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High expression of IRE1 in lung adenocarcinoma is associated with a lower rate of recurrence

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Abstract

Objective: Recent reports have shown that endoplasmic reticulum stress is associated with cancer. However, the impacts of endoplasmic reticulum stress on the prognosis of lung cancer are unknown. Therefore, in this study, we sought to reveal the relationship between the expression of endoplasmic reticulum stress-related genes (endoplasmic reticulum oxidoreductase 1L, protein kinase RNA-like endoplasmic reticulum kinase, activating transcription factor 6 and inositol-requiring kinase 1) and the outcome of lung adenocarcinoma.

Methods: One hundred and twenty-six patients with surgically resected lung adenocarcinomas were subjected to an endoplasmic reticulum stress-related mRNA expression analysis using quantitative RT-PCR. The following parameters were analyzed for all the study patients: age, sex, disease stage, smoking status, lymph node invasion (ly), vascular invasion (v) and EGFR mutation status. We assigned patients to either a high-expression group or a low-expression group according to the expression levels of endoplasmic reticulum stress-related genes.

Results: High expressions of endoplasmic reticulum stress-related genes were observed in patients with lower stages of lung adenocarcinoma and minimal vascular invasion. A Kaplan–Meier analysis showed significant differences in recurrence-free survival and overall survival between high-expression group and low-expression group. High inositol-requiring kinase 1 expression was an independent predictor of recurrence-free survival among patients with lung adenocarcinoma (hazard ratio, 0.396; 95% confidence interval, 0.188–0.834; $P = 0.015$).

Conclusions: Inositol-requiring kinase 1 may be a useful biomarker to predict recurrence in surgically resected lung adenocarcinoma patients.

Key words: non-small-cell lung cancer, lung adenocarcinoma, endoplasmic reticulum stress, inositol-requiring enzyme 1 (IRE1), prognosis

Introduction

Non-small-cell lung cancer (NSCLC) is the most common cause of cancer-related mortality worldwide (1). Despite advances in chemotherapy, radiation and surgery, the prognosis of NSCLC is generally poor, with a 5-year survival rate of 44% (2–4). Multiple processes are involved in the development of NSCLC, such as carcinogenesis, proliferation, invasion and the distant metastasis of cancer cells (5,6). Various types of stresses are exerted on cancer cells through these processes, resulting in the accumulation of unfolded proteins in the endoplasmic reticulum (ER), as demonstrated by reports that

ER stress markers are overexpressed in cancer (7). To overcome ER stress, cells upregulate unfolded protein responses (UPR) (8). UPR is an ER-specific cellular stress response that has been found to be conserved in eukaryotic cells. Recent reports have shown that ER stress is associated with cancer. High proliferation rates and mutated gene products lead to the accumulation of unfolded proteins in the ER, and adaptation to ER stress is essential for the survival of cancer cells. The disruption of ER homeostasis triggers UPR, which arrests protein translation and activates signaling pathways for molecular chaperones to assist protein folding and to direct the degradation of misfolded proteins (9,10). The prolongation of UPR can also lead to apoptosis in a caspase-dependent manner. UPR involves the activation of several proteins, including protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring kinase 1 (IRE1) (11). The activation of PERK phosphorylates eukaryotic translation initiation factor-2α (eIF-2α), which suppresses protein synthesis (12). The activation of RNase IRE1 initiates the splicing of X-box transcription factor-1 (XBP-1) mRNA into spliced variant XBP-1, which is subsequently translated into a potent transcription factor. The combination of ATF6 and the spliced variant XBP-1 positively regulates a wide variety of UPR target gene expressions, including several ER resident chaperones. ER oxidoreductase 1L (ERO1L) has been identified as a reoxidizer of protein disulfide isomerases (PDIs), which functions as a disulfide-introducing enzyme for secretory and cell-surface molecules in the cell. The ER is where proteins form disulfide bonds through an efficient electron relay driven by the family of PDIs. During this process, PDIs directly oxidize new proteins and are themselves reduced.

Recent studies have shown that ER stress has a dual role, either promoting cell survival or triggering cell death depending on the imbalance between the ER protein folding load and capacity (13). Moderate ER stress promotes cancer cell survival and enhances chemotherapeutic resistance; however, severe ER stress leads to cancer cell apoptosis (14). In addition, ER stress and autophagy are involved in the apoptosis induced by cisplatin in lung cancer cells (15). In the present study, we aimed to characterize the expression of ER stress-related genes (*ERO1L, PERK, ATF6* and *IRE1*) in surgically resected lung adenocarcinoma.

Table 1. Patient characteristics

	N	%
Age		
Median (range)	68 (36–86)	
Sex		
Male	63	50.0
Female	63	50.0
Stages		
IA	43	34.1
IB	43	34.1
II	18	14.3
III	16	12.7
IV	6	4.8
Smoking status		
Smoker	61	49.2
Never and light smoker	63	50.8
ly		
ly (−)	95	77.2
ly (+)	28	22.8
v		
v (−)	69	56.1
v (+)	54	43.9
EGFR mutation		
Mt	46	36.5
(EGFR-TKI use	16	12.7)
WT	68	54.0
Unknown	12	9.5
Adjuvant chemotherapy		
UFT	26	20.6
Platinum doublet	20	15.9
None	80	63.5

Patients and methods

Study participants

Data were retrospectively collected from 126 patients with lung adenocarcinoma who underwent lung resection at the University of Tokyo Hospital (Tokyo, Japan) between March 2007 and June 2011. All the patients were followed up until March 2016. The following parameters were recorded and analyzed for all the study patients: age, sex, disease stage, smoking status, lymph node invasion (ly), vascular invasion (v) and EGFR status. Recurrence-free survival (RFS) was defined as the time period from the date of lung resection until the date of radiologic evidence of disease recurrence. Overall survival (OS) was defined as the time period from the date of lung resection until the date of death or last recall. This study was approved by the Institutional Review Board at the University of Tokyo Hospital, and informed consent was obtained from each patient.

Measurements of ER stress-related gene expression using quantitative RT-PCR

Lung specimens were fresh frozen tissues collected from lung adenocarcinoma patients. Total RNA was isolated using RNAiso plus

Table 2. Associations between clinicopathological features and expressions of ER stress-related genes in 126 lung adenocarcinoma patients

	ERO1L			PERK			ATF6			IRE1		
	High	Low	*P* value	High	Low	*P* value	High	Low	*P* value	High	Low	*P* value
Patient number	62	62		63	62		61	61		62	62	
Sex (male/female)	31/31	32/30	0.852	32/31	31/31	0.929	34/27	28/33	0.277	32/30	31/31	0.857
Stages (IA, IB/II, III and IV)	45/17	40/22	0.334	50/13	36/26	0.010*	50/11	34/27	0.002*	48/14	37/25	0.033*
Smoking status (never and light/smoker)	30/31	31/30	0.856	30/32	32/29	0.652	29/32	31/28	0.584	34/28	27/33	0.277
ly (ly−/ly+)	49/13	45/14	0.715	50/11	45/16	0.276	51/9	43/16	0.105	51/10	43/17	0.115
v (v−/v+)	41/21	27/32	0.024*	44/17	25/36	0.001*	46/13	22/37	<0.001*	42/19	26/34	0.005*
EGFR status (Mt/WT)	25/31	21/35	0.442	24/33	22/34	0.760	24/30	21/36	0.415	26/30	20/36	0.249

*$P < 0.05$, the proportion was significantly different between the groups when examined using a Pearson chi-square (χ^2) test.

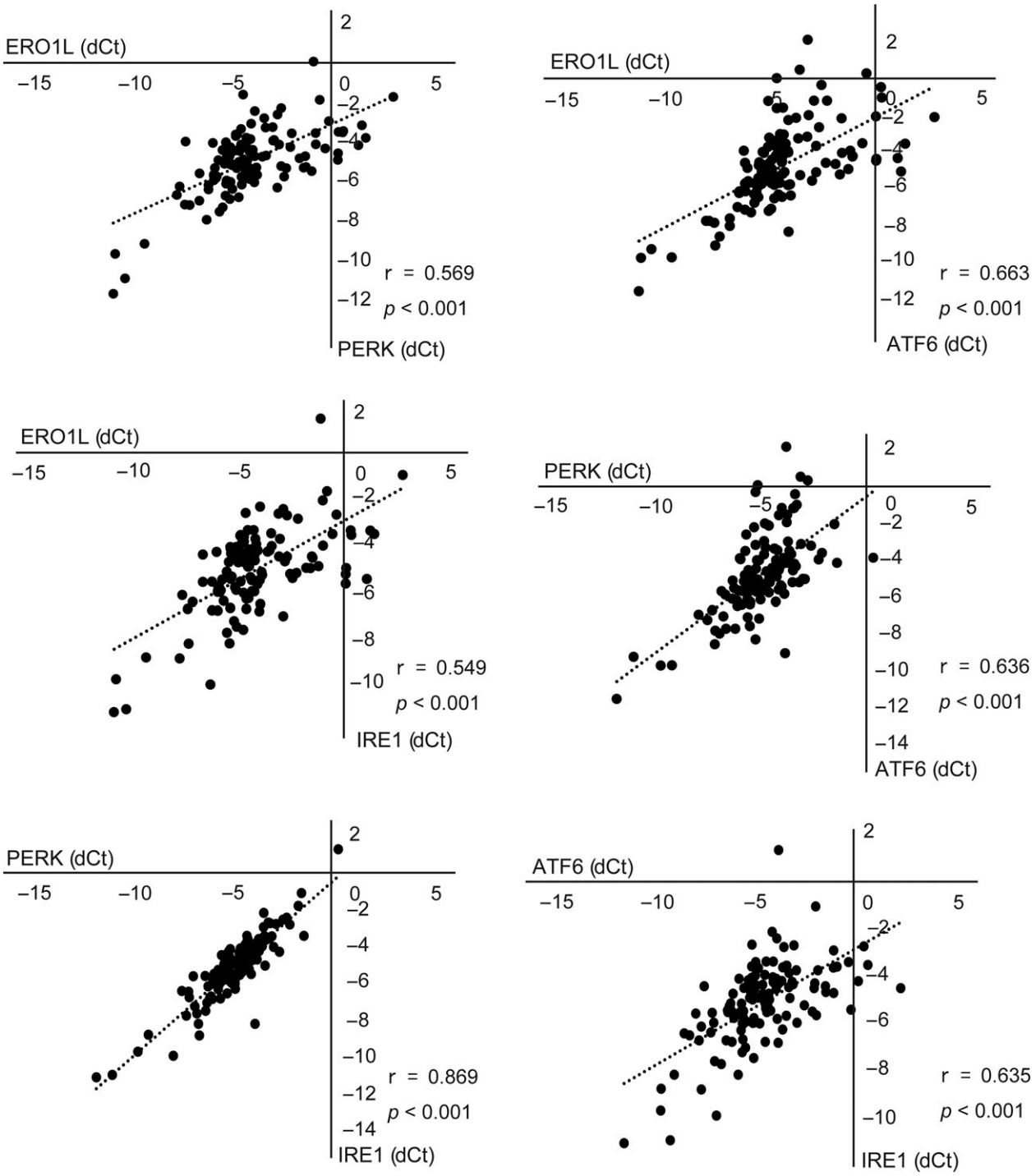

Figure 1. mRNA expression levels of different endoplasmic reticulum (ER) stress-related genes. The scatter plot suggests a definite relationship between the two different gene expressions. A positive correlation between the two variables appears to exist when examined using the Pearson correlation coefficient. Of note, the relationship between the two variables appears to be linear ($P < 0.001$).

reagent (TaKaRa Bio, Japan), according to the manufacturer's instructions. A total of 1 μg of total RNA was reverse-transcribed into cDNA using SuperScript III (Thermo Fisher Scientific, Waltham, MA). Optimized primers targeting each gene and GAPDH were designed using the Primer Analysis Software (OLIGO; Molecular Biology Insights, Inc.). cDNA was amplified using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The comparative quantification cycle threshold (Ct) method was used to determine the relative expression levels of the target genes using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primer sets (final concentration for each primer, 400 nM) were used in a final volume of 16 μL per well. The thermal profile used for qRT-PCR was 95°C for 1 min, 35 cycles of 95°C for 15 s and 60°C for 30 s, and 72°C for 45 s. Dissociation curves were obtained after the last PCR cycle. Background corrected

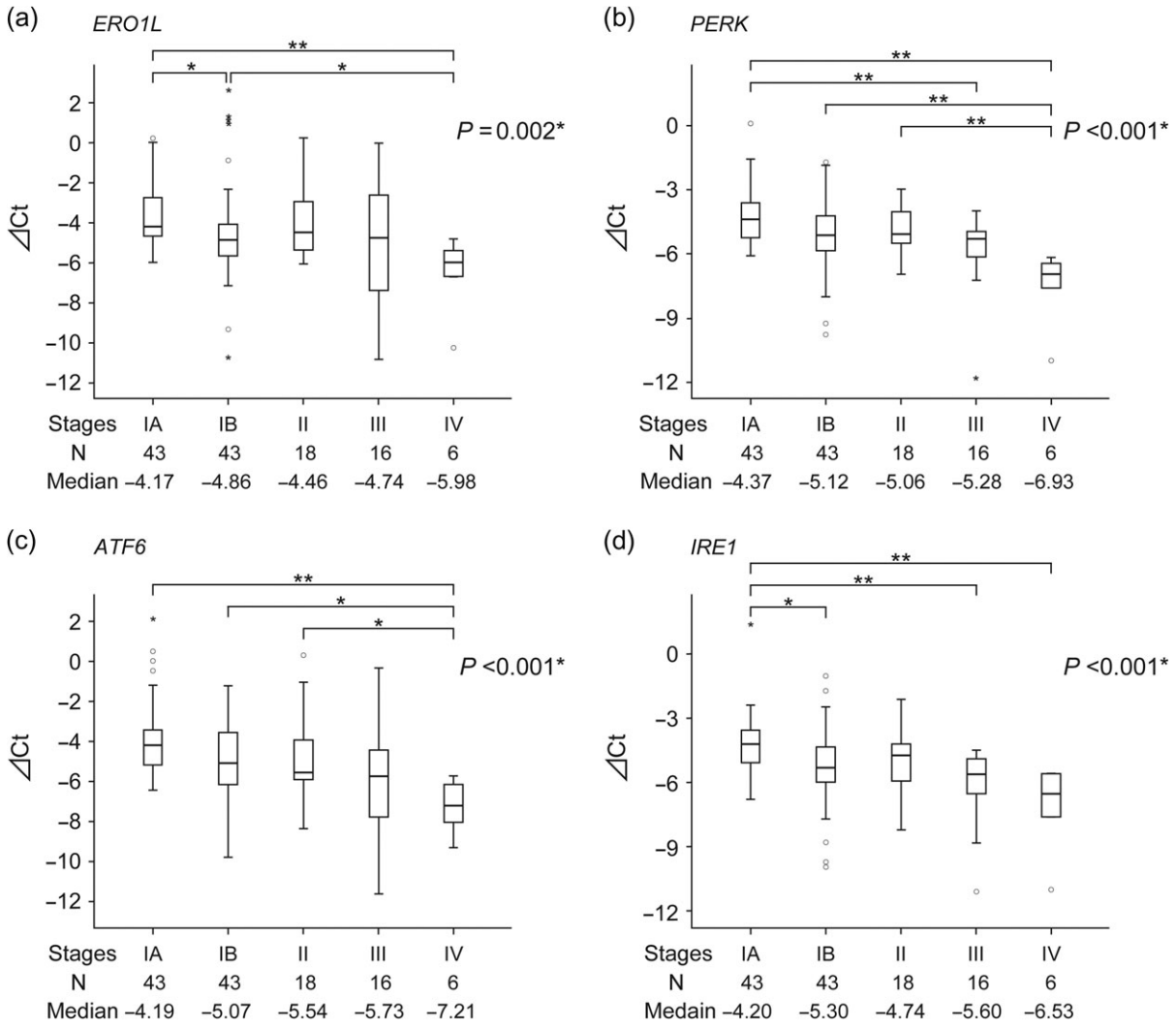

Figure 2. mRNA expression level of ER stress-related genes according to each disease stage. The box plot suggests a definite relationship between disease stage and the mRNA expression levels of ER stress-related genes: (a) the *ERO1L* expression level, (b) the *PERK* expression level, (c) the *ATF6* expression level and (d) the *IRE1* expression level. Significant differences between the means for different disease stages were observed using the Kruskal-Wallis test, Mann–Whitney *U* test and Bonferroni correction (*$P < 0.05$, **$P < 0.01$).

raw fluorescence data were analyzed using 7500 software v2.3. The relative expression level of each sample was determined after normalization to GAPDH using the ddCt method (16). The cycle number difference (dCt) was calculated for each replicate. Relative target gene expression values were calculated using the mean dCt of three replicates. qRT-PCR was performed using the following primer sets: GAPDH (F, CACCACCAACTGCTTAGCAC; R, TGGCAGG TTTTCTAGACGG), ERO1L (F, GACTTATATCTGGCCTACATG CAA; R, GGGCGCTCGAAGAATGGTAAC), PERK (F, GGCCAC TTTGAACTTCGGTAT; R, CTCCCTTCTTACTGAATGCCATAA CT), ATF6 (F, TCAGACAGTACCAACGCTTATGC; R, TAGGACA GGTTTAGTCACGGAAAG) and IRE1 (F, CTCCAGACAGA CCTGCGTAA; R, GAAGCGAGATGTGAAGTAGCAC).

Statistical analysis

The statistical analysis was performed using the SPSS statistical package, version 20 (SPSS, Inc., Chicago, IL). The Pearson chi-square (χ^2) test was used for multiple comparisons of different expressions. One-way ANOVA and Tukey HSD was used for multiple comparisons of the mRNA expression levels of ER stress-related genes for each disease stage. We confirmed the data were normally distributed before performing one-way ANOVA. The Kaplan–Meier method was used to analyze RFS and OS, and the log-rank test was used to examine any differences in survival. Univariate and multivariate analyses were used to study the associations among variables (age, sex, disease stage, smoking status, ly, v, EGFR mutation status and ER-related genes mRNA expressions). The multivariate analysis was performed using the Cox proportional hazards model. Differences were considered significant when the *P* value was <0.05.

Results

Clinical features of lung adenocarcinoma patients

The clinical features of all 126 patients are shown in Table 1. The median age was 68 years (range, 36–86 years), and 63 patients (50.0%) were male. The most common clinical stages were Stage IA and Stage IB (43 patients each, 34.1%). Smokers accounted for

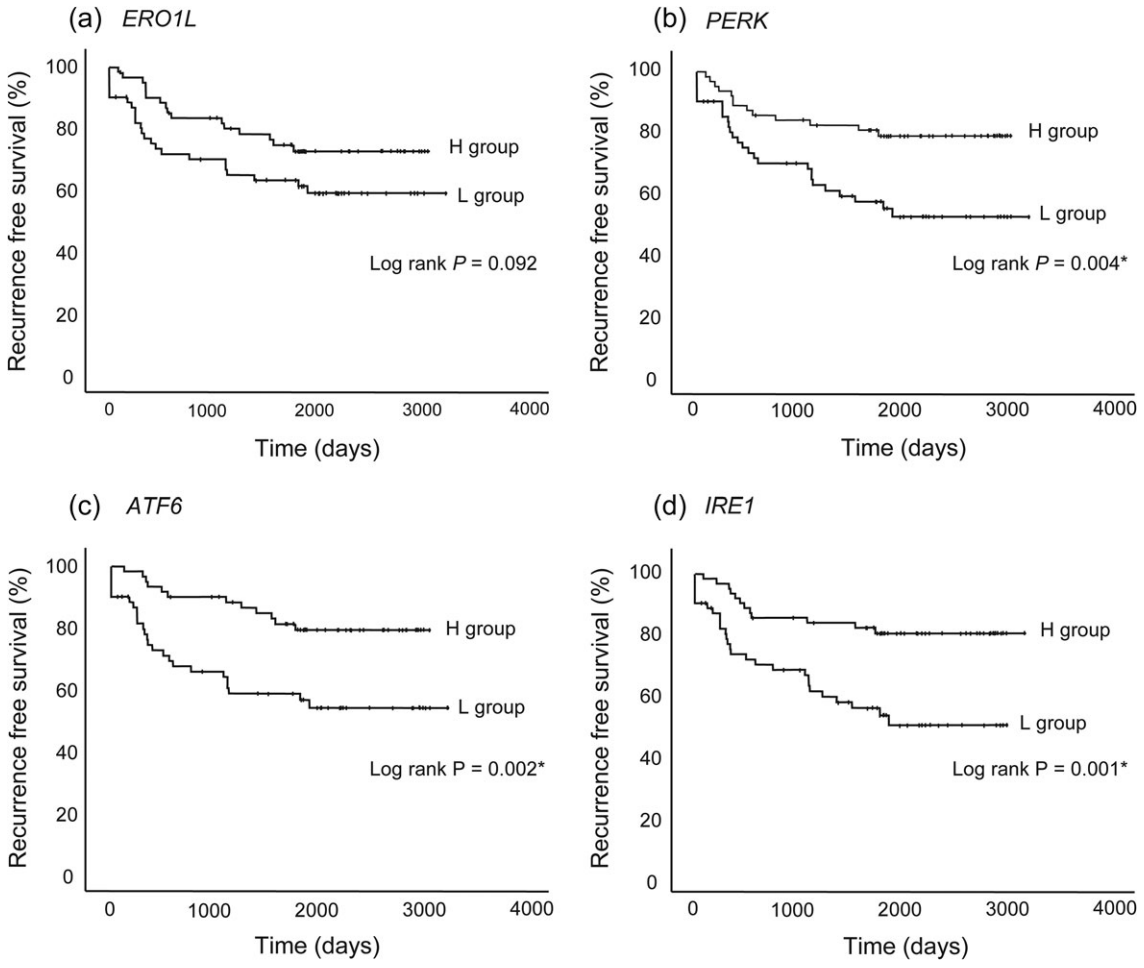

Figure 3. Recurrence-free survival (RFS) analysis of 126 lung adenocarcinoma patients stratified according to (a) the *ERO1L* expression level, (b) the *PERK* expression level, (c) the *ATF6* expression level or (d) the *IRE1* expression level (*$P < 0.05$).

approximately half of all the patients. Positive ly results were seen in 28 patients (22.8%), and positive v results were seen in 54 patients (43.9%). Forty-six patients (36.5%) had EGFR gene mutations, 68 patients (54.0%) had wild-type EGFR and 10 patients (9.5%) had an unknown EGFR mutation status. Of the 46 patients who had EGFR gene mutations, 16 patients (12.7%) used EGFR tyrosine kinase inhibitors (EGFR-TKI) after recurrence or because they were Stage IV. Postoperative adjuvant chemotherapy was administered to patients according to established guidelines. UFT was administered to 26 patients (20.6%) who mostly had Stage IB disease, platinum combination therapy was administered to 20 patients (15.9%) who mostly had Stage II or IIIA disease.

Expression of ER stress-related genes is associated with stage and vascular invasion

The associations between clinicopathological features and the expressions of ER stress-related genes in 126 lung adenocarcinoma patients are shown in Table 2. We assigned patients to either a high expression group (H group) or a low expression group (L group) according to their expression levels of ER stress-related genes: patients with an expression level higher than the median value were assigned to the high expression group, while those with an expression level lower than the median were assigned to the low expression group. We found that the tumor stage and v factor were significantly associated with the mRNA expression levels of ER stress-related genes. No correlations between the mRNA expression levels of ER stress-related genes and sex, smoking status, ly or EGFR mutation status were seen.

Next, we examined the relationships between the expression levels of ER stress-related genes using the Pearson correlation coefficient (Fig. 1). A scatter plot suggested a positive relationship between different gene expressions. A strong correlation was observed between the expression levels of PERK and IRE1 ($r = 0.899$, $P < 0.001$).

Additionally, the expression levels of ER stress-related genes tended to decrease as the disease stage increased (Fig. 2). Each gene showed statistically significant differences between the means of groups with different disease stages using one-way ANOVA (ERO1L, $P = 0.016$; PERK, $P < 0.001$; ATF6, $P = 0.001$; IRE1, $P < 0.001$). Therefore, we suspected that there might be an association between the prognosis of lung adenocarcinoma patients and the expression of ER stress-related genes.

IRE1 mRNA expression is a predictor of recurrence

A Kaplan–Meier analysis showed a significant difference in RFS and OS between groups H and L (Figs 3 and 4). Notably, even among

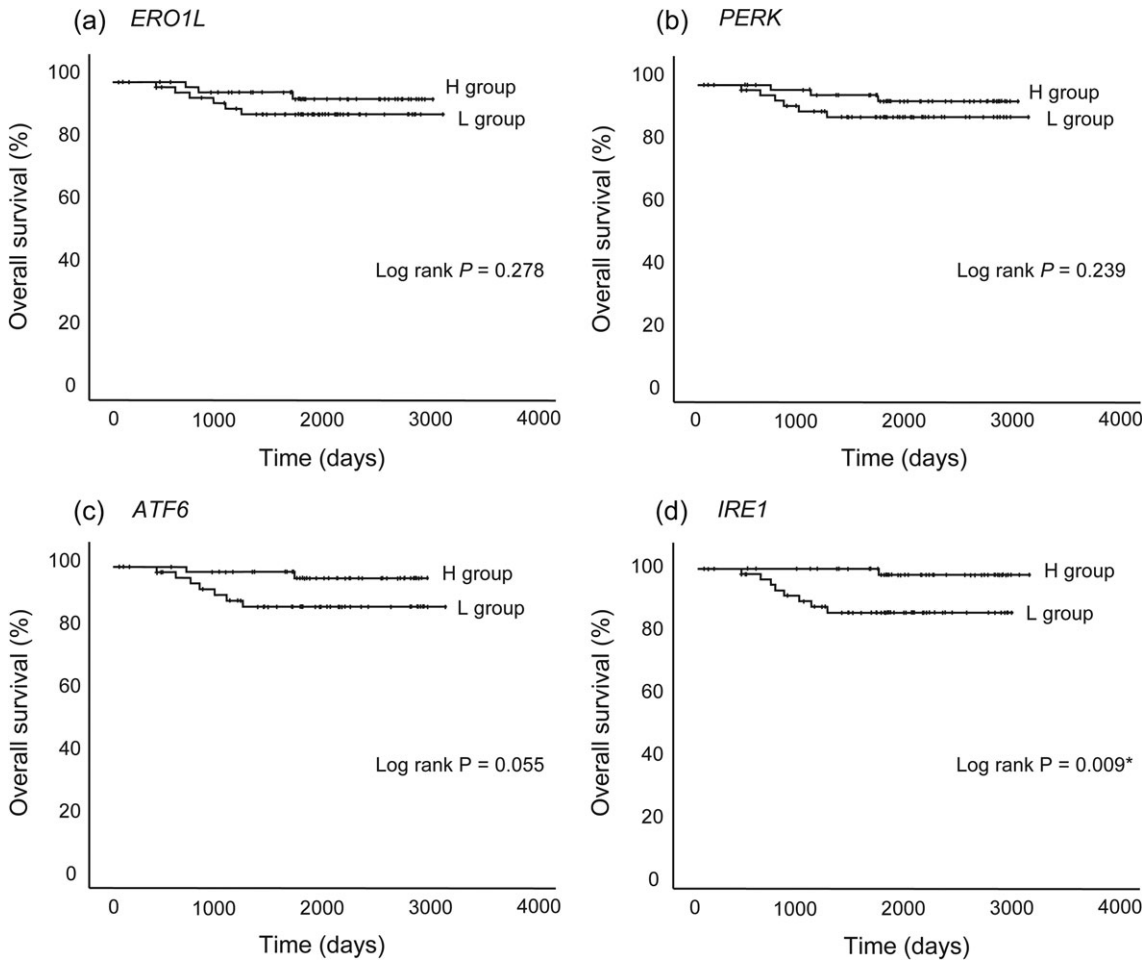

Figure 4. Overall survival (OS) analysis of 126 lung adenocarcinoma patients stratified according to (a) the *ERO1L* expression level, (b) the *PERK* expression level, (c) the *ATF6* expression level or (d) the *IRE1* expression level (*$P < 0.05$).

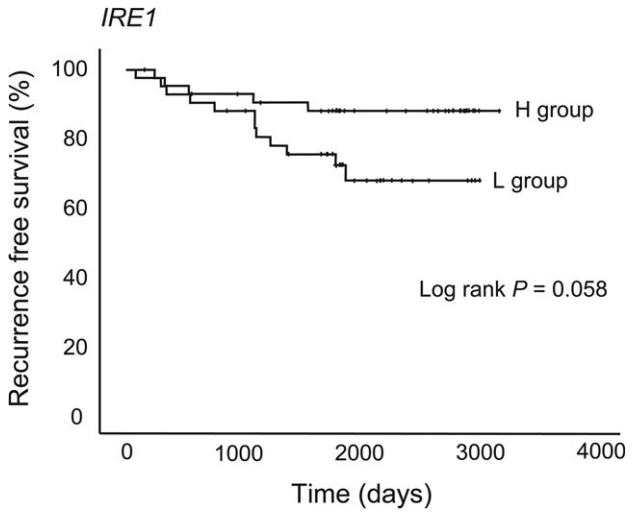

Figure 5. RFS analysis of 86 Stage I lung adenocarcinoma patients stratified according to the *IRE1* expression level.

patients with Stage I lung adenocarcinoma, our results indicated that a low IRE1 expression level might be a predictor of a poor prognosis ($P = 0.058$, Fig. 5). Patients with high PERK, ATF6 and IRE1 expressions had a significantly longer RFS, and patients with a high IRE1 expression had a significantly longer OS. Similar results were obtained in the population without EGFR-TKI treatment or adjuvant chemotherapy (data not shown), excluding the influence of such anti-cancer drug therapies on RFS and OS.

Univariate analysis indicated that disease stage, ly, v, PERK, ATF6 and IRE1 were statistically significant risk factors for poor RFS (Table 3, Fig. 3), while disease stage, v and IRE1 were statistically significant risk factors for poor OS (Table 3, Fig. 4).

Multivariate analysis of RFS was performed using PERK, ATF6, IRE1, disease stage, ly and v. As a result, IRE1, v and disease stage showed significant differences, with hazard ratios of 0.4-fold, 2.8-fold and 3.8-fold, respectively ($P = 0.015$, 0.008 and <0.001, respectively, Table 4). Moreover, a multivariate analysis of OS was performed for IRE1, v and disease stage; only v showed a significant difference, with an 11.8-fold increase in the hazard ratio ($P = 0.020$).

Discussion

In this study, we analyzed the expressions of ER stress-related genes in surgically resected specimens obtained from patients with lung adenocarcinoma. Our Kaplan–Meier survival analysis indicated that lung adenocarcinoma patients with high ER stress-related gene

Table 3. Univariate analyses of RFS and OS in lung adenocarcinoma patients

	RFS				OS			
	N	Mean (days)	95% CI	*P* value	*N*	Mean (days)	95% CI	*P* value
Age								
≥70	57	2396	2111–2681	0.137	57	2804	2632–2977	0.432
<70	69	2172	1860–2486		69	3071	2935–3207	
Sex								
Male	63	2158	1859–2458	0.560	63	2848	2688–3008	0.636
Female	63	2400	2087–2713		63	3060	2914–3206	
Stages								
I	86	2754	2548–2960	<0.001*	86	3135	3047–3224	0.010*
II, III, IV	40	1282	907–1657		40	2529	2271–2786	
Smoking status								
Smoker	61	2299	2008–2590	0.522	63	2815	2645–2985	0.261
Never and light	63	2284	1963–2604		63	3097	2967–3226	
ly								
ly (−)	95	2679	2462–2897	<0.001*	95	3090	2983–3196	0.079
ly (+)	28	1031	664–1397		28	2748	2414–3081	
v								
v (−)	69	2692	2488–2896	<0.001*	69	3012	2955–3070	0.004*
v (+)	54	1671	1305–2036		54	2835	2594–3077	
EGFR								
MT	46	2216	1817–2614	0.770	49	3103	2954–3253	0.322
WT	68	2233	1957–2508		68	2843	2691–2508	
ERO1L								
H group	62	2425	2159–2690	0.092*	62	3006	2884–3128	0.278
L group	62	2130	1785–2475		62	2963	2773–3154	
PERK								
H group	63	2527	2275–2779	0.004*	63	3013	2898–3127	0.239
L group	62	2003	1658–2348		62	2957	2762–3152	
ATF6								
H group	61	2600	2373–2828	0.002*	61	2971	2880–3062	0.055
L group	61	1994	1638–2351		61	2906	2692–3119	
IRE1								
H group	62	2711	2452–2971	0.001*	62	3184	3128–3241	0.009*
L group	62	1853	1524–2181		62	2723	2517–2928	

RFS, recurrence-free survival; OS, overall survival, *$P < 0.05$.

Table 4. Multivariate analyses of RFS in lung adenocarcinoma patients

	RFS			
	N	Relative risk	95% CI	*P* value
v				
v (+)	54	2.824	1.315–6.064	0.008*
v (−)	69			
Stages				
II, III, IV	40	3.758	1.907–7.407	<0.001*
I	86			
IRE1				
High expression	62	0.396	0.188–0.834	0.015*
Low expression	62			

*$P < 0.05$.

expressions had a significantly longer survival period. High IRE1 expression levels in lung adenocarcinoma were also identified as an independent predictor of a favorable prognosis based on the results of a multivariate Cox hazard regression analysis. Our results indicated that IRE1 might be a useful marker for predicting survival in patients with surgically resected lung adenocarcinoma. Notably, even among patients with Stage I lung adenocarcinoma, our results indicated that a low IRE1 expression level might be a predictor of a poor prognosis.

High IRE1 expression level was also strongly correlated with high expression levels of other ER stress-related gene. Interestingly, the correlation between IRE1 expression and PERK expression was strongest. IRE1 and PERK are both transmembrane proteins and the structures are very similar. In addition, the activation of both IRE1 and PERK is caused by the withdrawal of chaperon protein, BiP/GRP78. One report has indicated that the dynamics of IRE1 and PERK signaling events is critical to determining cellular outcome (17). Our finding again show that IRE1 and PERK may be the two ER stress pathways most closely regulated.

It remains controversial whether ER stress-related genes correlate with promoting cancer cell survival or tumor regression. Some reports have indicated that a high ERO1L expression level is associated with a poor prognosis in patients with breast cancer or gastric cancer (18,19). Another report indicated that an IRE1 inhibitor reversed drug sensitivity in breast cancer (20).

In contrast, ER stress may be associated with a favorable prognosis in NSCLC. For example, expression of the ER stress chaperon protein calreticulin in NSCLC was reported to correlate with a favorable prognosis (21). In addition, expression of ER

stress-related genes in tumor cells may promote the activity of anti-tumor immune cells (21,22). Our experimental results are consistent with these reports.

These conflicting results on prognosis likely reflect differences in the role of ER stress-related genes in different organs, and our results indicating that expression of ER stress-related genes correlates with vascular invasion may provide a clue. Unlike breast cancer and gastric cancer, lung cancer has a much poorer survival rate, indicating that vascular invasion by tumor cells and subsequent metastasis occur more frequently. Suppression of vascular invasion through higher expression of ER stress-related genes may lead to better prognosis in NSCLC but not in breast or gastric cancer because vascular invasion occurs less frequently. Some reports have indicated that ER stress-related genes are associated with angiogenesis (18,23) and extracellular matrix (ECM) production (24). ER stress-related genes may promote vascular invasion through angiogenesis or ECM production.

The relationship between ER stress and the prognosis of lung adenocarcinoma remains unclear. The prognosis of patients with lung adenocarcinoma has never been analyzed using such a large number of clinical specimens. Here, we showed that in lung adenocarcinoma, a high expression of ER stress-related genes is associated with a lower rate of recurrence.

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Conflict of interest statement

None declared.

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