

## **Immunohistochemical GRP78 as a tumor biomarker may predict poor prognosis in patients with non-small cell lung cancers: a retrospective study.**

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### **Abstract**

**Purpose:** To examine the correlation of GRP78 and GRP94 protein levels with clinicopathological variables and prognosis in primary Non-Small Cell Lung Cancer (NSCLC) patients.

**Methods:** A total of 89 primary patients with NSCLC were included. Their surgically-resected tissues were studied by immunohistochemistry to assess GRP78 and GRP94 expression. Student's t-test, Kaplan-Meier estimator with log rank test and Cox regression analyses were performed to examine the associations of GRP78/GRP94 levels with clinicopathological variables and survival.

**Results:** GRP78 and GRP94 expressions increased significantly in tumor samples of NSCLC patients. High GRP78 positive was associated with pathologic TNM status ( $p=0.003$ ) and pathologic N status ( $p=0.013$ ); while high GRP94 positive was associated with squamous-cell carcinoma subtype ( $p=0.001$ ). There is no correlation between GRP78 and GRP94 levels ( $p=0.086$ ). Various levels of GRP78 proteins were associated with survival of NSCLC patients ( $p=0.016$ ). A higher GRP78 level marked a shorter survival time ( $p=0.043$ ). Various levels of GRP94 proteins were not associated with survival of NSCLC patients ( $p=0.610$ ).

**Conclusions:** High expression of GRP78 can be related to a poor prognosis. Expression level of GRP78 protein may be a potential predictor for NSCLC prognosis.

**Keywords:** GRP78, GRP94, NSCLC, Immunohistochemistry, Prognosis, Predictor.

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### **Introduction**

NonSmall Cell Lung Carcinoma (NSCLC) is one of the primary causes for cancer-related death worldwide, in spite of obvious progress in surgery, chemotherapy, radiotherapy and biologically targeted therapy [1]. Research into novel prognostic biomarkers and therapeutic targets in NSCLC is still a focus of interest.

Glucose-Regulated Proteins (GRPs) are originally discovered as they are induced by glucose starvation, and GRPs belong to the Endoplasmic Reticulum (ER) chaperone families [2]. Under pathological conditions, such as acidosis, hypoxia or hypothermia, expression levels of GRPs are up-regulated [3].

Recently, knowledge about the correlation of GRPs with cancer has been improved. GRPs have been studied in cultured cancer cells [4-7] and clinic cancers, for instance colorectal carcinoma [8], pediatric hepatoblastoma [9], hepatitis B virus-related hepatocellular carcinomas [10], gastric carcinomas [11], esophagus adenocarcinomas [12] and lung cancer [13].

Novel GRPs-targeted therapeutic methods have been developed into preclinical tests and recommended for cancer

treatment to some extent [14-17]. Among GRPs, either GRP78 or GRP94 is one of the central regulators of ER function because of their roles in protein folding and activation of transmembrane ER-stress sensors. High GRP78 and GRP94 levels have been found to relate with high pathologic TNM status and aggressive behavior of cancers [11].

Ma et al. suggest that serum GRP78 level may be a tumor marker that predicts poor prognosis in late-stage NSCLC patients [18]. This hints that GRPs have great potentials to be tumor markers. But this previous study only including small sample size fails to find the correlation of GRPs with clinicopathological variables. Therefore, more investigations are worthy of doing to reveal the correlation of GRPs with tumor, clinicopathological factors and prognosis.

The current investigation was designed to detect the expression levels of GRP78 and GRP94 proteins by immunohistochemistry, and analyze the association with clinicopathological variables and prognosis.

## Materials and Methods

### Patients

A total of 89 patients with primary NSCLC underwent surgical resection in China Japan Union Hospital of Jilin University in June 2011 to October 2013. All patients did not undergo any anti-tumor therapy prior to surgery. The follow-up period was defined as the time from surgery to the last observation for censored cases, or death for complete observations. Patients without a study end date and who were lost to follow-up were considered censored. The minimum postoperative follow-up time was 15 months. The median survival time was 32 months. According to the current pathological Tumor Node Metastasis (TNM) classification, pT status and pN status of tumors were determined [19]. According to the WHO classification, histological subtype was determined [20]. All patients gave written informed consent. Study was approved by the Jilin University Ethics Committee.

### Immunohistochemistry

Specimens (paired normal tissue and cancer mass) were removed from each of 89 NSCLC patients and were embedded by paraffin. Typical regions of paraffin-embedded specimens were marked. Using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI), specimens were cut into 2 µm thick slices of 0.6 mm diameter. Then slices were dewaxed and rehydrated. Heat-induced antigen retrieval was performed routinely. Slices were cooled, and incubated with the primary antibodies at a final concentration of 0.20 mg/ml: either GRP78 monoclonal antibodies (Cell Signaling Biotechnology, Inc., Beverly, MA) or GRP94 monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Using fast red as the reaction indicator, the color was developed with the labeled streptavidin-biotinylated alkaline phosphatase system. Slices were counterstained with hematoxylin, followed by dehydration. Plasma cell staining was used as internal positive control. The immunoreactivity of either GRP94 or GRP78 was classified into five categories: -, no positive cells or only single positive cell; +, positive cells accounted for less than 10%; ++, positive cells accounted for 10-50%; +++, positive cells accounted for 50-80%; and +++++, positive cells accounted for above 80% [21]. The + and ++ protein levels denoted lower expression; the +++ and +++++ protein levels denoted higher expression.

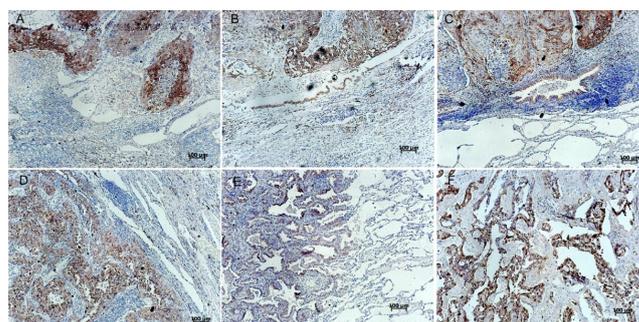
### Statistical analysis

SPSS 19.0 statistical software was used for statistical analysis. Spearman correlation analysis was used to assess correlation. Student's t-test was used for comparison between groups. Kaplan-Meier estimator and log rank test was used for survival analysis. Univariate and multivariate Cox regression analyses were performed using the Cox proportional hazards model. A two-tailed  $p < 0.05$  was considered statistically significant difference.

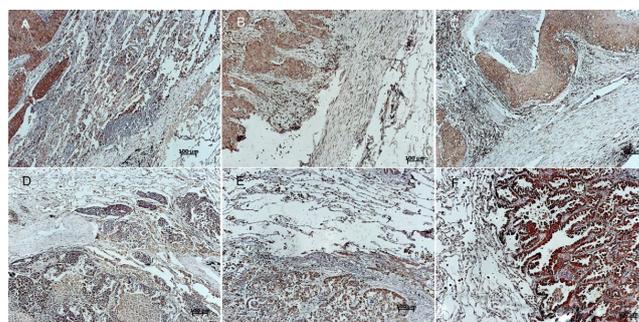
## Results

### GRP78 and GRP94 expressions

Immunohistochemistry was performed to examine the expression levels of GRP78 and GRP94 proteins. Both GRP78 and GRP94 were seen positive expression in cytoplasm and the staining intensities in cancer tissues were stronger than in normal tissues (Figures 1 and 2). The staining intensities of cancer tissues were ++ to +++++, while the intensities of the paired normal tissues were + to ++. There were statistically significant differences between them ( $p=0.000$  and  $p=0.034$ , respectively) (see Table 1). GRP78 expression was not associated with GRP94 expression (Spearman correlation coefficient 0.421,  $p=0.086$ ) (Table 2).



**Figure 1.** Examples of resected NSCLC tissues with GRP78 protein expression. A. poorly differentiated squamous-celled carcinoma; B. moderately differentiated squamous-celled carcinoma; C. well differentiated squamous-celled carcinoma; D. poorly differentiated adenocarcinoma; E. moderately differentiated adenocarcinoma; F. well differentiated adenocarcinoma.



**Figure 2.** Examples of resected NSCLC tissues with GRP94 protein expression. A. poorly differentiated squamous-celled carcinoma; B. moderately differentiated squamous-celled carcinoma; C. well differentiated squamous-celled carcinoma; D. poorly differentiated adenocarcinoma; E. moderately differentiated adenocarcinoma; F. well differentiated adenocarcinoma.

### Clinicopathological variables

Clinicopathological data were listed in table 3. The correlation of clinicopathological factors with either GRP78 or GRP94 expressions was tested. Positive expression of GRP78 was associated with pathologic TNM status ( $p=0.003$ ) and pathologic N status ( $p=0.013$ ). Positive expression of GRP94

*GRP78 relates to poor NSCLC prognosis*

was associated with squamous cell carcinoma (SCC) subtype (p=0.001) (Table 3).

**Table 1.** GRP78 and GRP94 expressions in paired NSCLC mass and normal tissues.

Tissues	Cases	GRP78				p value		GRP94					p value
		-	+	++	+++	-	+	++	+++	++++			
Tumor mass	89	0	34	34	21	0.000*	0	34	10	41	38	0.034*	
Normal tissues	89	65	24	0	0		38	43	8	0	0		

Note:\* p<0.05. Student's t -test.

**Table 2.** Spearman correlation between GRP94 and GRP78 protein in NSCLC patients.

GRP94	GRP78			Correlation coefficient	p value
	+	++	+++		
++	5	2	3	0.421	0.086
+++	14	22	5		
++++	15	10	13		

**Table 3.** Clinicopathological variables of NSCLC patients and correlation with either GRP78 or GRP94 protein levels. (\*p<0.05)

Clinicopathological variables	Cases	GRP78			P value	GRP94			P value	
		+	++	+++		++	++	++		
Number	89	34	34	21		10	41	38		
Sex	Male	67	27	26	14	0.322	10	31	26	0.082
	Female	22	7	8	7		0	10	12	
Smoking	Yes	47	15	20	12	0.258	7	21	19	0.263
	No	32	13	11	8		1	15	16	
	Unknown	10	6	3	1		2	5	3	
pTNM status	I	40	15	14	11	0.003	5	14	21	0.425
	II	32	12	14	6		4	16	12	
	III	17	7	6	4		1	11	5	
pT status	T1	31	11	12	8	0.779	4	15	12	0.055
	T2	47	21	14	12		3	20	24	
	T3	11	2	8	1		3	6	2	
pN status	N0	53	15	24	14	0.013	6	25	22	0.823
	N1	21	8	6	7		3	8	10	
	N2	13	10	3	0		1	6	6	
	N3	2	1	1	0		0	2	0	
Differentiation	Low	21	8	9	4	0.153	2	12	7	0.742

Moderate	55	23	21	11	6	23	26			
Well	8	3	2	3	2	3	3			
Unknown	5	0	2	3	0	3	2			
Pathology	SCC	55	24	22	9	0.057	9	30	16	0.001
	AC	34	10	12	12		1	11	22	
Local infiltration	Yes	26	8	11	7	0.388	0	13	13	0.137
	No	63	26	23	14		10	28	25	

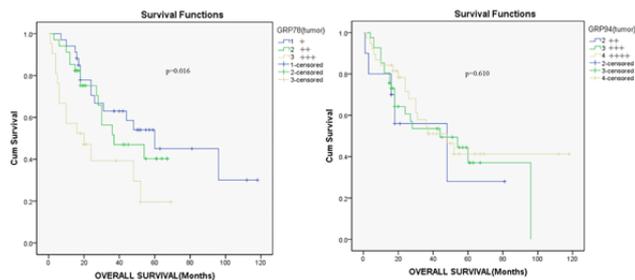
pTNM: pathological tumor-node metastasis, pT: pathological tumor, pN: pathological node, SCC: squamous cell carcinoma, AC: adenocarcinoma.

**Survival analysis**

The minimum postoperative follow-up time was 15 months. The median survival time was 32 months. The 1-, 2-, 3-, and 5-year survival rates were 88.7%, 67.3%, 53.7% and 38.7% for overall NSCLC patients with +, ++ and +++ GRP78; 86.7%, 66.1%, 57.3% and 36.7% for overall NSCLC patients with ++, +++ and ++++ GRP94. Correlation of either GRP78 or GRP94 protein expressions with survival of NSCLC patients was analyzed as shown in Kaplan-Meier survival curve (Figure 3). Various levels of GRP78 proteins were associated with survival of NSCLC patients (p=0.016. Figure 3A). According to the setting of the current study, the + and ++ protein levels denoted lower expression; the +++ and ++++ protein levels denoted higher expression. The patients with a higher GRP78 protein level had shorter survival times compared with the patients with a lower GRP78 level (median overall survival, 36.1 [+++ patients] versus 47.2 months [+ and ++ patients]; RR: 0.371, 95% CI: 0.135-1.019; p=0.043). Various levels of GRP94 proteins were not associated with survival of NSCLC patients (p=0.610) (Figure 3B).

**Prognostic factors for NSCLC patients**

The + and ++ protein levels denoted lower expression; the +++ and ++++ protein levels denoted higher expression. Therefore, patients were divided into lower and higher groups. A univariate analysis was performed to examine the clinicopathological factors that predict prognosis (Table 4).



**Figure 3.** Kaplan-Meier survival curves for NSCLC patients (n=89) in relation to either GRP78 or GRP94 expression.

**Table 4.** Univariate analysis of prognostic factors for NSCLC patients (n=89).

Clinicopathological variables	Hazard ratio	95%CI	p value
Sex (male vs. female)	3.264	1.188-8.966	0.015
Smoking (yes vs. nope)	1.258	0.458-3.456	0.026
pTNM status (stages I-II vs. III)	0.533	0.194-1.464	0.425
pT status(T1-2 vs. T3)	0.297	0.108-0.816	0.035
pN status(N0 vs. N1-3)	0.682	0.248-1.873	0.523
Differentiation(well and moderate vs. low)	3.252	1.184-8.933	0.014
Pathology (SCC vs. AC)	0.154	0.056-0.423	0.131
Local infiltration (yes vs. nope)	0.268	0.098-0.736	0.037
GRP78 level (low vs. high)	0.371	0.135-1.019	0.043
GRP94 level (low vs. high)	0.285	0.104-0.783	0.254

pTNM: pathological tumor-node metastasis, pT: pathological tumor, pN: pathological node.

**Table 5.** Multivariate analysis of independent prognostic factors for NSCLC patients (n=89).

Clinicopathological variables	Hazard ratio	95%CI	p value
Sex (male vs. female)	3.042	1.107-8.356	0.042
Smoking (yes vs. no)	1.865	0.679-5.123	0.006
pT status(T1-2 vs. T3)	0.493	0.179-1.354	0.105
Differentiation(low vs. well and moderate)	0.168	0.061-0.461	0.004
Local infiltration (Yes vs. no)	0.121	0.044-0.332	0.138
GRP78 level (low vs. high)	0.276	0.101-0.758	0.043

pT: pathological tumor

Among the factors analyzed, sex, smoking, pathological T, differentiation, local infiltration and GRP78 level were

significant prognostic factors (p=0.015, p=0.026, p=0.035, p=0.014, p=0.037 and p=0.043, Table 4). Then six factors were subjected to multivariate analysis to evaluate independent prognostic factors, including sex, smoking, pathological T, differentiation, local infiltration and GRP78 level. The results of multivariate analysis illustrated that sex, smoking, differentiation and GRP78 level were four independent prognostic factors in these NSCLC patients (Table 5).

### Discussion

There were 3,093,039 new lung cancer patients and 1,956,622 lung cancer deaths in China, 2010 [22]. Most patients are advanced lung cancer at the time they are diagnosed [23]. Thus we need specific biomarkers to evaluate the prognosis of NSCLC patients.

GRP78 and GRP94 are ER-retentive chaperones that are induced by glucose starvation [24]. They play an important role in tumorigenesis [25]. GRPs' expression is associated with tumorous development. Overexpression of GRP78 and GRP94 can be detected in oesophageal adenocarcinomas, and the coexpression is related with poor prognosis [21]. In colorectal carcinoma cell lines, GRP78 and GRP94 expressions seem to be associated with malignancy [8]. In gastric cancer cells, GRP78 and GRP94 expressions are correlated with BCL2, BAX, ERBB2 and CASP3 protein expressions, and involve in the process of apoptosis [26]. In gastric carcinomas, overexpression of GRP78 and GRP94 is associated with aggressive behavior and poor prognosis [11].

In Japanese gastric adenocarcinomas, GRP78 and GRP94 expressions are weaker in signet ring cell carcinoma [27]. In children with hepatoblastoma, overexpression of GRP78 and GRP94 is more frequently present in the patients with lymph node metastasis and higher clinic stage [28]. In esophagus adenocarcinoma, higher GRP78 is related with early pT stage, well differentiation and good prognosis; GRP94 expression is significantly associated with earlier tumor stage and less lymph node involvement; GRP94 seems to be correlated with GRP78 protein expression inversely [29].

Ma et al. suggest that GRP78 is highly enriched in late-stage lung cancer and may be a prognostic marker for non-small cell lung cancer [18]. Inconsistence of GRP78 or GRP94 expression in various tumors demonstrates that either GRP94 or GRP78 is tumor type-specific in various tumors. Thus the correlation of GRP78 and GRP94 with grade of differentiation, stage of cancer and prognosis in NSCLC patients should be explained specifically.

In the current investigation, we detected GRP78 and GRP94 protein expressions in NSCLC patients by immunohistochemistry. The results showed that overexpression of GRP78 and GRP94 proteins was seen in tumor tissues, but there was no correlation between them. GRP78 was associated with pTNM and pN. GRP94 was correlated with SCC subtype. While, only GRP78 was correlated with poor survival. These results suggest that GRP94 may be a biomarker to distinguish pathological subtype, and GRP78 may be a biomarker to assess

prognosis. The inconsistency between our results and others may imply a different GRP regulation due to the pathology subtype of tumors or the heterogeneity of patients included.

The current investigation had another interesting finding that GRP78 protein expression was associated with pTNM status and pN status, suggesting that GRP78 may be involved in tumor development and lymph node metastasis. Further analysis illustrated that high GRP78 protein expression was observed more frequently in pTNM II stage and pN0/N1 stage, and high GRP94 protein expression was detected more frequently in SCC subtype. This finding may be elucidated by different mechanisms of GRP regulation: overexpression in early stages of lung cancer may indicate an early response of the host's immune system, while overexpression in advanced stages may be correlated to dissimilar stress factors such as glucose starvation, hypoxia or other immune reactions towards lung cancer [30].

Furthermore, the current investigation examined the prognostic factors via univariate and multivariate analyses. We found that GRP78 level was one of four independent prognostic factors for NSCLC and a higher GRP78 expression was associated with a shorter overall survival time in the NSCLC patients included. This result was consistent with the results of Ma et al.'s study [18].

In the current investigation, the valid of outcome was limited by the small size of cases included. We did not track postoperative treatment, which may impact the survival of the patients. The operation manner that we did not discriminate may also affect prognosis. In addition, protein expression can also be analyzed using ELISA that provides more quantitative data. In the next investigation, we would investigate how the activated GRP78 and/or GRP94 impact the tumor subtyping and patients' outcome. A larger number of NSCLC patients would be included. ELISA would be preferred to quantify GRP78/GRP94 expression. The response of GRP78 to chemotherapy and radiotherapy in late-stage NSCLC would also be examined so that guides the treatment of NSCLC.

In conclusion, high expression of GRP78 protein is related to a poor prognosis, and GRP78 may be a predictor for NSCLC prognosis. Larger and more detailed follow-up studies are required.

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