The Prognostic Significance of Eukaryotic Elongation Factor 1 Alpha-2 in Non-Small Cell Lung Cancer

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Abstract. *Background: Eukaryotic elongation factor 1 alpha-2 (eEF1A2) has been recently shown to be a putative oncogene of lung cancer. Materials and Methods: We analyzed the expression and prognostic significance of eEF1A2 in 69 primary non-small cell lung cancer (NSCLC) cases. We also suppressed eEF1A2 expression using RNA interference and then analyzed cell proliferation, migration and invasion of five adenocarcinoma cell lines. Results: eEF1A2 protein expression was positive in 84.1%. Negative immunostaining for eEF1A2 was shown to be an independent prognostic factor and significantly correlated with lymph node metastasis. There was no significant correlation between eEF1A2 protein and mRNA expression levels. Among the five examined cell lines, transfection of eEF1A2 siRNA inhibited cell migration in only one cell line while it did not change cell proliferation and invasion. Conclusion: Negative immunostaining of eEF1A2 predicted for poor prognosis of NSCLC. The mechanism of this result could not be elucidated by cell proliferation, migration and invasion assays.*

Eukaryotic elongation factor 1 alpha (eEF1A) plays an important role in protein synthesis (1-3). eEF1A binds amino-acylated tRNAs and transfers them to ribosomes. The two isoforms of eEF1A, termed eEF1A1 and eEF1A2, were identified. They are 92% identical at the amino acid sequence and perform the same function (4, 5), but their expression patterns are markedly different. eEF1A1 is expressed ubiquitously, whereas eEF1A2 expression is limited to the heart, skeletal muscle and brain (4, 5). Moreover, *eEF1A2* is considered as a putative oncogene because of its ectopic expression in several kinds of cancers; eEF1A2 was found overexpressed in 30% of ovarian, 30-60% of breast and 80% of pancreatic cancers, examined through numerous studies (6-9). In non-small cell lung cancer (NSCLC), Li *et al*. reported that eEF1A2 was overexpressed in 28% of examined cases but their samples were limited to only stage I cancers (10). In addition to its canonical role in protein synthesis, eEF1A2 is involved in cell proliferation, migration and invasion by means of actin remodeling (11, 12) and phosphatidylinositol signaling (13, 14). A prognostic significance of eEF1A2 in malignancies has also been reported. High eEF1A2 expression was a marker for good outcome in ovarian and breast cancer and a marker for poor survival in pancreatic cancer (7, 15, 16). It has never been examined whether eEF1A2 expression has any prognostic value in NSCLC.

In the present study, we investigated the expression of eEF1A2 in stage IA-IIIB of NSCLC and revealed its prognostic significance. Furthermore, we examined the effect of eEF1A2 expression on NSCLC cell proliferation, migration and invasion using *in vitro* assays.

Materials and methods

Tumor and normal lung tissue samples. Sixty-nine primary tumor specimens and 46 normal lung tissues were obtained from patients with NSCLC who had undergone complete lung resection (lobectomy or pneumonectomy and systematic lymphadenectomy) between 2000 and 2004 in the Tohoku University Hospital. These 69 patients were postoperatively followed for more than five years, which enabled us to analyze long-term survival. Informed consent was obtained from all patients included in the study.

Immunohistochemistry. The NSCLC sections underwent immunostaining by the Linked Streptavidin-Biotin method using the Histofine SAB-PO(R) kit, (Nichirei Biosciences, Tokyo, Japan). Antigen retrieval was achieved by heating the slides in a microwave oven at 500 W in 0.01% EDTA pH 8.0 for 15 min. Anti-eEF1A2 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a primary antibody at a dilution of 1/50.

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Evaluation of immunoreactivity. All slides were examined and scored independently by two of the authors (M.K. and C.E.) who were blinded to the clinicopathological data of the patients. The intensity of staining (Intensity score: IS) was 0 for no staining, 1 for weak-positive, 2 for moderate and 3 for strong staining. The percentages of stained cancer cells (Proportion score: PS) was scored 0 for no staining, 1 for <25%, 2 for 25-50% and 3 for 50-100%. The cases were defined as positive if both of IS and PS ≥2.

Cell lines. Lung adenocarcinoma cells A549, II-18 (Riken bioresource center, Ibaraki, Japan), PC9 (Immuno-biological laboratories, Gunma, Japan), H1975 (American Type Culture Collection, Manassas, VA, USA) and LCSC#1 (Cell resource center, Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan), lung squamous cell carcinoma cells LK2 (Cell resource center, Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan) and normal human bronchial/tracheal epithelial cells NHBE (Lonza Walkersville, Basel, Switzerland) were grown according to instructions by the provider.

Quantitative real-time RT-PCR (qRT-PCR). Total RNA was isolated from tumor samples and cell lines and 1 μg of RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan Gene Expression Assay (the sequence is not open, Applied Biosystems) was used for EEF1A2 (Assay ID: Hs00951278_m1) and GAPDH (Assay ID: Hs02758991_g1). Threshold cycles of eEF1A2 probe were normalized to GAPDH and translated to relative values using the Delta-Delta Ct method (17).

Western blotting. Protein samples were obtained from cultured cells and 10 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Anti-eEF1A2 antibody (1 μg/mL, Abcam, Cambridge, UK) and anti-β-actin antibody (1/1000, Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies. Immunoreactive bands were detected using ECL western blotting detection system with ImageQuant LAS 4000mini (GE healthcare Bio-sciences, Piscataway, NJ, USA), according to the manufacturer's instructions.

RNA interference. Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. Silencer select siRNA (Applied Biosystems, Foster city, CA, USA) was used for *EEF1A2* (siRNA ID: s4480, sence: 5'-ACCGCGACUUCAU CAAGAAtt-3', antisence: 5'-UUCUUGAUGAAGUCGCGGUgg-3') and Negative Control #1 (the sequence is not open). Transfection of siRNA was carried out by incubating cells with Lipofectamine RNAiMAX at 3 μL/mL and siRNA at 5 nM for 48 h.

Cell proliferation, migration and invasion assays. In proliferation assays, cells were transfected to siRNA in a 96-well plate. 48 h after transfection, culture media were exchanged and cells were cultured for an additional 24 h. Cell proliferation assay was performed using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Fitchburg, WI, USA). Transwell migration and invasion assays were performed using CytoSelect 24-well Cell Migration and Invasion Assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

Table I. *Patients' characteristics*.

Number of patients	69
Age (years)	30-82 (median: 69)
Gender	
Male	50
Female	19
Histology	
Ad	50
Sq	19
pT	
1	26
2	30
3	5
4	8
pN	
0	43
1	10
2	16
Lymphatic invasion	
Positive	26
Negative	43
Microvascular invasion	
Positive	31
Negative	38
Pathological stage	
I	37
II	12
III	20
Recurrence	27
Death	34
Observation period (months)	3.8-138.4 (median: 52.5)
5-year overall survival (%)	63.5

Ad, Adenocarcinoma; Sq, squamous cell carcinoma.

Statistical analysis. The Chi-square test, Student's t-test and Wilcoxon rank-sum test were used to compare among ratios, means and medians respectively. Univariate survival analysis was performed using Kaplan-Meier method and log-rank test. Multivariate survival analysis was performed using Cox's proportional hazards model. Survival time was defined as the period from the day of operation to the day of death by any cause. $p<0.05$ was considered to be statistically significant. All statistical analyses were performed using the JMP 10 software (SAS Institute, Cary, NC, USA).

Results

Characteristics of patients. Out of 69 cases, 37 were pathological Stage I, 12 were in pStage II and 20 were in pStage III. 5-year overall survival of 69 cases was 63.5%. The patients' characteristics were summarized in Table I.

Expression of eEF1A2 protein. Representative photographs of the IS and PS are shown in Figure 1. Fifty-eight cases of 69 NSCLCs (84.1%) showed positive eEF1A2 protein expression. Two out of 37 cases (94.6%) in pStage I, 7 of 12 cases (58.3%) in pStage II and 16 of 20 cases (80.0%) in pStage III showed positive protein expression. The staining was specific

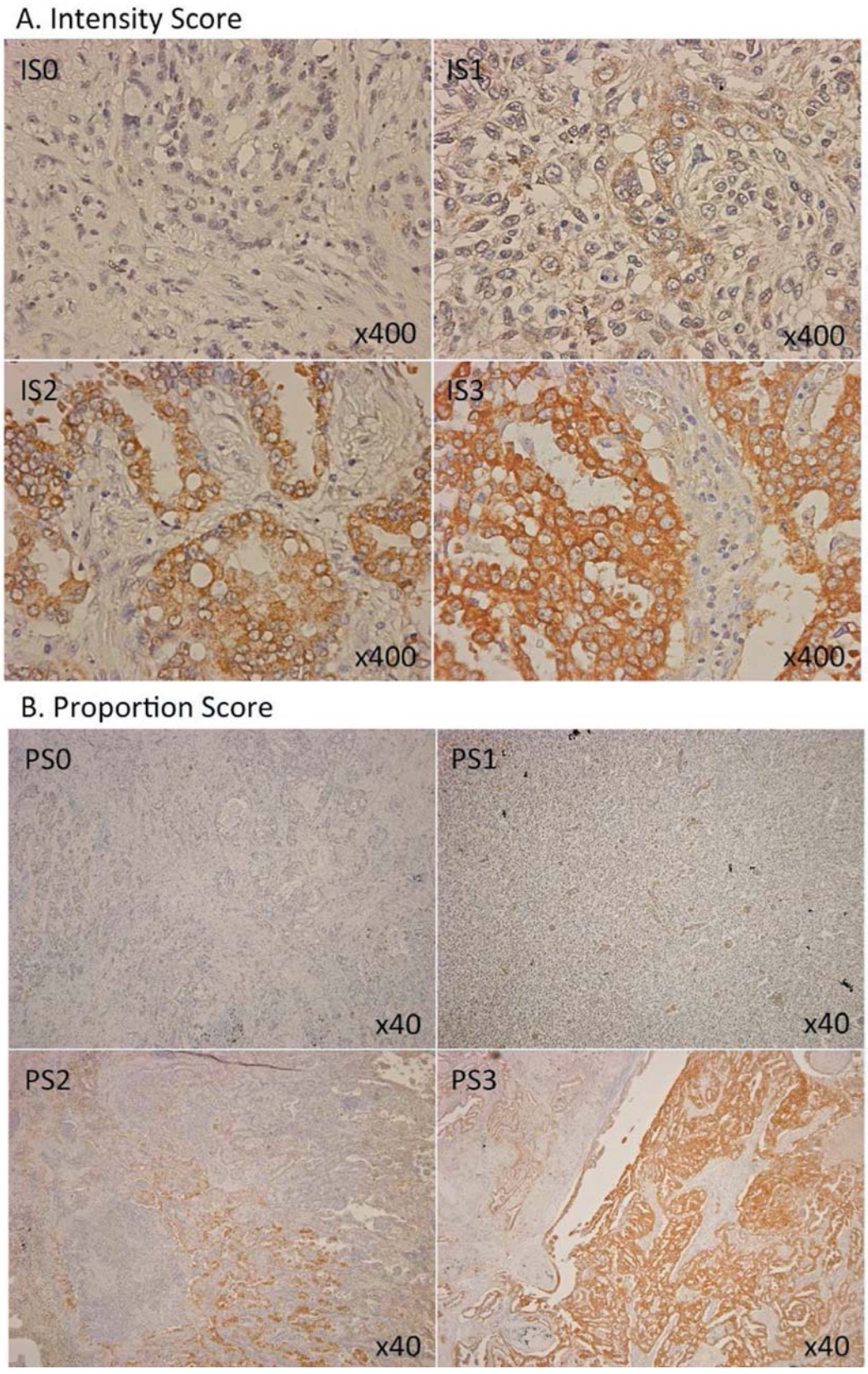

Figure 1. *Immunohistochemistry of eEF1A2 in NSCLC. A: Intensity score. B: Proportion score.*

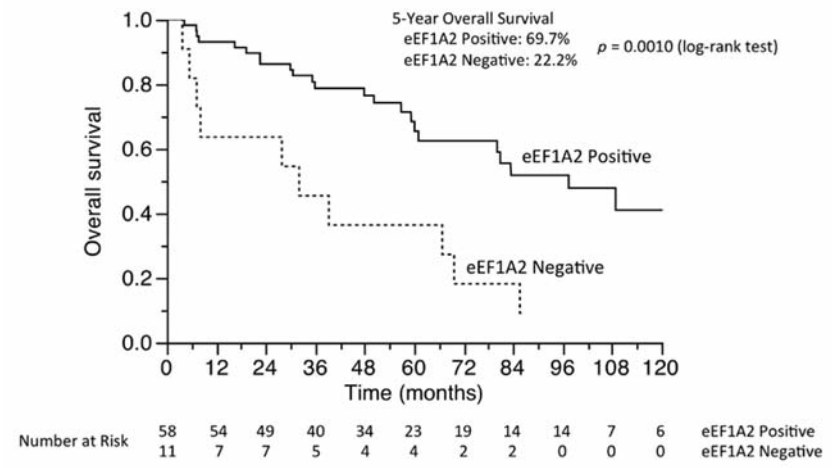

Figure 2. *Kaplan-Meier survival curves of NSCLC patients. The eEF1A2-negative group (dotted line) showed significantly worse prognosis compared with the eEF1A2-positive group (solid line).*

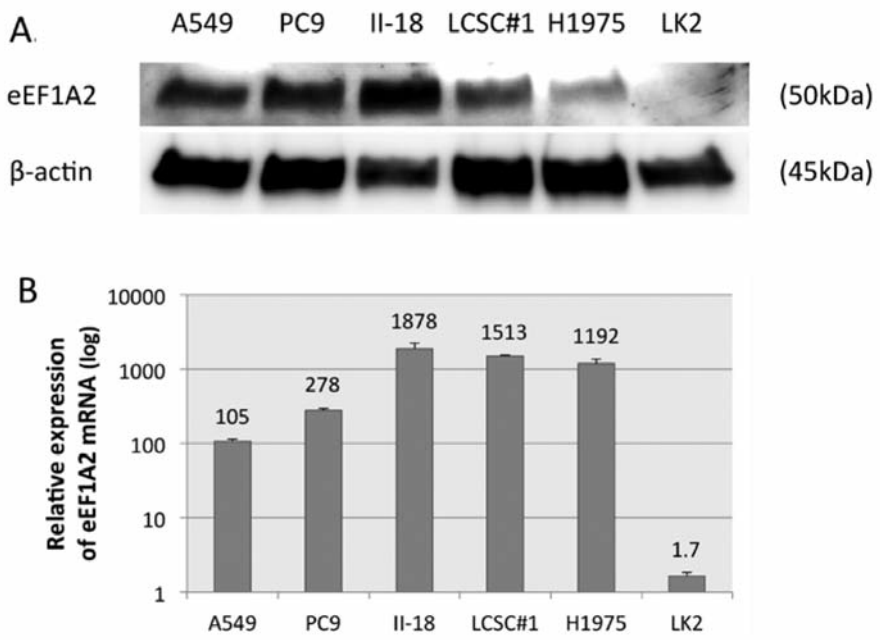

Figure 3. *eEF1A2 Protein and mRNA expression in NSCLC cell lines. A: All five adenocarcinoma cells showed eEF1A2 signal, yet squamous cell carcinoma cell LK2 did not. B: eEF1A2 mRNA relative expression of all five adenocarcinoma cells was more than 100, whereas that of LK2 was only 1.7.*

in the cytoplasm in all cases and only 2 showed positive staining of nuclei. In many instances, eEF1A2-positive cells existed in the part of the tumor as well. The statistical analysis showed significant correlations of eEF1A2 protein expression with survival (p=0.0026), lymph node metastasis (p=0.0097) and lymphatic invasion (p=0.0097). In contrast, eEF1A2 protein expression was not significantly correlated with age, sex, histology, T-factor or microvascular invasion (Table II).

Expression of eEF1A2 mRNA. eEF1A2 mRNA relative expression to normal lung tissue was 0.0028-1515 (median: 0.55) (Table II). Overexpression of *eEF1A2* mRNA did not significantly correlate with eEF1A2 protein expression (p=0.6404). *eEF1A2* mRNA expression level was higher in adenocarcinoma than in squamous cell carcinoma (p=0.0078) but did not significantly correlate with survival or lymph node metastasis (Table II).

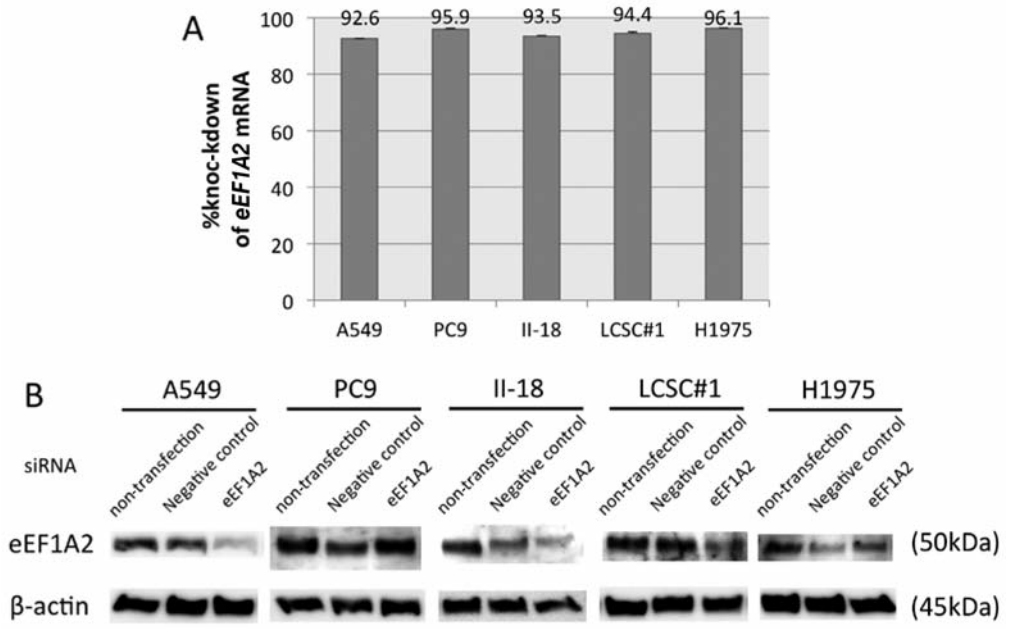

Figure 4. *Effect of RNA interference in eEF1A2 expression. A: Transfection of eEF1A2 siRNA achieved more than 90% reduction of eEF1A2 mRNA expression in all of five adenocarcinoma cells. B: Transfection of eEF1A2 siRNA also decreased the eEF1A2 protein expression in A549, II-18 and LSCS#1 but it was not reduced in PC9 and H1975.*

Table II. *Expression of eEF1A2 Protein and mRNA in NSCLC.*

Factor	eEF1A2 Immunohistochemistry				*eEF1A2* mRNA Relative Expression			
	Positive	Negative	Positive rate (%)	†*p*	First quartile	Median	Third quartile	††*p*
All cases	58	11	84.1		0.09	0.55	5.91	
Age				0.6265				0.4314
Under median	27	6	81.8		0.07	0.38	5.06	
Above median	31	5	86.1		0.15	0.60	9.02	
Gender				0.9830				0.9571
Male	42	8	84.0		0.10	0.56	5.20	
Female	16	3	84.2		0.09	0.32	10.88	
Histology				0.4487				0.0078*
Ad	41	9	82.0		0.14	1.54	12.90	
Sq	17	2	89.5		0.04	0.11	0.60	
pT				0.9921				0.9900
1	21	4	84.0		0.07	0.98	12.07	
2-4	37	7	84.1		0.09	0.53	6.19	
pN				0.0097*				0.8575
0	40	3	93.0		0.09	0.57	3.18	
1-2	18	8	69.2		0.10	0.42	10.12	
Lymphatic invasion				0.0097*				0.8868
Positive	18	8	69.2		0.10	0.42	11.15	
Negative	40	3	93.0		0.09	0.57	4.78	
Microvascular invasion				0.4843				0.6995
Positive	25	6	80.6		0.11	0.38	3.84	
Negative	33	5	86.8		0.08	0.56	14.26	
Death				0.0026*				0.0807
Yes	10	24	29.4		0.18	1.60	12.90	
No	34	1	97.1		0.07	0.17	2.31	

Expression of eEF1A2 protein was correlated with nodal involvement, lymphatic invasion and overall survival. However, relative expression level of *eEF1A2* mRNA was not correlated with them. Ad, adenocarcinoma; Sq, squamous cell carcinoma. †Chi-square test, ††Wilcoxon rank-sum test.

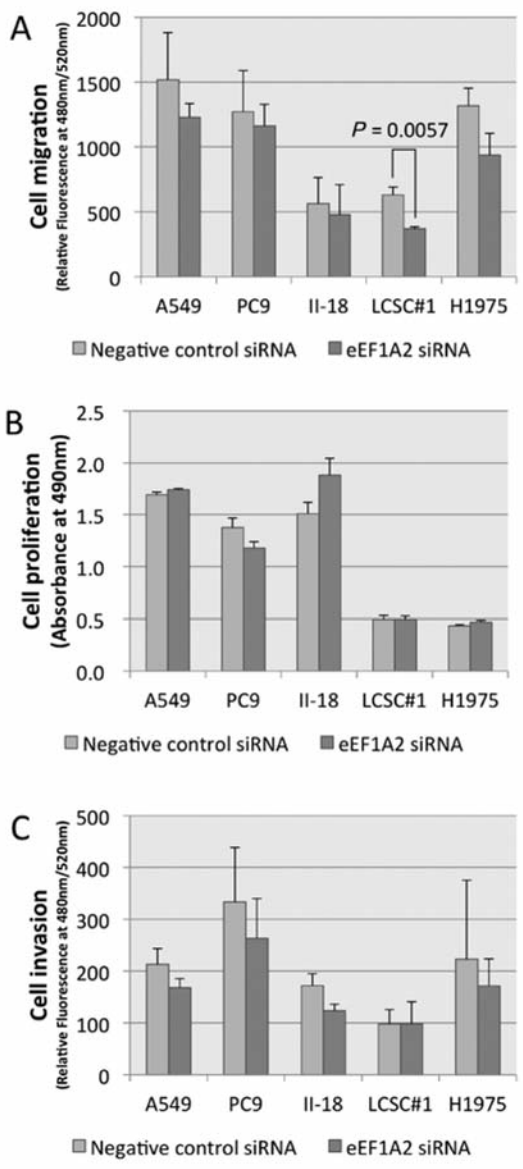

Figure 5. *Cell Proliferation, migration and invasion assays of the five cell lines examined. A: Transfection of eEF1A2 siRNA inhibited cell migration significantly in only LCSC#1. B: Proliferation and invasion (C) were not affected. Student's t-test was performed for statistical analysis.*

Survival analysis. Log-rank test showed eEF1A2 protein expression (p=0.0010) and lymph node metastasis (p=0.0244) significantly correlated with 5-year overall survival (Table III). As shown in Figure 2, the 5-year overall survival rate of eEF1A2-negative NSCLC patients was 22.2%, which was significantly worse than that of eEF1A2-negative patients (69.7%, p=0.0010). As shown in Table IV, multivariate survival analysis with Cox's proportional hazards model indicated that negative eEF1A2 immunostaining was an independent prognostic factor (hazard ratio: 2.71, 95% confidence interval 1.08-6.66, p=0.0335), as well as lymph node metastasis (hazard ratio: 4.07, 95% confidence interval: 1.06-14.63, p=0.0408).

eEF1A2 expression of NSCLC cells. Western blotting showed that all of five adenocarcinoma cells had eEF1A2 signal but squamous cell carcinoma cell LK2 did not (Figure 3A). *eEF1A2* mRNA relative expression to NHBE are shown in Figure 3B. Relative expression of all five adenocarcinoma cells was more than 100, whereas that of LK2 was only 1.7. Moreover, eEF1A2 protein signals of A549 and PC9 cells were stronger than those of H1975 (Figure 3A) although *eEF1A2* mRNA expression in A549 and PC9 was lower than in H1975 (Figure 3B). This meant a discrepancy between eEF1A2 protein and mRNA expression levels, which is compatible with the results of NSCLC clinical samples, as described above.

Influence of eEF1A2 suppression on cell proliferation, migration and invasion. Transfection of *eEF1A2* siRNA achieved more than 90% reduction of *eEF1A2* mRNA expression in all of five adenocarcinoma cells compared to negative-control siRNA (Figure 4A). eEF1A2 protein expression was also inhibited in A549, II-18 and LSCS#1 but it was not reduced in PC9 and H1975 (Figure 4B). Migration assay showed transfection of eEF1A2 siRNA inhibited cell migration significantly in only LCSC#1 (p=0.0057) (Figure 5A). Inhibition of eEF1A2 did not show significant effects on cell proliferation and invasion in lung adenocarcinoma cells (Figure 5B and 5C).

Discussion

This study has been the first to investigate the role of eEF1A2 in all stages of NSCLC. We showed that eEF1A2 protein expression was positive in more than 80% of all NSCLC cases and in 94% of Stage I cases. On the other hand, Li *et al*. reported that 28% of stage I NSCLC cases showed positive eEF1A2 protein expression (10). Their definition of positive staining was >10% of cancer cells to be stained, which was not as stringent as our criteria. One of the reasons of this discrepancy probably comes from the focal existence of eEF1A2-positive cells, which we observed. We immunostained and evaluated each one of the whole tumor sections but Li *et al*. stained only 2 tissue cylinders of 1.5-mm diameter obtained from each of the tumors. In addition, they used another eEF1A2 antibody and different method for antigen retrieval from ours, which is considered to affect the immunostaining intensity. In the present study we employed a more precise definition of positive staining for eEF1A2 and all stages of resected NSCLC cases were investigated. Our results should be more precise and reliable from the viewpoint of the positive staining proportion of eEF1A2.

Table III. *Univariate survival analysis*.

Factor	5-Year Overall Survival (%)	$p^{\dagger\dagger\dagger}$
Age		0.5127
Under median	62.9	
Above median	64.0	
Gender		0.3007
Male	68.8	
Female	51.7	
Histology		0.0755
Ad	59.9	
Sq	73.7	
pT		0.3556
1	79.5	
2-4	55.0	
pN		0.0244*
0	73.4	
1-2	48.2	
Lymphatic invasion		0.0750
Positive	52.8	
Negative	70.2	
Microvascular invasion		0.8554
Positive	58.6	
Negative	68.8	
eEF1A2 Immunohistochemistry		0.0010*
Positive	69.7	
Negative	22.2	

Ad, Adenocarcinoma; Sq, squamous cell carcinoma. †††Log-rank test.

Table IV. *Multivariate survival analysis*.

Covariate	Hazard ratio	95% Confidence Interval	$p^{\dagger\dagger\dagger\dagger}$
eEF1A2 Immunohistochemistry			0.0335*
Positive	1.00		
Negative	2.71	1.08-6.66	
pN			0.0408*
0	1.00		
1-2	4.07	1.06-14.63	
Age			0.1117
Under median	1.00		
Above median	1.84	0.87-4.04	
Histology			0.2075
Ad	1.00		
Sq	1.84	0.73-5.40	
Lymphatic invasion			0.2286
Positive	1.00		
Negative	2.30	0.59-8.56	

Ad, Adenocarcinoma; Sq, squamous cell carcinoma. ††††Multivariate analysis with Cox's proportional hazards model.

Negative eEF1A2 immunostaining is an independent prognostic factor for shorter survival and significantly correlated with lymph node involvement. Recent studies have demonstrated that some cancers showed positive correlation between eEF1A2 expression and prognosis or nodal involvement, and others showed a negative correlation; eEF1A2 expression was considered to be a marker for better prognosis in breast and ovarian cancer but a poor prognostic marker in pancreatic cancer (7, 15, 16). eEF1A2 expression significantly correlated with lymph node metastasis in pancreatic (16), but not in breast cancer (7). The reason why several kinds of cancer show different correlations of eEF1A2 expression and prognosis or lymph node involvement has yet to be elucidated.

We focused on and investigated the effect of eEF1A2 on cell proliferation, migration and invasion to elucidate the mechanism on how eEF1A2 affects prognosis and lymph node metastasis. Transfection with *eEF1A2* siRNA did not affect cell proliferation, so it was unlike that low *eEF1A2* mRNA expression accelerated lung cancer cell proliferation, resulting in short survival of NSCLC patients. Furthermore, among the five examined cell lines, transfection with *eEF1A2* siRNA inhibited cell migration in only one cell line and did not affect cell invasion. Although eEF1A2 has been reported to be involved in PTEN/PI3K/Akt signaling pathway in breast cancer cells, which promotes cell migration and invasion (13, 14), this effect might be negligible in NSCLC.

We also showed that there was no significant correlation between eEF1A2 protein and mRNA expression levels. It has been known that expression levels of protein and mRNA sometimes show discrepancy. Chen *et al*. reported that only 21.4% (21/98) of genes showed significant correlation between protein and mRNA expression levels in 76 cases of lung adenocarcinoma (18). Furthermore, Tomlinson *et al*. suggested a discrepancy of eEF1A2 protein and mRNA expression in 13 cases of ovarian cancer (19). We have not found any prognostic importance of *eEF1A2* mRNA expression in NSCLC.

There are several speculations which elucidate the mechanism on how eEF1A2 affects prognosis and lymph node metastasis. Firstly, eEF1A2 could affect the efficacy of chemotherapy. It has been reported that etoposide, a topoisomerase I inhibitor, acetylated eEF1A2 (20), suggesting that eEF1A2 is involved in the mechanism of action of etoposide. Secondary, eEF1A2 could inhibit angiogenesis, which shortens the survival of eEF1A2-negative NSCLC cases. Thirdly, there may exist a true prognostic factor that regulates eEF1A2 expression and eEF1A2 is just a surrogate marker. For example, Raf has been known to have an oncogenic role in the Ras/Raf/ERK1/2 signaling pathway and it modulates phosphorylation and expression of eEF1A2 (21-23). Further investigations are necessary to elucidate the reason why eEF1A2 expression seems to be correlated to prognosis or nodal involvement in NSCLC.

Conclusion

In the present study we showed that eEF1A2 protein expression was positive in more than 80% of NSCLC cases and negative eEF1A2 immunostaining was an independent prognostic factor for shorter survival. Transfection with *eEF1A2* siRNA did not alter cell proliferation, migration and invasion of lung adenocarcinoma cells.

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