

ORIGINAL ARTICLE

High expression of eukaryotic elongation factor 1-alpha-2 in lung adenocarcinoma is associated with poor prognosis

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Funding information

Japan Society for the Promotion of Science London, Grant/Award Numbers: 19K16578, 21K15398

Abstract

Eukaryotic elongation factor 1 alpha 2 (eEF1A2) encodes an isoform of the alpha subunit of the elongation factor 1 complex and is responsible for the enzymatic delivery of aminoacyl tRNA to the ribosome. Our proteomic analysis has identified eEF1A2 as one of the proteins expressed during malignant progression from adenocarcinoma in situ (AIS) to early invasive lung adenocarcinoma. The expression level of eEF1A2 in 175 lung adenocarcinomas was examined by immunohistochemical staining in relation to patient prognosis and clinicopathological factors. Quantitative PCR analysis and fluorescence in situ hybridization (FISH) were performed to evaluate the amplification of the eEF1A2 gene. Relatively high expression of eEF1A2 was observed in invasive adenocarcinoma (39/144 cases) relative to minimally invasive adenocarcinoma (1/10 cases) or AIS (0/21 cases). Among invasive adenocarcinomas, solid-type adenocarcinoma (15/32 cases, 47%) showed higher expression than other histological subtypes (23/92, 25%). Patients with eEF1A2-positive tumors had a significantly poorer prognosis than those with eEF1A2-negative tumors. Of the five tumors that were eEF1A2-positive, two cases showed amplified genomic eEF1A2 DNA, which was confirmed by both qPCR and FISH. These findings indicate that eEF1A2 overexpression occurs in the course of malignant transformation of lung adenocarcinomas and is partly due to eEF1A2 gene amplification.

KEYWORDS

adenocarcinoma in situ (AIS), clinicopathological analysis, eukaryotic elongation factor 1-alpha-2 (eEF1A2), gene amplification, immunohistochemistry, lung adenocarcinoma

Abbreviations: AIS, Adenocarcinoma in situ; eEF1A1, Eukaryotic elongation factor 1-alpha-1; eEF1A2, Eukaryotic elongation factor 1-alpha-2; FISH, Fluorescence in situ hybridization; MIA, Minimally invasive adenocarcinoma.

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INTRODUCTION

Lung cancer is the most common form of cancer worldwide and accounts for the highest cancer-related mortality.¹ Among lung cancers, adenocarcinoma is the most common histological subtype. Adenocarcinoma is considered to progress stepwise from atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA) and lepidic adenocarcinoma.² In 1995, Noguchi et al. demonstrated that AIS has an extremely favorable outcome, with a 5-year survival rate of 100%, whereas invasive carcinoma, even in the early stage, has a poorer outcome.³ To clarify the molecular mechanisms involved in the malignant progression of early-stage lung adenocarcinoma we compared the differences in gene expression profiles between AIS and early but invasive adenocarcinoma. Using quantitative proteome analysis, Dai et al. have shown that eukaryotic elongation factor 1-alpha-2 (eEF1A2) is overexpressed in early but invasive adenocarcinoma, in comparison to AIS.⁴

eEF1A2 and eEF1A1 are two isoforms of eukaryotic elongation factor 1 alpha (eEF1A). eEF1A is a subunit of the eukaryotic elongation factor 1 complex, and its main function is delivering aminoacylated tRNAs to the A site of the ribosome in the peptide chain elongation phase. eEF1A2 is located on chromosome 20q13 whereas eEF1A1 is located on chromosome 6q13. eEF1A1 and eEF1A2 have 93% identical amino acid sequences, but the expression pattern of the isoforms differs. eEF1A1 is expressed almost ubiquitously, except in neurons, heart and skeletal muscle, in the fetal phase and then its expression declines gradually and is replaced by eEF1A2 during development.^{5–7} Although the reason for this organ-specific distribution of the two isoforms remains largely unknown, Chambers and Newbery have shown that, in mouse, absence of eEF1A2 leads to degeneration of motor neurons and early death.^{7,8} On the other hand, eEF1A2 mutation has been reported in human patients with epileptic encephalopathy/intellectual disability.⁹

Among various malignancies, eEF1A2 is overexpressed in breast cancer,^{10,11} ovarian cancer,^{12,13} hepatocellular carcinoma,^{14,15} prostate cancer,¹⁶ plasmacytoma,¹⁷ pancreatic cancer^{18,19} and lung cancer.^{20–24} Gene amplification of eEF1A2 has been demonstrated in ovarian tumors^{12,13} and lung cancer,^{21,22} and its amplification has also been reported in prostate cancer cell lines.²⁵ eEF1A2 has been considered as active oncogene, as it is overexpressed in various malignant tumor tissues, and has been shown to confer tumorigenicity in a fibroblast cell line.¹² Many studies have indicated that eEF1A2 has an anti-apoptosis function,^{17,26–29} and is responsible for actin cytoskeletal rearrangement,^{30–32} acinar morphogenesis,³³ and epithelial-mesenchymal transformation.²⁴ Some studies have indicated that eEF1A2 activates the Akt pathway.^{14,15,17,19,27,30}

The relationship between eEF1A2 overexpression and prognosis in lung cancer is still under debate, as various studies have shown poorer^{20,21,24} or favorable²³ outcome in patients with tumors over-expressing eEF1A2. However, as these studies examined advanced cases, they did not include early-stage lung adenocarcinoma, such as AIS.

In the present study, we investigated eEF1A2 protein expression in surgically resected lung adenocarcinomas using immunohistochemistry, and compared the results between AIS, MIA, and invasive adenocarcinoma. We also studied the clinicopathological implications of eEF1A2 expression. In addition, we demonstrated eEF1A2 gene amplification in lung adenocarcinoma using fluorescence in situ hybridization (FISH) analysis.

MATERIALS AND METHODS

Clinical samples

Clinical samples of heart, skeletal muscle, lung, liver and kidney were obtained from two autopsy cases at the University of Tsukuba Hospital, for Western blot analysis. Frozen surgical specimens of lung adenocarcinoma used in Western blot analysis were obtained from materials that had been resected between 2014 and 2017 at the University of Tsukuba Hospital (Ibaraki, Japan) (Supporting Information S1: Table S1). All the samples had been obtained at University of Tsukuba Hospital with appropriate informed consent. The fresh tissues had been frozen and stored at -80°C . For immunohistochemical analysis, we prepared tumor tissue microarray (TMA) slides consisting of 175 formalin-fixed and paraffin-embedded (FFPE) tumor tissues that had been resected between 1999 and 2007 (Table 1). Histopathological classification was based on the WHO Classification of Thoracic Tumors, 5th edition. Invasive non-mucinous adenocarcinomas were classified into subgroups (lepidic, acinar, papillary, micropapillary, solid) on the basis of the predominant histological patterns. The pathological stages of these 175 tumors were evaluated according to the UICC TNM Classification of Malignant Tumors, 8th edition. Epidermal growth factor receptor (EGFR) mutation profiles and the results of ALK oncoprotein immunohistochemistry were collected from the clinical database (Supporting Information S1: Table S2). This study was approved by the ethics committee of the University of Tsukuba Hospital (No. H27-205).

Confirmation of antibody specificity

Western blotting was performed using the protocol reported previously.⁴ To verify the specificity of the antibodies against eEF1A1 and eEF1A2, Western blotting was performed using monoclonal anti-eEF1A1 antibody (ab157455; Abcam), polyclonal anti-eEF1A2

TABLE 1 Eukaryotic elongation factor 1 alpha 2 expression and clinicopathological features in patients with lung adenocarcinoma.

Clinicopathological features	Cases(n)	Positive	Negative	P-value
All patients	175	40	135	
Mean age (years)	66.8	66.0	67.0	
Sex				0.001
Male	104	33	71	
Female	71	7	64	
Histology (AIS, MIA vs others)				0.004
Adenocarcinoma in situ	21	0	21	
Minimally invasive adenocarcinoma	10	1	9	
Lepidic	44	10	34	
Papillary	26	7	19	
Acinar	18	5	13	
Solid	32	15	17	
Micropapillary	4	1	3	
Invasive mucinous adenocarcinoma	20	1	19	
Pathological stage (0,1 vs others)				0.626
pStage 0	21	0	21	
pStage I (IA1, IA2, IA3, IB)	94	25	69	
pStage II (IIA, IIB)	26	6	20	
pStage III (IIIA, IIIB, IIIC)	31	6	25	
pStage IV (IVA)	3	3	0	
pT factor (Tis, T1 vs others)				0.021
pTis	21	0	21	
pT1 (pT1a, pT1b, pT1c)	64	13	51	
pT2 (pT2a, pT2b)	63	22	41	
pT3	17	4	13	
pT4	10	1	9	
Lymph node metastasis				0.102
pN0/pNX	131	26	105	
pN1/pN2	44	14	30	
Pleural invasion				0.022
pI0	118	21	97	
pI1-3	57	19	38	
Lymphatic permeation				0.146
Ly0	109	21	88	
Ly1	66	19	47	
Vascular invasion				0.001
V0	101	14	87	
V1	74	26	48	

TABLE 1 (Continued)

Clinicopathological features	Cases(n)	Positive	Negative	P-value
History of smoking				<0.001
Never smokers	56	3	53	
Ever smokers	108	34	74	

Note: Correlation of eEF1A2 expression with each clinicopathological feature was analyzed by chi-squared test.

antibody (GTX102326, GeneTex), and mouse monoclonal anti-GAPDH antibody (sc-32233, Santa Cruz Biotechnology). eEF1A1 and eEF1A2 recombinant proteins (Abnova) were used as positive controls.

Cell culture and transfection using siRNA

A549 (lung adenocarcinoma cell line) was maintained in DMEM (Fujifilm Wako Pure Chemical) supplemented with 10% FBS under 5% CO₂ at 37°C. eEF1A2-specific siRNA (Stealth RNAi, Thermo Fisher Scientific) (Supporting Information S1: Table S3), lipofectamine RNAiMAX (Thermo Fisher scientific), and OPTI-MEM (Thermo Fisher Scientific) were added to the well and incubated for 20 min at room temperature. A549 cells were then seeded into the well containing the siRNA complex. The final siRNA concentration was 10 nM. The cells were incubated at 37°C in a CO₂ incubator for 48 h, and then collected for protein extraction. Stealth RNAi™ GCDuplex #2 (Thermo Fisher) was used as a siRNA negative control.

Immunohistochemical analysis

The TMA consisting of 175 lung adenocarcinoma specimens was subjected to immunohistochemical and clinicopathological analysis. A 2-mm core of each tumor was sampled and made into a tumor tissue array block. When a tumor showed mixed histological patterns, the tumor core was sampled from the predominant histological pattern. For example, the tumor tissue core of "lepidic adenocarcinoma" was sampled from the lepidic component of the tumor tissue. Then, 3-μm-thick sections were cut from the array blocks and deparaffinized and dehydrated. Antigen retrieval was performed by autoclave in 10 mM citric acid buffer (pH 6) at 115°C for 10 min. Immunohistochemistry was performed using a Histostainer 36 A (Nichirei Biosciences). The sections were incubated with anti-eEF1A1 antibody diluted 1:200, or with anti-eEF1A2 antibody diluted 1:100, for 30 min at room temperature. These antibodies against eEF1A1 and eEF1A2 were also used for Western blotting. The sections were subsequently incubated with the secondary antibody (Dako REAL EnVision Detection System; Agilent Technologies) and detected with DAB (Dako,

DAB + Liquid, Agilent Technologies), and counterstained with hematoxylin. In cases where more than 10% of the tumor cells were stained, we judged the tumor to be "positive," as reported previously elsewhere.¹⁵

Quantitative genomic PCR

Genomic DNA was extracted from 10-μm-thick paraffin embedded sections by digestion with proteinase K (Qiagen), followed by use of a magLEAD (Precision System Science). Oligonucleotide primers for EEF1A2 were designed using Primer3 (<http://primer3.sourceforge.net/>). GAPDH was used as an internal control gene for normalization. Quantitative PCR analysis was carried out using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio). The PCR reactions were carried out using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. A ratio (tumor / normal) of ≥1.5 was defined as representing gene amplification.

Fluorescence in situ hybridization (FISH)

FISH was performed using the protocol reported previously.³⁴ The 5-μm-thick serial sections from FFPE tissues were subjected to dual-color FISH using an EEF1A2-CEN20p probe (GSP Lab.). Using a fluorescence microscope (Keyence) with single interference filter sets for green (FITC), red (TexRed), and blue (DAPI), FISH signals were enumerated in non-overlapping tumor cell nuclei.

Statistical analysis

For statistical analysis, we used the SPSS Statistics package Version 26 (IBM). Correlations with clinicopathological features were analyzed using the chi-squared test. Survival curves were calculated using the Kaplan-Meier method and assessed using the log-rank test. The endpoint of this study was disease-free survival. Survival time was from the date of resection to the date of recurrence. The relationship between eEF1A2 amplification by qPCR and immunohistochemical positivity was examined with the Mann-Whitney test. Univariate and

multivariate analyses were carried out using the Cox proportional hazards model.

RESULTS

Confirmation of antibody specificity

eEF1A2 and its isoform eEF1A1 have very high sequence homology (93%) at the amino acid level. First, we validated the specificity of the antibody. Both antibodies against eEF1A1 and eEF1A2 detected their recombinant proteins specifically and separately on Western blots (Figure 1a). Each antibody also detected the organ-specific distribution of each isoform in protein extracts of normal human organs; eEF1A1 was distributed in lung, liver, and kidney, whereas eEF1A2 was distributed in heart and skeletal muscle (Figure 1a).

Lung cancer cell line A549 expressed both eEF1A1 and eEF1A2 (Figure 1a-b). After transfection with si-eEF1A2 on A549, eEF1A2 expression was suppressed but eEF1A1 showed no change (Figure 1b).

eEF1A2 expression in lung adenocarcinoma and clinicopathological features

Western blot analysis was conducted on two specimens of surgically resected lung adenocarcinoma. One specimen showed expression of both eEF1A1 and eEF1A2, while the other showed expression of only eEF1A1 (Figure 1b). Immunohistochemical analysis of normal lung tissue and AIS demonstrated scattered eEF1A2 expression in a few alveolar cells (Figure 2a-b), whereas eEF1A1 showed cytoplasmic expression in alveolar and bronchial cells (Figure S1), and in inflammatory cells such as

macrophages and lymphoid cells. Lung adenocarcinoma showed cytoplasmic expression of both eEF1A1 and eEF1A2 (eEF1A1: Figure S1, eEF1A2: Figure 2c). Invasive tumors with mixed histological patterns tend to show similar expression within the tumor, including a lepidic pattern. For example, eEF1A2-positive solid adenocarcinoma showed eEF1A2 positivity in both the solid pattern and the lepidic pattern (Figure S2).

We examined 175 lung adenocarcinomas immunohistochemically. Forty adenocarcinomas were positive for eEF1A2 (Table 1). Among them, 39 cases were diagnosed as invasive adenocarcinoma, and the remaining one as MIA (Table 1). Histopathologically, among the eEF1A2-positive adenocarcinomas, 10% (1/10) were MIA, 23% (10/44) were lepidic, 27% (7/26) were papillary, 28% (5/18) were acinar, 47% (15/32) were solid, 25% (1/4) were micropapillary, and 5% (1/20) were invasive mucinous adenocarcinoma. All 20 specimens of AIS were negative for eEF1A2 (Table 1). We investigated the correlation between eEF1A2 expression and the clinicopathological features of the patients using chi-squared test. eEF1A2 expression was significantly associated with sex ($P=0.001$), histology (AIS, MIA vs invasive carcinomas, $P=0.004$), pT factor (pTis, pT1 vs others, $P=0.021$), pleural invasion (pI0 vs pI1-3, $P=0.022$), vascular invasion ($P=0.001$) and history of smoking ($P<0.001$). EGFR mutation status and ALK oncoprotein expression did not show any significant association with eEF1A2 expression (Supporting Information S1: Table S2). In terms of disease-free survival, Kaplan-Meier curve analysis indicated that patients with eEF1A2-positive tumors had a significantly poorer prognosis than those with eEF1A2-negative tumors (Figure 3. Log rank test ($P=0.046$)). Univariate Cox analysis demonstrated that sex ($P=0.001$), pathological stage (0,1 vs Others) ($P<0.001$), pT factor (pTis, pT1 vs Others) ($P<0.001$), lymph node metastasis (pN0 vs pN1) ($P<0.001$), pleural invasion (pI0 vs Others)

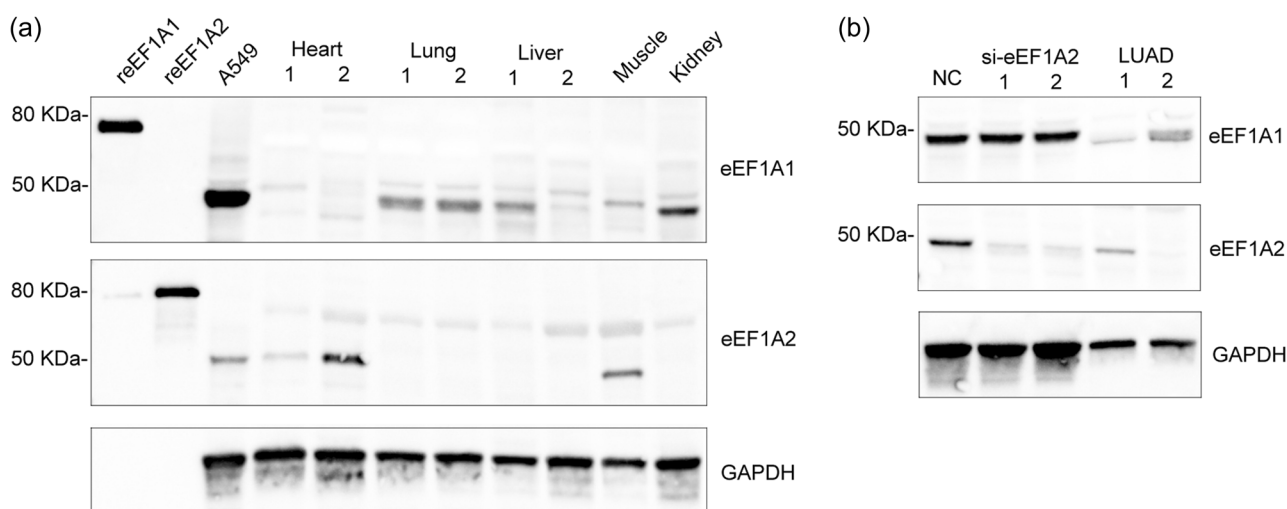


FIGURE 1 (a) Western blot of recombinant protein, protein extracts from the A549 cell line, and from normal human organs. reEF1A1 and reEF1A2 are recombinant proteins. Caption under organ name “1” and “2” mean two cases were examined for heart, lung and liver. (b) Western blot of A549 cells treated with si-eEF1A2. Scrambled RNA was used for the negative control (NC). LUAD indicates lung adenocarcinoma tissues.

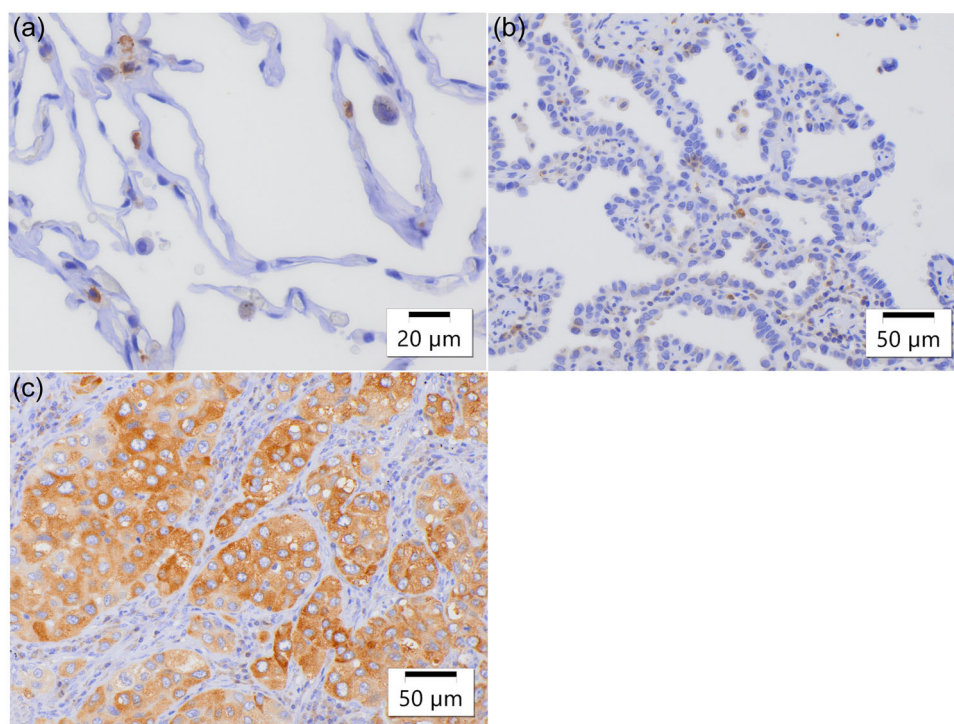


FIGURE 2 Immunohistochemistry of eukaryotic elongation factor 1 alpha 2 (eEF1A2) in normal lung tissue and in lung adenocarcinoma. (a) Normal lung tissue. (b) Adenocarcinoma in situ. (c) Solid adenocarcinoma with eEF1A2 expression.

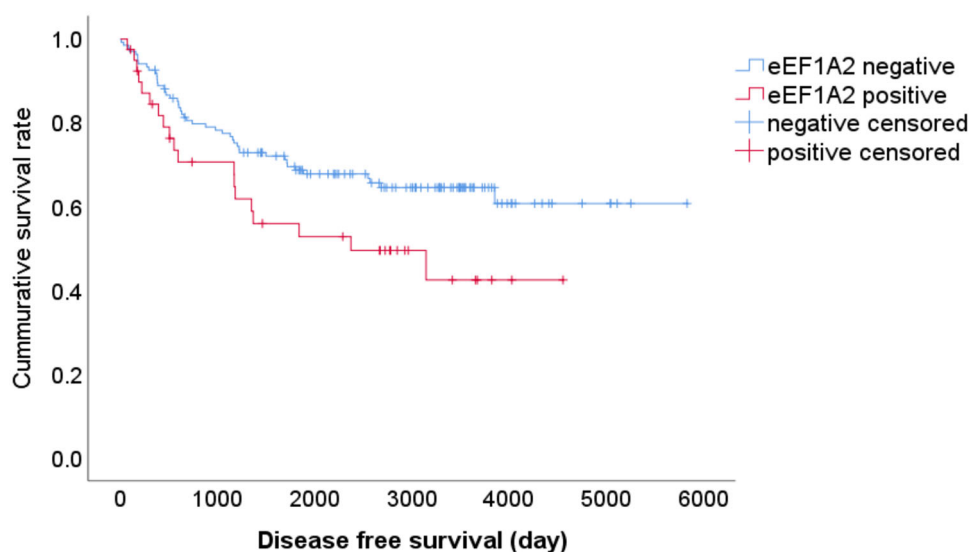


FIGURE 3 Kaplan-Meier curve of disease-free survival of patients with eukaryotic elongation factor 1 alpha 2 (eEF1A2).

($P < 0.001$), lymphatic permeation (Ly0 vs Ly1) ($P < 0.001$), vascular invasion (V0 vs V1) ($P < 0.001$), history of smoking ($P = 0.001$) and eEF1A2 (negative vs positive) ($P = 0.048$) had statistically significant correlations (Table 2). In multivariate Cox analysis, pT factor ($P = 0.026$), lymph node metastasis ($P = 0.016$), lymphatic permeation ($P = 0.015$), and vascular invasion ($P = 0.022$) remained significant, but eEF1A2 was not selected as an independent prognostic factor (Table 2).

Quantitative genomic PCR and FISH analysis

Five tumors that were immunohistochemically positive for eEF1A2, and two that were immunohistochemically negative for eEF1A2 were subjected to qPCR analysis. Among 5 eEF1A2-positive tumors, 4 showed increases of genomic eEF1A2 DNA (tumor/normal ratio > 1.5) (Figure 4a). One eEF1A2-negative

TABLE 2 Univariate and multivariate analysis of adenocarcinoma ($n = 175$) using the Cox proportional hazards model.

Clinicopathological features	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age	1.01 (0.98–1.04)	0.456	1.024 (0.996–1.052)	0.093
Sex (Female vs Male)	2.59 (1.45–4.62)	0.001	0.906 (0.316–2.60)	0.855
Pathological stage (0,1 vs Others)	3.58 (2.18–5.86)	<0.001	-a	-a
pT factor (pTis, pT1 vs Others)	4.64 (2.60–8.30)	<0.001	2.08 (1.09–3.96)	0.026
Lymph node metastasis (pN0 vs pN1)	4.89 (2.99–8.00)	<0.001	2.05 (1.14–3.69)	0.016
Pleural invasion (pI0 vs Others)	3.87 (2.36–6.33)	<0.001	-a	-a
Lymphatic permeation (Ly0 vs Ly1)	4.65 (2.79–7.73)	<0.001	2.12 (1.15–3.90)	0.015
Vascular invasion (V0 vs V1)	6.76 (3.82–12.0)	<0.001	2.737 (1.13–4.97)	0.022
History of smoking	3.282 (1.669–6.456)	0.001	1.67 (0.51–5.48)	0.398
eEF1A2 (negative vs positive)	1.72 (1.00–2.93)	0.048	1.16 (0.64–2.08)	0.628

Note: a, omitted by multicollinearity with other factors (pT and pN).

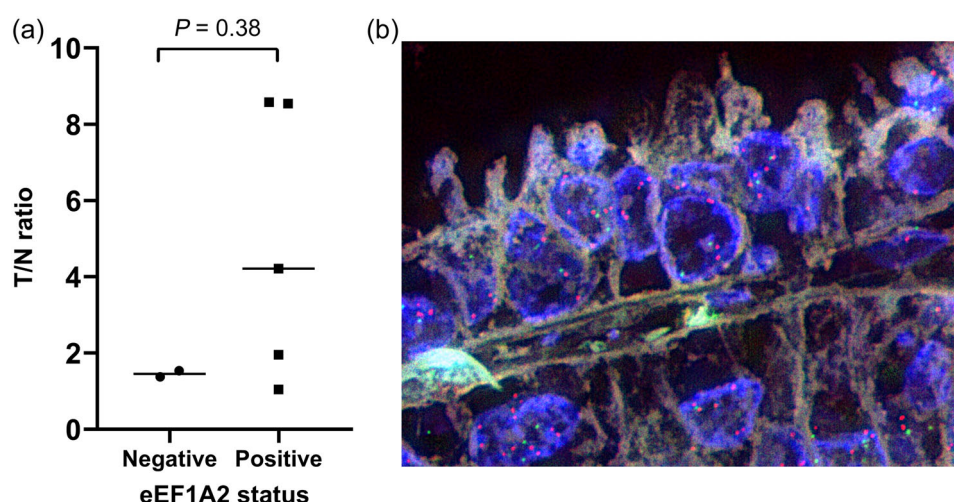


FIGURE 4 (a) Quantitative genomic PCR analysis of eukaryotic elongation factor 1 alpha 2 (eEF1A2) in lung adenocarcinoma. Five tumors immunohistochemically positive for eEF1A2 and two tumors immunohistochemically negative for eEF1A2 were subjected to the analysis. Each dot represents one tumor. A ratio (tumor/normal) of ≥ 1.5 was defined as representing gene amplification. (b) Fluorescence in situ hybridization of eEF1A2 in lung adenocarcinoma specimen. eEF1A2 amplification was detected in the tumor with increased expression of eEF1A2 DNA. Amplification of eEF1A2 was visualized as an increased number of red signals (eEF1A2) relative to green signals (Chromosome 20 centromeric probe: CEN20p).

tumor showed a mild increase of genomic eEF1A2 DNA (T/N ratio: 1.53) (Figure 4a). After the qPCR analysis, two specimens, including an eEF1A2-amplified tumor and a tumor without eEF1A2 amplification were selected and subjected to FISH analysis. This revealed that the eEF1A2-amplified tumor also showed eEF1A2 amplification (T/N ratio: 8.58), whereas the tumor without eEF1A2 amplification showed no eEF1A2 amplification (T/N ratio :1.373) (Figure 4b). The eEF1A2/CEN20p ratio was 2.19 (557/254) in the tumor with increased genomic

eEF1A2 DNA, whereas it was 1.50 in the other tumor without eEF1A2 amplification.

DISCUSSION

In this study we immunohistochemically validated the relatively high expression of eEF1A2 in invasive adenocarcinoma (39/144 cases) relative to MIA (1/10 cases) or AIS (0/21 cases). This confirmed our previous study involving proteome analysis.⁴ Although several studies

have demonstrated overexpression of eEF1A2 in invasive lung adenocarcinoma,^{20–24} the present study is the first to have compared the expression of eEF1A2 among AIS, MIA and invasive adenocarcinoma. Among the histologic subtypes, eEF1A2 expression was detected frequently in solid-type adenocarcinomas relative to other subtypes (papillary, acinar, micropapillary etc.). Since solid-type adenocarcinoma is the representative histological subtype of poorly differentiated adenocarcinoma, eEF1A2 expression may be associated with histological differentiation. Furthermore, it was noteworthy that the expression rate in lepidic adenocarcinoma (23%, 10/44) was markedly higher than in AIS and MIA (3%, 1/31). Since AIS and MIA are composed of lepidic-type adenocarcinoma, this finding suggests that overexpression of eEF1A2 occurs at the lepidic adenocarcinoma stage (well differentiated but invasive adenocarcinoma).

The increased expression of eEF1A2 in invasive carcinoma relative to AIS or MIA suggests that eEF1A2 may have a functional role in invasion. In several tumor cell lines, including hepatocellular carcinoma,^{14,15} mouse plasmacytoma,¹⁷ pancreatic cancer,¹⁹ and breast cancer,³⁰ eEF1A2 expression activates the Akt pathway and this activation suppresses apoptosis and promotes cell proliferation. In addition to its primary function in peptide chain extension, eEF1A is also known to bind to actin and act on the cytoskeleton.³⁵ It has been reported that eEF1A2 promotes the formation of cell pseudopodia and enhances cell invasion and migration in breast cancer cell lines.^{30,31} In lung cancer, it has been reported that eEF1A2 interacts with HSP90AB1 and promotes epithelial-mesenchymal transition via the TGF- β /SMAD pathway.²⁴ Although these studies do not settle the issue of whether eEF1A2 has a direct functional role in lung adenocarcinoma invasion, there is a possibility that these oncogenic properties of eEF1A2 could play some roles in invasion, and this would be an interesting subject for future research.

It is interesting to note that in our experiment, while pure AIS specimens did not show eEF1A2 expression, specimens of eEF1A2-positive invasive adenocarcinoma tended to express eEF1A2 in both the lepidic pattern and in non-lepidic patterns (Figure S2). This finding suggests that eEF1A2 is expressed in an adenocarcinoma subset with invasive capability, but that eEF1A2 expression alone might not be sufficient to confer invasive capability on individual tumor cells. Considering the multistep nature of malignant progression of lung adenocarcinoma, the process of invasion may also be multistep. If so, future research to clarify the role of eEF1A2 in invasion would be a promising avenue.

Among the histological subtypes of invasive adenocarcinoma, the predominantly solid subtype showed a higher rate of eEF1A2 positivity (47%) than the other subtypes (5%–28%) (Table 1). The solid subtype is

known to have a poorer prognosis^{36–38} and a closer association with smoking history.^{37,38} In the present study, a high proportion of patients with eEF1A2-positive tumors had a history of smoking (91.8% (34/37), Table 1), indicating some common background with the solid subtype. Molecular targeted therapy of the solid subtype is still under debate, as while the solid subtype is known to have a lower frequency of EGFR mutations^{37,38} and a higher prevalence of KRAS mutations,³⁷ it also includes a higher proportion of “pan-negative” tumors lacking seven common driver mutations.³⁷ One study has demonstrated a higher tumor mutational burden in the solid subtype.³⁸ Although the number of cases we studied was limited (34 cases), there was no significant association between EGFR mutation status and eEF1A2 expression (Supporting Information S1: Table S2). The solid subtype might consist of heterogeneous tumor populations, and further study is needed to better clarify the relationship between a solid morphology and eEF1A2 expression.

Our disease-free survival analysis showed that tumors with eEF1A2 overexpression were associated with a significantly poorer outcome (Figure 3). We found four papers that had reported on the relationship between eEF1A2 expression and prognosis in lung cancer. Three of them stated that high eEF1A2 expression indicated a poorer prognosis than low eEF1A2 expression,^{20,21,24} while Kawamura et al. found that high eEF1A2 expression was associated with a better prognosis.²³ They performed immunostaining of 50 cases of lung adenocarcinomas and 19 cases of squamous cell carcinoma ranging from Stage I to Stage III²³ using an eEF1A2 antibody different from the one we used. 82% of the adenocarcinomas were eEF1A2-positive, which was much higher than the positivity rate in the present study (26%, 40/154, excluded Stage 0) and two others (28%, 64%).^{21,24} We listed three possible reasons for this difference. First, the antibodies used by Kawamura et al. and ours were different. If the eEF1A2 antibody had reacted with eEF1A1 protein to some extent, the positive rate may have been higher than that of an antibody that reacted specifically with eEF1A2. Secondly, there is a possibility that the immunostaining method had not been optimized and that non-specific positive images were had been detected. In this study, the antigen retrieval conditions and concentration were adjusted so that positivity was detected only in areas where eEF1A2 was thought to be specifically distributed, such as cardiac muscle, pancreatic islets of Langerhans, and cerebral neurons. Kawamura et al. performed antigen retrieval under high pH conditions, and it is possible that there were nonspecific reactions. Third, the case populations may have led to differences in prognostic analysis. For example, in a study of eEF1A2 expression and prognosis in breast cancer,¹¹ eEF1A2 was

often expressed in the luminal type, and the prognosis of the luminal type was better than that of the basal type, resulting in a better prognosis for eEF1A2-expressing patients. In the present study, all cases were lung adenocarcinoma, whereas Kawamura et al. used 50 cases of lung adenocarcinoma and 19 cases of squamous cell carcinoma for prognostic analysis.

Our qPCR and FISH analyses demonstrated an increase of genomic eEF1A2 DNA in specimens of invasive adenocarcinoma (Figure 4). This result was in accord with former studies employing comparative genomic hybridization, which showed an increase of eEF1A2 gene expression in lung adenocarcinoma.^{21,22} One of those studies found a correlation between eEF1A2 gene amplification and protein overexpression.²¹ Our data also suggested a tendency for a simultaneous increase of genomic DNA and protein, although the number of cases was too small to draw a definitive conclusion. Amplification of genomic DNA is thought to be one of the mechanisms of eEF1A2 overexpression in lung adenocarcinoma.

Clinically, eEF1A2 can be a therapeutic target for treatment of malignant tumors.³⁹ Plitidepsin, an inhibitor of eEF1A2, has recently been approved in Australia as a third- or fourth-line treatment for multiple myeloma,^{40,41} and another, Metarrestine, is being tested in a phase I clinical trial for treatment of metastatic solid tumors in the United States.⁴²

In conclusion, eEF1A2 shows overexpression during the course of malignant progression of lung adenocarcinoma, and disease-free survival analysis has revealed that patients with eEF1A2-overexpressing tumors have a significantly poorer prognosis. One possible reason for the overexpression of eEF1A2 in lung adenocarcinoma may be an increase of genomic eEF1A2 DNA.

AUTHOR CONTRIBUTIONS

Mariko Yamato: Conceptualization (equal); investigation (equal); writing—original draft (lead). **Tomoko Dai:** Conceptualization (equal); investigation (equal); writing—original draft; writing—review and editing (support). **Yoshihiko Murata:** Investigation (supporting); writing—original draft (support). **Tomoki Nakagawa:** Investigation (support). **Shinji Kikuchi:** Investigation (support). **Daisuke Matsubara:** Resources (support). **Masayuki Noguchi:** Conceptualization (equal); project administration (equal); Resources (lead); supervision (lead); writing review and editing (lead).

ACKNOWLEDGMENTS

This study was supported by the Japan Society for the Promotion of Science (KAKENHI: grant numbers 19K16578 and 21K15398).

CONFLICT OF INTEREST STATEMENT

Masayuki Noguchi and Daisuke Matsubara is an Editorial Board member of Pathology International

and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication. The remaining authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yamato M, Dai T, Murata Y, Nakagawa T, Kikuchi S, Matsubara D, et al. High expression of eukaryotic elongation factor 1- α -2 in lung adenocarcinoma is associated with poor prognosis. *Pathol Int*. 2024;74:454–63. <https://doi.org/10.1111/pin.13457>