

## Original Article

# Guanine nucleotide binding protein-like 3 predicts poor prognosis in patients with early stage lung squamous carcinoma

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**Abstract:** Lung cancer is one of the common tumors worldwide, being a leading cause of cancer death. Considering its complex and heterogenous molecular basis, to find out accurate diagnosis biomarker is very helpful to clinical treatment. In this study, we aim to investigate the relationship between guanine nucleotide binding protein-like 3 (GNL3) expression and the prognosis of patients with lung squamous carcinoma. For this purpose, 75 carcinoma and normal tissue pairs were analyzed by immunohistochemistry assay. The relationship between GNL3 expression and clinical pathological parameters and their effects on the prognosis of patients with lung carcinoma were analyzed. The immunostaining results show that the expression levels of GNL3 in lung carcinoma tissues were significantly higher than those in paracancerous tissues. There was no association between GNL3 and clinicopathological features of patients. GNL3-positive patients had significantly shorter survival times than those with GNL3-negative tumors among patients with TNM stages I and II. Positive GNL3 was significantly related to worse survival time among patients with TNM stages I and II. In conclusion, high GNL3 expression is essential in the pathogenic process of lung squamous carcinoma and may be a useful prognosis biomarker and therapy target of patients with lung squamous carcinoma.

**Keywords:** Guanine nucleotide binding protein-like 3, nucleostemin, lung cancer, expression, prognosis

## Introduction

Lung cancer is the leading cause of cancer death among males in both more and less developed countries, and has surpassed breast cancer as the leading cause of cancer death among females in more developed countries [1]. The two major forms of lung cancer are non-small cell lung cancer (about 85% of all lung cancers) and small-cell lung cancer (about 15%) [2]. The early detection of lung cancer is critical to lung cancer treatment. This is due to the evidence that complete resection of lung cancer is associated with significantly longer survival remission. However, despite advances in early detection, only about 25% of patients

are candidates for surgical treatment at the time of diagnosis [3]. In addition, accurate staging of cancer at the time of diagnosis, especially at early stage, also plays a very important role in lung cancer treatment. It improves the precision of treatment options and prognosis prediction clinically.

Lung cancers are characterized by molecular alterations [4]. DNA mutations have been detected on several genes in lung cancer, which cause the activation of oncogenes, such as k-ras [5-7] and EGFR [8], and the inactivation of tumor suppressor gene, including p53 [9] and PTEN [10]. What's more, there are increasing evidence that some oncogenes are highly

**Table 1.** Clinicopathological characteristics of patients with lung squamous carcinoma

Characteristics	No. of Patients	%
Age (years)		
Median	62.0	
Range	31.0-71.0	
Sex		
Female	69	92.0
Male	6	8.0
Histologic grade		
1	2	2.7
2	53	70.7
3	20	26.7
TNM stage		
I	28	37.3
II	36	48.0
III	11	14.7
Lymph node status		
Negative	46	61.3
Positive	28	37.3
Unknown	1	1.3
Tumor size (cm)		
Median	5.0	
Range	2.5-12.0	

expressed in lung cancer, such as MYC, EGFR and ERBB2 [11]. A better understanding of molecular mechanism in carcinogenesis can probably improve the treatment of prevention of lung cancer.

Guanine nucleotide binding protein-like 3 (GNL3), also known as nucleostemin, is a GTP-conjugated protein preferentially expressed in stem cells [12], cancer cell lines [13, 14], and highly proliferating cells [15]. GNL3 is also found overexpressed in cancer tissues. In patients with oral squamous cell carcinoma (OSCC), a high GNL3 tumor expression level significantly correlated with an advanced T-stage and N-stage [16]. In breast cancer, patients with GNL3-positive tumors showed significantly shorter disease-free survival [17]. However, the expression of GNL3 in lung cancer is still unclear so far. In addition, GNL3 is a multi-functional protein, and participates in many biological processes. It is reported that GNL3 not only promotes cell proliferation in lung cancer [18] and breast cancer cell lines [19], but also enhances the invasion of some cancer cell lines [16, 19]. Knockdown of GNL3 causes cell

cycle arrest [13, 20-22] and cell apoptosis [21, 23, 24] in several different kinds of cell lines. These clues suggest that GNL3 has an impact on cancer malignancy, and may serve as an oncogene in lung cancer.

In this study, we investigated the expression levels of GNL3 in lung squamous carcinoma and adjacent normal tissues, as well as their relationship to clinical pathological parameters and prognosis. The results may help us uncover the role of GNL3 in lung cancer carcinogenesis, and also let us better understanding the correlation between GNL3 expression and clinical characteristics in patients with lung squamous carcinoma.

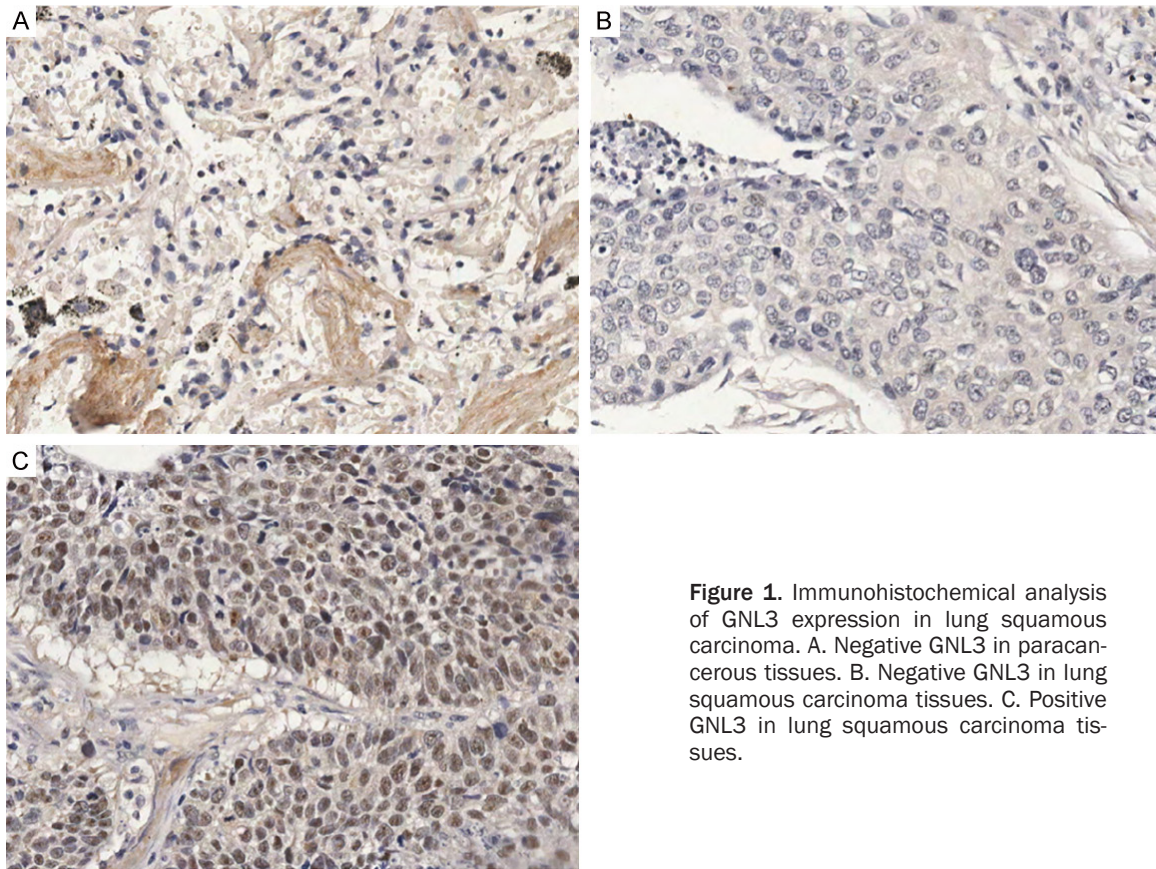
## Material and methods

### Sample collection

Seventy five pairs of lung squamous carcinoma and adjacent normal tissues were selected from the Biobank of National Engineering Center for Biochip at Shanghai. All samples were fixed with 4% formaldehyde, followed by being embedded in a routine paraffin wax. Tissue samples were further confirmed by pathologic examination as full clinical data. All cases were diagnosed as squamous cell carcinomas. Information consent forms had been filled by all the patients participating this study. This study was approved by the Ethics Committees of Subei People's Hospital and National Engineering Center for Biochip at Shanghai. Follow-up information was conducted through phone call and out-patient review. All cases in this study had complete follow-up information, and their survival time was calculated from the date of surgery up to the last date of follow-up time (July, 2012), or death date.

### Tissue microarray construction

The sample used for tissue microarray construction was collected from representative lung squamous carcinoma and its adjacent benign lung tissues. The specimens were formalin-fixed and paraffin-embedded. Microarray blocks were constructed using an automated tissue microarray instrument. First, the instrument was used to make holes with a diameter of 0.6 mm and a depth of 2 mm on the paraffin block. Second, the tissue mass of each group



**Figure 1.** Immunohistochemical analysis of GNL3 expression in lung squamous carcinoma. A. Negative GNL3 in paracancerous tissues. B. Negative GNL3 in lung squamous carcinoma tissues. C. Positive GNL3 in lung squamous carcinoma tissues.

were obtained through the fine hollow needles of the instrument, which was then pressed into the paraffin block holes. The region of the sample selected was decided based on the microscopic test of pathological sections through H&E staining by pathologist. In such a way, serial sections (0.66  $\mu$ m thick) were made from the arrayed paraffin block and placed into glass slides for the paraffinized tissue microarray. The samples were arrayed according to lung squamous carcinoma and adjacent benign tissues. Finally, the tissue microarray was validated by two pathologists using H&E staining.

## Immunohistochemistry (IHC) and scores

The expression of GNL3 in lung squamous carcinoma and benign lung tissues were observed by IHC, with a protocol using streptavidin-biotin-peroxidase complex method. Experimental procedures were performed based on the agent instructions. First, to rewarm the tissue microarray, the microarray was taken out from the refrigerator and placed in a glass slide rack, followed by a heating at 65°C for 1 hr to melt the seal wax away from the surface. Subse-

quently, the slide was soaked two times in xylene for 15 min, followed by in absolute alcohol for 7 min, and then in 95% ethanol for another 5 min, and lastly soaked at 70% ethanol for 5 min to remove the paraffin wax. For antigen retrieval, the slides were treated under high temperature with microwave in 10 mm sodium citrate buffer (pH 6) for 10 min, and then incubated at room temperature for 30 min. After blocking of non-specific binding, the sample was added with a drop of GNL3 antibody (Abcam, USA) at a dilution of 1:800. After that, slides were incubated with primary antibody overnight in a humid chamber at 4°C, and then washed three times with PBS for 1 min. To have a negative control, one of the slide was treated with PBS buffer instead of primary antibody at this step. Afterwards, the sample was treated with a drop of biotin-labeled second antibody, incubated at 37°C for 30 min, and washed three times with PBS buffer for 1 min. Subsequently, the sample was incubated at 37°C for 30 min with a drop of horseradish peroxidase-conjugated streptomycin working solution, and washed three times with PBS buffer



**Table 2.** Associations between GNL3 expression and the clinicopathological features of patients with lung squamous carcinoma

Variables	GNL3 expression		P value
	Negative	Positive	
Age (years)			
≤ 60	19	14	1
> 60	23	18	
Sex			
Male	38	31	0.689
Female	4	2	
Tumor size (cm)			
≤ 4.5	17	20	0.106
> 4.5	25	13	
Histologic grade			
1	2	0	0.446
2	29	24	
3	11	9	
Lymph node status			
Positive	15	13	0.809
Negative	27	19	
TNM stage			
I	15	13	0.480
II	19	17	
III	8	3	

for 5 min. Finally, the sample was stained with DAB/H<sub>2</sub>O<sub>2</sub> reaction, and fully washed with water. After several routine steps including counterstaining with hematoxylin (Sigma, USA), dehydrating, transparentizing, drying, and sealing, the IHC results were blindly determined by two independent pathologists.

#### Statistical analyses

The relationship between GNL3 expression and clinical pathological parameters was compared using Fisher's exact test and the  $\chi^2$  test. The Kaplan-Meier method and the Log-rank test were used to compare the survival rates among different groups. The Cox proportional hazard model was used for survival analysis.  $P < 0.05$  was considered statistically significant. The statistical analysis was carried out using the SPSS 20.0 (SPSS Inc., Chicago, USA).

#### Results

##### Clinicopathological features

The clinicopathological features of 75 patients with squamous carcinoma were summarized in

**Table 1.** Among 75 patients, 6 were female (8.0%), and 69 were male (92.0%). The median of age was 62.0 years (range 31.0-77.0 years). Two cases (2.7%) were well differentiation, 53 (70.7%) were moderate, and 20 (26.7%) was poor. Twenty eight cases (37.3%) were stage I, 36 (48.0%) were stage II, and 11 were stage III. Additionally, 46 cases (61.3%) of all didn't suffer from regional lymph node metastasis (LNM), whereas the other 28 cases (37.3%) had regional lymph node metastasis. No distant metastasis occurred in any cases. The tumor size ranges from 2.5 cm to 12.0 cm, with 5.0 cm as median.

##### Relationship between GNL3 expression and clinicopathological features of patients

The IHC results showed that GNL3 was localized in the nucleus. The percent positive stain of GNL3 in lung squamous carcinoma and adjacent normal tissues were 56.0% and 0.0%, respectively (**Figure 1**). Lung squamous carcinoma tissues had higher expression levels of GNL3 compared with paracancerous tissues ( $P < 0.001$ ). No association was observed between GNL3 expression and clinicopathological features of patients ( $P > 0.05$ , **Table 2**).

##### Survival analysis

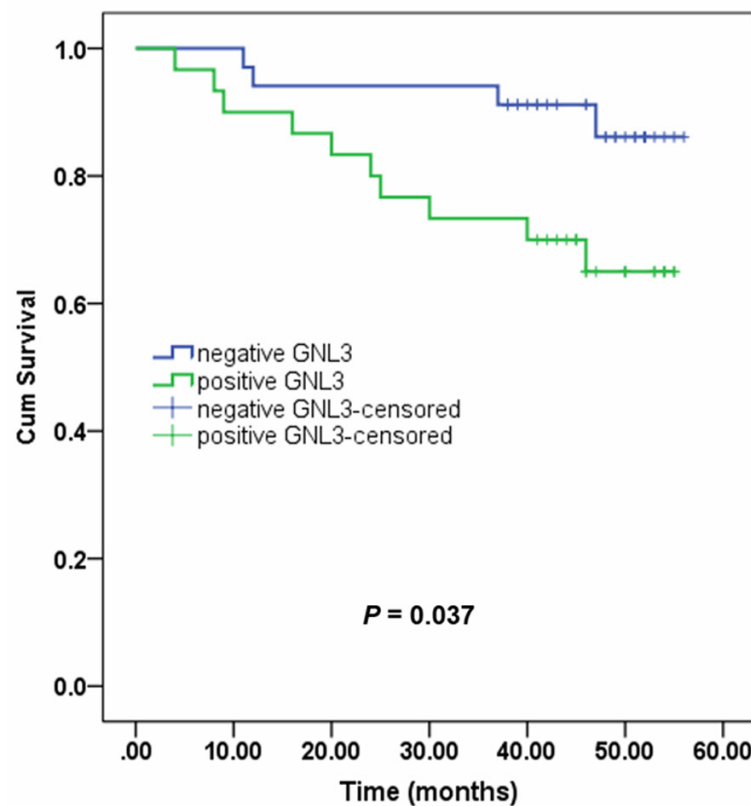
The median survival time (MST) was 46.4 months. During 56.0 months follow-up, 21 (28.0%) died as a result of disease progression. The MST of GNL3-positive patients was 48.4 months, whereas MST of GNL3-negative patients was 43.3 months. However, no statistical significance was detected between two groups ( $P = 0.164$ ). Further stratification analysis showed that GNL3 was associated with prognosis of patients with TNM stage I and II (HR = 3.218, 95% CI: 1.008-10.273,  $P = 0.048$ ) (**Table 3**). GNL3-positive patients had shorter survival time than those with GNL3-negative tumors (**Figure 2**).

#### Discussion

Lung cancer has a complex and heterogenous molecular basis. Better understanding the molecular mechanism of lung cancer carcinogenesis and identifying novel biomarkers may improve the diagnosis, prognostication and treatment of lung cancer. To date, more and more molecular abnormalities have shown up in lung cancer, including gene mutations and

**Table 3.** Univariate Cox regression analysis of overall survival in patients with TNM stages I and II

Variables	Univariate analysis	
	HR (95% CI)	P value
Age (years), > 60 vs. ≤ 60	3.042 (0.848-10.911)	0.088
Sex, female vs. male	0.043 (0.000-170.462)	0.458
Histologic grade, 3 vs. 1+2	1.517 (0.508-4.528)	0.455
Tumor size (cm), > 4.5 vs. ≤ 4.5	1.021 (0.358-2.913)	0.969
LN, positive vs. negative	0.703 (0.196-2.521)	0.588
TNM, II vs. I	1.008 (0.349-2.909)	0.988
GNL3 expression, positive vs. negative	3.218 (1.008-10.273)	0.048



**Figure 2.** Kaplan-Meier estimates of overall survival among 64 patients with TNM stages I and II according to GNL3 expression.

gene expression level variation [11, 25]. Besides the previously known mutations happen on KRAS, EGFR, and TP53 genes [26], new gene mutations are found by using whole genome sequencing methods. The results show that somatic mutations frequently appear on CSMD3 gene in non-small cell lung cancer [27], and on KDR, NTRK, NF1, APC, RB1 and ATM genes in lung adenocarcinomas [28]. Another two studies reveals gene mutation rates are extremely high in lung cancer [29,

30]. The advanced sequencing technologies are providing more sophisticated insights into the molecular aberrations underlying lung cancer. In addition, in lung cancer, aberrations are also found at epigenetic level, involving DNA methylation, histone tail modification and non-coding, microRNA regulation [31]. These findings imply that more attentions have been paid to understanding molecular level of lung cancer, and these genes may be potential diagnosis markers and clinic drug targets in the future. The current study identified that GNL3 expression was inverse associated with overall survival in patients with early-stage lung squamous carcinoma. This result indicated that GNL3 may be a valuable biomarker for early-stage lung cancer.

Many studies have found that GNL3 plays a very important role in cell proliferation, a considerable biological process in carcinogenesis [14, 16, 18, 19]. There are evidences showing that GNL3 promotes cell proliferation through different mechanisms. In nucleus, GNL3 interacts with and stabilizes MDM2, an important negative regulator of the tumor suppressor gene p53, thus decreases the level of p53

and increases cell proliferation rate [20-22, 32]. However, other voices are raised that GNL3 also regulates cell proliferation via a p53-independent manner. Upon knockdown of GNL3, the expression of cyclin D1 and survivin is shutted off in stromal stem cells [33]. In a leukemia cell line HL-60, knockdown of GNL3 causes inhibition of PI3K-AKT pathway, JAK-STAT pathway, RAS-RAF-MEK-ERK1/2 pathway and activation of JNK pathway, p38 MAPK pathway [24].

Given that GNL3 localizes to nucleolus [34-36], a organelle for ribosome biosynthesis [37], it is tempting to speculate that GNL3 plays a role in some ribosomal functions. Previous studies have demonstrated that GNL3 participates in several steps of pre-rRNA processing. Knockdown of GNL3 delays the processing of 32S pre-rRNA into 28S rRNA in human cells [34], and disturbs the integrity of small nucleolar ribonucleoproteins (snoRNPs), which finally reduce cellular level of pseudouridine modification of rRNA [35]. In zebrafish, knockdown of GNL3 impairs 60S large ribosomal subunit formation, and further leads to a reduction of total protein synthesis [38]. It is known that enhancing a couple of key steps of ribosomal synthesis promote cell proliferation, including rDNA transcription [39, 40], pre-rRNA processing [41] and snoRNPs assembling [42]. Moreover, many ribosomal function-related protein are found highly expressed in lung cancer [43], and inhibition of some ribosomal proteins reduces the proliferation rate of lung cancer cells [44, 45]. These findings provide a clear clue that GNL3 may promote cell proliferation via regulating ribosomal biosynthesis in lung cancer.

Considering GNL3 is a multi-functional protein, GNL3 may regulate lung cancer carcinogenesis through other mechanisms besides cell proliferation, such as cancer cell invasion [16, 19] and cell cycle [13, 22]. Intriguingly, GNL3 is required for self-renew in stem cells [23, 46], and plays an unexpected role in safeguarding the genome integrity of stem and cancer cells [23, 46-48]. Additionally, GNL3-enriched mammary tumor cells exhibit stronger tumorigenic activities and express higher levels of CD133 and Oct4 [49], markers of stem cell. GNL3 over-expression seems to have a causative role in gastric tumorigenesis and progression [50]. GNL3 may provide self-renew capability to some cancer cells, and increase the potential for cancer-stem cell formation, which plays a critical role in malignant transformation and tumor-initiation. In this study, we also found that early-stage patients with positive GNL3 had poor prognosis. Since there are few samples at advanced stage, further large-scale studies are warranted to validate these results.

In conclusion, our study revealed that GNL3 expression was correlated to the prognosis of patients with early-stage lung squamous carcinoma. GNL3 is a potential novel biomarker for lung cancer. Further investigations are needed

to better understanding the molecular function of GNL3 in lung cancer, and to find out the specific drugs targeting GNL3 in clinical therapy.

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## Disclosure of conflict of interest

None.

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