

# HRCT Scans of Peripheral Non-Small Cell Lung Cancers and their Relationship with Cyclin D1 Expression: a Longitudinal Study

Shuhua Ma<sup>1,2</sup>  
Linfa Wu<sup>1</sup>  
Xiaomao Xu<sup>3</sup>  
Hongbo Le<sup>1,2</sup>  
Xiaoling Cheng<sup>1,2</sup>  
Huihong Zhang<sup>1,2</sup>  
Zhengyu Sun<sup>4</sup>  
Zhaoxin Wang<sup>1,2</sup>  
Min Wu<sup>1</sup>  
Wei Mei<sup>1,2</sup>  
Zhiguo Hu<sup>1,2</sup>  
Yuguang Li<sup>1</sup>

<sup>1</sup> Department of Radiology, First Affiliated Hospital, Medical College of Shantou University, Shantou 515041, Guangdong Province, China.

<sup>2</sup> Guangdong Key Laboratory of Medical Molecular Imaging, Shantou 515041, Guangdong Province, China.

<sup>3</sup> Department of Radiology, Second Affiliated Hospital, Medical College of Zhongshan University, Guangzhou 510120, Guangdong Province, China.

<sup>4</sup> Department of Radiology, First Hospital of Harbin, Harbin 150010, Heilongjiang Province, China.

Correspondence to: Shuhua Ma  
Tel: 86-754-8526 432  
E-mail: mashuhua6699@sina.com

This work was supported by grants from Natural Science Foundation of Guangdong, China (No.06301077), Administration of Traditional Chinese Medicine of Guangdong, China (No.2060134), the Health Department Foundation of Guangdong, China (No.A2007421), the Shantou Technology Bureau Science Foundation of China (No.Shantou Government Technology [2006] 85).

Received October 26, 2007; accepted May 13, 2008.

CJCO <http://www.cjco.cn>  
E-mail: 2008coccr@gmail.com  
Tel (Fax): 86-22-2352 2919

**OBJECTIVE** To investigate cyclin D1 expression in peripheral lung cancers and its relationship with CT signs and prognosis.

**METHODS** Cyclin D1 expression in peripheral lung cancers and its relationship with the CT imaging and prognosis were analyzed retrospectively by means of SP immunohistochemistry and spiral CT scanning in 92 patients with peripheral lung cancer verified by pathology.

**RESULTS** Cyclin D1 expression was related to deep lobulation, spiculate protuberance, short thin spinules sign and mediastinal lymph node metastasis. Cyclin D1 expression was not related to tumor size, cavity, pleural indentation, histological type, differentiation, tumor TNM stage, age or sex. Cyclin D1 was a negative prognostic factor whose over-expression indicated a poor prognosis.

**CONCLUSION** Cyclin D1 expression may play an important role in the occurrence, progress and CT scan results of lung cancers. Cyclin D1 was a negative factor whose over-expression implied a poor prognosis. Detection of the cyclin D1 and observation of the CT scan can be considered as indexes of clinical diagnosis and prognostic evaluation.

**KEY WORDS:** lung neoplasms, cyclin D1, computed tomography.

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## Introduction

Growing patterns, characteristic shapes, degree of malignancy, relapse, and metastasis of cancers are mainly determined by their molecular biology. A change in the shape of a tumor, influenced by its biological behavior, may be detected by a CT scan. A pathological change links a CT image to molecular biology, thus making it possible to investigate the internal relationship between a CT scan and biological behavior. As a type of oncogene, cyclin D1 is an important cyclin protein in the G1 phase of the cell cycle<sup>[1-3]</sup>. The expression of cyclin D1 in peripheral non-small cell lung cancer has been studied using streptavidin peroxidase (SP) immunohistochemistry. Because of the relationship between cyclin D1 expression and CT imaging, the biological behavior and prognosis of peripheral lung cancer patients can be investigated. CT scans and expression of proteins both can be used to recognize macro/micro changes in lung cancers.

## Materials and Methods

### General clinical data

From May of 1998 to December of 2001, 92 patients with peripheral, non-small cell lung cancer—52 men and 40 women, ranging in age, 32–73 (mean age 56.3) with no prior chemical and/or radiation treatment before surgery—were studied with their consent. All had

undergone lung lobectomy, or a single lung removal and extensive lymph node ablation. Prior to surgery all had a CT scan. All records—age, sex, histological type, differentiation, tumor TNM stage, mediastinal lymph node metastasis, and the longevity—were stored in the records of the Department of Chest Surgery. CT imaging data for all the cases were stored in the Department of Radiology.

The study was performed with the approval of the human subjects ethics committee of the First Affiliated Hospital, Medical College of Shantou University.

### **Group and classification**

The histologic classification of the lung cancers was based on the guidelines of the World Health Organization (1999). Thirty-seven cases were squamous cell carcinoma and 55 cases were adenocarcinoma. According to the tumor grading standard (tissue structure, atypia, multiple nuclei), all the patients were classified as high, middle or low-differentiated groups. The tumor TNM stage was based on the guidelines of the International Union Against Cancer (UICC, 1997), and the cases were divided into Stages I, II, III, and IV. The 92 cases were assessed at 6, 12, 18, 24, 30, 36, 42 and 48 months after surgery. Of the 92 cases, 19 patients were dropped from the study due to death, or other diseases. Using the CT scan results, the patients were divided into 2 groups; one whose tumor size was less than 3 cm, and the other, whose tumor size was greater than 3 cm. Further classification was conducted based on whether the following conditions were present, or absent—deep lobulation, spiculate protuberance, short thin spinules, cavity, pleural indentation, and mediastinal lymph node metastasis.

### **HRCT examination technology**

Before surgery, all patients were examined, using a Toshiba Xpress/GX spiral CT machine; from the apex of the lung to the diaphragmatic dome in 10 mm sections, with one pitch and reconstruction thickness of 10 mm. After the regular spiral scanning, 4 slices of a high resolution scan (2 mm thickness and 4 mm spacing) were taken to observe the tumor edge and changes inside the lung and mediastinal lymph nodes.

### **Standards for CT evaluation**

Deep lobulation refers to a tumor margin with a protrusion whose cord length is 2.5 times or more of that of the perpendicular arc-cord distance. A short thin spinule sign refers to a rigid and small spinule protuberance of the tumor margin. Spiculate protuberance means an angular protuberance, with the distal end much narrower than proximal end. Mediastinal lymph node metastasis means that the shortest diameter of the mediastinal lymph nodes is more than 10 mm.

The analyses of the CT imagines from a double-blind test by two experienced radiologists were mostly consistent—92.5% of each other. Total consensus was reached

after further discussion with a third radiologist.

### **Immunohistochemical assay**

Tissue in each case was fixed by formalin and embedded in paraffin. Paraffin sections (5  $\mu$ m thickness) were used for immunohistochemistry using a streptavidin peroxidase (SP) kit and a hematoxylin and eosin staining kit (from Fuzhou Maixin Co., China). The slices were deparaffinized and dehydrated in graded alcohols. Heat-induced antigen retrieval was performed by using a microwave oven and citrate buffer (pH, 6.0; 10 mol/L). All samples were immunostained using the SP protocol with the monoclonal rabbit antibodies cyclin D1 (1:100 dilution, from Fuzhou Maixin Co., China). The positive breast cancer paraffin sections supplied with the SP kit were taken as positive controls, and the samples with primary antibody replaced by PBS were taken as negative controls.

### **Standards for immunohistochemical evaluation**

Cyclin D1-positive brown staining was located in the nucleus. The cyclin D1 staining was graded in terms of its extent and intensity as shown by Mattern et al.<sup>[4]</sup> To evaluate the cyclin D1 expression, a score corresponding to the sum of both (a) staining intensity (0 = negative; 1 = weak; 2 = intermediate; 3 = strong) and (b) percentage of positive cells (0 = 0% positive cells; 1  $\leq$  25% positive cells; 2 = 25–50% positive cells; 3  $\geq$  50% positive cells) was established. The sum of (a) and (b) reached a maximum score of 6. Ten fields of vision were observed and a score calculated as follows: negative, 0–2; positive, > 2; weakly positive 3–4; strongly positive expression, 5–6.

Cyclin D1 expression and CT scans were compared and was amenable to statistical analyses. The weakly and strongly positive expressions were considered positive in the study. Because Mattern's method is comparatively standardized, the immunohistochemical results were not analyzed and the gray scale value of every visual was not determined by computer.

Cyclin D1 expression was assessed by two experienced pathologist in lung pathology with 93.8% agreement. Where needed, consensus was reached after discussion with a third experienced pathologist.

### **Statistical analysis**

The chi-square test was used to compare two or multiple groups. The  $\chi^2$  value and *P* value were calculated by the Pearson method. The cumulative survival rate and median survival period (survival time corresponding to 50% of the survival rate) were estimated by life-span tables. The Kaplan-Meier and Log-rank test were used to evaluate the prognosis. The Cox regression model (enter method) was used to analyze relationships between multiple factors and the prognosis. All statistical analyses were performed using statistical software, SPSS 13.0 for Windows.

## Results

### Cyclin D1 expression

Of the 92 cases of peripheral lung cancer, 50 cases were positive and 42 were negative, the cyclin D1 positive rate was 54.35%.

### Relationship between cyclin D1 expression and clinical biological characteristics

The relationship of the histological type, differentiation, tumor TNM stage, age and sex compared to the positive rate of cyclin D1 expression showed no significant difference,  $P > 0.05$  (Table 1).

### Relationship of cyclin D1 expression with CT images

In the groups with deep lobulation, spiculate protuberance, short thin spinule images and mediastinal lymph node metastasis, the positive rate of cyclin D1 expression showed significant differences,  $P < 0.05$ . The level of cyclin D1 expression was obviously higher in those without the above mentioned images. In the groups with tumor size, cavity and pleural indentation, the positive rate of cyclin D1 expression did not show significant differences,  $P > 0.05$  (Table 2, Figs.1 and 2).

### Survival of patients with positive and negative cyclin D1 expression

Among the 92 cases of peripheral non-small cell lung cancer, 19 patients were lost for follow-up, including some who died from other diseases. The cumulative survival rate and median survival period were estimated from a life-span table for cyclin D1 positive and negative expression. The median survival period for the negative group was 34.2 months, and the median survival period for the positive group was 22.3 months. Cumulative survival rates and median survival period were lower in the positive group compared to those in the negative group. The comparison using the Log-rank test showed that the  $\chi^2$  was 4.763, and  $P$  value,  $0.029 < 0.05$ . The highly significant difference found between the survival periods of the 2 groups indicated that cyclin D1 was a negative prognostic factor (Table 3, Fig.3).

**Table 1. Relationship between cyclin D1 expression and the clinical biological characteristics of peripheral non-small cell lung cancer.**

Characteristic	<i>n</i>	Positive (%)	Negative	$\chi^2$	<i>P</i>
Age, years				1.104	0.293
$\geq 50$	60	35 (58.33)	25		
$< 50$	32	15 (46.88)	17		
Sex				1.896	0.169
Male	52	25 (48.08)	27		
Female	40	25 (62.50)	15		
Histology				1.523	0.217
Squamous cell carcinoma	37	23 (62.16)	14		
Adenocarcinoma	52	27 (49.09)	28		
Differentiation				0.391	0.822
High	35	19 (54.29)	16		
Middle	37	19 (51.35)	18		
Low	20	12 (60.00)	8		
Stage				0.040	0.998
I	33	18 (54.55)	15		
II	16	9 (56.25)	7		
III	26	14 (53.85)	12		
IV	17	9 (52.94)	8		

**Table 2. Relationship between cyclin D1 expression and CT images of peripheral non-small cell lung cancer.**

Characteristic	<i>n</i>	Positive (%)	Negative	$\chi^2$	<i>P</i>
Size				0.224	0.636
$> 3$ cm	55	31 (56.36)	24		
$\leq 3$ cm	37	19 (51.35)	18		
Deep lobulation				6.139	0.013
Yes	48	32 (66.67)	16		
No	44	18 (40.91)	26		
Spiculate protuberance				4.727	0.030
Yes	42	28 (66.67)	14		
No	50	22 (44.00)	28		
Short thin spinule sign				4.756	0.029
Yes	55	35 (63.64)	20		
No	37	15 (40.54)	22		
Cavity				0.004	0.952
Yes	26	14 (53.85)	12		
No	66	36 (54.55)	30		
Pleural indentation				0.411	0.522
Yes	34	17 (50.00)	17		
No	58	33 (56.90)	25		
Mediastinal lymph node metastasis				5.220	0.022
Yes	47	31 (65.96)	16		
No	45	19 (42.22)	26		

### The significance of the Cox regression model and CT scans on the of prognosis

The results of Cox regression model with multiple factor analyses (enter method) showed that cyclin D1 expression, spiculate protuberance, short thin spinule sign and mediastinal lymph node metastasis revealed a statistical significance,  $P < 0.05$ . The coefficient value of cyclin D1 expression, spiculate protuberance, short thin spinule sign and mediastinal lymph node metastasis were positive, indicating that they were independent hazardous prognostic factors. Spiculate protuberance, a short thin spinule sign, cyclin D1 expression and mediastinal lymph node metastasis had a bigger OR value, which indicated that they were closely related to the prognosis of lung cancer (Table 4).

### Discussion

The main cause of death for lung cancer patients after an operation is local relapse or metastasis. Therefore, the estimation of the probability of relapse, or metastasis after surgery and treatment based on the level of cyclin D1 expression has become an important issue<sup>[5-8]</sup>.

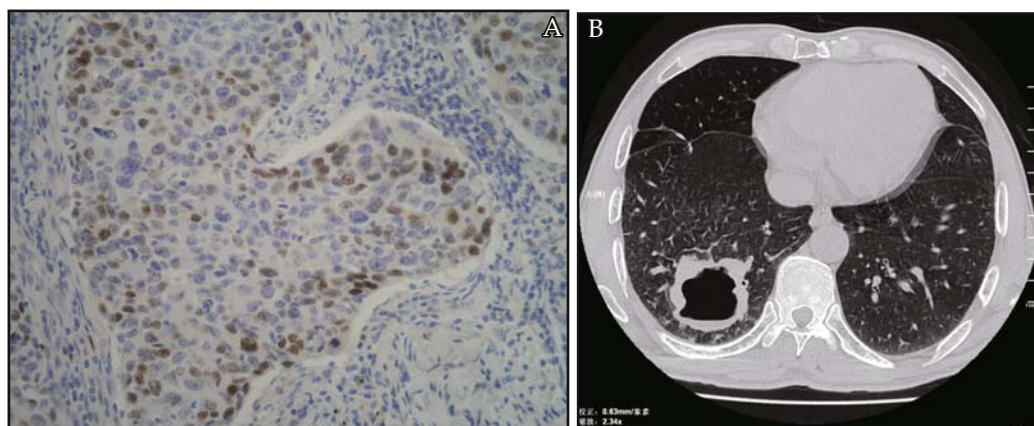
Cyclin D1 can induce a positive-regulatory effect in the cell cycle, especially in the G<sub>1</sub>-S transition. It can initiate a cell cycle, promote DNA synthesis, and accelerate cell generation. Therefore it is an important checkpoint for the G<sub>1</sub>~S in G<sub>1</sub>, S, G<sub>2</sub> and M phases of the cell cycle. Cells no longer need to depend on a surrounding stimulus and can finish division quickly once they enter the S from G<sub>1</sub>. Cyclin D1 can promote the cells to enter

**Table 3. Survival rate and median survival period related to cyclin D1 positive and negative expression.**

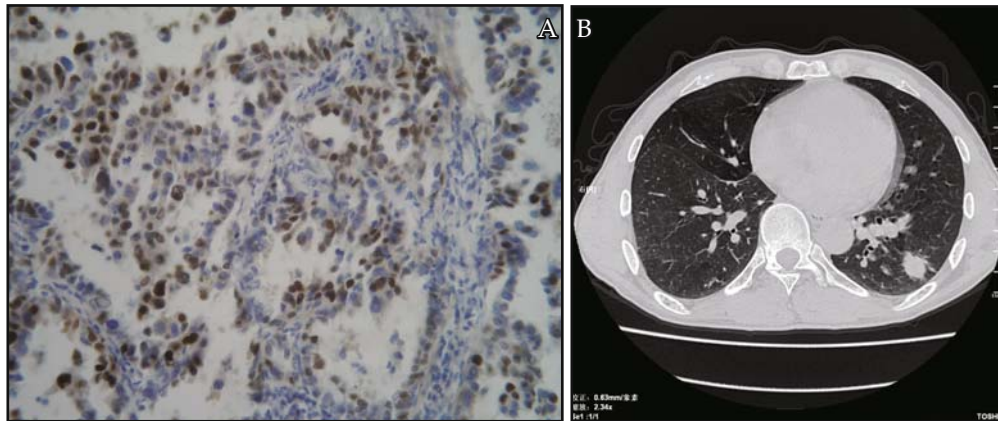
Cyclin D1	Survival rate (%)								
	6 months	12 months	18 months	24 months	30 months	36 months	42 months	48 months	Median months
Positive	74.00	58.00	49.56	42.31	32.16	16.08	0	0	22.30
Negative	85.71	80.95	69.05	61.90	49.77	34.84	27.10	27.10	34.20

**Table 4. Results of analysis using the Cox regression model.**

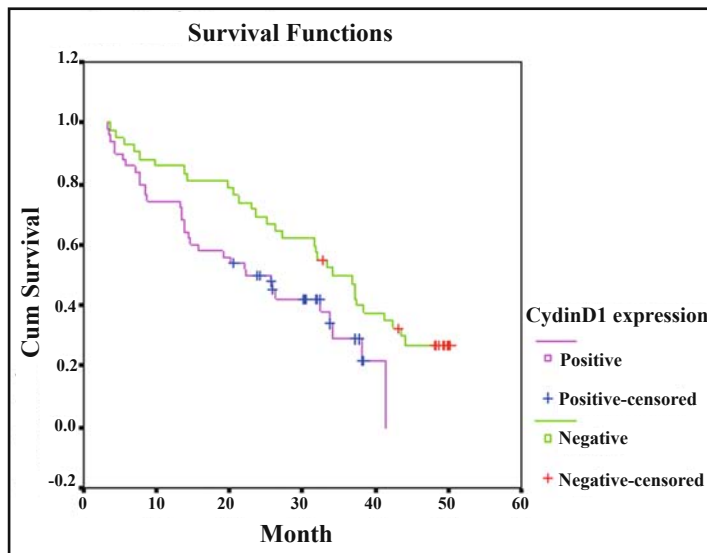
Characteristic	B (coefficient value)	Wald ( $\chi^2$ )	Sig ( $P$ )	Exp (B) (OR value)
Cyclin D1	0.813	4.918	0.027	2.255
Age	-0.839	3.068	0.080	0.432
Sex	-0.900	3.605	0.058	0.407
Histology	0.559	1.758	0.185	1.748
Differentiation	-0.075	0.085	0.771	0.928
TNM stage	-0.260	2.188	0.139	0.771
Size	0.656	2.068	0.150	1.926
Deep lobulation	1.031	3.159	0.075	2.804
Spiculate protuberance	3.038	10.713	0.001	20.865
Short thin spinule sign	2.227			9.273
Cavity	0.584	2.384	0.123	1.794
Pleural indentation	0.115	0.069	0.792	1.122
Mediastinal lymph node metastasis	1.217	4.831	0.028	3.378



**Fig.1. High-differentiation squamous cell carcinoma in the posterior basal segment of the inferior lobe of the right lung of a 57-year-old male.** A, S-P immunohistochemical staining shows a strong positive expression of cyclin D1 ( $\times 400$ ), the positive brown staining is located in the nucleus, the staining is strong, and positive cell percentage is more than 50%. B, Lung window of HRCT shows that there is an irregular cavity in the nodule, a short thin spinule sign, deep lobulation and a spiculate protuberance in the edge of the nodule.



**Fig.2.** A high-differentiated adenocarcinoma in the outer and posterior basal segment of the inferior lobe of the left lung of a 63-year-old male. A, S-P immunohistochemical staining shows strongly positive expression of cyclin D1 ( $\times 400$ ), the positive brown staining is located in the nucleus, the staining is strong, and the percentage of positive cells more than 50%; B, Lung window of high-resolution CT displays a short thin spinule sign, deep lobulation and spiculate protuberance are observed in the edge of the nodule.



**Fig.3.** Cumulative survival function curve of patients with positive and negative cyclin D1 expression, indicating that cumulative survival rate and median survival period were lower in the positive group compared with in negative group.

the S phase from  $G_1$ . Under normal circumstances, cyclin D1 is stable, and maintains a very lower level in the G1 phase. Cyclin D1 over-expression can cause the G1 phase to shorten, and stimulate the cells to over proliferate which ultimately causes cell growth to get out of control and promote tumor development<sup>[9-13]</sup>.

Our study indicated that and cyclin D1 expression was not significantly related to the clinical biological characteristics such as age, sex, histological type, differentiation, or tumor TNM stage. This is consistent with the findings of Masuda et al.<sup>[14]</sup> Betticher et al.<sup>[15]</sup> showed that cyclin D1 over-expression was not related to the histological type or TNM stage, which is consistent with our study. However, there was one difference in that latter study—cyclin D1 over-expression was not related to a different degree of the tumor.

HRCT examinations are one of the best imaging ways to evaluate characteristics such as the form and density of lung cancers. The variety of CT images of lung cancer is determined by the different growing patterns of

the tumors, which are closely related to cyclin D1.

The formation of lobulation images not only related to differentiation and a difference in the rate of growth, but also the indentation of the tumor caused by the spacing of pulmonary connective tissue, blood vessels, bronchial branching, and connective tissue growth of the tumor<sup>[16]</sup>. Deep lobulation signs play an important role in the diagnosis of peripheral lung cancers. Benign tumors show mainly shallow lobulation if lobulation does exist. In the research of 48 cases with deep lobulation, the level of cyclin D1 expression was significantly higher compared to those without this sign, indicating that deep lobulation indicated a higher degree. It also demonstrated one of the basic signs of peripheral lung cancer, and that it is vital for diagnosis of this tumor.

The pathological basis of a spiculate protuberance is infiltrating growth of malignant cells into the cancer nest, or lung parenchyma proximal to the tumor. This results in edema, fibrous degeneration, thickening and formation of a spiculate protuberance. The different

blood supply in various parts of the tumor tissue result in some parts growing faster, and malignant cells infiltrating the nearby connective tissue, proximal bronchus and lung artery, or extending along lymphatic vessels.

In our research of 42 cases with a spiculate protuberance, the positive rate of cyclin D1 expression was higher than those without this sign, which revealed that a spiculate protuberance was an indication of increased cellular proliferation.

Spinule signs are divided into short thin spinules, and long thick spinules. A short thin spinule is shorter, thinner, straighter and larger. It appears mainly in lung cancer. Its pathological basis is the infiltrated growth, effusion, proliferation reaction and inflammation of lympho-capillary vessels. Long thick spinules are longer, thicker, softer and less. They appear mainly in inflammation. Their pathological basis is the interlobular septum thickening by inflammation cell infiltration or the dragged micro blood vessel. In our research of 55 cases with a short thin spinule sign, the level of cyclin D1 expression was obviously higher than those without the sign, which revealed that short thin spinule signs pointed to more malignant characteristics of the peripheral lung cancer.

Mediastinal lymph node metastasis is an important index of a tumor prognosis. CT scanning of our 47 cases with mediastinal lymph node metastasis, showed the level of cyclin D1 expression was markedly higher than those without the sign, indicating that cyclin D1 expression was related to mediastinal lymph node metastasis.

Only a few reports have been published concerning the relationship between cyclin D1 expression and medical imaging of peripheral lung cancers. Our study indicated that cyclin D1 expression was related to mediastinal lymph node metastasis which was consistent with Masuda et al.<sup>[14]</sup> However, Mate et al.<sup>[17]</sup> showed that cyclin D1 expression was not related to lymph node metastasis, which is different from our study.

Using the SP immunohistochemical method, Hu and Wang<sup>[18]</sup> studied the relationship between cyclin D1 expression and CT signs in peripheral lung cancer. They found that cyclin D1 expression was related to deep lobulation, spiculate protuberance, rather than pleural indentation or histological type, which is consistent with this study. However, they reported that cyclin D1 expression was related to tumor size and differential degree, and denied a relationship between cyclin D1 expression with spinule signs and lymph node metastasis, which is not consistent with our study.

Our study indicated that the cumulative survival rate and median survival period were significantly lower in the positive group than in the negative group. Cyclin D1 over-expression implied a worse prognosis. This is consistent with Masuda's report<sup>[14]</sup>, but not completely in agreement with Nishio's study<sup>[19]</sup> who found that cyclin D1 expression was related to the prognosis of adenocarcinoma patients, rather than squamous cell carcinoma. However, the survival period of adenocarcinoma pa-

tients was shorter in the negative group.

There are some differences between the results of the above-mentioned researchers and ours. The inconsistency may be that the tumor location is more accurate, but was not quantified by means of immunohistochemistry detecting cyclin D1 protein expression. Immunohistochemistry is a substrate-catalyzed reaction by a tagged antibody. Distribution and amount of protein are indirectly determined observed by noting the distribution and amount of colored material formed. The immunohistochemical results are evaluated from both staining intensity and percentage of positive cells. However, the staining intensity is more affected by human factors. Therefore, the quantification is not accurate. These immunohistochemical results need to be validated further by Western-blot and PCR studies in the future.

The Cox regression model with multiple factor analyses was used to analyze 13 variants in this study. The results showed that cyclin D1 expression, spiculate protuberance, short thin spinule signs, and mediastinal lymph node metastasis were best correlated with the prognosis of lung cancer, rather than other factors. A positive coefficient value for cyclin D1 expression, spiculate protuberance, short thin spinule sign and mediastinal lymph node metastasis, indicated that they were independent risk prognostic factors. If the cyclin D1 expression, spiculate protuberance, short thin spinule signs, and mediastinal lymph node metastasis had a large OR value, this indicated that they were closely related to the lung cancer prognosis.

Briefly, the purpose of investigating cyclin D1 expression in peripheral non-small cell lung cancers and its relationship with the HRCT scans and prognosis, is to better understand the relationship between the degree of the biological factors involved in the prognosis of lung cancer, growing patterns as well as the characteristic shapes by contrast study between genes and imaging. This is beneficial for the timely treatment of peripheral non-small cell lung cancer.

Cyclin D1 expression may play an important role in the occurrence, progression, and HRCT scans of lung cancers. Cyclin D1 was a negative factor, which when over-expressed, implied a poorer prognosis. Detection of cyclin D1 and observation of the HRCT sign can be considered as indices of clinical diagnosis and prognostic evaluation.

## Acknowledgements

The authors are grateful to Tao Huang M.D. for excellent technical assistance; Lihua Sun for immunohistochemical studies; Baojun Huang M. D. for conducting the mass analyses of CT images. The English editorial assistance of John K. Lim, M.D. from the USA is gratefully acknowledged.

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