Original Article

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, 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 (EROS1L) 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 (EROS1L, PERK, ATF6 and IRE1) in surgically resected lung adenocarcinoma.

### 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 (vi), 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 1. Patient characteristics

| N | %  |
|---|----|
| Age | 68 (36–86) |
| Median (range) | |
| 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 |
| vi | |
| vi (−) | 69 |
| 56.1 |
| vi (+) | 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 |

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

| ERO1L | PERK | ATF6 | IRE1 |
|---|---|---|---|
| Patient number | High | Low | P value | High | Low | P value | High | Low | P value | High | Low | P value |
| 62 | 62 | 0.902 | 63 | 62 | 0.929 | 61 | 61 | 0.877 | 62 | 62 | 0.877 |
| Sex (male/female) | 31/31 | 32/30 | 0.852 | 31/31 | 32/30 | 0.892 | 50/13 | 36/26 | 0.010* | 48/14 | 37/25 | 0.033* |
| Stages (IA, IBII, III and IV) | 45/17 | 40/22 | 0.334 | 50/13 | 36/26 | 0.010* | 48/14 | 37/25 | 0.033* | 51/9 | 43/16 | 0.105 |
| 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−−) | 49/13 | 45/14 | 0.715 | 50/11 | 45/16 | 0.276 | 51/9 | 43/16 | 0.105 | 51/10 | 43/17 | 0.115 |
| vi (vi−−) | 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 (χ²) test.
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 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).
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. 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, TGGCAGATTCTAGACGG), ERO1L (F, GACTTATATCTGGCCTACATGCAA; R, GGGCGCTCGAAGAATGGTAAC), PERK (F, GCCACATTGGAGATGTGAAGTAGACA; R, CTCCCTTCTTACTGAATGCCATA), ATF6 (F, TAGGAGTTGAGATGTGAAGTAGACA) and IRE1 (F, CTCCAGACAGACCTGCGTAA; R, GAAGCGAGATGTGAAGTAGACA).

Statistical analysis
The statistical analysis was performed using the SPSS statistical package, version 20 (SPSS, Inc., Chicago, IL). The Pearson chi-square ($\chi^2$) 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
approximately half of all the patients. Positive results were seen in 28 patients (22.8%), and positive 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
patients with Stage I lung adenocarcinoma, our results indicated that a low IRE1 expression level might be a predictor of a poor prognosis \( (P = 0.058, \text{Fig. 5}) \). 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 \( (P = 0.015, 0.008 \text{ and } <0.001, \text{respectively, Table 4}) \). 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
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

| 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  | 2399        | 2008–2590  | 0.522   | 61  | 3071        | 2935–3207  | 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  |         |
| EGFR                       |     |             |            |         |     |             |            |         |
| MT                         | 46  | 2216        | 1817–2614  | 0.770   | 46  | 3013        | 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        | 2989–3127  | 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                       |     |             |            |         |     |             |            |         |
| High expression            | 62  | 2711        | 2452–2971  | 0.001*  | 62  | 3184        | 3128–3241  | 0.009*  |
| Low expression             | 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 |
| ly                         |     |               |            |         |
| 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.
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 corre-
lates 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 angiogen-
esis (18,23) and extracellular matrix (ECM) production (24). 
ER stress-related genes may promote vascular invasion through angi-
genesis or ECM production.

The relationship between ER stress and the prognosis of lung 
adenoarcinoma 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 adeno-
carcinoma, 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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