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TTF-1 Expression Predicts the Merit of Additional Antiangiogenic Treatment in Non-squamous Non-small Cell Lung Cancer

AKIRA TAKEUCHI[1], TETSUYA OGURI[1,2], YORIKO YAMASHITA[3], KAZUKI SONE[1], SATOSHI FUKUDA[1], OSAMU TAKAKUWA[4], TAKEHIRO UEMURA[5], KEN MAENO[1], KENSUKE FUKUMITSU[1], YOSHIHIRO KANEMITSU[1], HIROTSUGU OHKUBO[1], MASAYA TAKEMURA[1], YUTAKA ITO[1] and AKIO NIIMI[1]

[1]*Department of Respiratory Medicine, Allergy and Clinical Immunology,*
[2]*Department of Education and Research Center for Community Medicine,*
[3]*Department of Experimental Pathology and Tumor Biology,*
[4]*Department of Education and Research Center for Advanced Medicine,*
Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan;
[5]*Department of Respiratory Medicine, Aichi Cancer Center Hospital, Nagoya, Japan*

Abstract. *Background/Aim: To investigate whether TTF-1 expression predicts a beneficial response of non-squamous non-small-cell lung cancer (NS-NSCLC) patients to bevacizumab. Patients and Methods: We retrospectively screened 118 advanced NS-NSCLC patients who were treated with pemetrexed plus platinum derivatives alone (Bev(–)) or with bevacizumab (Bev(+)), and investigated the relationship between expression of TTF-1 and treatment outcomes. Results: Among the 92 TTF-1-positive patients, clinical outcomes in the Bev(+) group were significantly better than those in the Bev(–) group (response rate, 51.4% vs. 27.3%, p=0.027; median progression-free survival, 216 days vs. 137 days, p=0.012). Overall survival in the Bev(+) group tended to be longer than that in the Bev(–) group. However, the addition of bevacizumab to the standard treatment of 26 TTF-1-negative patients offered no clinical benefit. Conclusion: TTF-1 expression may serve as a predictive marker to identify patients who may benefit from the addition of bevacizumab to platinum doublet therapy.*

Thyroid transcription factor-1 (TTF-1) is a homeodomain-containing transcription factor, that is essential for the morphogenesis and differentiation of the thyroid, lung, and ventral forebrain. TTF-1 controls the expression of select genes in the thyroid, lung, and the central nervous system. In the lung, TTF-1 regulates the expression of surfactant proteins that are essential for lung stability and lung host defense (1). Expression of TTF-1 has been documented in neoplasms arising from cells that normally produce this transcription factor. Furthermore, TTF-1 is a lineage-survival oncogene in lung adenocarcinoma (2).

TTF-1 has been used widely as a diagnostic marker for primary and metastatic lung cancer, and, in particular, for identification of lung as the primary site of metastatic adenocarcinoma. Among primary non-small-cell lung cancers (NSCLC), TTF-1 immunoreactivity was significantly correlated with lung adenocarcinoma, but not with squamous cell carcinoma (3). Also, TTF-1 expression was recently shown to be a good prognostic factor of survival of NSCLC patients who received cytotoxic chemotherapy or EGFR-TKIs (4-6).

Vascular endothelial growth factor (VEGF) signaling pathway plays a crucial role in angiogenesis and is a promising target for cancer drug development. Anti-VEGF therapy, including bevacizumab, is therefore a major therapeutic option for targeting angiogenesis, especially in the context of advanced non-squamous (NS)-NSCLC (7, 8). However, biomarkers that help predict the efficacy of add-on bevacizumab therapy to cytotoxic agent chemotherapy in patients with NS-NSCLC are yet to be identified. In this study, we retrospectively examined the efficacy of addition of bevacizumab to platinum drugs and pemetrexed chemotherapy in patients with NS-NSCLC. Furthermore, we also investigated whether TTF-1 expression may serve as a predictive marker for addition of bevacizumab to platinum drugs and pemetrexed chemotherapy.

Correspondence to: Tetsuya Oguri, Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Tel: +81 528538216, e-mail: t-oguri@med.nagoya-cu.ac.jp

Key Words: TTF-1, cisplatin, carboplatin, pemetrexed, bevacizumab.

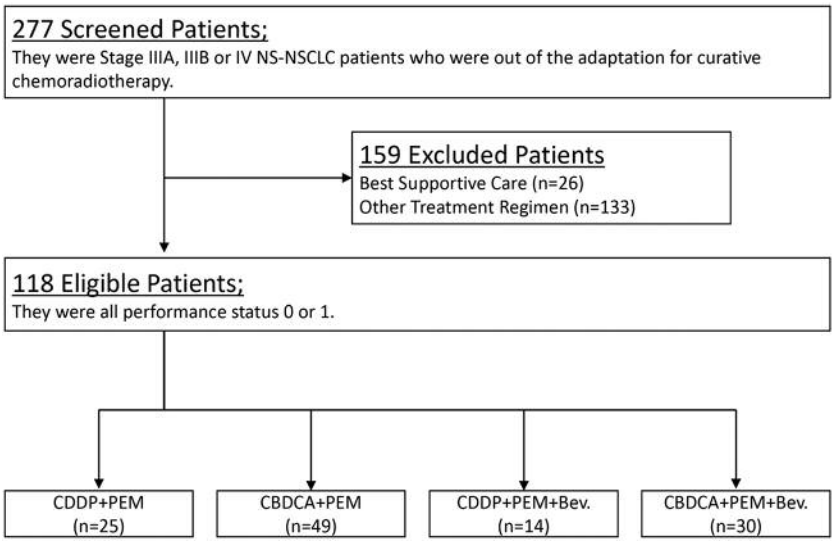

Figure 1. *A flow chart of total cases, excluded cases and treatment regimen of eligible cases. CDDP: Cisplatin; PEM: pemetrexed; CBDCA: carboplatin; Bev: bevacizumab.*

Patients and Methods

Patients and chemotherapy. We retrospectively identified patients with advanced NS-NSCLC who were treated with or without the addition of bevacizumab to platinum derivatives and pemetrexed therapy at the Nagoya City University Hospital (Japan) between October 2009 and May 2017. Patients received four to six cycles of bevacizumab (Bev) (15 mg/kg) and/ or cisplatin (CDDP) (75 mg/m^2) or carboplatin (CBDCA) (Area Under Curve [AUC] 5) and pemetrexed (PEM) (500 mg/m^2). Patients who achieved complete response (CR), partial response (PR), or stable disease after induction therapy also received maintenance therapy (pemetrexed or pemetrexed plus bevacizumab) to the extent possible in. Both therapies were continued until detection of progressive disease (PD) or development of intolerable toxicity. Dose reduction or interruption was permitted in case of treatment-related toxicity. Our Institutional Ethics Committee approved the protocol of the study (IRB number: 1115), and all medical data were anonymized.

Immunohistochemical analysis of TTF-1 expression in NS-NSCLC. We used NS-NSCLC tissue samples obtained at the time of diagnosis from surgery, bronchoscopy, or computed tomography-guided biopsy. All samples were paraffin-embedded and 2-4 μm thick sections were prepared. Antigen retrieval was performed by autoclaving the sections at 97˚C for 20 min in citrate buffer (pH 6.0). The sections were then incubated with mouse monoclonal anti-TTF-1 antibody clone 8G7G3/1 (Dako, Santa Clara, CA, USA, 1/100 dilution) for 2 h at room temperature. Primary antibody binding to the tissue sections was detected using EnVision FLEX kit (DAKO, Agilent). A pathologist (Y.Y.) and a pulmonologist (A.T.) who were blinded to the clinical information reviewed the immunostained sections, which were considered as positive when cytoplasmic or nuclear staining was evident.

Statistical analysis. Response rate (RR) was defined as the sum of CR and PR rate. RR was compared using the Fisher's exact test and $p<0.05$ was considered as statistically significant. Progression-free survival (PFS) was defined as the time from the first day of chemotherapy to the date of disease progression, death, or most recent follow-up. Overall survival (OS) was defined as the time from the first day of chemotherapy to the day of death or most recent follow-up. PFS and OS were analyzed using the Kaplan–Meier method and compared using the log-rank test. $p<0.05$ was considered as statistically significant. Multivariate analysis using a Cox proportional hazards model was performed to identify the association between clinical characteristics and survival. In this analysis, a probability of $p=0.10$ in the log-rank test was the threshold for the addition or removal of a covariable from the model, and $p<0.05$ again was considered as significant. All statistical analyses were performed with EZR (Saitama Medical center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics (9).

Results

We screened Stage IIIA, IIIB or IV NSCLC patients who were treated at the Nagoya City University Hospital between October 2009 and May 2017. Total of 277 patients were enrolled in the study, and they were all not indicated for curative radiotherapy. One hundred fifty-nine patients were excluded; 26 patients chose best supportive care, and 133 patients received other cytotoxic treatment regimens. The remaining 118 patients were reviewed. They were all performance status 0 or 1 and received chemotherapy: CDDP/PEM, CBDCA/PEM, CDDP/PEM/ Bev, or CBDCA/PEM/ Bev (Figure 1).

Table I. *Patients characteristics*.

Patient characteristics			TTF-1 expression						
			Total n=118		Positive n=92		Negative n=26		*p*-Value
Age	Median [min-max]		67	[37-79]	67	[37-76]	66.5	[49-79]	0.76
Gender	(%)	Male	79	(67)	59	(64)	20	(77)	0.25
		Female	39	(33)	33	(36)	6	(23)	
Smoke history	(%)	Former or current	84	(71)	64	(70)	20	(77)	0.63
		Never	34	(29)	28	(30)	6	(23)	
Pathology	(%)	Adenocarcinoma	114	(97)	90	(98)	24	(92)	0.21
		Large cell carcinoma	4	(3)	2	(2)	2	(8)	
TTF-1 expression	(%)	Positive	92	(78)					
		Negative	26	(22)					
EGFR mutation	(%)	Exon 19 deletion	12	(10)	12	(13)	0	0	0.04
		Exon 21 L858R	14	(12)	13	(14)	1	(4)	
		Wild type	92	(78)	67	(73)	25	(96)	
ALK fusion	(%)	Positive	3	(3)	3	(3)	0	0	1
		Negative	115	(98)	89	(97)	26	(100)	
Platinum	(%)	CDDP	39	(33)	33	(36)	6	(23)	0.25
		CBDCA	79	(67)	59	(64)	20	(77)	
Platinum times	Median [min-max]		4	[1-6]	4	[1, 6]	4	[1, 6]	0.92
Bevacizumab	(%)	Additional	44	(37)	37	(40)	7	(27)	0.26
		Without	74	(63)	55	(60)	19	(73)	
CEA	(%)	≤5.0 ng/ml	42	(36)	30	(33)	12	(46)	0.25
		<5.0 ng/ml	76	(64)	62	(67)	14	(54)	
CYFRA	(%)	≤3.5 ng/ml	61	(52)	52	(57)	9	(35)	0.07
		<3.5 ng/ml	57	(48)	40	(44)	17	(65)	

TTF-1: Thyroid transcription factor-1; EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; CDDP: cisplatin; CBDCA: carboplatin; CEA: carcinoembryonic antigen; CYFRA 21-1: cytokeratin 19 fragment.

The clinicopathological characteristics of 118 patients are shown in Table I. Presence of epidermal growth factor receptor (*EGFR*) mutation in patients with TTF-1-positive NS-NSCLC was significantly higher than that in the TTF-1 negative group. Patients treated with bevacizumab added on pemetrexed and platinum derivatives (Bev(+) regimen) exhibited significantly higher RR compared with those treated with pemetrexed and platinum derivatives only (Bev(–) regimen) [47.7% *vs*. 25.7%, p=0.017]. PFS of patients treated with Bev(+) regimen was significantly longer than that of patients treated with Bev(–) regimen; however, no significant difference was observed with respect to OS [median PFS, 206 days *vs*. 137 days, p=0.028 (Figure 2A); median OS, 579 days *vs*. 420 days, p=0.475 (Figure 2B), respectively].

In patients with TTF-1-positive NS-NSCLC, use of Bev(+) regimen significantly improved the RR [51.4% *vs*. 27.3%, p=0.027]. Furthermore, Bev(+) regimen was associated with significantly prolonged PFS and a tendency for longer OS compared with that with Bev(–) regimen [median PFS, 216 days *vs*. 137 days, p=0.012 (Figure 3A); median OS, 646 days *vs*. 503 days, p=0.471 (Figure 3C), respectively]. On the other hand, in patients with TTF-1 negative NS-NSCLC, Bev(+) regimen did not show any clinical benefit over Bev(–) regimen [RR, 28.6% *vs*. 21.1%, p=1; median PFS 124 days *vs*. 131 days, p=0.374 (Figure 3B); median OS, 351 days *vs*. 354.5 days, p=0.543 (Figure 3D), respectively].

We performed univariate analysis to evaluate the influence of background characteristics on PFS of TTF-1 positive patients. The addition of bevacizumab and normal plasma level of cytokeratin 19 fragment (CYFRA 21-1) were associated with longer PFS (Table II). Finally, multivariate analysis was performed using the addition of bevacizumab, plasma levels of CYFRA 21-1 and carcinoembryonic antigen (CEA). Among the three covariables, only the addition of bevacizumab was a statistically significant factor [hazard ratio (HR), 0.54, p=0.011] (Table III).

Discussion

In this study, patients with TTF-1 positive NS-NSCLC who received Bev(+) regimen showed better RR and PFS as compared with those who received Bev(–) regimen. The clinical benefit of Bev(+) regimen in these patients was also

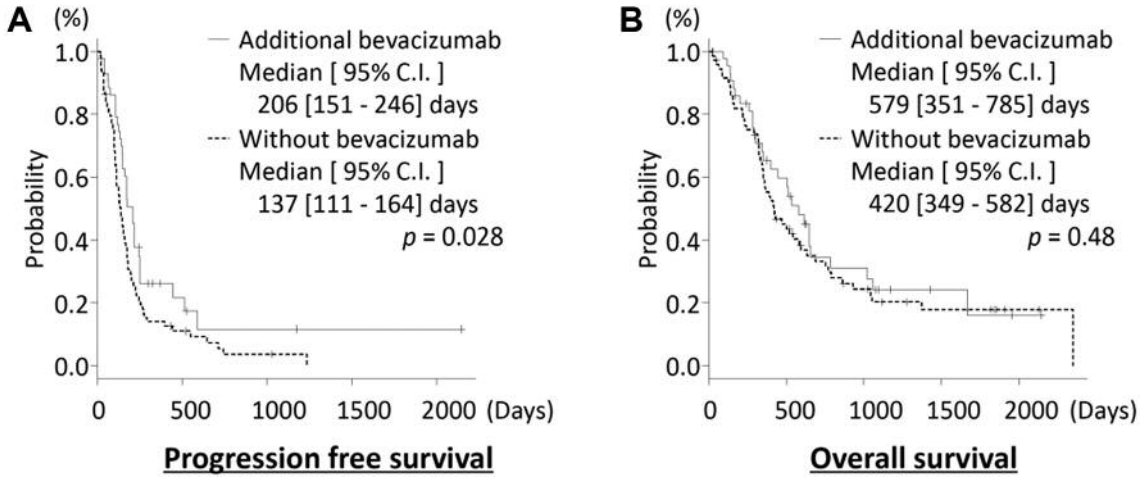

Figure 2. *Kaplan–Meier curve of progression-free survival (A) and overall survival (B) in all non-squamous non-small cell lung cancer patients who received platinum doublet and bevacizumab or platinum doublet. Both curves were compared by log-rank test. C.I.: Confidence interval.*

shown on multivariate analysis. On the other hand, the antitumor effect of Bev(+) and Bev(–) regimens showed no significant difference in patients with TTF-1-negative NS-NSCLC. Our findings suggest that TTF-1 expression may serve as a predictive marker to identify patients who may benefit from the addition of bevacizumab to platinum doublet therapy.

High expression of VEGF in NSCLC tissue is associated with poor prognosis (10). VEGF plays a key role in tumor proliferation by promoting tumor angiogenesis (11). Excess pro-angiogenic factors, including VEGF, induce abnormal tumor vessel formation, which results in tumor hypoxia and reduces the efficacy of anticancer treatment or irradiation (12). In addition, VEGF influences the tumor microenvironment by suppressing tumor-directed immune response (13). Bevacizumab, an anti-VEGF monoclonal antibody, not only suppresses and normalizes tumor vascularity but is also believed to re-engineer the tumor-immune microenvironment (14). Indeed, clinical trials have shown that the addition of bevacizumab improves the prognosis of patients with NS-NSCLC (7). Use of some biomarkers to predict the benefit of additional bevacizumab has been suggested in various cancers; these include, circulating endothelial progenitor cells in NSCLC (15), serum VEGF-A in breast cancer/pancreatic cancer/gastric cancer (16), and serum VEGF-D in colorectal cancer (17). However, these are preliminary findings, and their applicability to clinical practice is not clear. Our study suggests that TTF-1 expression in tumor tissues may potentially predict whether the addition of bevacizumab influences lung cancer treatment outcomes. VEGF promoter contains TTF-1 responsive elements, and TTF-1 positive cells have higher intracellular VEGF mRNA levels and secrete VEGF protein as compared with TTF-1 negative cells; in addition, reduction in TTF-1 was shown to downregulate these functions (18). It may be possible that tumor TTF-1 expression level controls its VEGF level and results in good clinical outcomes.

TTF-1 expression in NSCLC is known as a good prognostic factor for survival (19). However, the underlying mechanism of this correlation is not well understood. In normal lungs, TTF-1 is mainly expressed in alveolar type II and Clara epithelial cells and is a potential lineage-survival oncogene (2). TTF-1 expression has been shown to correlate with adenocarcinoma rather than squamous cell carcinoma (20). Although most lung adenocarcinomas express TTF-1, the frequency of TTF-1 expression in invasive mucinous adenocarcinoma (IMA), formerly mucinous bronchioloalveolar carcinoma, is lower than that in invasive non-mucinous adenocarcinoma (INMA) (21). Adenocarcinoma was categorized as terminal respiratory unit (TRU) type and non-TRU type, and IMAs are considered as non-TRU type, unlike many INMAs (22). TRU and non-TRU types exhibit differences with respect to expression of several genes which are associated with prognosis (23). TTF-1 expression in adenocarcinoma was proposed as a lineage marker of TRU (24); therefore, it is possible that TTF-1 positive and negative lung adenocarcinoma originate from different cellular lineages. This may influence the cellular proliferation pattern and sensitivity to chemotherapy, which results in different outcomes.

In this study, we assessed the efficacy of bevacizumab added to only pemetrexed and platinum combination therapy. Previously we have shown that serum cytokeratin 19 fragment (CYFRA 21-1) level is a predictive and prognostic marker of

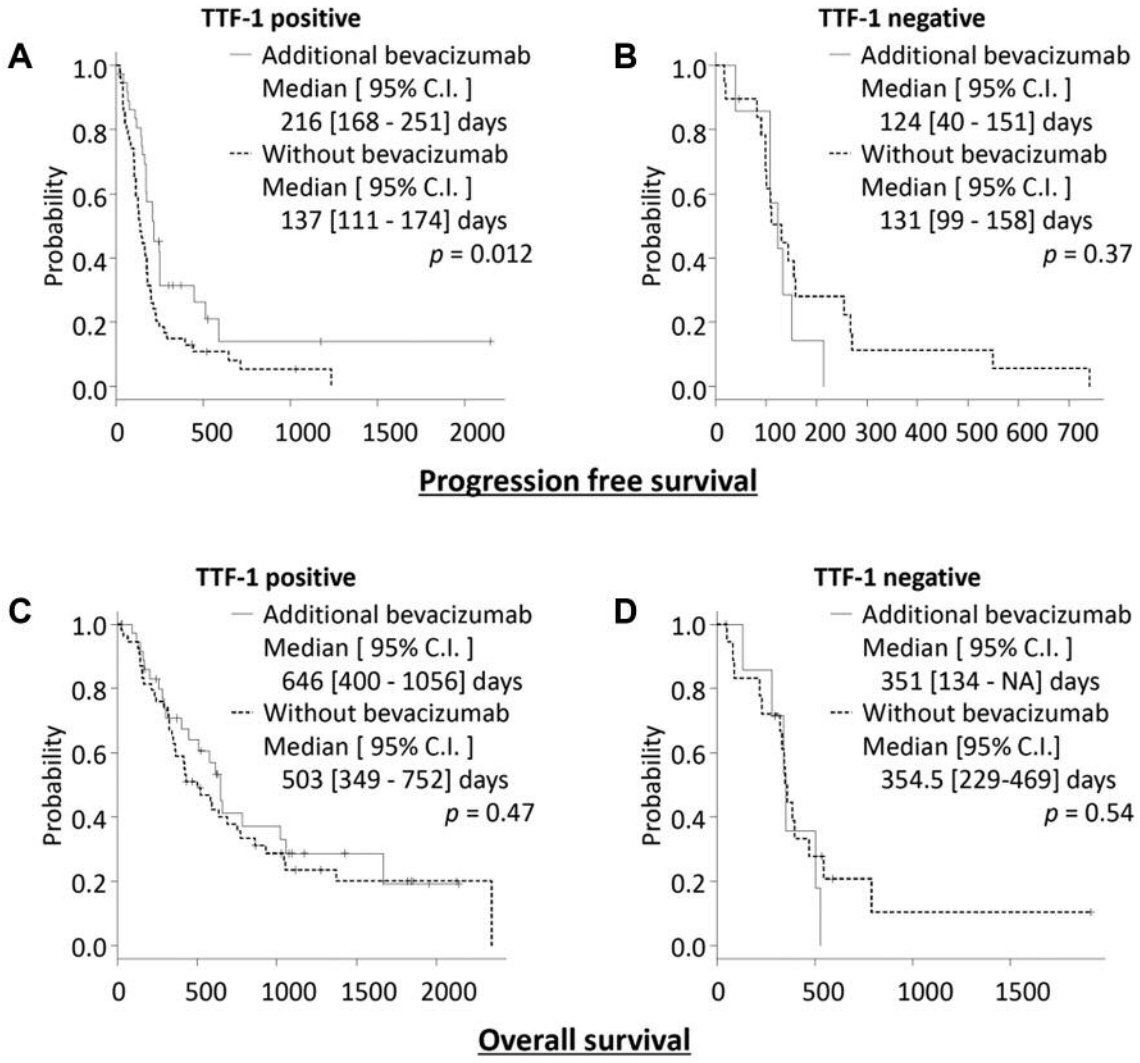

Figure 3. *Kaplan–Meier curve of Progression free survival (A, B) and overall survival (C, D) in thyroid transcription factor-1 positive (A, C) or negative (B, D) non-squamous non-small cell lung cancer patients who received platinum doublet and bevacizumab or platinum doublet. Both curves were compared by log-rank test. TTF-1: Thyroid transcription factor-1; C.I.: confidence interval.*

pemetrexed and platinum combination therapy in NS-NSCLC (25). CYFRA 21-1 is a specific marker of lung histological type of squamous cell carcinoma (26) which does not show a survival advantage with pemetrexed treatment (27); this suggests that elevated levels of serum CYFRA 21-1 indicate the presence of squamous cell components. Furthermore, we have also shown that CYFRA 21-1 predicts the efficacy of EGFR tyrosine kinase inhibitor treatment in patients with *EGFR* mutation-positive lung cancer (28). *EGFR* mutation is specific for TRU-type adenocarcinoma, which is often associated with TTF-1 expression (29). Collectively, these results and the findings from the present study suggest that the addition of bevacizumab would improve the prognosis of NS-NSCLC patients who are more sensitive to pemetrexed-containing chemotherapy, rather than improve poor prognosis.

Some limitations of this study need to be acknowledged. First, since this is a retrospective, single institute study, our results may be affected by selection bias. Clinical outcomes were almost similar to those in previous reports (30, 31), so we think the bias will not significantly influence the result. Second, the number of TTF-1 negative patients were quite lower than that of TTF-1 positive patients; therefore, the effect of additional bevacizumab may have been underestimated in the former group. However, the positivity rate of TTF-1 in primary pulmonary adenocarcinoma was shown to be 86% (3); therefore, the difference in the number

Table II. *Univariate analysis about PFS in TTF-1-positive patients.*

Category	Patient n=92	PFS MST	PFS (95%CI)	*p*-Value
Age				
<70	64	162	(128-174)	0.49
≤70	28	198	(116-246)	
Gender				
Male	59	144	(111-210)	0.4
Female	33	175	(162-208)	
Smoke history				
Former or current	64	149	(111-198)	0.18
Never	28	175	(162-226)	
Pathology				
Adenocarcinoma	90	170	(137-198)	0.68
Large cell carcinoma	2	171.5	(164-NA)	
EGFR status				
Mutated	25	171	(111-208)	0.63
Wild type	67	168	(137-206)	
ALK fusion				
Positive	3	174	(116-NA)	0.46
Negative	89	170	(137-198)	
Platinum				
CDDP	33	172	(112-228)	0.38
CBDCA	59	162	(137-198)	
Bevacizumab				
Additional	37	216	(168-251)	0.01
Without	55	137	(111-174)	
CEA				
≤5.0 ng/ml	30	171	(116-510)	0.07
5.0 ng/ml <	62	170	(137-198)	
CYFRA				
≤3.5 ng/ml	52	198	(168-250)	0.03
<3.5 ng/ml	40	141	(103-164)	

PFS: Progression-free survival; TTF-1: thyroid transcription factor-1; MST: median survival time; C.I.: confidence interval; EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; CDDP: cisplatin; CBDCA: carboplatin; CEA: carcinoembryonic antigen; CYFRA 21-1: cytokeratin 19 fragment.

of cases is acceptable. Third, TTF-1 is mainly expressed in primary lung cancer; we cannot use this method to predict whether bevacizumab may be effective in the context of other cancers.

In conclusion, TTF-1 expression may predict favorable outcomes with the addition of bevacizumab therapy to platinum derivatives. Immunohistochemical analysis for TTF-1 expression in NSCLC is commonly used; therefore, this method is useful in many institutes to predict whether bevacizumab produces superior outcomes or not. Further validation with use of more rigid and larger controls is warranted.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Table III. *Multivariate analysis about PFS in TTF-1 positive patients.*

Category	Hazard ratio	(95%CI)	*p*-Value
Bevacizumab	0.5401	(0.3353-0.8701)	0.01
CEA	1	(1-1.001)	0.07
CYFRA	1.016	(0.9824-1.051)	0.35

PFS: Progression-free survival; TTF-1: thyroid transcription factor-1; C.I.: confidence interval; CEA: carcinoembryonic antigen; CYFRA 21-1: cytokeratin 19 fragment.

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