Biomedical Research (Tokyo) **41** (3) 149–159, 2020

Prognostic significance of NAP1L1 expression in patients with early lung adenocarcinoma

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(Received 6 December 2019; and accepted 11 March 2020)

ABSTRACT

NAP1L1 is a key regulator of embryonic neurogenesis but its role in lung cancer remains unexplored. In this study, we investigated the relationship between NAP1L1 expression and the clinicopathological parameters and prognosis of non-small cell lung cancer patients. To this end, the expression of NAP1L1 in tumor samples was evaluated by immunohistochemistry. NAP1L1 expression was significantly associated with reduced differentiation ($P = 0.00014$), higher pathological TNM stages ($P < 0.00001$), lymph node metastasis ($P < 0.00001$), intrapulmonary metastasis ($P = 0.02955$), lymphatic invasion ($P = 0.00019$), vascular invasion ($P = 0.00008$) and poorer prognosis ($P = 0.0008$) of patients with adenocarcinoma. Moreover, multivariate analyses using the Cox-proportional hazards model confirmed that NAP1L1 expression increased the risk of death after adjusting for other clinicopathological factors (HR = 2.46, 95% CI, 1.22–4.96). Furthermore, NAP1L1 expression was identified as an independent poor prognostic factor in patients with resectable stage I lung adenocarcinoma. NAP1L1-siRNA-treated lung adenocarcinoma-derived A549 cells showed significant suppression of proliferation, migration, and invasion abilities. These findings suggest that NAP1L1 may be a novel predictive and prognostic marker in lung adenocarcinoma, particularly in those with stage I of the disease.

INTRODUCTION

Primary lung cancer is the leading cause of cancer deaths and the incidence of lung cancer subtype adenocarcinoma (AC) has increased gradually in recent decades (Travis *et al.* 2011; Torre *et al.* 2012). There have been important advances in the treatment of lung cancer that have improved the survival of patients but further research is essential to identify more effective therapies (Johnson *et al.* 2014). Therefore, a better understanding of the mechanisms involved in AC progression and the identification of useful prognostic molecular markers to accurately predict the clinical outcome of AC patients are needed.

Nucleosome assembly protein-like-1 (NAP1L1) is a member of the NAP1L family that has been reported to participate in nucleosome assembly, histone transport, histone eviction, transcriptional regulation, and cell cycle progression (Zlatanova *et al.* 2007). The human NAP1L family includes NAP1L1, NAP1L2, NAP1L3, NAP1L4, NAP1L5, and NAP1L6. NAP1L1 and NAP1L4 are expressed ubiquitously in human tissues, whereas NAP1L2, NAP1L3, and NAP1L5 are expressed predominantly

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in the brain. NAP1L1 can be detected in most human tissues and cell lines but increased levels are often found in rapidly proliferating cells (Attia *et al.* 2013). An elevated expression of NAP1L1 is detected in many several neuroendocrine tumors and it has been suggested that NAP1L1 may play a role in tumor proliferation and metastasis (Kidd *et al.* 2006; Modlin *et al.* 2006; Schimmack *et al.* 2014). Recently, Qiao *et al.* reported that NAP1L1 played essential roles in the proliferation and differentiation of neural progenitors during embryonic neurogenesis (Qiao *et al.* 2018). Therefore, NAP1L1 may be involved in nerve and neuroendocrine cell development and differentiation. In non-neuroendocrine tumors, such as hepatocellular carcinoma (HCC), NAP1L1 is a poor prognostic marker (Le *et al.* 2019). To date, there are no reports of the association of NAP1L1 expression with clinicopathological parameters and patient outcomes in cancers other than HCC.

In the present study, we determined the expression and localization of NAP1L1 in non-small cell lung cancer (NSCLC) tissues using immunohistochemistry. Additionally, the relationships between clinicopathological characteristics and the prognostic significance of NAP1L1 expression in AC patients were statistically evaluated. Moreover, we investigated the effects of the NAP1L1 molecule on the proliferation, migration, and invasion behaviors of lung AC cells using a NAP1L1-siRNA knockdown study.

MATERIALS AND METHODS

Patients and tissue specimens. In this retrospective cohort study, we analyzed tissues from 193 NSCLC patients that had undergone complete resection from January 2002 to September 2005 at the Kitasato University Hospital. No preoperative chemotherapy and/or radiotherapy cases were included. The tissues were fixed with 10% formalin, embedded in paraffin, and sectioned. The histological diagnosis was based on the criteria of the World Health Organization Classification of Lung and Pleural Tumors (Travis *et al.* 2015). Each case was reassigned a TNM classification and pathological stage based on the IASLC staging system (Goldstraw *et al.* 2007; Sobin *et al.* 2009). The 147 ACs consisted of 18 acinar (12.2%), 51 lepidic (34.7%), 9 micropapillary (6.1%), 50 papillary (34.0%), 14 solid (9.5%), and 5 invasive mucinous (3.4%) subtypes. The following clinical and pathological parameters were retrospectively reviewed in each case: age at surgery, gender, smoking habits, histological type, tumor differentiation, p-TNM stage, intratumoral vascular invasion, intratumoral lymphatic invasion, pleural invasion, adjuvant chemotherapy, viability status, and survival time after surgery. Viability status was determined based on whether or not an NSCLC-related death had occurred and survival time was defined as the duration from the date of surgery to the date of death or the end of the follow-up. The prognostic analysis was used only in cancer-related deaths and excluded deaths from other causes.

All samples were collected in accordance with the ethical guidelines and written informed consent from all patients was received. This study was approved by the Ethics Committee of Kitasato University School of Medicine (B07-06) and followed the Declaration of Helsinki protocol. All patients were approached following the approved ethical guidelines and those who agreed to participate in this study were required to sign consent forms. At any time, patients could refuse entry or discontinue their participation. All participants provided written informed consent.

Immunohistochemical staining for NAP1L1. Paraffin-embedded 3-μm sections of the harvested samples were deparaffinized in xylene, rehydrated in a descending ethanol series, and treated with 3% hydrogen peroxide for 10 min. Tissues were subjected to antigen retrieval by incubation with 0.01 mol/L citrate buffer (pH 6.0) in 0.1% Tween 20 at 121°C for 10 min. After blocking with 2% normal swine serum for 10 min, the sections were incubated with an anti-NAP1L1 monoclonal antibody (1 : 500; Abcam, Cambridge, UK) for 2 h at room temperature (RT). The sections were then rinsed in Tris-buffered saline (0.01 M Tris-HCl pH 7.5, 150 mM NaCl) three times for 5 min each and incubated with an HRP-labeled polymer (EnVision+ Dual Link System-HRP kit; Dako, Glostrup, Denmark) for 30 min at RT. The sections were subsequently incubated with a stable DAB solution (Invitrogen, Carlsbad, CA, USA) and counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemical staining. For NAP1L1, cytoplasmic staining in tumor cells was considered to be positive. The staining of bronchial epithelial cells was used as an internal positive control. NAP1L1 staining was determined by multiplying the percentage of positive tumor cells and staining intensity (Nagashio *et al.* 2008). The percentage of positive tumor cells was categorized into

four groups: 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%. The staining intensity was categorized into three groups by comparing the staining intensity of tumor cells with bronchial epithelial cells: 1 (weak) = weaker than epithelial cells, 2 (moderate) = the same as epithelial cells, and 3 (strong) = stronger than epithelial cells.

Two investigators (RN and YS) separately evaluated all of the specimens in a blinded manner. Case in which the scores of the examiners were different were reviewed and discussed until a consensus was reached.

Statistical analyses for IHC. Continuous variables were presented as the median (range), whereas numerical variables were given as *n* (%). The relationships between NAP1L1 expression and clinicopathological parameters were assessed by the Mann-Whitney *U* test. The cumulative survival of patients was estimated using the Kaplan-Meier method and statistical significances between the survival rate of the NAP1L1-high expression (score ≥ 4) and NAP1L1-low expression (score < 4) groups were tested using the log-rank test. Multivariate analysis was performed by using the Cox-proportional hazards regression model to examine the interactions between NAP1L1 expression and other clinicopathological variables and estimate the independent prognostic effect of NAP1L1 on survival by adjusting for confounding factors. The conventional *P*-value of less than 0.05 was considered significant. All reported *P*-values are two-sided. Analyses were performed using StatFlex software version 6.0 (Artech Co., Ltd., Osaka, Japan).

Survival analysis using the TCGA database. The Cancer Genome Atlas (TCGA) data were downloaded from the National Cancer Institute and National Human Genome Research Institute website (https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp). NAP1L1 mRNA expression data were divided into two groups according to the best cut-off value. We performed the log-rank survival analysis focusing on stage I lung AC patient data ($n = 268$) using StatFlex software version 6.0.

Cell culture. The A549 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). A549 cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals, Inc., Santa Ana, CA, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO_2 and 95% humidified air.

siRNA transfection. For siRNA transfection, two NAP1L1 siRNA sequences (Flexi Tube GeneSolution siRNAs #1 SI03019296 and #2 SI04248160; QIAGEN, Venlo, Netherlands) and an AllStars Negative Control siRNA (QIAGEN) were used. A total of 2.5×10^4 A549 cells were cultured in a 24-well plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), followed by transfection using the HiPerFect Transfection Reagent (QIAGEN). Final concentrations of 10 nM of each siRNA for the *NAP1L1* gene and of the negative-control siRNA were used.

Quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA from siRNA-treated A549 cells was extracted using an miRNeasy Mini Kit (QIAGEN) and reverse transcribed into cDNA using a Prime Script RT reagent kit (Takara Bio Inc., Shiga, Japan). qRT–PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus; Takara) on a LightCycler® 96 System (Roche, Mannheim, Germany). All experiments were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative RNA expression. The following primers were used in this experiment: NAP1L1 forward, 5′-AGG GAC GTG GGA CAG TTC GTA-3′ and reverse, 5′-TTT CGA AGT CTG CAG CAA GGA TAG-3′; and GAPDH forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-TGG TGA AGA CGC CAG TGG A-3′ (TaKaRa Bio Inc.).

Westtern blot analysis. siRNA-treated A549 cells were lysed with the M-PER Reagent (Thermo Fisher Scientific). Total proteins were quantified using a BCA protein assay kit (Thermo Fisher Scientific). Protein samples (2 μg) were separated on 5%–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto polyvinylidene fluoride (PVDF; Merck-Millipore, Darmstadt, Germany) membranes. The membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with the indicated horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark) for 45 min at RT. Subsequently, the membranes were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore) and detected using the Cool Saver System (ATTO, Tokyo, Japan). The results of this experiment were semi-quantified using the ImageJ software (1.46; National Institutes of Health, Bethesda, MD, USA). The anti-nucleosome

assembly protein 1 like 1 (NAP1L1) primary antibody was obtained from Abcam (EPR11845; Cambridge, MA, USA) and the anti-GAPDH primary antibody (MAB2344) was purchased from Abnova (Taipei City, Taiwan).

MTS assay. Cell proliferation was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA) at 0, 24, 48, and 72 h after transfection. Transfected A549 cells (1×10^3 cells per well) were seeded onto a 96-well plate. MTS solution (20 μL) was added into each well at the same time and the plates were incubated for 2 h at 37°C. The resulting product was measured at 492 nm and at the reference wavelength of 630 nm using a ChroMate 4300 instrument (Awareness Technology Inc., Palm City, FL, USA).

Wound-healing assay. A wound-healing assay was performed to measure cell migration ability. siRNA-treated A549 cells were cultured in 24-well plates and grown to 80% to 90% confluence in the culture medium. The cell monolayer was scratched gently with a 10 μL pipette tip. Subsequently, PBS (−) was used to wash the wounded cells, which were then incubated with culture medium supplemented with 10% FBS for 24 h. The wound-healing process was imaged using a DP12 digital camera (Olympus, Tokyo, Japan). After images of each well were acquired, wound-closure events were measured manually.

Transwell migration and invasion assays. For migration and invasion assays, siRNA-treated A549 cells were resuspended at 5×10^4 cells per 500 μL in serum-free RPMI-1640 medium 48 h after transfection and added to the upper chambers of 24-well Transwell inserts (Corning, Corning, NY, USA) coated with 50 μL of Matrigel (Corning) dissolved in serum-free RPMI-1640 medium, for the invasion assay, or non-coated ones, for the migration assay. The wells in both assays were filled with 750 μL of RPMI1640 medium supplemented with 10% FBS. After 20 h of culture, the cell culture inserts were removed, the upper surfaces of the filters were cleaned thoroughly with cotton swabs, and the lower surfaces were fixed with 10% formalin. Subsequently, the cells on the lower surfaces were stained with hematoxylin and eosin. Cells in three random high-power fields were counted in triplicate. Student's *t*-test was used for statistical analysis.

Table 1 *Characteristics of the patients*

Characteristics	*n* = 193 (%)	
Age		
<65	83	(43.0)
≧65	110	(57.0)
Gender		
Male	126	(65.3)
Female	67	(34.7)
Smoking habit		
Never smoker	71	(36.8)
Smoker	122	(63.2)
Histology		
AC	147	(76.2)
SCC	46	(23.8)
Tumor differentiation		
Well	83	(43.0)
Moderately/Poorly	110	(57.0)
p-TNM stage		
Stage I	104	(53.9)
Stage II	46	(23.8)
Stage III	43	(22.3)
Receiving adjuvant chemotherapy		
Yes	29	(15.0)
No	164	(85.0)
Vital status		
Alive	103	(53.4)
Lung cancer-related death	63	(32.6)
Other causes of death	16	(8.3)
Unknown	11	(5.7)

Data are presented as No. (%).
AC = adenocarcinoma; p-TNM = pathological TNM; SCC = squamous cell carcinoma.

RESULTS

Patient characteristics

The clinicopathological characteristics of the patients are summarized in Table 1. This study included 126 male and 67 female patients that were 34 to 82 years old (median = 63). Of these, 122 patients (63.2%) were smokers. There were 104 (53.9%) stage I (69 stage IA and 35 stage IB), 46 (23.8%) stage II (24 stage IIA and 22 stage IIB), and 43 (22.3%) stage III (40 stage IIIA and 3 stage IIIB) patients, including 147 (76.2%) with ACs and 46 (23.8%) with squamous cell carcinomas (SCCs). Twenty-nine (15.0%) of the patients had received adjuvant chemotherapy. The overall follow-up durations ranged from 3 to 127 months (median = 59). A total of 103 patients were alive at the end of the follow-up, while 63 patients died of lung cancer, 16 patients died from other causes, and 11 patients were lost to follow-up.

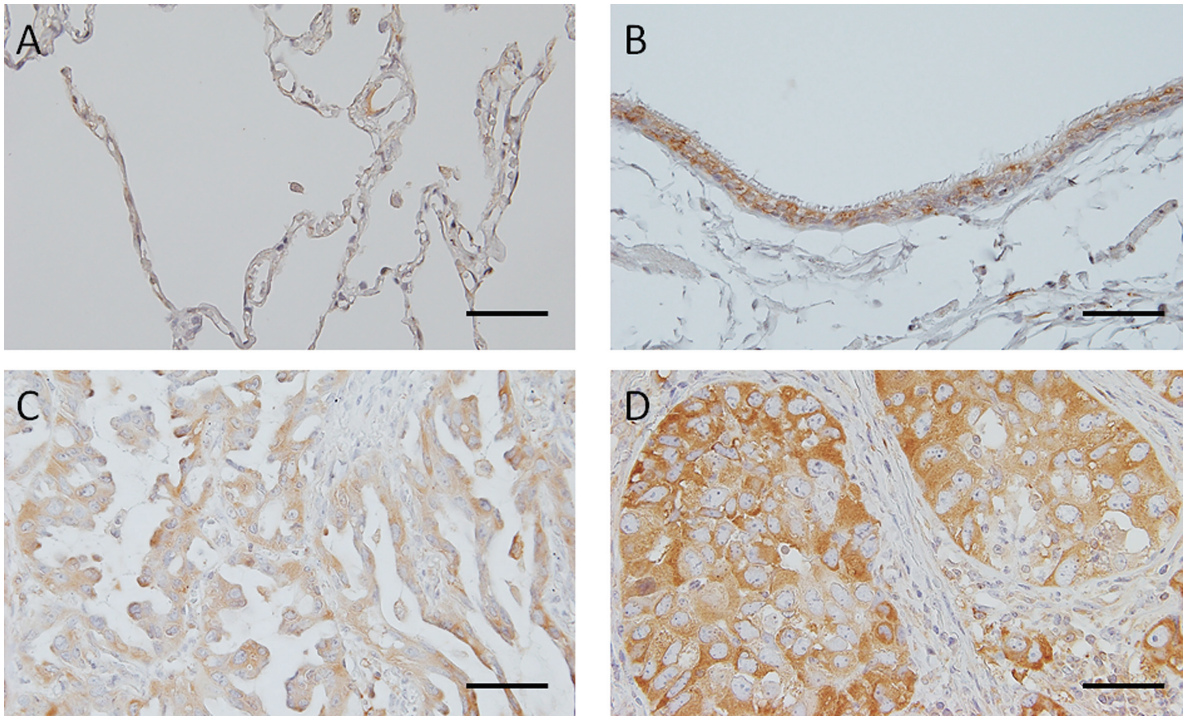

Fig. 1 NAP1L1 expression in normal lung tissues and lung adenocarcinomas. (**A**) No staining was observed in normal alveolar epithelial cells. (**B**) Low to moderate expression was observed in the cytoplasm of bronchial epithelial cells. (**C**) Moderate and (**D**) strong expression of NAP1L1 in lung adenocarcinoma cells (original magnification: A to D ×400). Scale bar indicates 50 μm.

NAP1L1 expression in ACs and SCCs

Cytoplasmic expression of NAP1L1 was detected mainly in tumor cells and was uniformly expressed throughout the tumor mass (Fig. 1). Low to moderate expression of NAP1L1 was often observed in the bronchial epithelial cells, which served as an internal control (Fig. 1B). NAP1L1 expression was observed in 187/193 of the NSCLC tissues (96.9%). Specifically, it was expressed in 141/147 (95.9%) ACs and 46/46 (100%) SCCs, which exhibited mean NAP1L1 staining scores of 3.6 and 5.3, respectively. The NAP1L1 staining score in histologic subtypes of lung AC was 3.9 in acinar, 3.5 in lepidic, 5.0 in micropapillary, 3.4 in papillary, 4.2 in solid, and 2.0 in invasive mucinous. Significant differences were observed between micropapillary and lepidic ($P = 0.0392$), and micropapillary and invasive mucinous ($P = 0.0308$) subtypes.

Relationship between NAP1L1 expression and clinicopathological characteristics in lung ACs and SCCs

The relationships between NAP1L1 expression and clinicopathological characteristics in ACs and SCCs are summarized in Table 2. There was no significant relationship between NAP1L1 expression and clinicopathological parameters in SCCs. On the other hand, NAP1L1 expression was significantly associated with reduced differentiation ($P = 0.00014$), higher pathological TNM stages ($P < 0.00001$), lymph node metastasis ($P < 0.00001$), intrapulmonary metastasis ($P = 0.02955$), lymphatic invasion ($P = 0.00019$), and vascular invasion ($P = 0.00008$) in ACs. Although it was not significant, NAP1L1 expression was related to tumor size ($P = 0.05399$). There were no significant associations between NAP1L1 expression and age, gender, smoking habit, and pleural invasion.

Kaplan-Meier estimate of survival of AC patients with high and low NAP1L1 expression

The overall follow-up periods ranged from 4 to 127 months (median = 58) and the mean survival time was 76 months. We divided the patients into two groups that had a score that was either below or above 4, which was the median score in ACs. The five-year cumulative cancer-specific survival probability was 60.0% for the NAP1L1-high expression group (score ≥ 4), which was significantly lower ($P = 0.0008$) compared to 88.6% for the NAP1L1-low expression group (score < 4) (Fig. 2A). More-

Table 2 *Relationship between NAP1L1 expression and clinicopathological parameters*

Characteristics	ACs			SCCs		
	Total	Ave. score	*P*-value	Total	Ave. score	*P*-value
Age, y						
<65	77	3.8	0.43512	12	6	0.23642
≧65	70	3.4		34	5.1	
Gender						
Male	83	3.6	0.91463	43	5.3	0.67021
Female	64	3.7		3	5	
Smoking habit						
Never smoker	68	3.5	0.45792	3	4.7	0.50769
Smoker	79	3.7		43	5.4	
Tumor differentiation						
Well	78	2.9	0.00014	5	4	0.15377
Moderate/Poorly	69	4.4		41	5.5	
Tumor size						
<5 cm	128	3.5	0.05399	30	5.3	0.84576
≧5 cm	18	4.6		16	5.4	
p-TNM stage						
I	88	2.8	0.00001	16	5.3	0.98043
II, III	59	4.8		30	5.3	
Nodal status						
No	108	3.1	0.00001	23	5.1	0.14823
Yes	39	5.1		23	5.5	
Pleural invasion						
No	98	3.5	0.39542	18	5.4	0.57494
Yes	49	3.9		27	5.1	
Intrapulmonary metastasis						
No	138	3.5	0.02955	45	5.3	n/a
Yes	9	5		1	6	
Lymphatic invasion						
No	79	2.9	0.00019	9	5.2	0.73089
Yes	43	4.7		22	5	
Vascular invasion						
No	77	2.8	0.00008	8	5.8	0.79275
Yes	54	4.7		28	5.5	

Data are presented as an average score.
See Table 1 legend for expansion of abbreviations.

over, in stage I lung AC patients, the NAP1L1-high expression group was also significantly associated with a poorer outcome compared to the NAP1L1-low expression group ($P = 0.0309$, Fig. 2B).

NAP1L1 mRNA expression levels and prognosis in stage I lung AC patients

We analyzed NAP1L1 mRNA expression levels in stage I lung AC patients using the TCGA database and compared this to the data we obtained from the immunohistochemistry analysis. According to the log-lank survival analysis, the high NAP1L1 mRNA expression group showed a significantly poorer outcome than the low NAP1L1 expression group ($P = 0.048$, Fig. 3).

Effect of NAP1L1 expression on survival determined by multivariate analysis

The Cox-proportional hazards model was used to estimate the effects of NAP1L1 expression on patient survival. The crude hazard ratio (HR) of the NAP1L1-high expression group (score ≥ 4) compared to the NAP1L1-low expression group (score < 4) was 2.460 (95% CI, 1.22–4.96; $P = 0.0118$), which indicated that in patients with high NAP1L1 expression the risk of lung cancer-related death was increased. Multivariate analysis revealed that NAP1L1 expression, tumor size, lymphatic invasion, and pleural invasion were significantly associated with patient survival (Table 3). Moreover, NAP1L1 expression was also an independent prognostic indica-

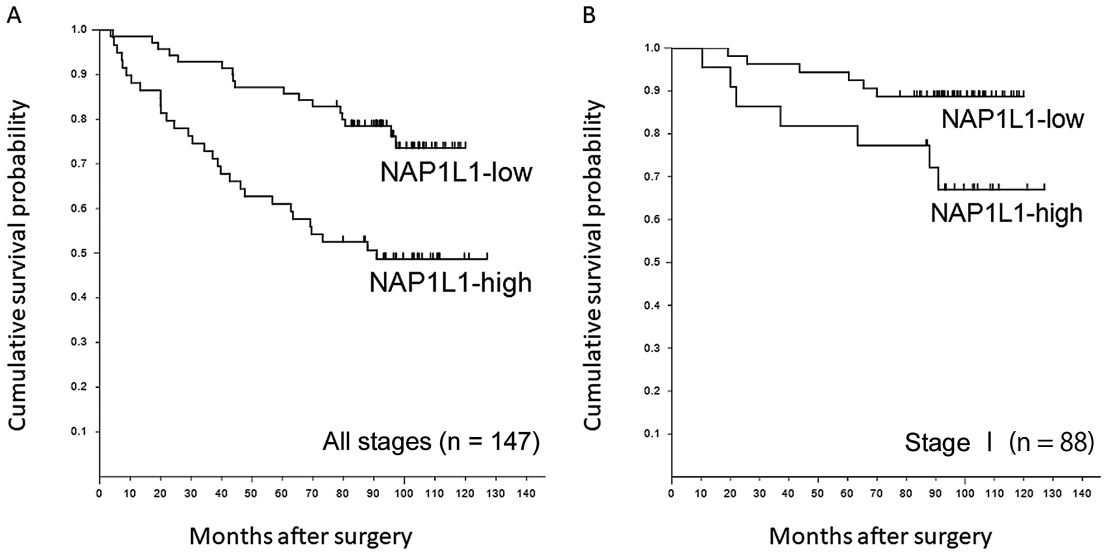

Fig. 2 The cumulative cancer-specific survival of patients with lung adenocarcinoma estimated by the Kaplan-Meier method. Patients with other causes and those lost to follow-up were treated as censored cases. The NAP1L1-high expression group was significantly correlated with poorer survival compared with the NAP1L1-low expression group in adenocarcinomas (A; $P = 0.0008$) and stage I lung adenocarcinomas (B; $P = 0.0309$).

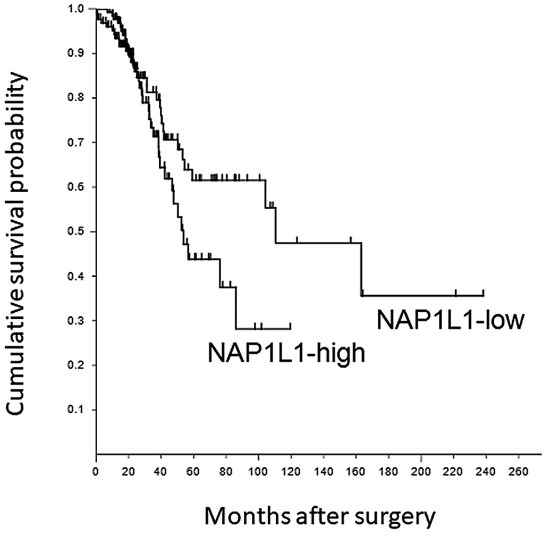

Fig. 3 The prognostic significance of NAP1L1 mRNA expression in patients with stage I adenocarcinoma based on TCGA data and estimated by the Kaplan-Meier method. The high-NAP1L1 mRNA expression group showed significantly poorer overall survival than the low-NAP1L1 mRNA expression group ($P = 0.048$).

tor for stage I lung AC in patients ($P = 0.0095$, Table 4).

NAP1L1 siRNA transfection and cell proliferation, migration, and invasion assays

To investigate the role of NAP1L1 in lung adenocarcinoma cells, cell proliferation and Transwell assays were performed after the transfection of A549 cells with a NAP1L1 siRNA. Transfection of A549 cells with the NAP1L1 siRNAs #1 and #2 significantly suppressed NAP1L1 mRNA levels compared with the negative-control-siRNA-treated cells. Similarly, no obvious protein expression was observed in NAP1L1-siRNA-treated cells (Fig. 4A).

The proliferation capability of cells treated with NAP1L1-siRNAs #1 and #2 at 72 h was significantly reduced compared with the negative-control-siRNA-treated cells, respectively (Fig. 4B). These results indicated that the downregulation of the *NAP1L1* gene significantly suppressed the proliferation of A549 cells. In the wound-healing migration assay, NAP1L1-knockdown cells exhibited delayed wound-healing compared with negative-control cells (Fig. 4C). Finally, Transwell assays demonstrated that cell migration with non-coated inserts and invasion with Matrigel-coated ones were significantly impaired in NAP1L1-knockdown cells (Fig. 4D and 4E). These results suggest that NAP1L1 is a key regulator of the aggressive phenotype in lung adenocarcinomas.

DISCUSSION

NAP1L1 is the human counterpart of the yeast NAP-I protein, which is a histone-binding factor involved in the maintenance of cumulative nucleosome formation (Ohkuni *et al.* 2003). In humans,

Table 3 *Univariate and multivariate analyses for the effects of NAP1L1 expression on patient survival in AC*

Factors	Univariate			Multivariate		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
NAP1L1 score						
High (≧4) vs. Low (<4)	2.58	1.41–4.69	0.002	2.46	1.22–4.96	0.0118
Age						
≧65 years vs. <65 years	0.91	0.51–1.62	0.7386	n/d	n/d	n/d
Gender						
Male vs. Female	1.73	0.95–3.15	0.0733	n/d	n/d	n/d
Smoking habits						
Smoker vs. Never smoker	1.59	0.88–2.85	0.1235	n/d	n/d	n/d
p-TNM stage						
Stage II and III vs. I	5.55	2.87–10.75	<0.0001	n/d	n/d	n/d
Differentiation						
MD/PD vs. WD	3.05	1.65–5.67	0.0004	n/d	n/d	n/d
Tumor size						
≧5 cm vs. <5 cm	4.64	2.38–9.04	<0.0001	13.83	5.88–32.53	<0.0001
Vascular invasion						
Yes vs. No	1.66	0.90–3.06	0.106	n/d	n/d	n/d
Lymphatic invasion						
Yes vs. No	4.08	2.13–7.83	<0.0001	3	1.30–6.91	0.0097
Pleural invasion						
Yes vs. No	3.01	1.68–5.40	0.0002	2.5	1.13–5.51	0.0235
Intrapulmonary Metastases						
Yes vs. No	2.81	1.19–6.68	0.019	n/d	n/d	n/d

n/d: not done.

Table 4 *Univariate and multivariate analyses for the effects of NAP1L1 expression on patient survival in stage I AC*

Factor	Univariate			Multivariate		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
NAP1L1 score						
High (≧4) vs. Low (<4)	3.12	1.05–9.30	0.0407	5.71	1.53–21.3	0.0095
Age						
≧65 years vs. <65 years	1.49	0.46–4.83	0.5101	n/d	n/d	n/d
Gender						
Male vs. Female	1.56	0.51–4.78	0.4351	n/d	n/d	n/d
Smoking habits						
Smoker vs. Never smoker	1.13	0.38–3.37	0.8253	n/d	n/d	n/d
Differentiation						
MD/PD vs. WD	4.4	1.43–13.5	0.0095	3.79	1.15–12.5	0.0289
Tumor size						
≧5 cm vs. <5 cm	3.83	0.50–29.7	0.1979	n/d	n/d	n/d
Vascular invasion						
Yes vs. No	5.32	1.71–16.5	0.0039	n/d	n/d	n/d
Lymphatic invasion						
Yes vs. No	3.2	0.98–10.4	0.0536	n/d	n/d	n/d
Pleural invasion						
Yes vs. No	5.43	1.81–16.2	0.0025	10.6	2.75–41.1	0.0006

n/d: not done.

NAP1L1 has been shown to play a role in the proliferation and metastasis in different types of cancer. Kidd *et al*. demonstrated that NAP1L1 mRNA is up-regulated in small intestinal neuroendocrine cell-derived neoplastic tissues compared to epithelial-derived tumor tissues in the gastrointestinal tract.

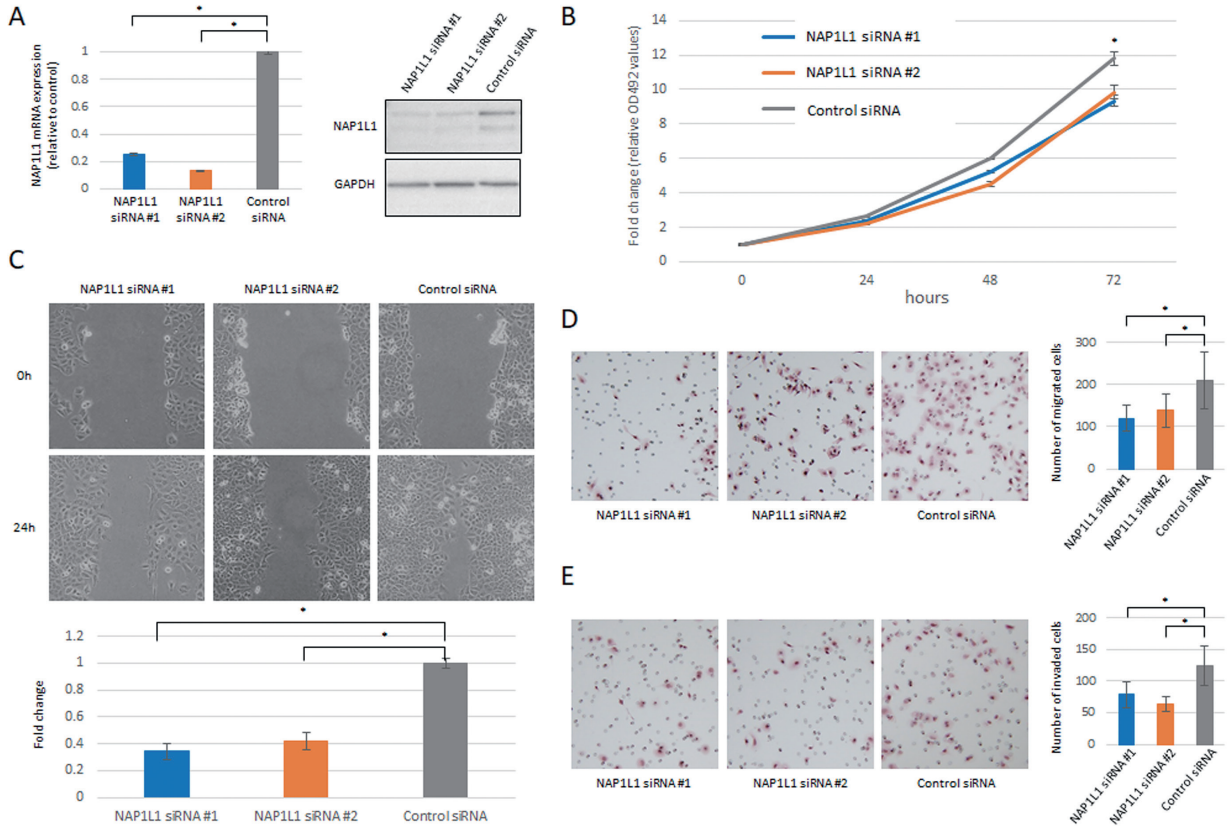

Fig. 4 Transfection of the NAP1L1 siRNA into lung adenocarcinoma-derived A549 cells, and cell proliferation, migration, and invasion assays. (**A**) The efficacy of NAP1L1 knockdown at the mRNA and protein levels was confirmed by real-time RT–PCR and Western blotting, respectively. A549 cells treated with NAP1L1 siRNAs #1 and #2 exhibited significant down-regulation of NAP1L1 at the mRNA level. No obvious protein expression was observed in cells treated with either of the NAP1L1 siRNAs. (**B**) The proliferation capability of cells treated with the two NAP1L1 siRNAs was significantly reduced at 72 h. (**C**) In the wound-healing migration assay, both NAP1L1-knockdown cells exhibited delayed wound-healing. (**D**) The migration and (**E**) invasion capacities were significantly impaired in NAP1L1-knockdown cells. $^{*}P < 0.05$.

Furthermore, tumors that had metastasized contained increased levels of NAP1L1 (Kidd *et al.* 2006). This suggests that NAP1L1 is a marker of small intestinal carcinoids and its expression correlates to the malignant behavior of these tumors. Overexpression of NAP1L1 in malignant neuroendocrine tumors has also been reported (Modlin *et al.* 2006; Schimmack *et al.* 2014). Schimmack *et al.* (2014) demonstrated that NAP1L1 was overexpressed in metastatic pancreatic neuroendocrine tumors and that it epigenetically promoted cell proliferation through inhibition of the mTOR pathway and the tumor suppressor p57Kip2. Malignant appendiceal carcinoids also have an elevated expression of NAP1L1 mRNA compared with benign appendiceal carcinoids, suggesting that NAP1L1 is a useful marker to differentiate malignant and benign carcinoids (Modlin *et al.* 2006). NAP1L1 expression and its potential role in the tumor pathogenesis have been mainly reported in malignant neuroendocrine tumors as above mentioned.

Recently, overexpression of NAP1L1 in several cancer types, including non-neuroendocrine tumors, has been reported using different approaches (Line *et al.* 2002; Guidi *et al.* 2012). Other than in HCC (Le *et al.* 2019), the correlation of NAP1L1 expression with clinicopathological characteristics and its prognostic significance in patients has not been elucidated. Le *et al.* (2019) described that high NAP1L1 expression in HCC tissues was associated with aggressive clinicopathological features and patients with high NAP1L1 expression had a poor overall survival rate in their cohort. Furthermore, in the extra-validation cohort analysis of the TCGA microarray dataset NAP1L1 was identified as an independent prognostic factor in HCC patients treated with radical resection. They also reported that the high NAP1L1 expression group, who experienced

recurrence after radical resection, showed a reduced overall survival after trans-arterial chemoembolization compared to the low NAP1L1 expression group. From these data, Le *et al.* stated that NAP1L1 is a prognostic biomarker and contributes to chemotherapy resistance in human HCC. Several neuroendocrine and non-neuroendocrine tumor studies revealed that NAP1L1 is highly expressed in malignant and aggressive tumors and suggested that NAP1L1 might be involved in oncogenesis. The present study found that cytoplasmic expression of NAP1L1 was observed in tumor cells in the majority of NSCLCs. Further, these expression levels were significantly associated with aggressive phenotypes of clinicopathological factors, such as reduced differentiation, higher pathological TNM stages, lymph node metastasis, lymphatic invasion, and vascular invasion of lung ACs. Also, we demonstrated that the NAP1L1-high expression group showed significantly poorer survival rates than the NAP1L1-low expression group, suggesting that NAP1L1 is an independent poor prognostic factor for cancer-specific survival in patients with AC, even in stage I. We demonstrated that NAP1L1 expression was also associated with aggressive clinicopathological characteristics and poor patient outcome in lung AC. These results suggest that NAP1L1 may be used as a potential biomarker to select patients who should receive adjuvant chemotherapy, even in early-stage lung AC.

A recent study revealed that the depletion of NAP1L1 in hepatocytes leads to the downregulation of 358 genes. At the top of this list were genes, involved in PI3K/AKT signaling (Cevik *et al.* 2017). Furthermore, Chen *et al.* (2018) demonstrated that the PRDI-BF1 and RIZ homology domain containing 8 (PRADM8), which is a key regulator of neural development and testis steroidogenesis, is downregulated in HCC and exhibited anti-tumor activities toward cancer cell progression by targeting NAP1L1 and regulating the PI3K/Akt/mTOR signaling pathway. Upregulation of PI3K/AKT/mTOR activity has been observed in 40–50% of HCC (Matter *et al.* 2014) and activation of this signaling pathway is associated with less differentiated tumors and an earlier recurrence (Villanueva *et al.* 2008). These data suggest that the PI3K/AKT/mTOR pathway occupies a central position in the network of dysregulated signaling pathways in HCC and neuroendocrine tumors (Schimmack *et al.* 2014). In lung AC-derived A549 cells, NAP1L1 regulates the NF-κB signaling pathway by modifying gene expression of the anti-apoptotic factor Mc1-1 (Tanaka *et al.* 2017). NF-κB regulates several genes that play important signal transduction roles in development, immunity, tissue homeostasis, inflammation, stress responses, cell survival, proliferation, epithelial-mesenchymal transition and invasion, and stemness (Taniguchi and Karin 2018). NF-κB is a promising molecular target for lung cancer stem cells as the deactivation of NF-κB using a kinase inhibitor effectively reduced their stemness, self-renewal, and migratory capabilities (Zakaria *et al.* 2018). NAP1L1 may contribute to the aggressive nature of the tumor cells through NF-κB and/or PI3K/AKT/mTOR signaling pathways in lung AC. In this NAP1L1-siRNA knockdown study, we demonstrated that A549 lung adenocarcinoma cells exhibited significantly reduced proliferation, migration, and invasion abilities compared with negative-control-siRNA-treated cells. A detailed analysis of the function of NAP1L1 and its associated signaling pathways in lung AC are required in the future.

In the micropapillary subtype of AC, the observation that tumor cells were arranged in small papillary tufts without an obvious fibrovascular core was first reported as an invasive ductal carcinoma variant of breast cancer (McDivitt *et al.* 1982). The extent of the micropapillary pattern in AC varies from minor to predominant and is detected in a substantial percentage of tumors. Additionally, lung AC with a micropapillary pattern had more frequent and severe vascular (lymphatic and/or venous) invasion, and more advanced lymph node involvement, and resulted in the poorer prognosis of patients compared to conventional AC without a micropapillary pattern (Hirano *et al.* 2014; Travis *et al.* 2015; Le *et al.* 2019). In this study, the average score of NAP1L1 expression in the micropapillary subtype tumor was higher than in the other types. It is speculated that increased expression of NAP1L1 contributes to the aggressive nature of these tumors. Lee *et al.* proposed that the increased expression of cancer stem cell markers, such as SOX2 and NOTCH3, in colorectal micropapillary carcinomas contributes to the poor prognosis of patients (Lee *et al.* 2013). NF-κB, which is regulated by NAP1L1, plays an important role in the maintenance of cancer stem cells in NSCLCs (Zakaria *et al.* 2018). Therefore, it is necessary to investigate the relationship between NAP1L1 expression and stem cells in the micropapillary pattern of lung AC.

In summary, NAP1L1 expression in lung AC was significantly correlated to various clinicopathological factors, and patients in the NAP1L1-high expression group had a significantly poorer overall survival. Multivariate analysis also revealed that NAP1L1 ex-

pression was an independent poor prognostic factor. Especially in patients with stage I lung AC, the expression of NAP1L1 is a risk factor for the poor prognosis of patients and may be used as a potential marker for a specific group of patients who should receive adjuvant chemotherapy. Therefore, the use of NAP1L1 as a biomarker may help improve clinical decision making and patient outcomes.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 15K21359 and a grant from Kitasato University School of Allied Health Sciences (Grant-in-Aid for Research Project, No. 2017-1006 and 2018-1014).

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