Prognostic Significance of Urine N^1,N^{12}-Diacetylspermine in Patients with Non-small Cell Lung Cancer

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Abstract. *Background: Recently N^1,N^{12}-diacetylspermine, a diacetylated polyamine derivative, was recognized as a tumor marker in patients with several kinds of cancers. However, the significance of its levels in urine as a prognostic factor has not been elucidated. In the present study, we examined whether the urine N^1,N^{12}-diacetylspermine levels can be used as a prognostic factor in patients with NSCLC. Patients and Methods: Urine samples from 251 patients with NSCLC were collected prior to surgery and the urinary N^1,N^{12}-diacetylspermine concentration was measured. Thereafter, all 251 patients underwent curative surgery and the analysis of prognosis was performed for over 10 years. Out of the 251 patients, 91 had recurrent disease. The significance of the urinary N^1,N^{12}-diacetylspermine level as a prognostic factor among all 251 patients and among the 91 patients with recurrence was evaluated. Results: Univariate analysis of all 251 patients showed that the level of urinary N^1,N^{12}-diacetylspermine was a significant prognostic factor for disease-free survival and overall survival; however, multivariate analysis showed it had no significance. Conversely, the univariate and multivariate analyses of post-recurrent survival of the 91 patients with recurrence showed that urinary N^1,N^{12}-diacetylspermine was an independent prognostic factor for post-recurrent survival. Conclusion: Patients with recurrence with positive urinary N^1,N^{12}-diacetylspermine should undergo more intensive care and determination of urinary N^1,N^{12}-diacetylspermine may contribute to improvement of prognosis of NSCLC.*

Lung cancer is one of the most deadly types of cancer worldwide (1). The prevalence of lung cancer and the resultant mortality are increasing. The rate of recurrence after curative resection is relatively high at 30-75%, and prognosis is poor (2, 3). Once the disease has relapsed after surgery, it is seldom curable, and the estimated median survival time after recurrence is 11.5 months (4). Because lung cancer comprises many histological types, reliable estimates of prognosis cannot be obtained using only one factor. Therefore, several types of convenient prognostic markers are urgently required to improve prognosis of lung cancer. Many prognostic factors for postoperative survival of patients with non-small cell lung cancer (NSCLC) have been described in a number of reports and include insulin receptor expression, forkhead box P3 (FOXP3) expression in NSCLC cells, vascular endothelial growth factor receptor 2 gene polymorphisms, human immunodeficiency virus infection, and the carcinoembryonic antigen (CEA) levels in pleural lavage fluid (5-9). In contrast, although some reports have shown that conventional factors such as early p-stage, resectable recurrent disease, female gender, pleural invasion, and extrathoracic recurrence are predictive prognostic factors for patients with recurrence (4, 10, 11), other unique prognostic factors for post-recurrent survival in patients with NSCLC have not been well-elucidated. This may be because longer follow-up periods are required for the analysis of post-recurrent survival.

Polyamines are alkylamines with multiple amino groups. They appear during mitosis, are acetylated in liver, and are excreted in urine (12). Among polyamine derivatives, N^1,N^{12}-diacetylspermine (hereafter referred to simply as diacetylspermine) and N^1,N^8-diacetylspermidine have recently attracted much attention in oncology research

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because they have been detected by conventional methods such as enzyme-linked immunosorbent assay (13-15). In particular, the significance of urinary diacetylspermine as a tumor marker has been reported in many types of cancers, including breast, colorectal, pancreatobiliary, urinary bladder, prostatic, and lung cancer, because of its high sensitivity (12, 16-21). Indeed, we showed that the sensitivity of predictions by urinary diacetylspermine level was 46.4% and that it was higher than the sensitivity of serum CEA and cytokeratin 19 fragment (CYFRA 21-1) levels (32.7% and 23.7%, respectively) in patients with lung cancer (12).

However, the significance of urine diacetylspermine as a prognostic factor has not been reported. In the present study, we evaluated whether diacetylspermine in urine can be used as a predictive prognostic factor for NSCLC.

Patients and Methods

Patients. Urine samples from a total of 251 patients with NSCLC were collected prior to surgery and the diacetylspermine concentration was measured. Thereafter all 251 patients underwent curative surgery between February 2003 and May 2012 at the Department of Thoracic Surgery, Hamanomachi Hospital (Fukuoka, Japan) and the analysis of prognosis was performed for more than 10 years. Of these 251 patients, 91 had recurrent disease. The TNM stage was classified using the Seventh Edition of the General Rule for Clinical and Pathological Record of Lung Cancer (22). We performed adjuvant chemotherapy in all patients excluding those with stage IA disease. Chemotherapy regimen after surgical resection at our Hospital is as a general rule: first-line: 1,1-cyclobutanedicarboxylato platinum(II) (carboplatin, CBDCA) with paclitaxel; second-line; cis-diamminedichloroplatinum with vinorelbine. After recurrence, patients with adenocarcinoma harboring an epidermal growth factor receptor (*EGFR*) mutation were treated with EGFR-tyrosine kinase inhibitor (TKI), while the other patients were treated with first-line therapy of CBDCA-plus-pemetrexed with or without bevacizumab, and second-line with CBDCA with paclitaxel. Few patients underwent re-operations because the recurrence pattern was often multiple. All patients enrolled in the present study had been given full explanations and provided their written informed consent before treatment.

Measurement of urinary diacetylspermine concentration. Urine samples were collected once in the early morning before breakfast and were frozen at −20°C until use. After one freeze/thaw cycle, the urine diacetylspermine concentrations were determined using an auto-diacetylspermine reagent kit (Alfresa Pharma Co., Osaka, Japan). The value derived was normalized by the urinary volume and serum creatinine (Cre) level. The cut-off value for urinary diacetylspermine, which was the mean +2×SD for healthy donors, was 250 nmol/g × Cre (13).

Statistical analysis. The cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. Survival was evaluated as the period from surgery to the occurrence of death, relapse or the date on which the patient was last seen. Post-recurrent survival was evaluated as the period from the date on which the patient was diagnosed with recurrence to the occurrence of death or the date on which the patient was last seen. A two-tailed *p*-value of <0.05 was judged to be statistically significant. The distribution of clinicopathological profiles was calculated by the Chi-square test.

Table I. *The clinicopathological profiles of 251 patients enrolled in this study.*

	Negative	Positive	*p*-Value
Age, years			
≤58	28	25	
59-62	16	13	
63-66	13	18	
67-70	22	15	
71-74	24	14	
≥75	33	30	0.6002
Gender			
F	57	46	
M	79	69	0.1921
Histology			
Adeno	112	61	
SCC	17	41	
Adeno-SCC	3	7	
Others	4	6	<0.0001*
TNM stage			
I	103	59	
II	8	20	
III	23	32	
IV	2	4	0.0005*
Lymph node metastasis			
Negative	104	72	
Positive	25	37	
Unknown	7	6	0.0387*
Lymphatic invasion			
Negative	106	73	
Positive	19	32	
Unknown	11	10	0.0208*
Venous invasion			
Negative	104	81	
Positive	20	24	
Unknown	12	10	0.4361
Pulmonary metastasis			
Negative	115	83	
Positive	5	10	
Unknown	16	22	0.048*

Mean±SD age=66.9±10.8 years. Adeno: adenocarcinoma; SCC: squamous cell carcinoma; Adeno-SCC: adeno-squamous cell carcinoma; F: female, M: male; *Statistically significant difference.

Results

Clinicopathological profiles of patients. The clinicopathological profiles of patients with diacetylspermine-negative and -positive urine among all 251 patients are shown in Table I. Significant differences were observed in histological type,

Table II. *The clinicopathological profiles of 91 patients with recurrence.*

	Negative	Positive	*p*-Value
Age, years			
≤59	6	14	
60-63	7	4	
64-67	3	8	
68-71	6	3	
72-75	10	9	
≥76	8	13	0.1985
Gender			
F	14	19	
M	26	32	0.8243
Histology			
Adeno	32	20	
SCC	4	20	
Adeno-SCC	3	6	
Others	1	5	0.0011*
TNM stage			
I	21	14	
II	3	7	
III	14	26	
IV	2	4	0.1104
Lymph node metastasis			
Negative	25	24	
Positive	12	26	
Unknown	3	1	0.0854
Lymphatic invasion			
Negative	26	26	
Positive	11	21	
Unknown	3	4	0.374
Venous invasion			
Negative	30	35	
Positive	7	12	
Unknown	3	4	0.7706
Pulmonary metastasis			
Negative	31	35	
Positive	5	6	
Unknown	4	10	0.4497

Mean±SD age=67.8±9.9 years. Adeno: Adenocarcinoma; SCC: squamous cell carcinoma; Adeno-SCC: adeno-squamous cell carcinoma; F: female; M: male; *Statistically significant difference.

Table III. *Univariate and multivariate analysis of disease-free survival in 251 patients.*

Univariate analysis	Hazard ratio	95% CI	*p*-Value
Age, years			
≤58 *vs.* ≥59	0.929	0.56-1.54	0.7737
≤62 *vs.* ≥63	0.908	0.584-1.412	0.6695
≤66 *vs.* ≥67	0.865	0.571-1.311	0.4943
≤70 *vs.* ≥71	0.773	0.51-1.17	0.2218
≤74 *vs.* ≥75	0.736	0.467-1.161	0.1857
Gender F *vs.* M	0.729	0.475-1.117	0.1448
Adeno *vs.* non-adeno	0.475	0.313-0.72	0.0003*
Stage			
I *vs.* II+III+IV	0.229	0.15-0.351	<0.0001*
I+II *vs.* III+IV	0.2	0.132-0.303	<0.0001*
I+II+III *vs.* IV	0.174	0.075-0.404	<0.0001*
Lymph node metastasis No *vs.* yes	0.325	0.212-0.498	<0.0001*
Lymphatic invasion No *vs.* yes	0.329	0.211-0.513	<0.0001*
Venous invasion No *vs.* yes	0.768	0.461-1.281	0.3106
Pulmonary metastasis No *vs.* yes	0.319	0.168-0.605	0.0002*
Diacetylspermine Negative *vs.* positive	0.592	0.391-0.896	0.0121*
Multivariate analysis			
Adeno *vs.* non-adeno	0.47	0.279-0.793	0.0047*
Stage			
I *vs.* II+III+IV	0.902	0.368-2.21	0.8218
I+II *vs.* III+IV	0.34	0.147-0.788	0.0118*
I+II+III *vs.* IV	0.458	0.184-1.144	0.0945
Lymph node metastasis No *vs.* yes	0.853	0.465-1.565	0.6084
Lymphatic invasion No *vs.* yes	0.467	0.273-0.8	0.0055*
Pulmonary metastasis No *vs.* yes	0.756	0.369-1.551	0.4456
Diacetylspermine Negative vs. positive	0.972	0.57-1.659	0.9173

CI: Confidence interval; F: female; M: male; Adeno: adenocarcinoma; Non-adeno: non-adenocarcinoma; *Statistically significant difference.

TNM stage, lymph node metastasis, lymphatic invasion and pulmonary metastasis (Table I). The clinicopathological profiles of the 91 patients with recurrence are shown in Table II. A significant difference was observed only in histological type; there were no significant differences in many other clinicopathological factors among subgroups, such as age, gender, TNM stage, lymph node metastasis, lymphatic invasion, and venous invasion (Table II).

Correlation of urinary diacetylspermine with disease-free survival (DFS). To evaluate the correlation of urinary diacetylspermine with DFS, all 251 patients who had undergone surgical treatment were examined. Univariate analysis showed that histological type, TNM stage, lymph node metastasis, lymphatic invasion, pulmonary metastasis and urine diacetylspermine level were significantly correlated with DFS (Table III). We next identified the independent predictive factors for DFS. Multivariate analysis among these eight factors revealed that histological type, TNM stage (I+II *vs.* III+IV), and lymphatic invasion were significantly correlated with DFS (p=0.0047, p=0.0118, and p=0.0055, respectively) (Table III). The significance of the urinary diacetylspermine level was completely lost (p=0.9173).

Correlation of urinary diacetylspermine with overall survival (OS). To evaluate the correlation of urinary diacetylspermine with OS, all 251 patients were examined. Univariate analysis showed that age, gender, histological type, TNM stage, lymph node metastasis, lymphatic invasion, and urinary diacetylspermine level were significantly correlated with OS (Table IV). We next identified the independent predictive

Table IV. *Univariate and multivariate analysis of overall survival in 251 patients.*

Univariate analysis	Hazard ratio	95% CI	*p*-Value
Age, years			
≤58 *vs.* ≥59	0.874	0.474-1.613	0.6673
≤62 *vs.* ≥63	0.835	0.486-1.434	0.5129
≤66 *vs.* ≥67	0.7	0.42-1.168	0.1695
≤70 *vs.* ≥71	0.478	0.29-0.789	0.0031*
≤74 *vs.* ≥75	0.45	0.268-0.755	0.0019*
Gender F *vs.* M	0.313	0.169-0.577	<0.0001*
Adeno vs non-adeno	0.26	0.157-0.431	<0.0001*
Stage			
I *vs.* II+III+IV	0.207	0.122-0.351	<0.0001*
I+II *vs.* III+IV	0.202	0.122-0.334	<0.0001*
I+II+III *vs.* IV	0.269	0.097-0.745	0.0067*
Lymph node metastasis No *vs.* yes	0.36	0.215-0.605	<0.0001*
Lymphatic invasion No *vs.* yes	0.36	0.21-0.616	0.0001*
Venous invasion No *vs.* yes	0.635	0.347-1.164	0.1384
Pulmonary metastasis No *vs.* yes	0.509	0.217-1.196	0.1141
Diacetylspermine Negative *vs.* positive	0.436	0.259-0.734	0.0013*
Multivariate analysis			
Age, years			
≤70 *vs.* ≥71	0.331	0.153-0.718	0.0051*
≤74 *vs.* ≥75	1.04	0.477-2.266	0.9216
Gender F *vs.* M	0.564	0.268-1.184	0.1302
Adeno vs non-adeno	0.347	0.173-0.697	0.0029*
Stage			
I *vs.* II+III+IV	0.804	0.28-2.311	0.686
I+II *vs.* III+IV	0.184	0.066-0.513	0.0012*
I+II+III *vs.* IV	0.915	0.312-2.685	0.8717
Lymph node metastasis No *vs.* yes	1.498	0.707-3.172	0.2911
Lymphatic invasion No *vs.* yes	0.536	0.281-1.023	0.0588
Diacetylspermine Negative *vs.* positive	0.703	0.365-1.353	0.2915

CI: Confidence interval; F: female; M: male; Adeno: adenocarcinoma; Non-adeno: non-adenocarcinoma; *Statistically significant difference.

Table V. *Univariate and multivariate analysis of post-recurrent survival in 91 patients.*

Univariate analysis	Hazard ratio	95% CI	*p*-Value
Age, years			
≤59 *vs.* ≥60	0.877	0.473-1.625	0.6763
≤63 *vs.* ≥64	0.919	0.533-1.585	0.7613
≤67 *vs.* ≥68	0.767	0.459-1.283	0.3102
≤71 *vs.* ≥72	0.606	0.364-1.01	0.0519
≤75 *vs.* ≥76	0.421	0.243-0.729	0.0014*
Gender F *vs.* M	0.224	0.120-0.421	<0.0001*
Adeno *vs.* non-adeno	0.313	0.185-0.530	<0.0001*
Stage			
I *vs.* II+III+IV	0.642	0.373-1.107	0.1079
I+II *vs.* III+IV	0.721	0.433-1.202	0.2078
I+II+III *vs.* IV	1.285	0.465-3.547	0.6274
Lymph node metastasis No *vs.* yes	0.989	0.584-1.676	0.9684
Lymphatic invasion No *vs.* yes	0.912	0.530-1.572	0.741
Venous invasion No *vs.* yes	0.665	0.355-1.247	0.2
Pulmonary metastasis No *vs.* yes	1.649	0.694-3.921	0.2525
Diacetylspermine Negative *vs.* positive	0.551	0.323-0.940	0.0263*
Multivariate analysis			
Age, years ≤75 *vs.* ≥76	0.55	0.315-0.961	0.0356*
Gender F *vs.* M	0.163	0.073-0.366	<0.0001*
Adeno *vs.* non-adeno	0.806	0.426-1.527	0.5089
Diacetylspermine Negative *vs.* positive	0.333	0.175-0.633	0.0008*

CI: Confidence interval; F: female; M: male; Adeno: adenocarcinoma; Non-adeno: non-adenocarcinoma; *Statistically significant.

Table VI. *The correlation of urinary diacetylspermine with recurrent rate.*

	Diacetylspermine test		
	Negative	Positive	*p*-Value
All cases			
No recurrence	96	64	
Recurrence	40	51	0.0142*
Adenocarcinoma			
No recurrence	80	41	
Recurrence	32	20	0.5634
Non-adenocarcinoma			
No recurrence	16	23	
Recurrence	8	31	0.0497*

*Statistically significant difference.

factors for OS. Multivariate analysis among these 10 factors revealed that age (≤70 years *vs.* ≥71 years), histological type and TNM stage (I+II *vs.* III+IV) were significantly correlated with OS (p=0.0051, p=0.0029 and p=0.0012, respectively) (Table IV). The significance of the urinary diacetylspermine level again completely disappeared (p=0.2915).

Correlation of urinary diacetylspermine with postrecurrent survival. To evaluate the correlation of urinary diacetylspermine with post-recurrent survival, the 91 patients with recurrence among the total 251 patients were examined. Univariate analysis showed that age ≤75 years, female gender, adenocarcinoma, and negative urinary diacetylspermine test were significantly correlated with better survival after recurrence (p=0.0014, p<0.0001, p<0.0001, and p=0.0263, respectively) (Table V, Figure 1). We next identified the independent predictive factors for

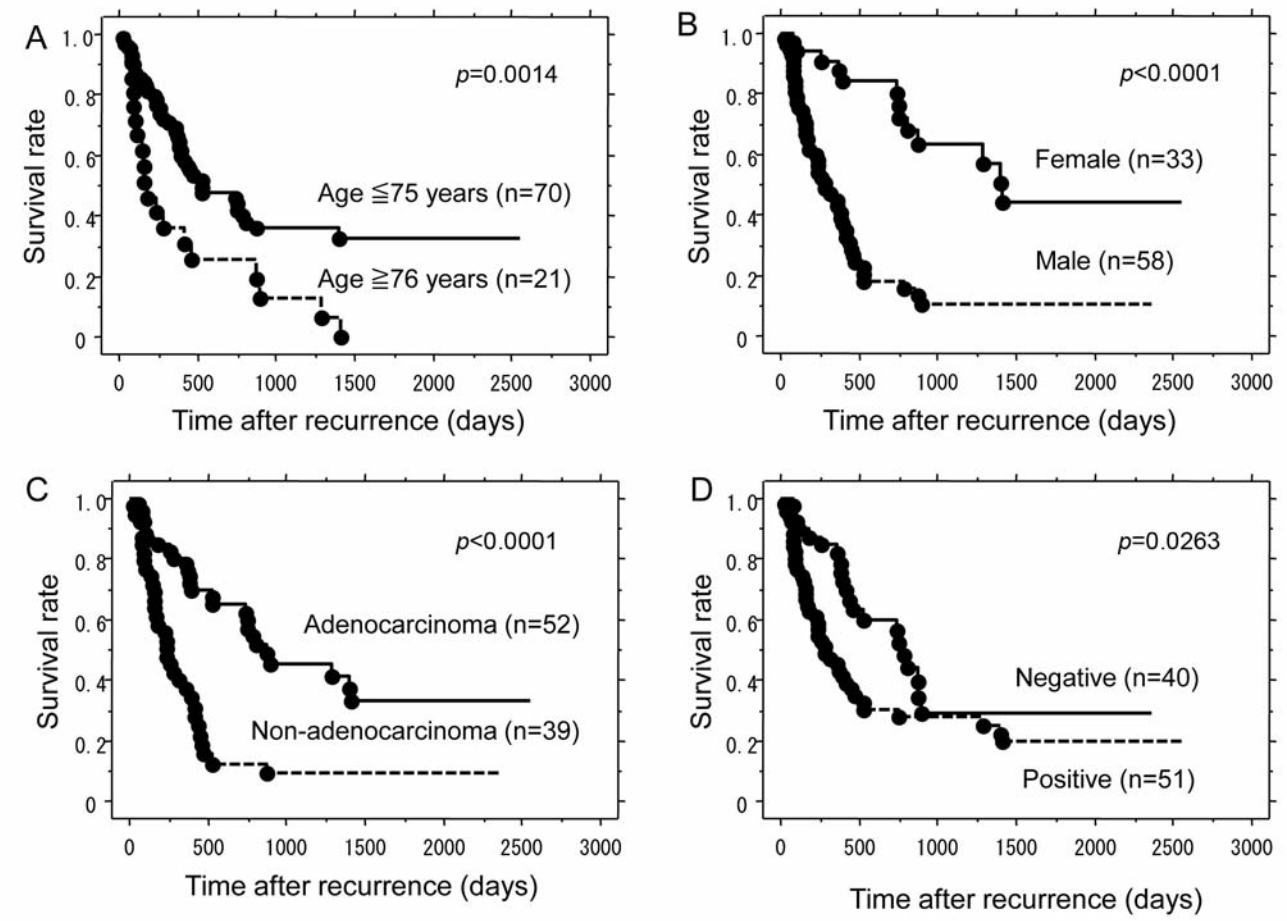

Figure 1. *A total of 91 patients with recurrent non-small cell lung cancer (NSCLC) were divided into two categories based on the indicated factors, and post-recurrent survival was evaluated by Kaplan–Meier analysis. The indicated factors were age (A), gender (B), tumor histology (C), and expression of urinary diacetylspermine (D).*

post-recurrent survival time. Multivariate analysis including age, gender, histology, and urinary diacetylspermine level revealed that age ≤75 years, female gender and negative urinary diacetylspermine test were significantly correlated with better postrecurrent survival (p=0.0356, p<0.0001 and p=0.0008, respectively) (Table V).

Correlation of urinary diacetylspermine with recurrence rate. To evaluate the correlation of urinary diacetylspermine with the recurrence rate, all 251 patients were examined. Patients with positive urinary diacetylspermine test had a significantly higher recurrence rate than patients with a negative test (p=0.0142) (Table VI). In particular, in patients with non-adenocaricoma, urinary diacetylspermine was a significant biomarker for recurrence (p=0.0497) (Table VI).

Discussion

In the present study, the 5-year survival rate after surgical resection was approximately 70%, which is almost the same as that previously reported (23). In addition, the recurrence rate in our series was 36.3% (91 patients out of 251 patients), which is relatively low and is very similar to the previously reported rates of 30-75% (2, 3). This may be the result of the same skilled surgeon performing surgery at one Institute and provides reassurance that our samples were good comparators in our analysis.

In this study, urine specimens were frozen at –20°C until use, and measurements were performed after one freeze/thaw cycle. Fresh specimens might have been more desirable for investigation of the urinary diacetylspermine concentration because freeze/thaw cycles may affect the results of protein analysis. However, in our

preliminary study, there was no significant difference in the urinary diacetylspermine-concentrations between specimens that had undergone freeze/thaw cycles and fresh specimens, even when the freeze/thaw cycle was repeated nine times (data not shown). Therefore, this issue does not appear to be clinically relevant. Importantly, urine specimens are easy to obtain from patients, and the measurement method for urinary diacetylspermine is easy to perform. It would be useful to examine the significance of the urinary diacetylspermine concentration in many types of cancers.

In the univariate analysis of OS and DFS among all 251 patients, urinary diacetylspermine was a prognostic factor for OS and DFS. However, in multivariate analysis, the significance of urinary diacetylspermine disappeared (Table III and IV). We believe that the reason for this may be that urinary diacetylspermine test was negative in many patients with adenocarcinoma whose prognosis was good and that it was positive in many patients with non-adenocarcinoma whose prognosis was poorer. The prognosis of adenocarcinoma was significantly improved by EGFR-TKI, gefitinib, and erlotinib in patients with *EGFR* mutation. Indeed, at our Institution, patients with adenocarcinoma and concurrent *EGFR* mutation have an extremely higher survival rate than do those with adenocarcinoma without *EGFR* mutation and other histological findings (data not shown). One of the mechanisms of the elevation of urinary diacetylspermine is that rapidly-growing cancer cells produce and excrete high amounts of diacetylspermine (24). Consistent with this theory, a higher diacetylspermine level was observed in colorectal cancer tissue specimens (24). Although it is still unclear whether the production of diacetylspermine correlates with the degree of malignant potential of cancer cells or histological types, our results showed that the urinary diacetylspermine level was higher in patients with non-adenocarcinoma histological findings, both among all 251 patients and among the 91 patients with recurrence (Table I and II), and that it was correlated with the recurrence rate in patients with non-adenocarcinoma (Table VI). These results suggest that adenocarcinoma cells produce less diacetylspermine and display a less aggressive phenotype than do other types of cancer, such as squamous cell carcinoma and adenosquamous cell carcinoma, and that in cases with a high TNM stage, lymph node metastasis, lymphatic invasion, and pulmonary metastasis, tumor cells may secrete a large amount of diacetylspermine. Importantly, although these differences in distribution were observed, urinary diacetylspermine was still an independent prognostic factor for post-recurrent survival according to the multivariate analysis.

In the analysis of survival time, an age of ≤70 years was a better prognostic factor for OS, and an age of ≤75 years was a better prognostic factor for post-recurrent survival. The reason that such an analysis can be performed at such a high age may be associated with progress in the development of surgical procedures. We began performing laparoscopic-assisted thoracic operations in 2001. With the introduction of this technique, the wound size and amount of resected costal cartilage decreased by half and patient recovery was faster. Therefore, the indications for this surgical operation were extended to elderly patients.

As we have previously shown, the sensitivity of urinary diacetylspermine as a tumor marker is higher than those of CEA and CYFRA21-1, and its specificity is almost identical (12). In the present study, we further demonstrated that urinary diacetylspermine is a new candidate as a predictive prognostic factor for post-recurrent survival in patients with NSCLC. We believe that the fact that urinary diacetylspermine is obtained by a very non-invasive method is also important for patients with cancer who are suffering from anemia and emaciation. Our results suggest that this new prognostic factor may contribute to the improvement of the prognosis of NSCLC and that patients with recurrent NSCLC with a positive urinary diacetylspermine test should undergo more intensive care.

Conflicts of Interest

All Authors declare no conflicts of interest.

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