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Amyloid precursor protein and its phosphorylated form in non-small cell lung carcinoma

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ABSTRACT

Amyloid precursor protein (APP) is a well-known to be involved in the development of Alzheimer's disease and harbors several phosphorylation sites in its cytoplasmic domain. APP has been also proposed as one of the molecules involved in cell proliferation and invasion in several human malignancies. However, the roles of APP including its phosphorylated form (p-APP) have remained largely unexplored in non-small cell lung carcinoma (NSCLC). Therefore, in this study, we first examined both APP and p-APP expressions and then explored the association between p-APP/APP status and clicopathological parameters in NSCLC. The number of APP-positive cases was 24/91 (26%) in adenocarcinomas (Ad) and 16/35 (46%) in squamous cell carcinomas (Sq), respectively. p-APP-positive cases in Ad and Sq were 28 (31%) and 17 (49%), respectively. In Ad cases, both APP and p-APP were significantly associated with clinical stages (APP and p-APP), pathologic T (p-APP), and pathologic N (APP and p-APP) of the cases examined. In Sq cases, there were no significant associations between APP status and any of the clinicopathological parameters examined with an exception of the significant correlation of p-APP with lymphatic invasion. APP status was not significantly associated with overall survival (OS) of Ad patients but a significant association was detected between p-APP-positive cases and OS of these patients ($p < 0.0001$). In Sq cases, both APP- ($p = 0.01$) and p-APP-positive ($p = 0.04$) groups were also significantly associated with adverse clinical outcome. These results did firstly demonstrate that APP, in particular, p-APP, is considered a potent prognostic factor for both Ad and Sq lung carcinoma patients. However, APP signaling including its phosphorylation signal are considered different between these two types of NSCC cells and further investigations are required for clarification.

1. Introduction

Lung cancer is the fifth lethal malignancy in 2015, and its incidence has increased markedly compared to that of 2010 [1]. Lung carcinoma is further histologically subclassified into small and non-small cell lung carcinoma (NSCLC). NSCLC, which accounts for approximately 80% of all lung carcinoma patients, generally responds poorly to chemotherapy compared to small cell carcinoma [2]. Therefore, various molecular targeted therapeutic agents have been developed in NSCLC. At this juncture, molecular targeted therapeutic agents toward driver gene mutations such as epidermal growth factor receptor (EGFR) gene mutation are widely administered in routine clinical settings but their side effects and eventual development of therapeutic resistance are usually considered unavoidable in the clinical course of these patients. Therefore, it is pivotal to search for further therapeutic targets for improving the clinical outcome of the patients with NSCLC.

Amyloid precursor protein (or amyloid β precursor protein) (APP) is known as a type I transmembrane protein, which is enzymatically cleaved into some fragments by α-, β-, and γ-secretase [3,4]. Aggregation of β-amyloid, one of the processed APP products, has been well-known as an amyloid plaque detected in the cerebral cortex of Alzheimer's disease [5]. However, results of subsequent mRNA data-

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base did reveal that APP was actually widely distributed in various non-neural tissues such as kidney, heart, pancreas, thyroid, prostate, and muscle, and has been also reported to be involved in the growth, differentiation, and adhesion of these cells [4,6]. APP has been also reported to play pivotal roles in the biological behavior in several human malignancies such as colon [7], pancreas [8], prostate [9,10], breast [11,12], stomach [13], and urinary bladder [14]. APP was also demonstrated to stimulate cell proliferation and migration, and to regulate epithelial-mesenchymal transition-related genes in prostate carcinoma cell line LNCap [9]. In addition, APP was also identified as a secretion protein from lung squamous cell carcinoma LC-I/sq [15]. The knock-down of APP expression by specific siRNA was also reported to induce G0 arrest in lung adenocarcinoma cell lines, H1650 and A549 [16]. However, the status of APP has not been examined at all in NSCLC tissues, and its significance has remained largely unknown.

APP is also known to harbor several phosphorylated sites in its cytoplasmic domain [17]. Among these APP phosphorylated sites of the molecule, the phosphorylation of APP at Thr668 (APP_{695} isoform numbering) has been extensively studied in neuronal tissues [17]. Results of these studied revealed that APP_{695} was mediated by neuronal cyclin-dependent protein kinase 5 (CDK5), p34cdc2 protein kinase (CDC2), glycogen synthase kinase 3β (GSK-3β), or c-jun N-terminal kinases (JNKs) [18–21]. However, the biological and/or clinical significance of APP_{695} as well as of other phosphorylated sites of APP molecule has also remained largely unknown in non-neural tissues including malignant tumors. Therefore, in this study, we firstly studied the status of both phosphorylated and un-phosphorylated or naïve forms of APP (p-APP and APP) in NSCLC tissues using immunohistochemistry. We then evaluated the associations between p-APP/APP status and clicopathological parameters of these cases in order to further explore the clinical significance of APP in NSCLC.

2. Materials and methods

2.1. Patients and tissues

A total of 126 cases of NSCLC (91 adenocarcinoma cases and 35 squamous cell carcinoma cases) were randomly retrieved from surgical pathology files of 2003 to 2005 in Tohoku University Hospital, Sendai, Japan with their relevant clinical information. In this study, only two female squamous cell carcinoma cases were available for evaluation. Previously published studies on Japanese patients with lung squamous cell carcinoma did reveal the sex or gender ratios similar to this study [22–24]. Therefore, the sex or gender ratio of lung squamous cell carcinoma in this study was reasonably considered to represent that of Japanese patients with lung squamous cell carcinoma. We reevaluated stage criteria according to the 7th edition of UICC. In addition, distant metastasis of the cases in this study was clinically evaluated primarily using computer tomography. Control tissues such as cerebrum and testis for immunohistochemistry were also retrieved from the autopsy pathology files of the Department of Pathology, Tohoku University Hospital. All the specimens had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee of the Tohoku University School of Medicine (2013-1-581).

2.2. Immunohistochemistry

Rabbit polyclonal antibodies for amyloid precursor protein (APP) and p-APP (Thr668) were commercially obtained from Cell Signaling Technology (CST, Danvers, MA, USA). A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), based upon the streptavidin-biotin amplification method, was also used. Immunostaining was performed according to the methods reported previously [12,25].

The antigen-antibody complex was visualized with 3,3-diaminobenzidine and counterstained with hematoxylin. As a positive control for APP and p-APP immunostaining, human cerebral tissue from a patient with Alzheimer's disease was used in this study [12]. Testis was also employed as positive control for APP and negative control for p-APP [26]. In the absorption control test, both APP and p-APP antigenic peptides (CST) were employed, and mixed with APP and p-APP antibodis diluted at 0.5 μg/μL solution, respectively. These mixtures were incubated for 1 h at 37 °C and then, centrifuged at 10,000*g* for 1 h. The supernatant was then used for further immunohistochemistry.

In our present study, carcinoma cells were tentatively classified into the following three categories based on relative immuno-intensity: 0, negative; 1 +, weak; 2 +, strong. When there were two or more scores in a case, a higher score was used as the score for that particular case. These scores were finally divided into two groups such as negative (score 0) and positive (score 1 + and 2 +), and the cases that had more than 10% positive staining carcinoma cells were considered positive in this study [12]. Results of preliminary analysis according to the 3 categories above did reveal no significant differences between any of these three categories and clinicopathological factors including overall survival (OS) examined in this study (unpublished data). Two of the authors (S.I. and Y.M.) independently evaluated the immunoreactivity of APP/p-APP. Discordant results were mainly due to differences in the evaluation of score 1 + or 0, which is considered as a background staining, and re-evaluated by the same two authors above.

2.3. Statistical analysis

An association between p-APP/APP immunoreactivity and clinicopathological factors was evaluated using Chi-squared or Fisher's Least Significant Difference (LSD) test. OS curves were generated according to the Kaplan–Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using the proportional hazard model (Cox).

3. Results

APP immunoreactivity was detected in the cytoplasm of nerve cells in the cerebral cortex and convoluted seminiferous tubules and Leydig cells in testis employed as positive controls (Fig. 1A,B). p-APP immunoreactivity was also detected in the cytoplasm of neuron of the cerebral cortex (Fig. 1A). APP and p-APP immunoreactivity detected in neurons were abolished in the absorption test described above (Fig. 1B). Relative immuno-intensity of p-APP in the testis was score 1 (Fig. 1C) used for lung carcinomas. Therefore, native form of APP was considered dominant in human normal testis.

Immunoreactivity of both APP and p-APP was detected in the cytoplasm of carcinoma cells in NSCLC (Fig. 2A,B) but both APP and p-APP were not detected in normal alveolar epithelial cells (Fig. 2C). These immunoreactivities were abolished in the absorption test using NSCLC tissues (data not shown). In this study, the number of APP-positive cases was 24 in 91 adenocarcinomas (26%) and 16 in 35 squamous cell carcinomas (46%), respectively. p-APP-positive cases in adenocarcinomas and squamous cell carcinomas were 28 (31%) and 17 (49%), respectively. The ratio of APP positive cases was significantly higher in squamous cell carcinomas ($p = 0.0367$) in this study. In

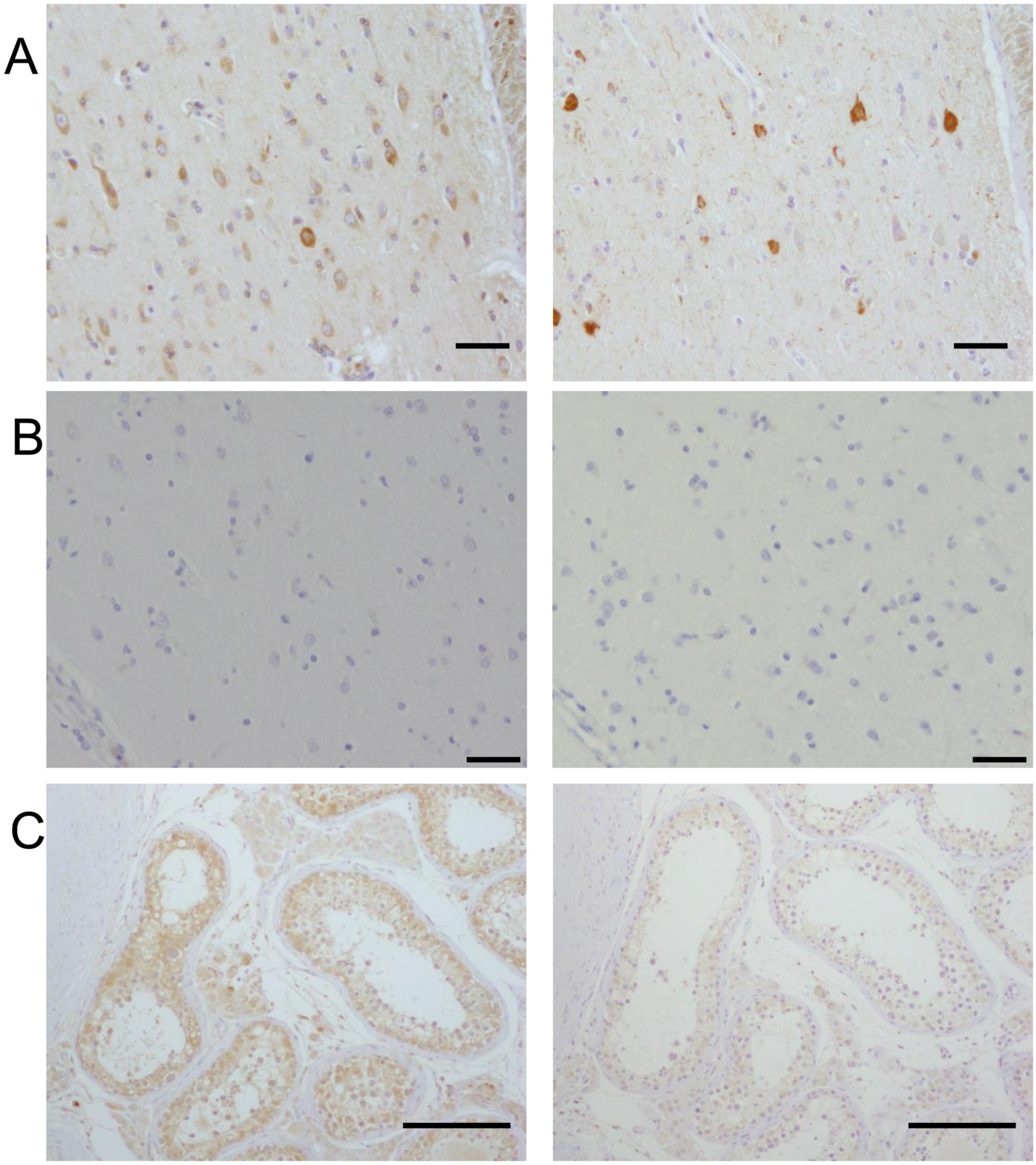

Fig. 1. Imunohistochemistry of amyloid precursor protein (APP) and phosphorylation of APP (p-APP) in cerebral cortex and testis examined as positive and/or negative staining controls in this study. (A) Both APP (*left*) and p-APP (*right*) immunoreactivity was detected in cerebral cortex. Scale bars, 10μm. (B) These immunoreactivities were not detected in absorption control using specific peptides (APP, *left*; p-APP, *right*). Scale bars, 10μm. (C) APP immunoreactivity was detected in testis (*left*). No immunoreactivity of p-APP was detected in testis (*right*). Scale bars, 50μm.

addition, the ratio of p-APP-positive cases also tended to be high in squamous cell carcinomas but the difference did not reach statistical significance ($p = 0.0618$).

The association between APP/p-APP and clinicopathological parameters of NSCLC cases examined were summarized in Table 1. Among 126 cases of NSCLC examined in this study, APP status in carcinoma cells was significantly associated with stage and pN of the patients (Table 1). p-APP status was also significantly associated stage, pT and pN of the patients examined (Table 1). Among 91 adenocarcinoma cases, APP and p-APP were significantly associated with stage (APP and pAPP), pT (p-APP), and pN (APP and pAPP) of the patients (Table 2). Among 35 squamous cell carcinomas examined, the status of p-APP but not APP was significantly associated with lymphatic invasion (Table 3).

Among 126 cases of NSCLC, both APP-positive ($p = 0.001$) and p-APP-positive ($p < 0.0001$) groups were significantly associated with adverse clinical outcome of the patients (Fig. 3A). In 91 adenocarcinoma cases, APP status was not significantly associated with OS ($p = 0.11$) but a significant association was detected between p-APP-positive groups and OS ($p < 0.0001$) (Fig. 3B). In 35 squamous cell carcinoma cases, both APP-positive ($p = 0.01$) and p-APP-positive

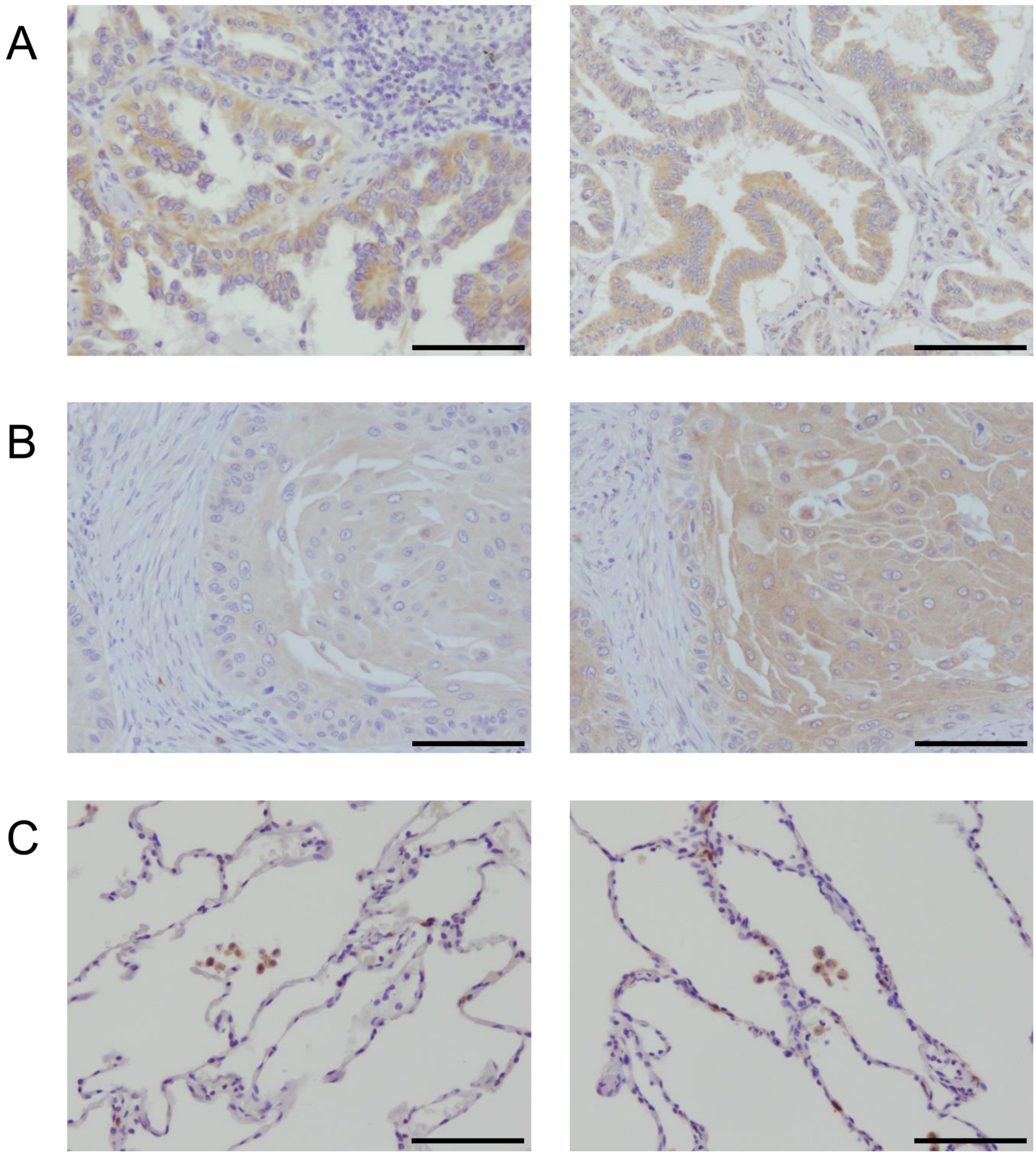

Fig. 2. Immunohistochemistry of amyloid precursor protein (APP) and phosphorylation of APP (p-APP) in non-small cell lung carcinomas. (A) APP (*left*) and p-APP (*right*) immunoreactivity in lung adenocarcinoma cases. (B) APP (*left*) and p-APP (*right*) immunoreactivity in lung squamous cell carcinoma cases. (C) APP (*left*) and p-APP (*right*) were not detected in alveolar epithelial cells. Positive immunoreactivity was also detected in alveolar macrophages. Scale bars, 100μm.

($p = 0.04$) groups of the patients were significantly associated with adverse clinical outcome of the patients (Fig. 3C).

Univariate analyses for OS did reveal APP, p-APP, sex, stage, pT, pN, and histological type as significant prognostic variables in total of 126 NSCLC cases (Table 4). Subsequent multivariate analysis further demonstrated that pN were independent prognostic factor predicting worse clinical outcome among total cases. Results of univariate analyses in 91 adenocarcinoma cases revealed that OS was associated with p-APP, sex, stage, pT, and pN, but only p-APP turned out independent factors in multivariate analysis (Table 4). Among 35 lung squamous cell carcinoma cases, APP, p-APP, stage, and pN were all significantly associated with OS of the patients but only APP turned out independent prognostic factor in multivariate analysis (Table 4). In our present study we evaluated randomly selected cases. Therefore no consideration was given to the presence or absence of adjuvant therapy in the clinical course of individual patients at the time of selecting the cases.

4. Discussion

This is the first study to demonstrate the status of APP and p-APP in human NSCLC. Results of our present study did indicate that p-APP was indeed increased in NSCLC compared to normal lung tissues, and the

Table 1
Amyloid precursor protein and phosphorylation of APP immunoreactivity and clinicopathological parameters in 126 patients with non-small cell lung carcinoma.

	APP immunoreactivity			Phospho-APP immunoreactivity		
	Positive (n = 40)	Negative (n = 86)	*p*-value	Positive (n = 45)	Negative (n = 81)	*p*-value
Age (years)						
Median ± SE	64.6 ± 1.5	65.4 ± 1.1		66.1 ± 1.4	64.6 ± 1.1	
70 >	14	30	0.99	19	25	0.20
70≤	26	56		26	56	
Sex						
Male	28	54	0.43	32	50	0.29
Female	12	32		13	31	
Stage						
1	20	59	**0.04**	15	64	< **0.0001**
2–4	20	27		30	17	
Pathological T factor (pT)						
pT0,1	17	52	0.06	14	55	< **0.0001**
pT2-4	23	34		31	26	
Lymph node metastasis (pN)						
Positive (pN1-3)	13	14	**0.04**	17	10	**0.0009**
Negative (pN0)	27	72		28	71	
Distant metastasis (M)						
Positive (M1)	4	2	0.06	5	1	**0.01**
Negative (M0)	36	84		40	80	
Smoking history						
Smoking	30	56	0.27	33	53	0.36
Non-Smoking	10	30		12	28	

APP, Amyloid precursor protein; p-APP, phosphorylation of APP; pT, pathologic T factor; pN, pathologic N factor.
Bold values indicate significant associations.

Table 2
Amyloid precursor protein and phosphorylation of APP immunoreactivity and clinicopathological parameters in 91 patients with lung adenocarcinoma.

	APP immunoreactivity			Phospho-APP immunoreactivity		
	Positive (n=24)	Negative (n=67)	*p*-value	Positive (n=28)	Negative (n=63)	*p*-value
Age (years)						
Median ± SE	63.0 ± 2.2	65.2 ± 1.3		65.4 ± 2.0	64.3 ± 1.3	
70 >	8	25	0.73	12	21	0.38
70≤	16	42		16	42	
Sex						
Male	13	36	0.97	15	34	0.97
Female	11	31		13	29	
Stage						
1	11	48	**0.02**	7	52	< **0.0001**
2–4	13	19		21	11	
Pathological T factor (pT)						
pT0,1	11	42	0.15	8	45	**0.0001**
pT2-4	13	25		20	18	
Lymph node metastasis (pN)						
Positive (pN1-3)	8	9	**0.03**	11	6	**0.0008**
Negative (pN0)	16	58		17	57	
Distant metastasis (M)						
Positive (M1)	3	2	0.08	4	1	**0.01**
Negative (M0)	21	65		24	62	
Lymphatic invasion[a]						
Positive	10	39	0.12	11	38	0.07
Negative	14	26		16	24	
Vascular invasion[b]						
Positive	11	40	0.25	13	38	0.33
Negative	12	25		13	24	
Histological Subtype						
lepidic	7	15	0.92	8	14	0.51
papillary	10	26		14	22	
acinor	2	7		2	7	
solid	2	8		2	8	
mucinous	3	9		2	10	
pleomorphic	0	2		0	2	
Smoking history						
Smoking	16	38	0.39	18	36	0.52
Non-Smoking	8	29		10	27	

APP, Amyloid precursor protein; p-APP, phosphorylation of APP; pT, pathologic T factor; pN, pathologic N factor.
Bold values indicate significant associations.
[a] There were two unknown cases.
[b] There were three unknown cases.

Table 3
Amyloid precursor protein and phosphorylation of APP immunoreactivity and clinicopathological parameters in 35 patients with lung squamous cell carcinoma.

	APP immunoreactivity			Phospho-APP immunoreactivity		
	Positive (n = 16)	Negative (n = 19)	*p*-value	Positive (n=17)	Negative (n=18)	*p*-value
Age (years)						
Median ± SE	67.0 ± 1.7	66.1 ± 1.6		67.4 ± 1.7	65.7 ± 1.6	
70 >	6	5	0.48	7	4	0.23
70≤	10	14		10	14	
Sex						
Male	15	18	0.90	17	16	0.16
Female	1	1		0	2	
Stage						
1	9	11	0.92	8	12	0.24
2-4	7	8		9	6	
Pathological T factor (pT)						
pT0,1	6	10	0.37	6	10	0.23
pT2-4	10	9		11	8	
Lymph node metastasis (pN)						
Positive (pN1-3)	5	5	0.74	6	4	0.39
Negative (pN0)	11	14		11	14	
Distant metastasis (M)						
Positive (M1)	1	0	0.27	1	0	0.30
Negative (M0)	15	19		16	18	
Lymphatic invasion[a]						
Positive	8	11	0.79	13	6	**0.016**
Negative	7	8		4	11	
Vascular invasion[b]						
Positive	10	12	0.81	14	8	0.08
Negative	5	5		3	7	
Differentiation grade						
Well	6	5	0.33	4	7	0.46
Moderate	7	6		8	5	
Poor	3	8		6	5	
Smoking history						
Smoking	14	18	0.45	15	17	0.51
Non-Smoking	2	1		2	1	

APP, Amyloid precursor protein; p-APP, phosphorylation of APP; pT, pathologic T factor; pN, pathologic N factor.
Bold values indicate significant associations.
[a] There was a unknown case.
[b] There were three unknown cases.

presence of increased p-APP in carcinoma cells also indicated its potential biological importance in NSCLC. APP mRNA expression was also reported to be significantly higher than that in adjacent normal tissues in 50 cases of lung adenocarcinoma tissues [27]. In this study, APP-positive cases were significantly more frequent in squamous cell carcinomas than adenocarcinomas. The expression of APP mRNA was reported to be down-regulated by microRNA (miR)-373-3p in adenocarcinoma cell line A549 [27]. In addition, the relative abundance of miR-373 was reported in lung adenocarcinoma compared to squamous cell carcinoma cells [28]. miR-20a was also reported to regulate APP expression in OVCAR3 ovarian cancer cell line [29]. Several regulatory mechanisms of APP expression were reported and microRNA expression profiles could be involved in the difference of APP expression depending on lung carcinoma histological types.

In lung adenocarcinoma cases examined in this study, the status of APP in carcinoma cells was significantly associated with lymph node metastasis of the cases. APP was also reported to stimulate cell migration and invasion as well as cell proliferation in several types of human malignancies such as prostate [9], nasopharynx [30], and thyroid [31]. In prostate carcinoma cell line, knockdown of APP by siRNA did decrease invasion-related genes such as *a disintegrin and metalloprotease* (*ADAM*) *10* and *ADAM17*, and EMT-related genes such as *Vimentin* and *SNAI2/Slug* [9]. In lung adenocarcinoma and squamous cell carcinoma, both *ADAM10* and *ADAM17* were reported to induce cell invasion or EMT [32–34]. Therefore, further investigations of analysis of downstream signaling of APP are required for clarifying the roles of APP as a therapeutic target in NSCLS.

In this study, APP was significantly associated with poor prognosis in squamous cell carcinoma but not in adenocarcinoma cases. In oral squamous cell carcinomas, the status of increased APP mRNA compared to normal was not associated with clinicopathological parameters such as differentiation, lymph node metastasis, and clinical stages of the patients [35] but increased APP was reported to be significantly associated with adverse clinical outcome by Kaplan-Meier analysis [35]. In our present study, p-APP but not APP status in lung squamous cell carcinomas was significantly associated with lymphatic invasion. In addition, there were significant associations between APP/p-APP status and lymph node metastasis in lung squamous cell carcinomas. APP was also reported to be identified as a secretory protein of lung squamous cell carcinoma cell line [15]. Therefore, APP could be involved in the degradation of cell matrix during the initial phase of invasion in lung squamous cell carcinoma but further investigations are required for clarification. Results of the database analysis using The Cancer Genome Atlas (TCGA)-based program [36] did reveal that high expression levels of APP tended to be associated with poor prognosis in lung adenocarcinoma (p = 0.078), whereas not necessarily with that of squamous

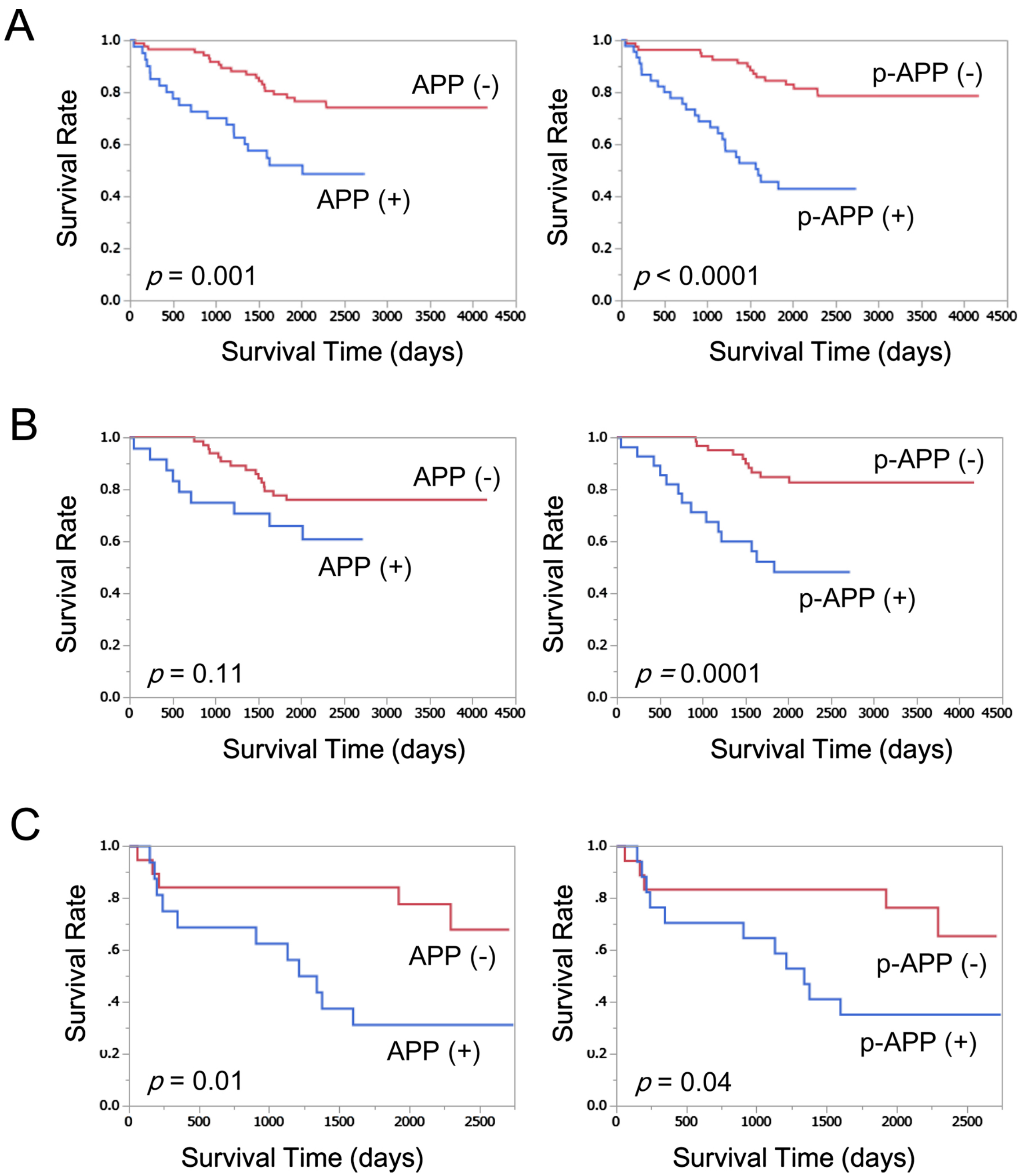

Fig. 3. Overall survival (OS) of non-small cell carcinoma patients according to amyloid precursor protein (APP) and phosphorylation of APP (p-APP) status. Kaplan-Meier curves of the total cases (A), lung adenocarcinoma cases (B), and lung squamous cell carcinoma cases (C) were demonstrated. APP (-), APP-negative group; APP (+), APP-positive group.

cell carcinoma (p = 0.908) (Fig. 4). In addition, according to the OS analysis by Kaplan-Meier plotter [37], the APP status was reported to be related to the poor prognosis in lung adenocarcinomas but not squamous cell carcinomas (Fig. 5). Furthermore, in lung adenocarcinoma cases, only APP status turned out an independent prognostic factor for OS of the patients but in lung squamous cell carcinoma cases, only p-APP, not APP, was an independent worse prognostic factor. These findings all indicated that APP plays different roles in adenocarcinoma and squamous cell carcinoma but further investigations including *in vitro* studies were required for clarification.

The status of APP immunoreactivity in carcinoma cells was reported to be correlated with adverse clinical outcome of the patients with adenocarcinomas such as prostate [10] and breast [12,38,39] cancers. However, APP status was not correlated with the clinical outcome of lung adenocarcinoma patients in this study. In addition we firstly evaluated the status of p-APP in NSCLC and clarified the correlation

Table 4
Univariate and multivariate analyses of non-small cell lung carcinoma -specific survival.

	Total cases (n = 126)			Adenocarcinoma cases (n = 91)			Squamous cell carcinoma cases (n = 35)		
	Univariate	Multivariate		Univariate	Multivariate		Univariate	Multivariate	
	p-value	*p*-value	Relative risk (95% CI)	*p*-value	*p*-value	Relative risk (95% CI)	*p*-value	*p*-value	Relative risk (95% CI)
APP	**0.002**	0.31		0.13			**0.01**	**0.03**	4.34 (1.19-17.63)
p-APP	< **0.0001**	0.22		**0.0004**	**0.03**	2.84 (1.13-7.43)	**0.04**	0.80	
Age (years)	0.08			0.23			0.13		
Sex	**0.003**	0.07		**0.04**	0.07		0.09		
Stage	< **0.0001**	0.24		**0.0001**	0.55		**0.009**	0.42	
Pathological T factor (pT)	**0.01**	0.87		**0.01**	0.58		0.52		
Lymph node metastasis (pN)	< **0.0001**	**0.04**	2.46 (1.05-6.20)	**0.001**	0.11		**0.002**	0.11	
Distant metastasis (M)	0.32			0.53			0.25		
Histological type	**0.03**	0.31		–			–		
Smoking history	0.21			0.36			0.71		

APP, Amyloid precursor protein; p-APP, phosphorylation of APP; pT, pathologic T factor; pN, pathologic N factor.
Bold values indicate significant associations.

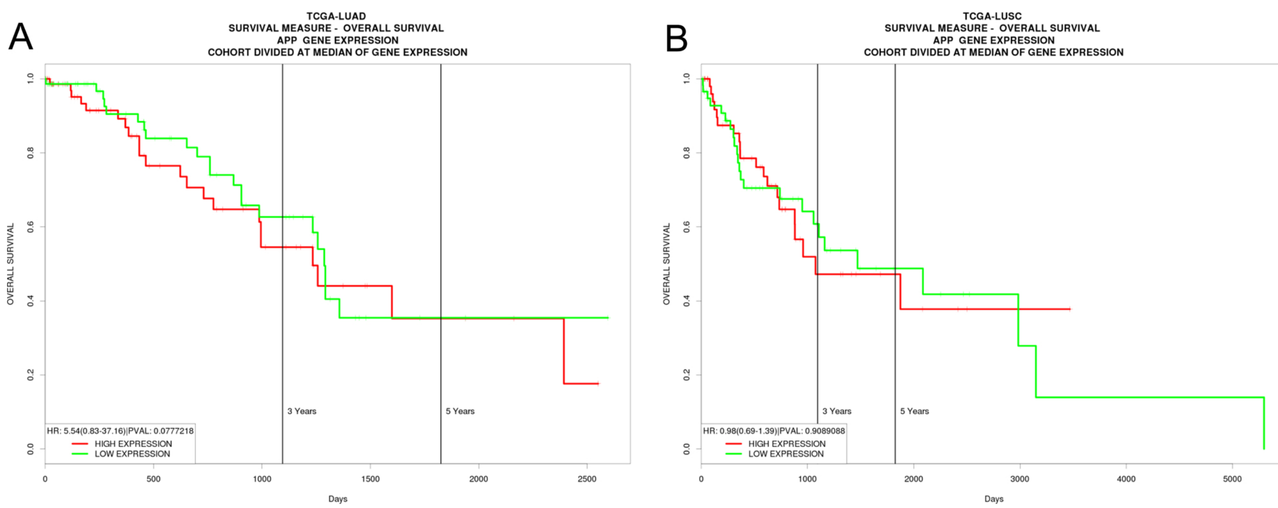

Fig. 4. Overall survival (OS) of non-small cell carcinoma patients according to amyloid precursor protein (APP) status. The Cancer Genome Atlas (TCGA) data about lung adenocarcinoma and squamous cell carcinoma cases was analyzed by PROGgeneV2 (http://www.compbio.iupui.edu/proggene). The expression of each gene was tentatively classified into two groups, low (LOW EXPRESSION) and high (HIGH EXPRESSION), according to their median values, respectively. Kaplan-Meier curves of lung adenocarcinoma cases (A) and lung squamous cell carcinoma cases (B) were demonstrated. HR, hazard ratio; PVAL, *p*-value.

between p-APP status and clinical outcome in lung adenocarcinoma cases. In this study, we could not evaluate the downstream pathways of APP/p-APP in NSCLC. However, in rat neuroblastoma cells, APP was reported to directly promote an activation of Ras-MAPK pathway, which is generally considered to stimulate proliferative signaling [40]. In addition, both p-APP and phosphorylation-tau were also reported to be induced by activation of ERK and GSK-3, and to result in neurodegeneration and ultimately neuronal death shown in Alzheimer's disease [40]. Therefore, these results indicated that both APP and p-APP could be possibly involved in the process of invasion and proliferation in NSCLC cells through the different intracellular signals but it awaits further investigations for clarification. Results of primary culture of rat cortical neuron demonstrated that p-APP at Thr668 was increased by treatment with ocadaic acid, a potent inhibitor of protein phosphatase-2A, and this increased p-APP was markedly reduced by c-jun N-terminal kinase (JNK) inhibitor [18]. Amyloid β precursor protein-binding, family B, member 1 (APBB1 also known as FE65) was reported to be bound to APP at endoplasmic reticulum and/or Golgi membrane and anchored there [41,42]. Nakaya et al. [43] also reported that FE65 was released from APP by its phosphorylation and trans-located to nucleus. Further investigation including the interaction between FE65 and APP/p-APP are required to clarify the significance that APP is phosphorylated in NSCLC cells.

Analyses of protein-protein interaction network have revealed possible interaction of APP with EGFR and/or ras-related C3 botulinum toxin substrate 1 (RAC1) [44]. RAC1 overexpression was reported to be positively correlated with lymph node metastasis, TNM stage, and poor differentiation in NSCLC patients [45]. In addition, RAC1 has been considered a resistant key factor against chemotherapy and EGFR-tyrosine kinase inhibitors in lung cancer patients [46]. Therefore, APP may be involved in the process of developing resistance to these therapeutic agents through these binding-factors, and phosphorylation form of APP could therefore play an important role in this particular binding-process above.

In summary, both APP and p-APP were detected in human NSCLC tissues. APP and its downstream signaling were considered to be

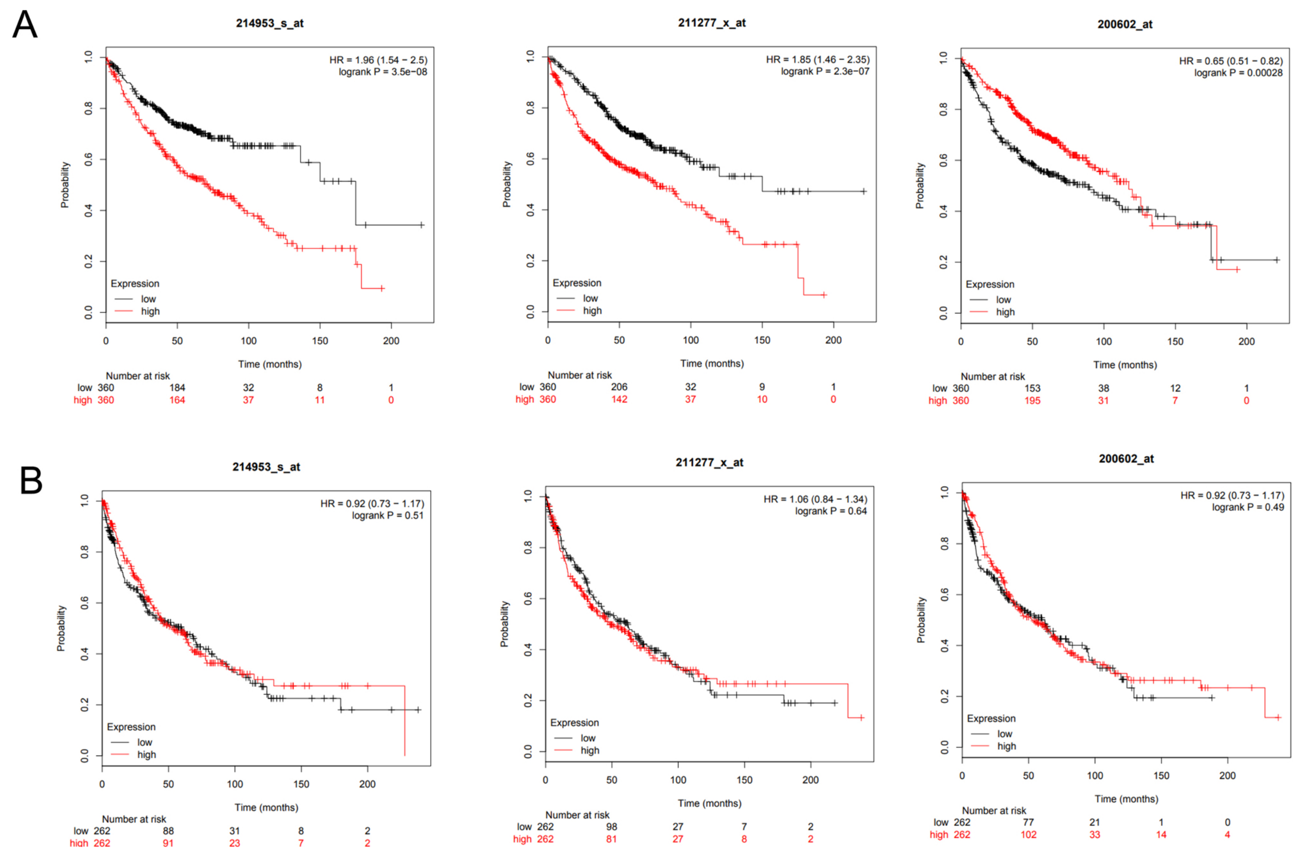

Fig. 5. Overall survival (OS) of non-small cell carcinoma patients according to amyloid precursor protein (APP) status. The Affymetrix microarray database about lung adenocarcinoma and squamous cell carcinoma cases was analyzed by Kaplan Meier plotter (http://kmplot.com/analysis/). The expression of each gene was tentatively subclassified into two groups, low and high, according to their median values, respectively. Kaplan-Meier curves of lung adenocarcinoma cases (A) and lung squamous cell carcinoma cases (B) were demonstrated. There were three Affymetrix probes (200602_at, 211277_x_at, and 214953_s_at) available about APP gene. HR, hazard ratio.

different between lung adenocarcinomas and squamous cell carcinomas, but p-APP status turned out potent prognostic factor in both histological types. Therefore, the detection of phosphorylated form of APP is required when evaluating the potential biological and clinical roles of APP in lung cancer patients.

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Conflict of interest

The authors declare that they have no conflict of interests regarding this research.

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