung epithelial cells (2-5). Conversely, the role of TTF-1 in lung tissues involves regulation of the surfactant and Clara cell secretory protein gene expression, but not in follicular thyroid cells (6-8).

Since the expression of TTF-1 is an early event during lung differentiation, the evaluation of TTF-1 expression in lung tumors may have an impact in clinical research and in the management of these neoplasms (9). Also, tissue-specific transcription factors contribute to the transcriptional activation of genes expressed in particular cell types, and thus the study of tran-

scription factors like TTF-1 in human lung tumors could be of benefit in our understanding of the molecular events of their neoplastic transformation (10, 11).

In lung cancers, several reports described the differences in TTF-1 expression between histologic types (11-17). They reported that TTF-1 was expressed in 62.5-90% of adenocarcinomas and 89-100% of small cell carcinomas, whereas it was not found or found at a very low frequency in squamous carcinomas (0-27%) and large cell carcinomas (0-25%). Most reports have emphasized the usefulness of TTF-1 in differential diagnosis of lung cancer from nonpulmonary cancers, whereas relatively few and contradictory data are available on the prognostic implication of TTF-1 immunoreactivity because its upregulation has been considered as a favorable or unfavorable predictor of survival in patients with non-small cell lung carcinoma (NSCLCs) (13, 14, 16, 18). Also, there are few studies referred to possible significance of TTF-1 expression in lung carcinogenesis, despite its crucial roles in lung development and maintenance of pulmonary function by means of surfactant apoprotein A and Clara cell antigen induction.

Other factors for risk stratification in patients with lung cancers include the tumor proliferative potential, which is commonly evaluated by Ki-67 antigen immunostaining, and the mutation in *p53* genes, the most common genetic alteration in lung cancers (14, 15, 18). Although the independent prognostic role of these prognostic factors in lung cancer patients still remains a matter of debate, there are no doubts the cell proliferation and the regulation of cell cycle are essential factors for tumor growth. However, the relationships between TTF-1 and cell proliferation or *p53* overexpression have been only rarely reported in human lung carcinomas, including NSCLC and small cell lung carcinoma (SCLC) (14, 15, 18).

This study was aimed at evaluating the prevalence and prognostic implication of TTF-1 immunoreactivity in 65 cases of NSCLC and 16 cases of SCLC patients, and at demonstrating its relationship with cell proliferation assessed by Ki-67 immunostaining and cell-cycle regulation by *p53* staining.

Our findings indicate that TTF-1 is expressed almost exclusively by adenocarcinomas and small cell carcinomas, and thus this transcriptional protein is likely to be involved in the growth of adenocarcinomas and small cell carcinomas, with inverse correlation with Ki-67 proliferative potential; and that TTF-1 immunoreactive NSCLCs show a trend for a favorable clinical course, although the TTF-1 immunoreactivity fails to confirm a statistically significant prognostic implication.

MATERIALS AND METHODS

Patients and tissue samples

Primary tumor specimens from 65 NSCLCs and 16 SCLCs were obtained by surgical resection and bronchoscopic biopsy, respectively, between 1996 and 2001 at the Dankook University Hospital. All hematoxylin-eosin stained slides of the tissue samples were reviewed, and the pathological diagnoses of the histologic grades and types were confirmed by a pathologist. Histologic typing was performed according to the World Health Organization diagnostic criteria for lung carcinomas (1999). The hospital records of all 81 patients (65 NSCLCs and 16 SCLCs) were reviewed to obtain the clinicopathologic variables such as age, sex, smoking history, tumor-node-metastasis (TNM) stage (in NSCLC), and tumor extent (in SCLC). The pathologic staging of NSCLC and SCLC was assessed according to the TNM Classification of AJCC staging system (1997), and the Veterans' Administration Lung Study Group (VALSG) staging system (1973), respectively. Death from lung cancer was the terminal event for survival calculations. All patients were followed for up to about 5 yr.

Immunohistochemical analysis

All specimens used in this study were 4 μ m-thick sections of paraffin-embedded tissue obtained at surgery and broncho-

scopic biopsy. The standard avidin-biotin-peroxidase complex method was used for immunohistochemical examination using monoclonal antibodies against TTF-1 (8G7G3/1, NeoMarkers, CA, U.S.A.), *p53* (DO7, Novocastra, Newcastle-upon-Tyne, U.K.), Ki-67 (AO47, DAKO, Carpinteria, CA, U.S.A.).

Deparaffinization of all sections were performed through a series of xylene baths, and rehydration was performed through graded ethyl alcohols. The sections were microwaved in 10 mM citrate buffer at 90°C for 10 min, and were treated with 3% H₂O₂-PBS solution to reduce endogenous peroxidase activity. Then, they were incubated with normal bovine serum to reduce nonspecific antibody binding and were subsequently subjected to the primary antibody reactions. The antibodies for TTF-1, *p53*, and Ki-67 proteins were reacted with the sections at room temperature for one hour at the same dilution (1:100). Detection of the immunoreactive staining was carried out by avidin-biotin-peroxidase complex method using the LSAB kit (DAKO). The sections were subjected to a color reaction with 3,3'-diaminobenzidine tetrahydrochloride containing 3% H₂O₂ in Tris buffer, and were lightly counterstained with Mayer's hematoxylin.

For evaluation of the expressions of TTF-1, *p53*, and Ki-67 proteins, the immunostained cells were considered positive only when distinct nuclear staining was identified. The extent of the positive cells was semiquantitatively scored as follows: 0, negative reaction; 1, <5% positive tumor cells; 2, 5-50%; 3, 51-100% of tumor cells. The staining intensity was evaluated with three categorized scores of weak 1, moderate 2, and strong 3. To exclude equivocal reactions, the staining was regarded as positive for TTF-1 expression when the combined scores were more than 2 (≥ 2). Cases were considered positive for Ki-67 expression, when more than 10% of tumor cells were reactive, because the median value of Ki-67 proliferative fraction was about 10% in all cases. Cases were considered positive for *p53* overexpression, when at least 50% of tumor cells showed nuclear immunopositivity because high *p53* expression would be expected if *p53* mutations existed (19). To obtain the survival curves, the TTF-1 nuclear staining was divided into as negative (scores 0-1) and positive (scores 2-9) groups based on the tumor nuclei with unequivocal staining.

Statistical Analysis

The comparison of TTF-1 immunoreactivity between several two-categorical clinicopathologic variables and the relationships of TTF-1 expression with Ki-67 proliferative activity and *p53* overexpression were analysed by chi-square test (SPSS 10.0). The survival period was calculated as the time from the date of surgery to the date of death or last follow-up. Postoperative survival curves were constructed using the Kaplan-Meier method and then compared by the log rank test. A *p* value less than 0.05 was defined as statistically significant.

RESULTS

Clinicopathologic data and correlation with TTF-1 expression

Patient profiles are shown in Tables 1 and 2. NSCLC group consisted of 49 men and 16 women, with median ages of 58.0 and 61.8 yr, respectively. The SCLC group included 14 men and 2 women with median ages of 60.6 and 62 yr, respectively. Overall, the median age at diagnosis was 59.9 yr (range: 31-83) in NSCLC and 60.8 yr in SCLC (range: 38-75). All 16 SCLC patients were smokers. The pathological staging of the 65 NSCLC cases revealed 25 cases of stage 1, 10 of stage 2, and 30 of stage 3. Pathological T stages were pT1 in 14 cases, pT2 in 31, pT3 in 16, and pT4 in 4. The 16 SCLC cases were staged as 8 limited and 8 extensive diseases. The histologic types of 65 NSCLCs included 30 cases of squamous cell carcinoma (SCC), 25 cases of adenocarcinoma (ADC), 3 cases of adenosquamous carcinoma (ASC), 2 cases of bronchioloalveolar carcinoma (BAC), 3 cases of large cell carcinoma

Table 1. Correlations between TTF-1 expression and clinicopathologic features in non-small cell lung carcinomas

Clinicopathologic features	No. of cases	TTF-1 immunoreactivity		<i>p</i> value*
		Positive (%)	Negative (%)	
Age				
≤59 yr	29	9 (31)	20 (72)	0.510
>59 yr	36	14 (42)	22 (58)	
Sex				
Male	49	14 (29)	35 (71)	0.044
Female	16	9 (56)	7 (44)	
Smoking history				
Smokers	48	14 (29)	34 (71)	0.078
Non-smokers	17	9 (53)	8 (47)	
Stage				
1	25	10 (40)	15 (60)	0.538
2-3	40	13 (33)	27 (67)	
pT				
1-2	45	17 (38)	28 (62)	0.545
3-4	20	6 (30)	14 (70)	
Grade				
1-2	42	17 (40)	25 (60)	0.096
3	13	2 (15)	11 (85)	
Histologic type				
SCC	30	0 (0)	30 (100)	0.000 [†]
ADC	25	19 (76)	6 (24)	
ASC	3	1 (33)	2 (67)	
BAC	2	1 (50)	1 (50)	
LCC	3	1 (33)	2 (67)	
PLC	2	1 (50)	1 (50)	
Total	65	23 (35)	42 (65)	

*chi-square test, [†]: *p* value between the percentages of TTF-1 immunoreactivity in squamous cell carcinoma versus adenocarcinoma, SCC: squamous cell carcinoma, ADC: adenocarcinoma, ASC: adenosquamous carcinoma, BAC: bronchioloalveolar carcinoma, LCC: large cell carcinoma, PLC: pleomorphic carcinoma.

(LCC), and 2 cases of pleomorphic carcinoma (PLC), based on the WHO classification (1999). The adenocarcinomas and squamous cell carcinomas were graded as 6 cases of grade 1 (well differentiated), 36 of grade 2 (moderately differentiated), and 13 of grade 3 (poorly differentiated). Clinicopathologic variables such as age, smoking history, tumor size, and histologic grades in NSCLCs were not significantly associated with TTF-1 expression, although TTF-1 expression tend to be higher in non-smokers and well-differentiated tumors. However, TTF-1 was expressed significantly higher in women than in men ($p < 0.05$) and in ADCs than SCCs ($p < 0.001$).

In SCLCs, any significant difference in TTF-1 expression was not found between two categorical groups of the clinicopathologic variables such as age, sex, and stage.

TTF-1 expression in normal lung, NSCLCs, and SCLCs

In non-neoplastic lung, nuclear TTF-1 immunoreactivity was limited in alveolar and bronchiolar epithelial cells; interstitial cells, lymphocytes, alveolar macrophages did not stain for TTF-1 (Fig. 1). No differences between non-neoplastic and malignant cells were observed in the staining pattern with TTF-1 antibody.

Overall, TTF-1 immunoreactivity was found in 23 (35%) of 65 NSCLCs, including 19 (76%) of 25 ADCs, 1 (33%) of 3 ASCs, 1 (50%) of 2 BACs, 1 (33%) of 3 LCCs, and 1 (50%) of 2 PLCs. The 30 squamous cell carcinomas were completely devoid of TTF-1 immunoreactivity (0%). No differences in the nuclear immunoreactivity were noted among the different tumor types, and there was no preferential distribution of TTF-1-reactive cells in perivascular, central, or peripheral viable areas of the neoplasms. Among the different histologic types of NSCLCs, ADCs showed TTF-1 expression in the majority of the cases (76%), which was significantly higher than SCCs (0%) ($p < 0.001$) (Fig. 2). TTF-1 expression did not vary with the morphologic subtypes of ADCs, which showed acinar and solid patterns. One of two BACs revealed TTF-1

Table 2. Correlation between TTF-1 immunoreactivity and clinicopathologic features in 16 small cell lung carcinomas (SCLCs)

Clinicopathologic features	No. of cases	TTF-1 immunoreactivity		<i>p</i> value*
		Positive (%)	Negative (%)	
Age				
≤59 yr	7	6 (86)	1 (14)	0.849
>59 yr	9	8 (89)	1 (11)	
Sex				
Male	14	12 (86)	2 (14)	0.568
Female	2	2 (100)	0 (0)	
Stage [†]				
Limited disease	8	8 (100)	0 (0)	0.131
Extensive disease	8	6 (75)	2 (25)	
Total	16	14 (88)	2 (12)	

*chi-square test, [†]16 cases of SCLC were staged as limited and extensive diseases.

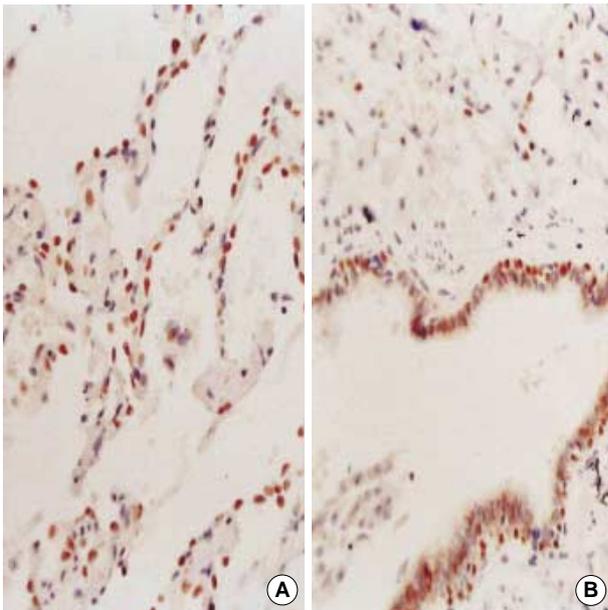


Fig. 1. Nuclear immunoreactivity for thyroid transcription factor-1 (TTF-1) in normal alveolar (A) and bronchiolar (B) epithelial cells ($\times 400$).

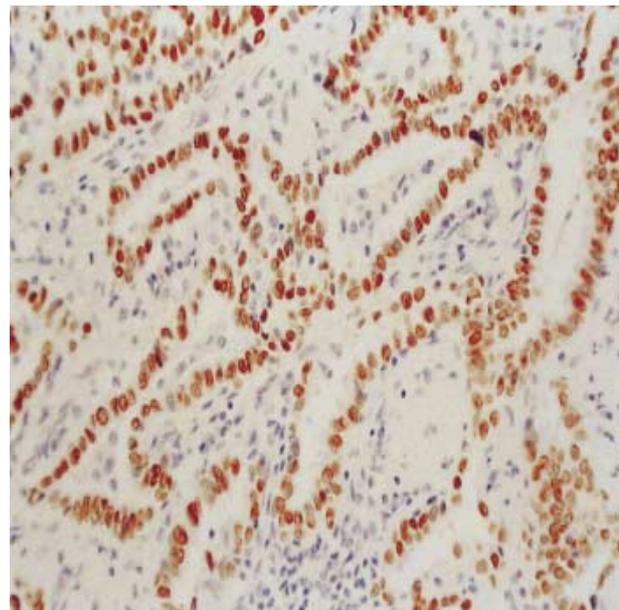


Fig. 2. Immunohistochemical finding of thyroid transcription factor-1 (TTF-1) in moderately differentiated adenocarcinoma, showing diffuse and strong nuclear immunoreactivity for TTF-1 ($\times 400$).

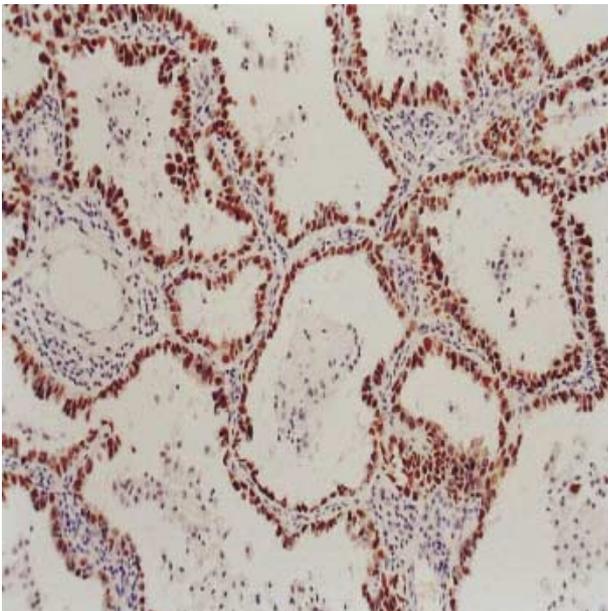


Fig. 3. A bronchioloalveolar carcinoma of non-mucinous type revealed high immunoreactivity for thyroid transcription factor-1 (TTF-1) ($\times 200$).

expression not in mucinous type but in the non-mucinous type (Fig. 3).

SCLCs examined in this study showed high frequency of TTF-1 expression (88%), which was higher than adenocarcinomas (76%) (Fig. 4).

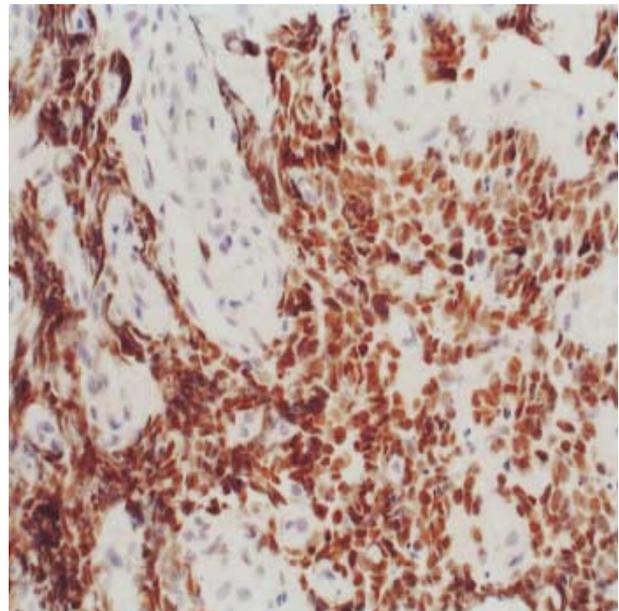


Fig. 4. Pulmonary small cell carcinoma showing high nuclear expression for thyroid transcription factor-1 (TTF-1) ($\times 400$).

Association of TTF-1 expression with p53 and Ki-67 proteins in 65 NSCLCs

The statistical analysis for the relationships was shown in Tables 3 and 4. Significant inverse correlation was found between TTF-1 expression and proliferative activity evaluated by Ki-67 protein ($p < 0.01$). However, there was no significant

Table 3. Correlation between TTF-1 expression and Ki-67 proliferative activity in non-small cell lung carcinomas

TTF-1 expression	No. of cases	Ki-67 immunoreactivity		p value*
		Positive (%)	Negative (%)	
Positive	23	9 (39)	14 (61)	0.003
Negative	42	32 (76)	10 (24)	

*: chi-square test.

Table 4. Correlation between TTF-1 expression and p53 overexpression in non-small cell lung carcinomas

TTF-1 expression	No. of cases	p53 overexpression		p value*
		Positive (%)	Negative (%)	
Positive	23	10 (43)	13 (57)	0.215
Negative	42	25 (60)	17 (40)	

*: chi-square test.

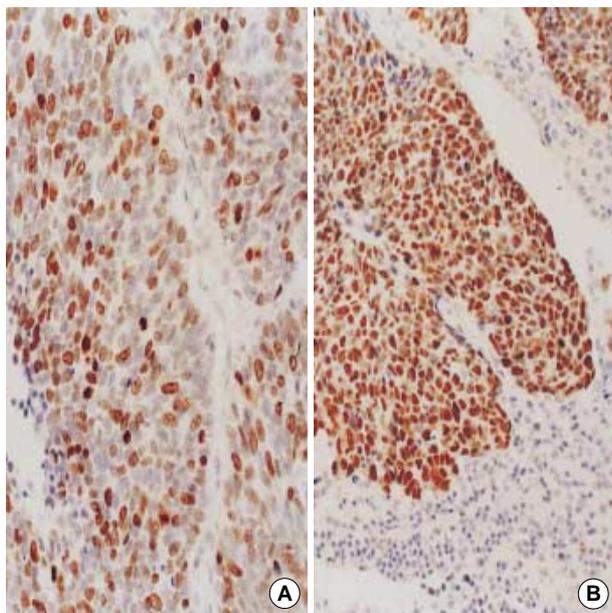


Fig. 5. A case of squamous cell carcinoma showing moderately brisk mitotic activity evaluated by Ki-67 immunostaining (A, $\times 400$) and diffuse nuclear immunopositivity for p53 protein (B, $\times 200$).

correlation between TTF-1 expression and the overexpression of p53 protein, a cell cycle regulator. Two representative cases are shown for the immunostaining results for Ki-67 proliferative activity and p53 overexpression in Fig. 5.

Due to the small number of cases, those relationships were not analyzed in 16 SCLCs.

Survival data and its comparison between TTF-1 (+) and TTF-1 (-) groups

Complete follow-up information was available on 36 pati-

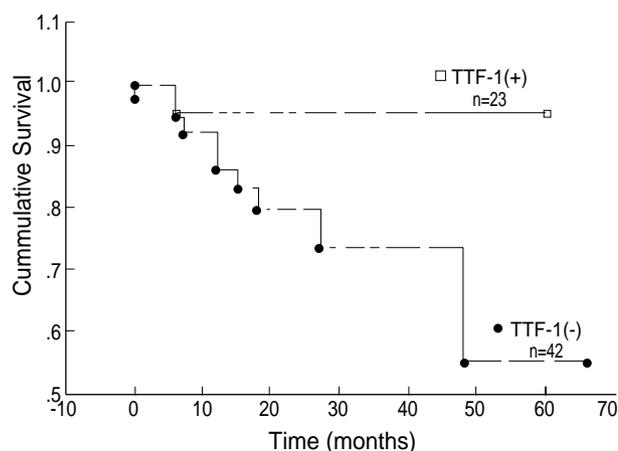


Fig. 6. Kaplan-Meier survival curves of patients with non-small cell lung carcinoma (NSCLC). Comparison between TTF-1 positive and TTF-1 negative groups shows a tendency for better survival in the TTF-1 positive group (log rank test; $p=0.0956$).

ents of 65 NSCLCs and 6 of 16 SCLCs, with a median follow-up duration of 29.7 months for living patients (range 12-66 months) in NSCLCs and 45 (range 24-66 months) in SCLCs. At the time of analysis, 26 (72.2%) and 2 (33%) patients were alive in NSCLCs and SCLCs, respectively. When all of 65 NSCLC patients analyzed for postsurgical survival were examined for the difference in survival time between TTF-1 (+) and TTF-1 (-) groups by Kaplan-Meier method and then log rank test, the group with a positive immunoreactivity for TTF-1 tended to reveal the better survival, compared with the group with a negative immunostaining for that (Fig. 6). When we also obtained the survival curves for the lower (stage 1, 2) and higher stage (stage 3) and compared them by log rank test, the patients with stage 3 revealed significantly lower survival time compared with those with stage 1, 2 ($p=0.0077$) (data not shown).

Due to the limited number of SCLC patients, the statistical analysis for the survival data between TTF-1 (+) and TTF-1 (-) groups was not performed.

DISCUSSION

Tissue-specific gene expression is mediated largely by transcriptional factors, and a master regulatory gene is thus a potential marker of cellular lineage (15, 20). TTF-1 is an example of such a lineage marker, which plays a crucial role in normal peripheral lung function and morphogenesis (15).

In the present study, normal lung tissue revealed a very limited expression for TTF-1 in the terminal airways such as alveolar and bronchiolar epithelial cells. The staining was quite uniform, and TTF-1 appeared to be useful for labeling the committed cells or lineage of the terminal respiratory unit. This result also indicates that TTF-1 could be used as a molecular marker of differentiation for neoplasms arising from these cell

types (9).

In the lung cancer group, TTF-1 expression was found in 23 NSCLCs of non-squamous cell type (35%) and 14 SCLCs (88%). Of the 23 NSCLCs, the majority (19 cases, 83%) was ordinary type of adenocarcinoma and the remaining consisted of 1 non-mucinous type of BAC, 1 ASC, 1 LCC, and 1 PLC. In a recent report (15), adenocarcinomas with terminal respiratory unit (TRU) morphology revealed significantly higher TTF-1 expression than those with non-TRU morphology. They suggested that TTF-1 expression could be used as a lineage marker for TRU type adenocarcinomas, and could play a role in the molecular pathogenesis of those adenocarcinomas. Although the adenocarcinomas were not classified into TRU and non-TRU type, the present study also suggested that TTF-1 could be a useful marker of adenocarcinomas in the diagnosis of poorly differentiated NSCLCs because there was a highly significant difference in TTF-1 expression between two major groups of NSCLCs, ADC and SCC (76% versus 0%, $p=0.000$). Also, TTF-1 expression in this study was significantly higher in the female than in male, and tend to be higher in non-smokers than in smokers. These results seem to be associated with the fact that pulmonary ADCs tend to be more prevalent in female non-smokers than SCCs. In our study, all 30 cases of SCCs revealed no TTF-1 immunoreactivity (0%). The rare occurrence or lack of TTF-1 immunoreactivity in SCC or metaplastic epithelium suggests that this protein is not involved in the differentiation of squamous cells. This fact is supported by a few studies showing that the lung-specific genes activated by TTF-1 are not expressed in SCCs (8, 17, 21). Pelosi et al. (14) also proposed a possibility of the synthesized TTF-1 with an aberrant function in SCCs.

SCLC has also been reported to express TTF-1 in most cases (89-100%) (9, 11, 12, 22), and our data confirmed the high frequency of TTF-1 expression in SCLC (14 of 16, 88%). Although this high frequency of TTF-1 expression in SCLC appears to conflict with the previously mentioned usefulness of TTF-1 as the cell lineage marker for adenocarcinoma, Yatabe et al. (15) explained it as follows; first, small cell carcinomas frequently produce TTF-1 aberrantly like ectopic hormones; second, SCLC is a type of neuroendocrine carcinoma and can make TTF-1 because TTF-1 is reported to be expressed in the the diencephalon area (1); and third, TTF-1 expression may be atavistic in these undifferentiated cells of SCLC.

Taken together, the present study suggests that TTF-1 may play an important role as the transcriptional factor in the molecular pathogenesis of adenocarcinomas and small cell carcinomas.

Most of the previous studies on TTF-1 expression focused on its usefulness as the immunohistochemical marker for diagnosis of lung tumors and for differential diagnosis of primary pulmonary adenocarcinomas from extrapulmonary adenocarcinomas metastatic to the lung (23). Few studies investigated the prognostic role of TTF-1 in NSCLC and showed contradictory data on the relationship between TTF-1 expression and survival (13, 14, 16, 18). In the current study, we found

that the patients with TTF-1 expression tend to survive longer than TTF-1 (-) group, although the difference was not statistically significant (log rank test, $p=0.096$). Potential sources of the conflicting results of TTF-1 expression and survival in the literatures have been analyzed, including the scoring system of immunohistochemical staining for TTF-1, the staining procedure, and statistical analysis of data (14, 16).

Although the independent prognostic role of p53 mutation and Ki-67 proliferative activity is still debatable in NSCLC, the importance of p53 mutation and cell proliferation in the pulmonary carcinogenesis seems to be obvious (14, 24). p53 is considered as the cell cycle regulator as it blocks progression through the cell cycle in cells that have sustained DNA damage (24). In general, p53 mutation or overexpression is considered as an indicator of poor prognosis. Also, Ki-67 is frequently used to evaluate the prognostic value of proliferative activity of lung cancers, and high Ki-67 expression was hypothesized to be correlated with a poor clinical outcome (25). However, the study on the relationship between TTF-1 expression and p53 overexpression or proliferative activity is very rare. Yatabe et al. (15) reported that p53 overexpression showed the negative correlation with TTF-1-positive adenocarcinoma. Puglisi et al. (18) performed a combined analysis of TTF-1 and MIB-1 proliferative activity in NSCLCs, showing no correlation between them but the poorest survival in the TTF-1-positive/MIB-1-positive group. The presence of a relationship between TTF-1 expression and cell proliferation has been reported on the basis of data showing that reduced proliferation rate of thyroid cells is induced by decrease in TTF-1 concentration by antisense procedures (26). Also, it has been suggested that homeodomain-containing nuclear proteins functionally similar to TTF-1 have positive effects on cell growth (27) as well as antiapoptotic effects demonstrated by loss of function experiment (28).

However, our data unexpectedly showed inverse relationship between the TTF-1 expression and Ki-67 proliferative activity in NSCLCs. This contradictory result seems to be difficult to be interpreted because of paucity of the similar study except for a study by Pelosi et al. (14). The investigation showed TTF-1 immunoreactivity correlated inversely with the tumor proliferative fraction assessed by Ki-67 immunostaining. But as an interpretation for the result, they commented only that the relationship of TTF-1 expression with cell proliferation of lung cancer remains to be unveiled. Therefore, our result for the inverse relationship between TTF-1 and proliferative activity in NSCLCs remains to be proved further.

In conclusion, we confirmed that TTF-1 appears to be a reasonably sensitive and highly specific diagnostic marker for pulmonary adenocarcinomas and small cell carcinomas; that TTF-1 may be used a good prognostic marker based on its negative correlation with Ki-67 proliferative activity and tendency for better survival; that the cell lineage marker of the lung may in part participate in the molecular pathogenesis of lung cancers at the level of transcription.

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