



DNMT3a expression pattern and its prognostic value in lung adenocarcinoma

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ABSTRACT

Objectives: DNA methyltransferases (DNMTs) are an important part of the methylation pathway that is highly correlated with the pathophysiology of cancers. Several studies have reported overexpression of DNMTs in human lung cancer, but none have compared the expression pattern to pathological features. In this study, we clarified the association of DNMT3a expression pattern with pathological features and prognosis of lung adenocarcinoma.

Materials and methods: 135 cases of surgically resected lung adenocarcinoma specimens were used for DNMT3a immunohistochemistry (IHC). IHC score was determined by counting the number of positive nuclei. The ROC curve was drawn to determine the best cut-off point of the score; this was set at 57.5. Western blot also implemented and confirmed the specificity of the antibody. Correlations between expression pattern and clinicopathological features and prognosis were analyzed using chi-squared method and Cox proportional hazards model respectively.

Result: Seventy-nine of the 135 cases (58.5%) showed strong positive reactivity to anti-DNMT3a. In terms of histological subtypes, among invasive lung adenocarcinomas 41 out of 53 lepidic adenocarcinomas (77%) were strongly positive, while among the other histological subtypes only 23 out of 66 cases (34.8%) showed a positive reaction. Among non-invasive lung adenocarcinomas 15 out of 16 cases (93.8%) were strongly positive. The level of DNMT3a expression was associated with patient outcome, and patients with weak expression of DNMT3a had a poorer outcome than those with strong expression. Multivariate analysis also indicated that DNMT3a is an independent prognostic marker in lung adenocarcinoma.

Conclusion: Our results indicate that DNMT3a expression in lung adenocarcinoma is associated with the histologically non-invasive type and lepidic subtype, and a favorable prognosis. We also showed that DNMT3a expression is an independent prognostic marker in lung adenocarcinoma. Since lack of DNMT3a is thought to facilitate tumor progression, DNMT3a might be clinically applicable as an indicator of favorable prognosis.

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1. Introduction

As the proportion of the aging population increases and cancer-promoting habits such as smoking still persist, lung cancer remains

one of the leading causes of cancer-related death worldwide [1]. Among the histological subtypes of lung cancer, adenocarcinoma is the most frequent and the number of cases is increasing [2]. Adenocarcinoma shows rapid progression and is associated with a high mortality rate [3]. Although the introduction of many targeted drugs has increased the survival time of patients, research on the pathobiology of adenocarcinoma is still important.

Epigenetic change, reflected in features such as methylation status, has been reported to correlate with the progression of adenocarcinoma [4,5]. Hypermethylation can silence the function of tumor suppressor genes, resulting in tumor progression [6]. On the other hand, global hypomethylation of genomic DNA can lead to

Abbreviations: DNMT, DNA methyl transferase; IHC, immunohistochemistry; AAH, adenomatous atypical hyperplasia; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma.

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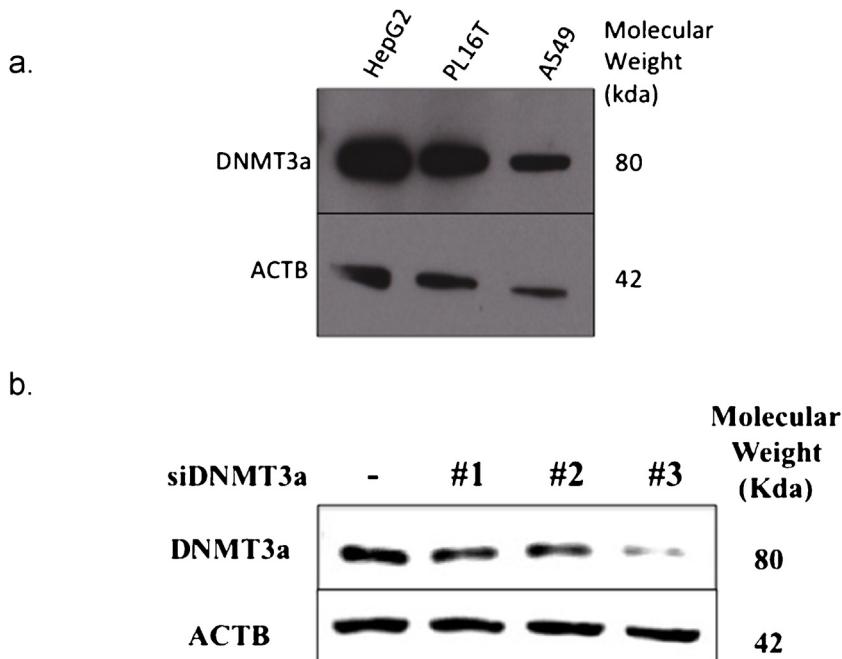


Fig. 1. (a) Western blot of DNMT3a in lung. HepG2 was used as a positive control. A549 and PL16T cell lines showed a single band specific for DNMT3a (80 kDa). (b) Western blot of DNMT3a using A549 transfected with siDNMT3a. Scrambled siRNA was used as a negative control.

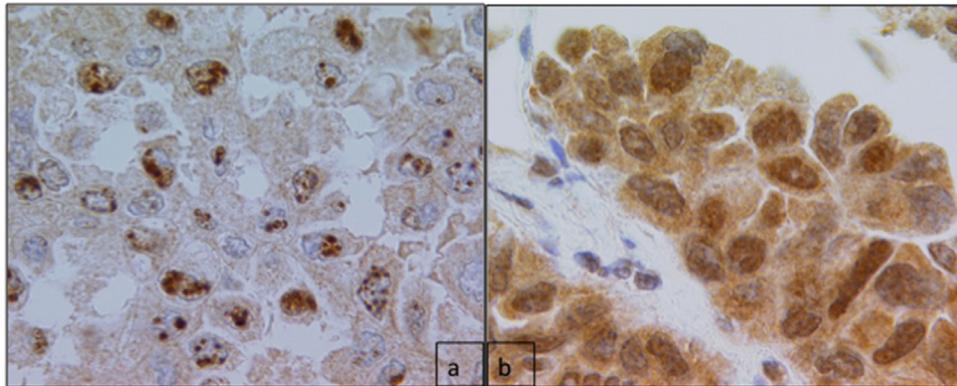


Fig. 2. Immunohistochemistry of DNMT3a in lung adenocarcinoma cells. DNMT3a was stained not only in the nucleus (a) but also in both the cytoplasm and nucleus (b).

genetic alteration, resulting in tumorigenesis [7]. One of the important components correlated with the methylation pathway are the DNA methyltransferases (DNMTs), which transfer a methyl group to the C-5 position on the cytosine ring of DNA [8]. DNMT1, 3a, and 3b have catalytic activity in the context of methylation status. DNMT1 acts mainly as maintenance methyltransferase, while DNMT3a and 3b play roles in de novo methylation [9].

Studies of DNMT expression and its clinicopathological correlations have been conducted on various human cancers, including ovarian cancer, retinoblastoma, and gastroenteropancreatic neuroendocrine tumors. It has been reported that DNMT3b cooperates with HDAC in controlling the progression of ovarian cancer, while overexpression of DNMT1, 3a, and 3b correlates with tumorigenesis in retinoblastoma and gastroenteropancreatic neuroendocrine tumors [10–12]. Although DNMT1 and 3b have been reported to play a specific role in tumorigenesis by silencing tumor suppressors [13–16], the role of DNMT3a in cancer pathophysiology has remained unclear. While it has been reported that DNMT3a expression is significantly associated with the prognosis of gastric and

hepatocellular carcinomas [17,18], its correlation with the histological features or prognosis of lung adenocarcinoma has not yet been clarified.

The purpose of the present study was to evaluate the expression pattern of DNMT3a in lung adenocarcinoma and its possible clinical application, such as its value as a prognostic marker.

2. Materials and methods

2.1. Patients and sample selection

One hundred thirty five lung adenocarcinomas surgically resected at Tsukuba University Hospital (Ibaraki, Japan) between 2002 and 2012 were selected and examined for this study. The patients comprised 71 males and 64 females ranging in age from 34 to 85 years with mean age of 67.04 years. Follow-up information obtained from medical records was available for all of the selected patients. Informed consent for this study had been obtained from

Table 1

DNMT3a expression and clinicopathological features in patients with lung adenocarcinoma.

Clinicopathological Features	Total Patients	DNMT3a Expression		
		Weak	Strong	P value
Total Patients	135	56	79	
Age (years)				0.323
≤60		17	18	
>60		39	61	
Sex				0.097
Male	71	32	39	
Female	64	22	42	
Pathological Stage				0.001
Stage I	85	25	60	
Stage II	21	11	10	
Stage III	27	18	9	
Stage IV	2	2	0	
Lymph Node Status				0.001
N0/Nx	95	31	64	
N1 and N2	40	25	15	
Pleural Invasion				<0.001
pI0	84	25	59	
pI1	27	18	9	
pI2	15	5	10	
pI3	9	8	1	
V				<0.001
+	68	44	24	
-	67	12	55	
Ly				0.051
+	52	27	25	
-	83	29	54	
Pathological Subtype				<0.001
Acinar Predominant	31	18	13	
Papillary Predominant	20	12	8	
Solid Predominant	15	13	2	
Lepidig Predominant	53	12	41	
AIS	14	1	13	
MIA	2	0	2	

Stage I includes IA and IB, stage II includes IIA and IIB, stage III includes IIIA and IIIB. Correlation between DNMT3a expression and clinicopathological features was analyzed using chi-squared test.

all of the patients. Formalin-fixed and paraffin-embedded samples were used for immunohistochemistry (IHC).

2.2. Cell lines and culture conditions

The cell lines HepG2 and A549 were purchased from RIKEN BRC (Ibaraki, Japan). PL16T cells were established in our laboratory from human lung atypical adenomatous hyperplasia (AAH) [19]. HepG2 was maintained in RPMI 1640 (Life Technologies, Carlsbad, California) supplemented with 5% fetal bovine serum (FBS) (Corning Inc., Corning, NY), and A549 was maintained in D-MEM/F12 (Life Technologies) supplemented with 10% FBS. PL16T was maintained in MCDB153HAA (Wako, Osaka, Japan) supplemented with 2% FBS. All cells were cultured in a 5% CO₂ incubator at 37 °C. All were used for Western blotting.

2.3. Immunohistochemistry (IHC)

For IHC, we used 4-micrometer-thick sections cut from formalin-fixed and paraffin-embedded blocks. The sections were deparaffinized and rehydrated. For antigen retrieval, we autoclaved the sections in 10 mmol/L Tris-EDTA buffer (pH 9.0) at 121 °C for 10 min. Further steps were performed on an automated stainer, Histostainer 48a (Nichirei Biosciences, Tokyo, Japan). Endogenous peroxidase was blocked with 3% hydrogen peroxide. The slides were incubated in a 1:100 dilution of rabbit polyclonal DNMT3a antibody (ABGENT, San Diego, CA) at RT for 1 h, and subsequently incubated in the secondary antibody at RT for 1 h. The signal was

detected using DAB (Dako REAL Envision Detection System; Dako, Glostrup, Denmark). Hematoxylin was used for counterstaining.

Two pathologists (REH and SI) evaluated all cases independently without prior knowledge of the clinicopathological data. One thousand tumor cells were evaluated for the most intense nuclear staining ("hot spot") in accordance with previous report [20] with minor modification. Surrounding non-neoplastic bronchial epithelium served as an internal negative control. DNMT3a IHC score was determined by counting the number of stained nuclei. The ROC curve was drawn to determine the best cut-off point of the score. (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.lungcan.2016.04.018) Since the aim of the study is to clarify the correlation between DNMT3a expression and the prognosis of the patient, we used DNMT3a expression and the outcome of the patient as variables to draw ROC curve. After drawing the curve we draw diagonal line from bottom right into top left corner and the cut off point is the coordinates where the diagonal line cross over the curve. Here, the coordinate falls in 57.5% (0.622 sensitivity and 0.351 (1-specificity)), thus it was adopted as the cut off point in this study. Counts below 57.5% were judged as weak expression, and those above 57.5% as strong expression.

2.4. Transfection using siRNA

A549 cells were seeded at a density of 6.0 × 10⁶/mL and incubated overnight in antibiotics-free D-MEM supplemented with 10% FBS. On the next day, the cells were washed with phosphate-buffered saline (PBS), and then OPTI-MEM reduced serum medium (Life Technologies) was added to the cells. Three sets of DNMT3a-specific siRNA (Life Technologies) (Supplementary Table 1) and a nucleic acid transferring agent, lipofectamine RNAiMAX (Life Technologies), were incubated together in OPTI-MEM reduced serum medium for 20 min at room temperature to form a siRNA-lipofectamine complex. The medium containing the siRNA-lipofectamine complex was added to the cells to give a final siRNA concentration of 20 nM. The cells were incubated at 37 °C in a CO₂ incubator for 48 h, and then western blotting was carried out with Stealth RNAi Negative Control Medium GC Duplex #2 (Life Technologies) was used as a negative control.

2.5. Western blotting

Total cell lysates were prepared on ice with M-PER (Life Technologies) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysates were centrifuged for 10 min at 4 °C, and the insoluble fractions were discarded. The total protein in the soluble lysates was measured using a BCA protein assay kit (Life Technologies). Twenty micrograms of protein was mixed with sample buffer and denatured at 95 °C for 5 min, followed by electrophoresis on a 7.5% Tris-HCl gel (Bio-Rad, Hercules, California). An iBlot gel transfer system (Life technologies) was used to transfer the protein. Rabbit polyclonal DNMT3a antibody (1:4000, ABGENT) and mouse monoclonal anti-beta actin antibody (1:5000, Sigma-Aldrich) were used as primary antibodies. To visualize the protein bands, Super Signal West Femto Maximum Sensitivity Substrate (Life Technologies) and care stream Kodak Biomax Light Film (Sigma-Aldrich) were used.

2.6. Statistical analysis

For all statistical analyses, SPSS 22 (SPSS, Chicago, Illinois) was used. For determining the cut-off point for IHC scoring, the ROC curve method was used. Correlation of clinicopathological features with DNMT3a expression was analyzed by chi-squared test. The Kaplan-Meier method was used for calculation of survival curves, and log-rank test was performed for comparisons. Multivariate

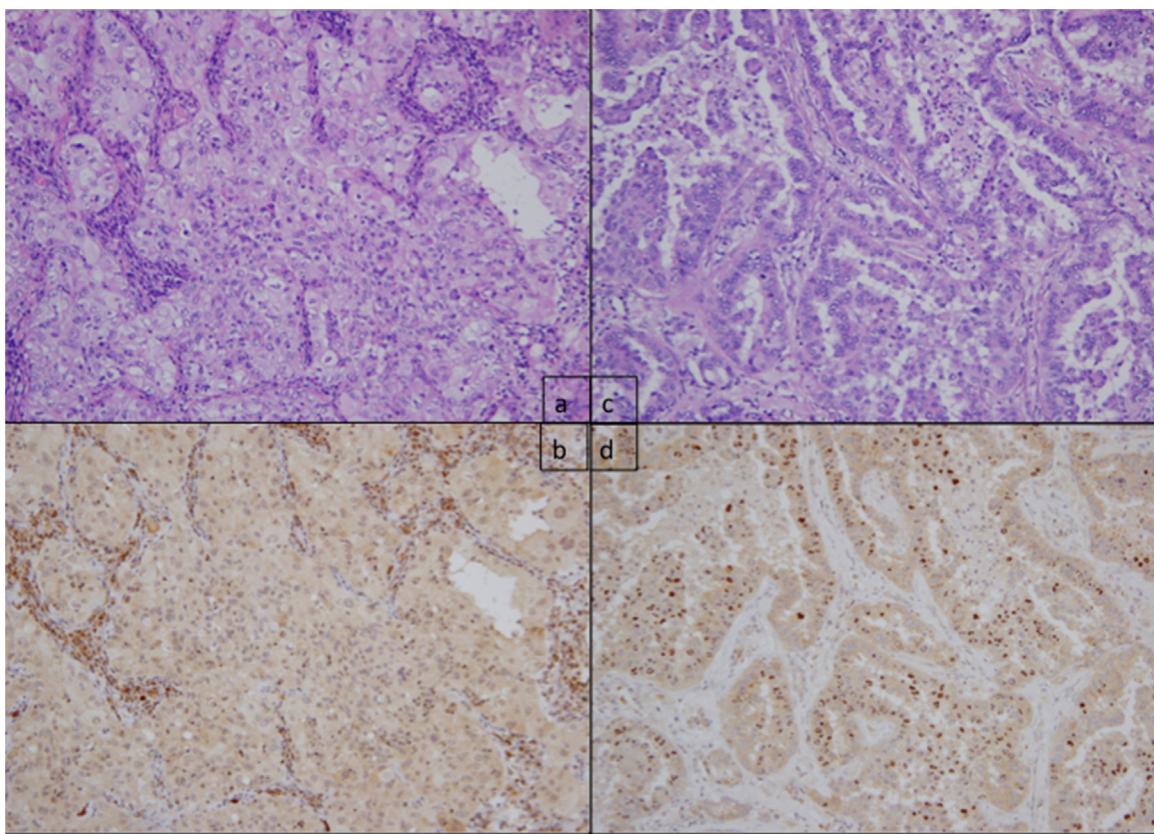


Fig. 3. Immunohistochemistry of DNMT3a in lung adenocarcinoma. (a) and (c) HE staining of the solid and lepidic subtype, (b) negative expression of DNMT3a, (d) strong expression of DNMT3a.

analysis was done using the Cox proportional hazards model. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Western blotting of DNMT3a

In accordance with a previous report [21], we confirmed a single band of DNMT3a in HepG2 used as a positive control. Also, we detected specific bands of DNMT3a in the lung adenocarcinoma cell line A549 and the AAH cell line PL16T. (Fig. 1a) To further analyze the specificity of the antibody, we carried out transfection using specific siRNA for DNMT3a (siDNMT3a). As a result, all siDNMT3a

shown suppression on the protein expression, thus demonstrating the specificity of the anti-DNMT3a antibody used for IHC (Fig. 1b).

3.2. Immunohistochemistry for DNMT3a

In lung adenocarcinoma, DNMT3a was stained not only in the nucleus but also in both the cytoplasm and nucleus (Fig. 2). In most cases, stronger positive staining was observed in nuclei. Since DNMT3a mainly functions in the nucleus, we adopted nuclear immunoreactivity for evaluation. Surrounding non-neoplastic bronchial epithelium was used as an internal negative control. Among the 135 samples, weak staining was found in 56 and strong staining in 79 (Table 1, Fig. 3).

Table 2

Multivariate analysis using the Cox proportional hazards model.

Clin. Features	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P value	HR	95% CI	Pvalue
Gender	0.91	0.541–1.518	0.708			
Age	1.025	0.998–1.052	0.069			
Pleural Invasion (0,1 vs other)	1.67	1.200–2.151	0.001	1.46	1.062–1.993	0.02
Vascular Invasion (+ vs -)	0.512	0.382–0.687	<0.001	0.77	0.543–1.093	0.14
Lymphatic Permeation (+ vs -)	0.53	0.401–0.688	<0.001	0.6	0.446–0.812	0.001
DNMT3a Exp (Strong vs Weak)	0.61	0.472–0.797	<0.001	0.72	0.529–0.974	0.033
Pathological Stg (1 vs other)	0.65	0.501–0.843	0.001	1.03	0.763–1.395	0.84

Adjusted for gender, age, DNMT3a expression, pleural invasion, vascular invasion, lymphatic permeation, and pathological stage.

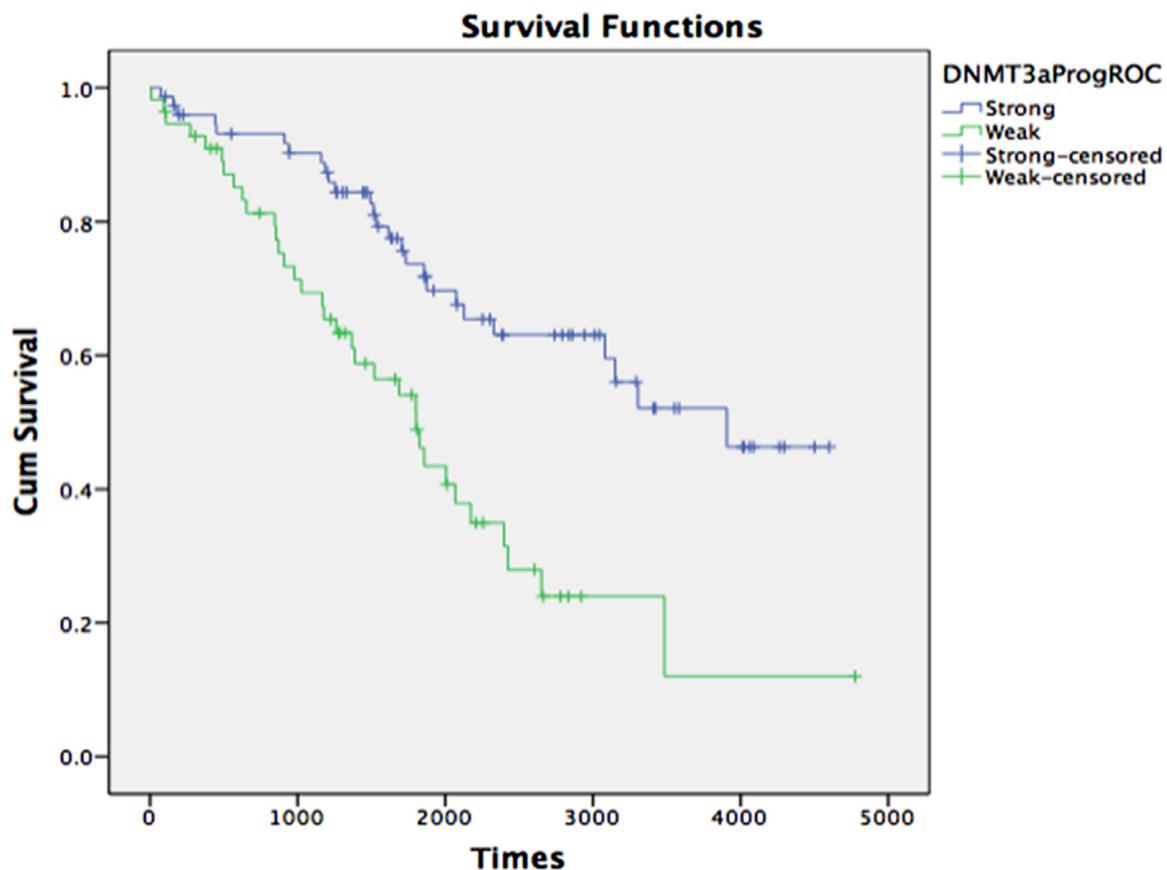


Fig. 4. Disease-free survival depicted as Kaplan-Meier curves shows the correlation between DNMT3a expression and outcome. Weak expression of DNMT3a was associated with poor prognosis relative to strong expression ($p < 0.001$).

3.3. Expression pattern of DNMT3a in lung adenocarcinoma

Among invasive lung adenocarcinomas, strong expression of DNMT3a was detected in 41/53 (77%) lepidic, 8/20 (40%) papillary, 13/31 (42%) acinar, and 2/15 (13%) solid adenocarcinomas. Among non-invasive lung adenocarcinomas, strong expression was detected in 13/14 (92.9%) AIS and 2/2 (100%) MIA. (Table 1, Fig. 3).

We also assessed the correlation between DNMT3a expression and clinicopathological features of the patients using the chi-squared method. Although DNMT3a expression showed no significant correlation with age, sex, or lymphatic permeation it was significantly correlated with the pathological stage, lymph node status, pleural invasion, vascular invasion, and pathological subtype of lung adenocarcinoma (Table 1). DNMT3a showed significantly higher expression in lepidic adenocarcinomas (41/53 cases, 77%) than in other adenocarcinomas (39/83 cases, 47%).

3.4. Correlation of DNMT3a expression with postoperative overall survival

The Kaplan-Meier curves indicated that the patients in the strong expression group had a significantly more favorable outcome than those in the weak expression group (Fig. 4, $p < 0.001$).

3.5. Multivariate analysis using the cox proportional hazards model

After adjustment for gender, age, pathological stage, pleural invasion, vascular invasion, lymphatic permeation, and DNMT3a expression, patients with strong expression of DNMT3a showed a significantly lower risk of lung cancer-related death than those with

weak expression (HR: 0.72, 95%CI: 0.529–0.974, P: 0.033). Multivariate analysis also indicated that lymphatic permeation, pleural invasion, and DNMT3a expression were independent prognostic factors indicative of poor survival in patients with lung adenocarcinoma (Table 2).

4. Discussion

Alteration of the methylation state is involved in tumor initiation and progression. DNMT3a is one of the molecules best known to play a crucial role in the regulation of methylation status. It has been suggested that DNMT3a expression in tumor cells is associated with the prognosis of some cancers including gastric and hepatocellular carcinoma [17,18]. Here, we found that nearly all non-invasive lung adenocarcinomas showed significantly strong expression of DNMT3a. Also, among invasive lung adenocarcinomas, the lepidic subtype showed significantly higher DNMT3a expression than other histological subtypes. Moreover, we identified a significant correlation between DNMT3a expression and prognosis. Patients who showed strong DNMT3a expression had a significantly better outcome. These findings reflected the fact that non-invasive lung adenocarcinoma and the lepidic subtype have a relatively more favorable prognosis than the other subtypes [22,23]. Thus it is suggested that low DNMT3a expression in lung adenocarcinoma might be associated with poor prognosis.

In the present study, we only evaluated the nuclear immunoreactivity of DNMT3a on the basis of previous reports [14,17]. However, the cytoplasm in tumor cells also showed DNMT3a expression. Since the function of DNMT3a in the cytoplasm is unclear, further analysis is required to clarify the relationship of cytoplasmic expression with DNMT3a function.

Alteration of tumor DNA methylation status involves two phenomena: hypermethylation and hypomethylation of DNA [5,24]. In many tumor tissues, widespread global hypomethylation and locus-specific or regional hypermethylation, particularly in tumor suppressor genes, have been simultaneously observed. It is generally said that reduced methylation at repetitive elements, such as short interspersed elements (SINES) and long interspersed elements (LINEs), which collectively constitute about 33% of the human genome [25], significantly contributes to global hypomethylation in cancer cells. In addition to global hypomethylation, gene-specific hypomethylation has also been reported for several loci, including MAGEA, TKT1, BORIS, DDR1, TMSB10, and stratifin (SFN) in lung adenocarcinoma [3,26–32]. This hypomethylation of certain genes leads to a change in gene expression and alteration of cell proliferation, angiogenesis, and cell adhesion, thus promoting tumor progression [3,33]. Although further analysis is required to clarify the mechanism in detail, DNMT3a is thought to be involved in this pathway. Our data suggest that low expression of DNMT3a in tumor tissue might lead to demethylation of oncogenes and subsequent cancer progression, resulting in poor prognosis. Although previous reports indicating that DNMT3a expression is an indicator of poor prognosis in gastric cancer have cast doubt on this possibility [17], Gao et al. have demonstrated that deletion of DNMT3a promotes lung tumor progression in a mouse model [33]. Their data suggested that DNMT3a might act as a tumor suppressor gene, and that suppression of DNMT3a expression might lead to progression of lung adenocarcinoma, in accordance with our data. Furthermore, although DNMT3a mutation is frequently detected in hematological tumors, comprehensive profiling of lung adenocarcinoma has shown that it has no DNMT3a mutation [34]. The loss of DNMT3a expression demonstrated in the present study is considered not to be associated with somatic mutation. Further study will be required in order to clarify the molecular mechanism underlying the functions of DNMT3a in tumors.

In conclusion, our results indicate that DNMT3a expression in lung adenocarcinoma is associated with the histologically non-invasive type and lepidic subtype, and a favorable prognosis. We also showed that DNMT3a expression is an independent prognostic marker in lung adenocarcinoma. Since lack of DNMT3a is thought to facilitate tumor progression, DNMT3a might be clinically applicable as an indicator of favorable prognosis.

Conflict of interest

The authors have no conflicts of interest to declare in association with this presentation.

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