Int J Clin Exp Pathol 2017;10(3):3315-3326
www.ijcep.com /ISSN:1936-2625/IJCEP0032832

Original Article

Endoplasmic reticulum stress sensor GRP78/BiP expression in lung adenocarcinoma: correlations and prognostic significance

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Received May 26, 2016; Accepted July 25, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Background: Endoplasmic reticulum (ER) stress sensor glucose-related protein 78 (GRP78)/BiP is an important member of the heat shock protein family 70 (HSPs70) that plays an essential role in tumor growth and progression. Although GRP78/BiP is highly expressed by various cancer cells, the clinicopathological significance of its expression in non-small cell lung cancer (NSCLC) remains unclear. The aim of the present study was to investigate GRP78/BiP expression in patients with lung adenocarcinoma. Patients and Methods: Two hundred and twenty patients with surgically resected lung adenocarcinoma were evaluated as an institutional cohort. Tumor sections were stained by immunohistochemistry for GRP78/BiP, PERK, Ki-67, phospho-mTOR (p-mTOR), and CD34 to assess microvessel density. Results: GRP78/BiP was highly expressed in 41% of patients and significantly associated with pleural invasion, lymphatic permeation, vascular invasion, cell proliferation, and p-mTOR phosphorylation. Multivariate analysis confirmed that GRP78/BiP expression was an independent factor for predicting poor progression-free survival and overall survival in patients with stage I disease. Conclusions: Increased GRP78/BiP expression is an independent prognostic factor for patients with early stage lung adenocarcinoma. Our study suggests that GRP78/BiP expression as an ER stress marker plays a crucial role in lung adenocarcinoma pathogenesis and development.

Keywords: BiP, endoplasmic reticulum stress, glucose-related protein 78, immunohistochemistry, non-small cell lung cancer, prognosis

Introduction

In 2012, lung cancer was the most frequently diagnosed cancer and the leading cause of cancer-related death in men worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, and more than 50% of patients with NSCLC are diagnosed at advanced disease stages [2]. The NSCLC group can be further divided into histologic subtypes with adenocarcinoma, squamous cell carcinoma, and large cell carcinoma being the most common at 40%, 27%, and 8% of all lung cancers, respectively [3]. Surgical removal is often the choice of treatment in primary lung cancer, but widespread dissemination of the cancer often defeats this treatment mode. At the time of diagnosis, identifying negative prognostic factors is useful for identifying the patients with tumors who are at high risk of treatment failure. Many pre-operative variables that affect the survival of patients with NSCLC have been identified [4]. However, no established biomarker has been identified as a post-treatment predictor in patients with NSCLC.

The glucose-regulated protein GRP78, a 78-kDa protein, also referred to as immunoglobulin heavy chain binding protein (BiP), is a major molecular chaperone at the endoplasmic reticulum (ER) that has been characterized on cell membranes and in the cytoplasm [5]. GRP78

was involved in the folding and assembly of newly synthesized proteins in the ER and increased resistance to ER stress-induced apoptosis [5-7]. The level of GRP78 is highly elevated in many cancer cells and human cancers and closely associated with malignancy, metastases, and chemotherapy resistance [6, 7]. There have been only a few studies about the prognostic significance of GRP78/BiP for various patients with breast, lung, gastric, hepatocellular, or prostate cancer [7]. In one study of lung cancer, no significant difference was observed between GRP78/BiP expression and sex, age at surgery, histological type, pathologic stage, pathologic T status, or pathologic N status [8]. However, that study indicated that positive GRP78/BiP expression is a significant factor for predicting a favorable prognosis in patients with lung cancer [8]. Based on these reports, it remains unclear whether positive GRP-78/BiP expression could predict more favorable prognosis than negative expression.

Controversy persists about the prognostic significance of positive GRP78/BiP expression. Moreover, PKR-like ER kinase (PERK) is considered a sensor of ER stress [9]. PERK reportedly induces apoptosis via CCAAT/enhancer-binding protein homologous protein (CHOP) accumulation under irremediable ER stress, and its inhibition leads to ER stress-driven cell death [9]. Vamdexynckel et al documented that the PERK pathway was activated during tumor progression and that the pro-apoptotic target CHOP was upregulated, and a small-molecule PERK inhibitor could be a promising target for cancer therapy [9]. Although GRP78/BiP and PERK are known ER stress markers, it remains undetermined whether GRP78/BiP can predict a favorable or unfavorable prognosis after surgery in patients with NSCLC. Although GRP78/BiP may work as a contrary mechanism among different cancer types, it remains obscure how it works to play a crucial role in carcinogenesis and pathogenesis. Its role in outcomes varies among histological types, which prevents us from reaching a conclusion.

Since the expression profile of GRP78/BiP in adenocarcinoma seems to be different from that of squamous cell carcinoma, the clinical significance of GRP78/BiP or PERK expression should be analyzed according to the histological types. Here we evaluated whether GRP78/BiP and PERK are ER stress-related markers for patients with lung adenocarcinoma. This clinicopathological study investigated GRP78/BiP expression in resected lung adenocarcinoma tissue samples. The aims of our study were to clarify whether GRP78/BiP expression was closely associated with post-treatment outcomes, explore the correlation between GRP-78/BiP and patient clinical characteristics, and assess the correlation between GRP78/BiP expression and PERK expression, the Ki-67 labeling index, microvessel density (MVD) (determined by CD34), and phospho-mTOR (p-mTOR) phosphorylation.

Patients and methods

Patients

Here we analyzed 233 consecutive patients with lung adenocarcinoma (stages I-III) who underwent resection by lobectomy or pneumonectomy with mediastinal lymph node dissection at Gunma University Hospital (Maebashi, Gunma, Japan) between July 2002 and December 2010. Of these patients, 13 were excluded from the subsequent analyses because their tissue specimens were not available; thus, a total of 220 patients were enrolled in this study. As a control, we stained samples from 30 patients with squamous cell carcinoma.

The authors' approach to tumor evaluation and resection was described previously [10]. As postoperative adjuvant chemotherapy, platinum-based regimens and the oral administration of tegafur (a fluorouracil derivative drug) were administered to 13 and 50 patients, respectively. Baseline characteristics, including age, sex, smoking status, tumor differentiation, pathological stage, pleural invasion, lymphatic permeation, and vascular invasion were retrospectively obtained from the patients' medical charts. The study protocol was approved by the institutional review board. The tumor specimens were histologically classified according to World Health Organization criteria. The pathological tumor-node-metastasis stages were established using the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer [11]. The day of the surgery was considered the first day after surgery. The follow-up duration was 69-3571 days (median, 1490 days).

Immunohistochemical staining

GRP78/BiP and PERK were detected using a rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA; 1:100 dilution) and a mouse polyclonal antibodies (Cell Signaling Technology; 1:100 dilution), respectively. The detailed immunostaining protocol was described elsewhere [12]. The GRP78/BiP and PERK expression scores were assessed by the staining extent as follows: 1, ≤10% of tumor area stained; 2, 11-25% stained; 3, 26-50% stained; 4, 51-75% stained; and 5, ≥76% stained. The tumors in which the stained tumor cells were scored as 3, 4, or 5 were defined as high-expression tumors.

For CD34 and Ki-67, immunohistochemical staining was performed according to the procedures described in a previous report [12]. Mouse monoclonal antibodies against CD34 (Nichirei, Tokyo, Japan; 1:800 dilution) and Ki-67 (Dako, Glostrup, Denmark; 1:40 dilution) were used. The number of CD34-positive vessels was counted in four selected hotspots in a 400× field (0.26 mm^2 field area). MVD was assessed using the criteria of Weidner *et al* [13]. The areas of highest neovascularization were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD34. Any brown-stained endothelial cells or endothelial cell clusters that were clearly separate from the adjacent microvessels, tumor cells, and other connective tissue elements was considered a single countable microvessel. Microvessels in sclerotic areas within the tumor, where microvessels were sparse, and the immediately adjacent areas of unaffected lung tissue were not considered in the vessel counts. The number of CD34-positive vessels was counted in four selected hot spots in a 400× field (0.26 mm^2 field area). The mean value of the two independent readings of the same specimen was calculated, and MVD was defined as the mean count of microvessels per 0.26 mm^2 field area [14].

For Ki-67, a highly cellular area of the immunostained sections was evaluated. All epithelial cells with nuclear staining of any intensity were defined as high-expression cells. Approximately 1000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median Ki-67 labeling index value was evaluated, and the tumors exceeding the median value were defined as high-expression tumors. The sections were assessed using light microscopy in a blinded fashion by at least two of the authors. For p-mTOR, a semi-quantitative scoring method was used: 1, ≤10% of tumor area stained; 2, 11-25% stained; 3, 26-50% stained; 4, 51-75% stained; and 5, ≥76% stained. Those tumors with a staining score >3 were considered strongly stained [15, 16]. Sections were evaluated by two investigators separately; in the case of discrepancies, both would evaluate the slide simultaneously and reach consensus in their final assessment. Neither investigator had knowledge of the patient outcomes.

Statistical analysis

Probability values <0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association between two categorical variables. The correlations between different variables were analyzed using the nonparametric Spearman's rank test. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Overall survival (OS) was determined as the time from tumor resection to death from any cause. Progression-free survival (PFS) was defined as the time between tumor resection and the first disease progression sign or death. Multivariate analyses were performed using the stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analyses were performed using JMP for Windows version 11 (SAS, Institute Inc., Cary, NC, USA).

Results

Immunohistochemical staining

Immunohistochemical analyses were performed of the 220 patients' data. **Figure 1** shows a representative imaging of GRP78/BiP. GRP78/BiP immunostaining was detected in carcinoma cells in tumor tissues and stained in the cytoplasm and membrane. GRP78/BiP and PERK were expressed in 41.8% (92/220) and 19.6% (41/220), respectively, demonstrating no significant difference (P=0.219). For comparison, we stained squamous cell carcinoma

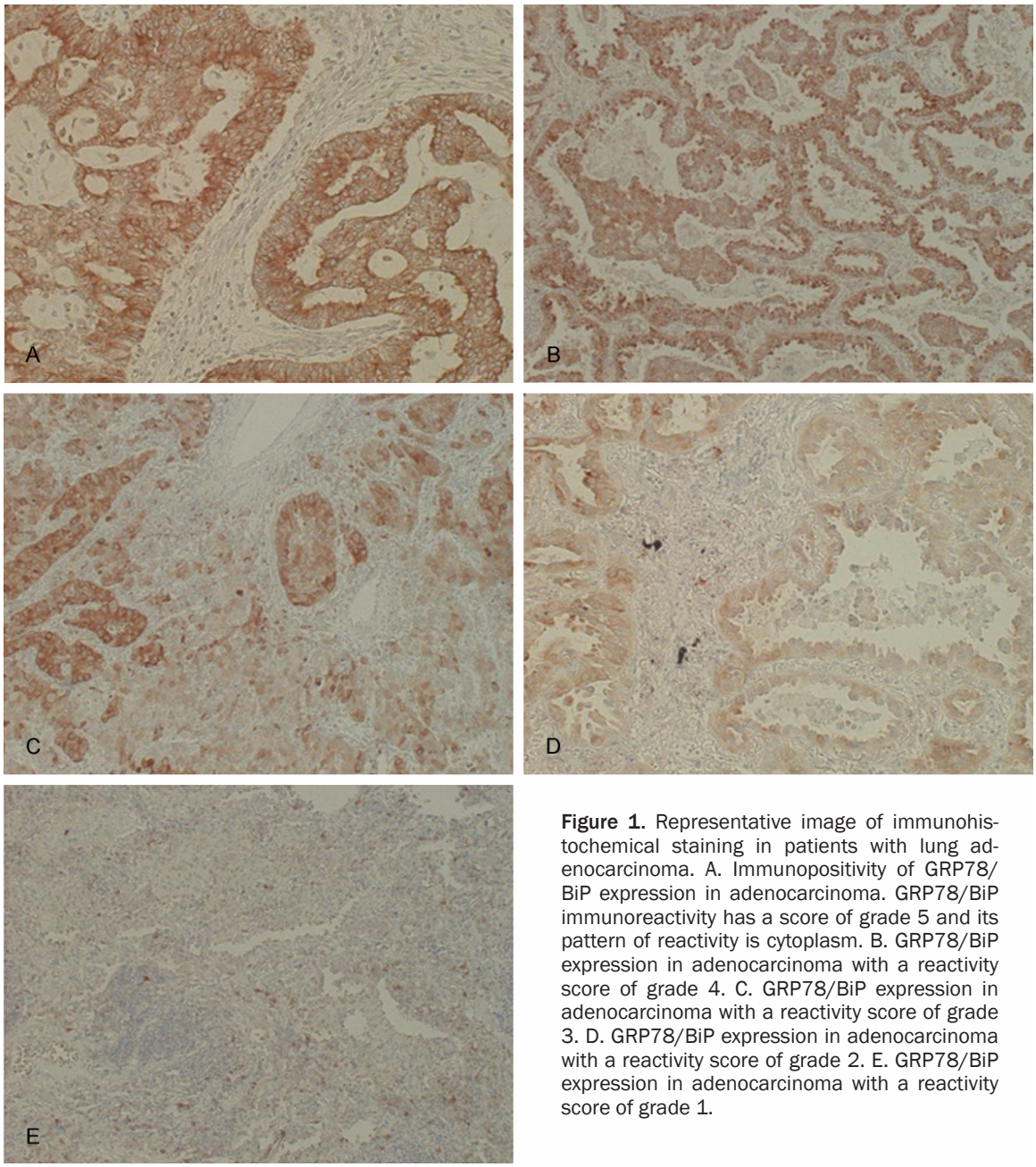

Figure 1. Representative image of immunohistochemical staining in patients with lung adenocarcinoma. A. Immunopositivity of GRP78/BiP expression in adenocarcinoma. GRP78/BiP immunoreactivity has a score of grade 5 and its pattern of reactivity is cytoplasm. B. GRP78/BiP expression in adenocarcinoma with a reactivity score of grade 4. C. GRP78/BiP expression in adenocarcinoma with a reactivity score of grade 3. D. GRP78/BiP expression in adenocarcinoma with a reactivity score of grade 2. E. GRP78/BiP expression in adenocarcinoma with a reactivity score of grade 1.

samples. The GRP78/BiP positivity rate was significantly higher in the squamous cell carcinoma group than in the adenocarcinoma group (63% vs. 41%, $P<0.001$). Moreover, the mean adenocarcinoma score (n=220) was 2.32 ± 1.27, whereas that of squamous cell carcinoma (n=30) was 2.86 ± 1.16 ($P=0.029$). The median value of the Ki-67 labeling index was 9% (range, 1-92), which was chosen as the cutoff point. The median number of CD34-positive vessels was nine (range, 1-45), which was chosen as a cutoff point. High Ki-67, CD34, and p-mTOR expressions were recognized in 55.9% (123-/220), 51.8% (114/220), and 32.2% (71/220) of patient samples, respectively. **Table 1** shows the patient demographics according to GRP78/BiP expression. High GRP78/BiP expression was significantly associated with pleural invasion, lymphatic permeation, vascular invasion, cell proliferation (Ki-67 labeling index), and p-mTOR phosphorylation.

Next, immunohistochemical staining of GRP78/BiP was performed on the samples from the

Table 1. Patient demographics by GPR78/BiP expression

Variable		Total	GRP78/BiP		*p* value
			High	Low	
		(n=220)	(n=92)	(n=128)	
Age	≤65/>65 years	76/144	29/63	47/81	0.473
Sex	Male/Female	103/117	45/47	58/70	0.681
Smoker	Yes/No	107/113	49/43	58/70	0.275
Differentiation	WD or MD/PD	184/26	77/15	107/11	0.143
T factor	T1/T2-4	118/102	42/50	76/52	0.055
N factor	N0/N1-2	171/49	67/25	104/24	0.143
Disease stage	I/II or III	162/58	64/28	98/30	0.278
Pleural invasion	Yes/No	63/157	34/58	29/99	**0.023**
Lymphatic permeation	Yes/No	48/112	42/50	36/92	**0.009**
Vascular invasion	Yes/No	69/151	41/51	28/100	**<0.001**
PERK expression	High/Low	41/179	21/71	20/108	0.219
Ki-67 expression	High/Low	123/97	67/25	56/72	**<0.001**
CD34 expression	High/Low	114/106	51/41	63/65	0.412
p-mTOR expression	High/Low	71/149	42/50	29/99	**<0.001**

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated. The bold entries show statistically significant differences.

Table A1. Patient demographics by GPR78/BiP expression in patients with stage I non-small cell lung cancer

Variable		Total	GRP78/BiP		*p* value
			High	Low	
		(n=162)	(n=64)	(n=98)	
Age	≤65/>65 years	58/104	22/42	36/62	0.867
Sex	Male/Female	78/84	35/29	43/55	0.2
Smoking	Yes/No	77/85	36/28	41/57	0.079
Differentiation	WD or MD/PD	73/89	29/35	44/54	>0.999
T factor	T1/T2-4	107/55	36/28	71/27	**0.042**
Pleural invasion	Yes/No	29/133	17/47	12/86	**0.034**
Lymphatic permeation	Yes/No	33/129	20/44	13/85	**0.009**
Vascular invasion	Yes/No	31/133	22/42	9/91	**<0.001**
PERK expression	High/Low	32/130	15/49	17/81	0.42
Ki-67 expression	High/Low	82/80	48/16	34/64	**<0.001**
CD34 expression	High/Low	74/88	35/29	39/59	0.076
p-mTOR expression	High/Low	43/119	24/40	19/79	**0.017**

WD, well differentiated; MD, moderate differentiated; PD, poorly differentiated; PERK, PKR-like endoplasmic reticulum kinase; p-mTOR, phospho-mTOR. The bold entries show statistically significant differences.

162 patients with stage I disease. Of them, GRP78/BiP was expressed in 39.5% (64/162). High Ki-67, CD34, and p-mTOR expressions were recognized in 50.6% (82/162), 45.6% (74/162), and 26.5% (43/162), respectively. **Table A1** shows the demographics according to GRP78/BiP expression of the patients with stage I disease. High GRP78/BiP expression was significantly associated with T factor, pleural invasion, lymphatic permeation, vascular invasion, cell proliferation (Ki-67 labeling index), and p-mTOR expression.

Correlation between GRP78/BiP expression and different variables

On Spearman's rank correlation analysis, GRP78/BiP was significantly correlated with Ki-67 (r=0.361, P<0.001), CD34 (r=0.184, P=0.006), and p-mTOR (r=0.353, P<0.001) but not PERK (r=0.1-04, P=0.124) (**Table 2**).

In patients with stage I disease, GRP78/BiP was significantly correlated with Ki-67 (r=0.437, P<0.001), CD34 (r=0.193, P=0.014), and p-mTOR (r=0.285, P<0.001) but not PERK (r=0.082, P=0.295) expression (**Table A2**).

Survival analysis

The 5-year survival rate and median survival time for all patients were 73% and not reached, respectively. We already reported detailed information about the survival analysis of various clinicopathological variables and several biomarkers in previous studies [10]. **Table 3** shows the univariate and multivariate analysis results of OS and PFS for all patients. **Figure 2A** and **2B** show the Kaplan-Meier survival curves for patients with high and low GRP78/BiP expression. Univariate analysis revealed that age, sex, smoking status, pathological stage, pleural invasion, lymphatic permeation, vascular invasion, GRP78/BiP, Ki-67 labeling index, and CD34 and p-mTOR

Table 2. Correlation between GRP78/BiP expression and cellular factor expressions

	Spearman γ	95% CI	*p* value
PERK	0.104	0.032-0.237	0.124
Ki-67	0.361	0.238-0.476	<0.001
CD34	0.184	0.049-0.313	0.006
p-mTOR	0.353	0.227-0.466	<0.001

95% CI, 95% confidence interval; PERK, PKR-like endoplasmic reticulum kinase; p-mTOR, phospho-Mtor. The bold entries show statistically significant differences.

Table A2. Correlation with GRP78/BiP expression in patients with stage I non-small cell lung cancer

	Spearman γ	95% CI	*p* value
PERK	0.082	0.077-0.238	0.295
Ki-67	0.437	0.299-0.557	<0.001
CD34	0.193	0.035-0.341	0.014
p-mTOR	0.285	0.132-0.425	<0.001

95% CI, 95% confidence interval; PERK, PKR-like endoplasmic reticulum kinase; p-mTOR, phospho-mTOR. The bold entries show statistically significant differences.

expressions were significant variables for OS, while age, smoking status, pathological stage, pleural invasion, lymphatic permeation, vascular invasion, GRP78/BIP, Ki-67 labeling index, and CD34 and p-mTOR expressions were significant prognostic factors for PFS. Multivariate analysis confirmed that age and pathological stage were independent prognostic factors for predicting negative OS. In the analysis of PFS, age, smoking status and pathological stage were identified as independent predictors.

Table 4 shows the univariate and multivariate analyses of OS and PFS in patients with stage I disease. **Figure 2C** and **2D** show the Kaplan-Meier survival curves for stage I patients with high and low GRP78/BiP expression. In patients with stage I disease, univariate analysis revealed that age, sex, smoking status, pleural invasion, lymphatic permeation, vascular invasion, GRP78/BiP, Ki-67 labeling index, and CD34 expression were significant variables for OS, while age, sex, smoking status, pleural invasion, lymphatic permeation, vascular invasion, GRP78/BiP, Ki-67 labeling index, and CD34 and p-mTOR expressions were significant prognostic factors for PFS. Multivariate analysis confirmed that age and GRP78/BiP were independent prognostic factors for predicting negative OS and PFS in patients with stage I disease. No statistically significant difference in OS and PFS was recognized between patients with stage II or III disease and high or low GRP78/BiP expression (**Figure 2E** and **2F**).

Discussion

This clinicopathological study evaluated the prognostic significance of GRP78/BiP expression in patients with lung adenocarcinoma. GRP78/BiP was expressed in 41% (92/220) and yielded a significant relationship with pleural invasion, lymphatic permeation, vascular invasion, cell proliferation, and p-mTOR expression. We found that high GRP78/BiP expression was an independent prognostic factor for predicting negative outcome in patients with lung adenocarcinoma incase with stage I disease. Our study suggests that GRP78/BiP expression as an ER stress marker plays a crucial role in lung adenocarcinoma pathogenesis and development.

It was recently described that GRP78 is anti-apoptotic and plays an important cytoprotective role in oncogenesis [7]. However, controversy persists about the prognostic significance of GRP78/BiP expression in various human neoplasms [7]. High GRP78/BiP expression in patients with hepatocellular, gastric, prostate, and renal cell carcinoma achieved a worse prognosis than those with low expression, whereas low GRP78/BiP expression yielded unfavorable survival for patients with esophageal and lung cancer [7]. In *in vitro* studies, GRP78/BiP was required for the tumor progression and highly metastatic cancer cell lines induced high GRP78/BiP expression [17]. In the present study, high GRP78/BiP expression was significantly associated with pleural invasion, lymphatic permeation, vascular invasion, cell proliferation (Ki-67 labeling index), and p-mTOR expression. Because microvascular density and mTOR phosphorylation are closely related cancer cell survival and metastasis, GRP78/BiP inhibition may suppress tumor growth by decreasing vascularization and mTOR phosphorylation. Our study findings suggest that GRP78/BiP plays a crucial role in lung cancer progression. This may contribute to poor survival as a negative predictor in lung adenocarcinoma. Further large-scale studies are needed to confirm these results.

Table 3. Univariate and multivariate analyses of overall and progression-free survival of all patients with non-small cell lung cancer

Variables	Overall survival					Progression-free survival				
	Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	*p* value	HR	95% CI	*p* value	5-year survival rate (%)	*p* value	HR	95% CI	*p* value
Age										
<65 years/≥65 years	86/65	<0.001	3.41	1.73-6.79	<0.001	80/58	0.001	2.91	1.68-5.37	<0.001
Sex										
Male/Female	67/77	0.039	0.878	0.56-1.31	0.54	64/69	0.245	1.18	0.82-1.65	0.352
Smoker										
Yes/No	66/79	0.004	1.34	0.86-2.05	0.193	56/75	0.003	1.61	1.09-2.24	0.016
p-Stage										
I/II-III	85/44	<0.001	4.89	2.88-8.43	<0.001	80/29	<0.001	5.47	3.39-8.91	<0.001
Pleural invasion										
Positive/Negative	49/84	<0.001				40/77	<0.001			
Lymphatic permeation										
Positive/Negative	48/86	<0.001				39/82	<0.001			
Vascular invasion										
Positive/Negative	47/85	<0.001				37/80	<0.001			
GRP78/BiP expression										
High/Low	64/79	0.017	1.27	0.98-1.66	0.075	53/75	0.003	1.26	0.99-1.61	0.055
PERK expression										
High/Low	71/73	0.885				68/66	0.901			
Ki-67 expression										
High/Low	61/85	<0.001				51/83	<0.001			
CD34 expression										
High/Low	65/81	0.003				56/77	0.005			
p-mTOR expression										
High/Low	64/78	0.066				48/75	0.001			

95% CI, 95% confidence interval; HR, hazard ratio; p-stage, pathological stage; PERK, PKR-like endoplasmic reticulum kinase; p-mTOR, phospho-mTOR. The bold entries show a statistically significant difference.

In a study evaluating the correlation between GRP78/BiP expression and the prognosis of 132 patients with lung cancer, high GRP78/BiP expression was detected in two-thirds of lung cancer patients. Those with high GRP78/BiP expression had better prognosis than those with low GRP78/BiP expression [8]. Thus, the report suggested that high GRP78/BiP expression may be a useful marker for predicting favorable prognosis in patients with lung cancer. However, our result was opposite, possibly due to GRP78/BiP being anti-apoptotic and playing an important cytoprotective role in oncogenesis [7]. One potential explanation is the difference in histological types in the patient groups. High GRP78/BiP expression is closely associated with poor outcome in hepatocellular cancer, gastric cancer, prostate cancer, and renal cell carcinoma. These studies focused primarily on adenocarcinoma. On the other hand, GRP78/BiP was significantly correlated with lower tumor stage and favorable survival in patients with esophageal carcinoma. The study focused mainly on squamous cell carcinoma. These studies showed that adenocarcinoma has poor prognosis, whereas squamous cell carcinoma has good prognosis. The previous lung cancer study included patients with adenocarcinoma and squamous cell carcinoma (57.5% [76/132] and 33.3% [44/132], respectively) [8]. On the other hand, all patients in our study had adenocarcinoma. That is, although the previous study population was heterogeneous, our study population was homogeneous. Our report focuses on lung adenocarcinoma as a homogeneous group of patients with stage I disease. That is to say, our result supports the notion that the ER stress marker GRP78/BiP could be a promising molecular target for the treatment of lung adenocarcinoma.

GRP78/BiP overexpression in the lung cancer tissue correlated with differentiation grade and tumor stage [18, 19]. There was stronger

Table 4. Univariate and multivariate analyses of overall and progression-free survival of patients with stage I non-small cell lung cancer

Variables	Overall survival					Progression-free survival				
	Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	*p* value	HR	95% CI	*p* value	5-year survival rate (%)	*p* value	HR	95% CI	*p* value
Age										
<65 years/≥65 years	94/78	0.003	2.78	1.77-5.32	0.002	96/71	<0.001	2.97	1.78-6081	<0.001
Sex										
Male/Female	75/92	<0.001	1.76	0.98-3.38	0.059	76/84	0.031	1.18	0.82-1.65	0.677
Smoker										
Yes/No	77/91	<0.001	0.84	0.43-1.53	0.577	71/89	0.001	1.61	1.09-2.24	0.151
Pleural invasion										
Positive/Negative	59/91	<0.001				50/87	<0.001			
Lymphatic permeation										
Positive/Negative	65/89	<0.001				53/87	<0.001			
Vascular invasion										
Positive/Negative	45/93	<0.001				45/88	<0.001			
GRP78/BiP expression										
High/Low	75/91	0.003	1.58	1.05-2.47	0.029	68/88	0.002	1.26	0.99-1.61	0.009
PERK expression										
High/Low	81/85	0.3				78/80	0.428			
Ki-67 expression										
High/Low	73/95	<0.001				66/93	<0.001			
CD34 expression										
High/Low	77/91	0.01				70/89	0.009			
p-mTOR expression										
High/Low	76/89	0.145				65/86	0.014			

95% CI, 95% confidence interval; HR, hazard ratio; p-stage, pathological stage; PERK, PKR-like endoplasmic reticulum kinase; p-mTOR, phospho-mTOR. The bold entries show statistically significant differences.

expression in poorly differentiated tumors than in well or moderately well differentiated tumors as well as stronger expression in stage III than in stages I and II tumors [18]. In other cancers, GRP78/BiP overexpression in both primary gastric cancer and metastatic lymph nodes was inversely correlated with patient survival, suggesting that GRP78/BiP serves as a prognostic parameter for gastric cancer. Moreover, GRP78/BiP expression was strongly correlated with lymph node metastasis status [20]. Patients with strong GRP78/BiP immunoreactivity in the primary tumor are at higher risk of clinical recurrence and death than patients with prostate cancer and weak GRP78/BiP expression [21]. High GRP78/BiP mRNA and protein expressions are related to large tumor size and high clinical stage in patients with renal cell carcinoma [22]. These results support the clinicopathological significance of GRP78/BiP expression as a tumor aggression and malignant formation.

A recent review described that high GRP78/BiP expression levels correlated with drug resistance, tumor recurrence, and poor survival [7]. High GRP78 expression was related to poor response and poor prognosis, which suggests a correlation between GRP78 and chemo- and radioresistance in locally advanced rectal cancer [23]. The down-regulation or inhibition of GRP78/BiP activity may be a potential molecular target for the treatment of various cancers. This review described the correlation between GRP78/BiP and drug resistance in various human neoplasms [7]. GRP78/BiP induction could be a target therapy against the drug-resistant cells of lung, bladder, and breast cancers, whereas GRP78/BiP inhibition is effective against the resistant cells of gastric cancer, transformed fibroblasts and epidermoid carcinoma. Although there have been many discussions regarding GRP78/BiP induction or inhibition as a potential therapeutic target, it is important that we discover any small molecules related to modulation of the ER stress signaling pathway and GRP78/BiP expression.

The heat shock protein (HSP) family is a group of molecular chaperones that assist in protein

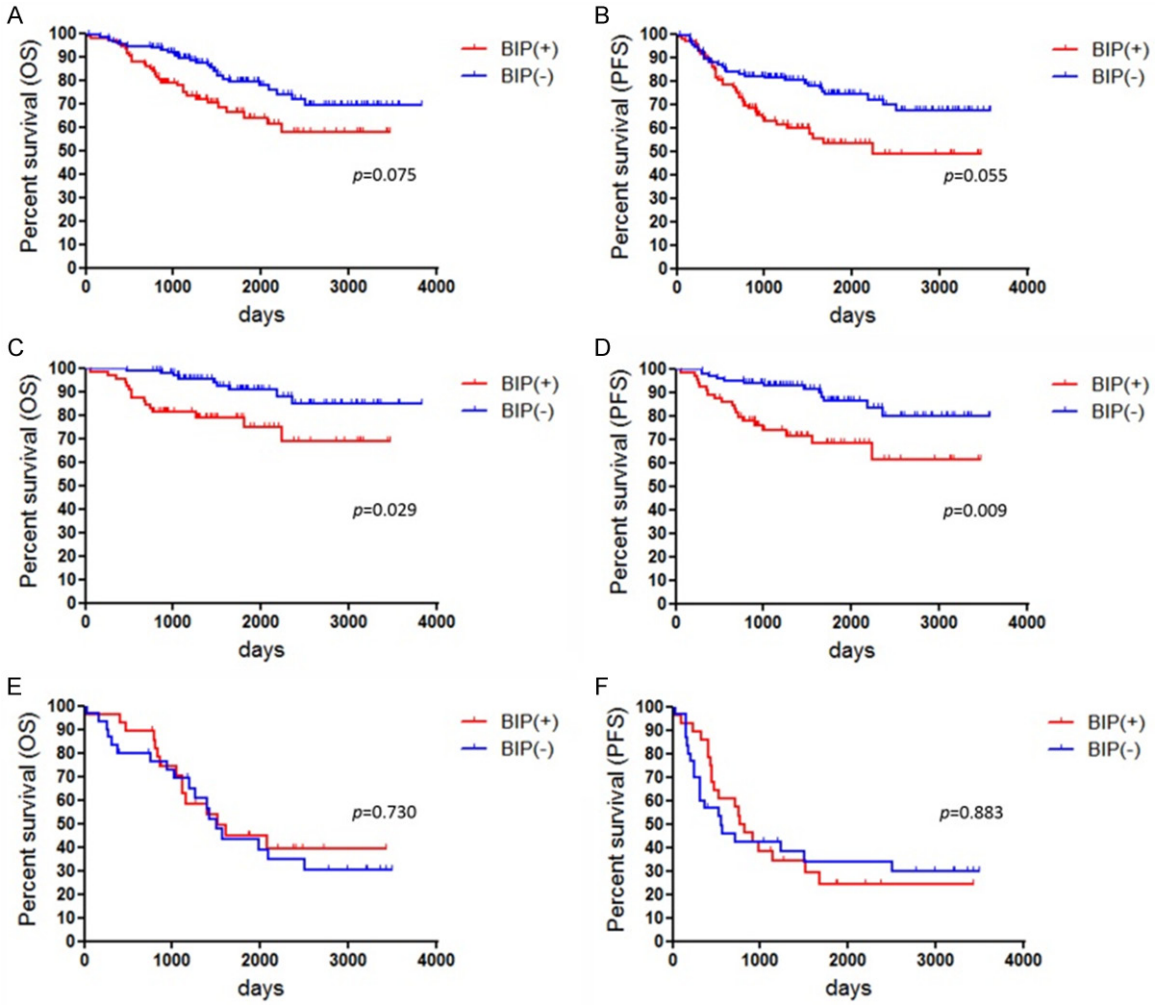

Figure 2. Kaplan-Meier survival curve of overall survival (OS) and progression-free survival (PFS) according to GRP78/BiP expression. No statistically significant difference in OS and PFS was recognized between patients with high and low GRP78/BiP expression [OS, *P*=0.075 (A); PFS, *P*=0.055 (B)], a statistically significant difference in OS and PFS was recognized between patients with stage I disease and high versus low GRP78/BiP expression [OS, *P*=0.029 (C); PFS, *P*=0.009 (D)], but no statistically significant difference in OS and PFS was recognized between patients with stage II+III disease and high versus low GRP78/BiP expression [OS, *P*=0.730 (E); PFS, *P*=0.883 (F)], respectively.

folding, modification, and transportation. Different members of the HSP family are essential for tumor cell survival by binding diverse client proteins and regulating homeostasis. A previous study indicated that the differential expression of HSP70 is associated with the malignant phenotype of NSCLC cell lines and plays an important regulatory role in NSCLC cell proliferation [24]. Moreover, a specific inhibitor of HSP70 significantly inhibits NSCLC as well as colon cancer proliferation and cell cycle progression [24, 25]. These reports showed that the specific HSP70 inhibitor can potentiate the apoptotic potential in certain cancer cell lines. These and our data suggest that the specific HSP70 and GRP78/BiP inhibitors are good candidates for NSCLC treatment and that the HSP machinery is a good target for developing NSCLC therapeutics.

Although PERK expression plays an important role in tumor progression as a marker of ER stress, it remains unclear whether high or low PERK expression is significantly associated with patient survival and lung cancer aggressiveness. In the present study, high GRP78/BiP expression was not significantly associated with PERK expression. The role of PERK as a prognostic predictor seemed to be markedly

weak compared to that of GRP78/BiP in lung cancer.

The present study has evaluated the prognostic significance of GRP78/BiP expression in stage I lung adenocarcinoma. Although a borderline statistically significant difference in OS and PFS was recognized between patients with high and low GRP78/BiP expression, the results clearly demonstrated that GRP78/BiP expression was a significant independent factor for predicting poor prognosis in patients with completely resected stage I lung adenocarcinoma. The major task of prognostic markers in early-stage NSCLC is to determine patients with high-risk features such as recurrence or metastasis after surgery and possibly to lead adjuvant treatment. In patients with stage II-IIIA NSCLC, adjuvant chemotherapy after surgical treatment with cisplatin-based regimens is now the standard of therapy based on the results of previous Phase III trials [26-28]. However, adjuvant chemotherapy with uracil-tegafur for cases with stage IB NSCLC remains contentious [29, 30]. Hence, we should research possible markers for the decision of whether adjuvant chemotherapy is needed in patients with stage I NSCLC.

The limitations of the current study must be addressed. One limitation is that the sample size in this study was relatively small, which may bias our results. Another limitation is that we focused on adenocarcinoma and did not investigate the other NSCLC subtypes such as squamous cell carcinoma, large cell carcinoma, and small cell carcinoma. Therefore, it remains unclear whether our study findings completely correspond to those of previous studies. Further investigations are warranted to evaluate the prognostic significance of the ER stress marker GRP78/BiP for patients with NSCLC using a large sample size.

In conclusion, GRP78/BiP is highly expressed in lung adenocarcinoma, and fabricated a significant relationship with pleural invasion, lymphatic permeation, vascular invasion, cell proliferation, and p-mTOR expression. On multivariate analysis, high GRP78/BiP expression was identified as an independent prognostic factor for predicting negative outcome in patients with stage I disease. The ER stress marker GRP78/BiP could be a promising molecular target for the treatment of lung adenocarcinoma in patients with stage I disease.

Acknowledgements

We thank Ms. Keiko Kogure and Drs. Tomohito Kuwako, Tomomi Masuda, Yosuke Kamide, Akihiro Ono, Yasuhiko Koga, Noriaki Sunaga, Takeshi Hisada, and Masanobu Yamada for their assistance in preparing this manuscript. We also deeply appreciate Prof. Masahiko Nishiyama of the Department of Molecular Pharmacology and Oncology for his critical review of this manuscript as well as Ms. Yuka Matsui for her technical assistance with the manuscript submission process. We also wish to thank Editage (www.editage.jp) for English language editing.

Disclosure of conflict of interest

None.

Authors' contribution

HI, KK, TY, and AS developed the initial research question and performed data analysis. HI and KK wrote the original manuscript. HI, KK, TY, AS, TN, YO, KO, TA, TO, and KS made all revisions to the manuscript. All authors read and approved the final manuscript.

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