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Rb/p16 pathway in non-small-cell lung cancers

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[**Abstract**] **Objective:** To investigate the expression of Retinoblastoma (Rb) and p16 in non-small-cell lung cancer (NSCLC) and their relationship to clinicopathological factors. **Methods:** Eighty cases of NSCLC were studied for expression of Rb and p16 by immunohistochemical technique SP method. **Results:** Fifty-six (70.00%) and 47 (58.75%) cases of 80 patients revealed loss of protein expression for RB and p16 respectively. The correlation was found between Rb and p16 ($P<0.05$). Loss of p16 expression was noted in most squamous and in a small fraction of adenocarcinomas ($P<0.05$). Notably, loss of Rb expression was associated with T stage ($P<0.05$). **Conclusions:** Our results suggest that disruption of Rb/p16 pathway is frequently invoved in NSCLC.

[**Key words**] lung neoplasms; Retinoblastoma; p16

Lung cancer is the most common cause of cancer death. It is generally believed that cancer is the end-result of a multistep process involving the activation of dominant oncogenes and the inactivation of tumor suppressor genes. Retinoblastoma (Rb) and p16 are tumour supressor genes, and play immportant roles in cell cycle regulation. Abnormalities of Rb/p16 tumour suppressor pathway in cell cycle in some cancers have been reported recently, such as gastic carcinoma, hepatocellular carcinoma, oral carcinoma. This study examined the resected samples of 80 non-small-cell lung cancers (NSCLC) patients, to determine the frequency of expression loss of Rb and p16 and their relations with clinicopathologic features by immunohistochemical method. We also investigated the roles of Rb/p16 pathway in lung tumorigenesis.

1 MATERIALS AND METHODS

1.1 *Patient material* Tumor blocks were obtained from 80 patients with primary NSCLC at the Pathology Department of Bengbu Medical College, who had been treated with curative resectional surgery. The samples were collected from Jan 2000 to June 2001. There were 64 males and 16 females, with an age range of 26-77 years. Histologcal subtype included 55 squamous cell carcinomas (SCC), 23 adenocarcinomas, 3 squamous-adenocarcinomas (SAC). The clinical data of these patients including sex, age, location of the tumor, histologic type, tumor size, lymph node metastasis and clinical stage was shown in Table 1.

1.2 *Immunohistochemistry* All surgical specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4 μm-thick sections. One section of each specimen was stained with H&E and used for histological identification. The rest were used for immunostaining.

For immunochemical demonstration of the Rb and p16 protein expression in the tumor tissue, the sections were dewaxed with xylene and rehydrated through a graded series of ethanol. Then 0.3% of H_2O_2 was used to block endogenous peroxidase activity. These were then incubated with goat serum to reduce nonspecific antibody binding. To enhance immunostaining, sections were treated with an antigen retrieval solution and heated in a microwave oven at high power for 7 min. Finally, slides were incubated with primary antibody Rb or p16 (Fuzhou Maxin Company, ready-to-use) in a humidified chamber overnight at 4 ℃. Then antibody bridge and enzyme labelled SP were added, colorized by DAB, stained by hematoxylin. Negtive controls with PBS replacing specific primary antibodies were included in each run.

Immunostaining was classified into negative and positive groups according to both intensity and extent. Two independent pathologists were involved in the assessment of expression.

1.3 *Statistical analysis* The correlation between various clinical or pathological parameters with the expression of Rb or p16 was anlaysed using chi-square test and rank sum test (Kruskal-Wallis method).

2 RESULTS

2.1 *Rb protein expression in NSCLC* In present

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study, Rb nuclear and/or cytoplasmic reactivity was detected in 24 out of the 80 lung cancers(Figure 1). A loss of Rb protein expression was observed more frequently in T1, T2 patients than in T3 patients, and such a difference was also statistically significant ($P<0.05$). However, there were no statistically significant correlations between the loss of Rb expression and patient age, sex, histologic type, gross type, lymph node metastsis and clinical stage (shown in Table 1).

Table 1 Relationships between expression of Rb and p16 and clinicopathological variables(n)

Factors	n	p16				Rb			
		−	+	χ^2	P	−	+	χ^2	P
sex									
male	64	39	25	0.63	>0.05	44	20	0.03	>0.05
female	16	8	8			12	4		
age(years)									
<40	8	5	3				7	1	
40~59	32	15	17	3.13△	>0.05	21	11	1.46	>0.05
≥60	40	27	13			28	12		
gross type location									
diffuse	3	1	2			2	1		
central	53	33	20	1.26△	>0.05	37	16	0.02△	>0.05
peripheral	24	13	11			17	7		
histologic type									
SCC	56	38	18			40	16		
AC	20	7	13	6.61△	<0.05	13	7	0.34△	>0.05
SAC	4	2	2			3	1		
T stage									
T1	14	6	8			9	5		
T2	39	24	15	1.78	>0.05	16	23	12.67	<0.005
T3	27	17	10			3	24		
N stage									
N0	44	25	19			31	13		
N1	17	10	7	0.22	>0.05	12	5	0.03	>0.05
N2	19	12	7			13	6		
Clinical stage									
I	27	15	12			16	11		
II	30	17	13	0.56	>0.05	23	7	2.29	>0.05
III+IV	23	15	8			17	6		

SCC, squamous cell carcinoma; AC, adenocarcinoma; SAC, squamous adenocarcinoma; △ value of Hc

2.2 *P16 protein expression in NSCLC* Forty-seven of 80 tumor samples (58.75%) showed abnormal immunoreactivity for p16 protein expression(Figure 2). There was no statistically significant correlation between the loss of p16 expression and clinicopathologic features except histologic type of tumor. Loss of p16 expression was noted in most squamous (38 out of 56) and in a small fraction of adenocarcinomas (7 out of 20, $P<0.05$).

2.3 *Correlation of Rb expression with p16 protein expression* Rb and/or p16 expression loss was observed in 66 (82.5%) out of 80 patients. The positive rate of both Rb and p16 was 17.5% (14/80). The negative rate expression of both Rb and p16 proteins was 46.25% (37/80). There was a reverse correlation between Rb and p16 expression in 80 NSCLC ($P<0.05$) (Table 2).

Table 2 Correlation of Rb and p16 proteins expression

	p16+	p16−	Total	χ^2	P
Rb+	14	10	24		
Rb−	19	37	56	4.13	<0.05
Total	33	47	80		

3 DISCUSSION

It is now widely accepted that carcinogenesis and progression of lung cancer are related to the activation of pro-oncogenes and/or the inactivation of anti-oncogenes. Both Rb and p16 genes are tumor suppressor genes. They play important roles in the regulation of the cell cycle. The proteins of these two genes, Rb and p16, inhibit cell progression from G1 to S phase. Dephosphorylation of Rb inactivates the transcription factors such as E2F1, an important factor for the transition from G1 to S phase, thereby arresting cells in G0/G1 phase, resulting in suppressed cell division and proliferation. When Rb protein is phosphorylated, several transcription factors are released, which induce the cell from G1 to S phase rapidly, resulting in excessive proliferation of cells. P16 has been shown to exert its function through inhibition of cyclin-dependent kinase 4 (CDK4) mediated phosphorylation of Rb. Functional loss of p16 might reslut in nonregulation of CDK4 activity, leading to persistent Rb phosphorylation and uncontrolled cellular proliferation.

The p16(INK4A) belongs to the G1 control gene involving the "Rb pathway", and the inactivation of p16 gene has been detected in various human malignancies. Several studies have reported that the aberrant p16 expression occurred in 27-62.1% of NSCLC, but its prognostic significance in NSCLC remains controversal[1-3]. In the study of Huang CJ et al[4], the alteration of p16 was considered as a significant factor

for poor prognosis in squamous cell carcinoma. Contrary to this kind of result, abnormal expression of p16 was observed in 58.75% of the cases in our study, which is consistent with the data reported by some others previously[1~3,5]. However, there was no significant association between loss of p16 with clinicopathological parameters except histological type. Loss of p16 expression was found to be significantly greater in squamous cell carcinoma than in adenocarcinoma cases (65% vs 32.14%) ($P<0.05$). Similar data were obtained from previous IHC study[6], and suggested that loss of p16 is a relatively early event in the development of some NSCLC involving tumor differentiation.

Controversial results were reported on the effect of Rb on survival. Although Rb expression loss was found to be an independent prognostic factor in previous reports[6], there was no relation between Rb and age, sex, tumor histology, tumor stage, or nodal status[7,8] reported in others. In this study, the rate of Rb expression loss was 58.25%, nine specimens (64.28%) exhibited alteration of Rb in T1 stage, whereas only 3 cases (11.11%) Rb loss in T3 stage. Rb protein loss was related to tumor stage ($P<0.05$). The possible reason for this result is that physiological inactivation of Rb gene in early stage of lung cancer induces cell progression from G1 to S phase, resulting in excessive cell proliferation. This explanation also confirmed the conclusion about tumor growth fraction. Tumor growth is normally fast in early stage due to active division of malignant cells. As the tumor grows, most malignant cells go into G0 stage and have low growth fraction.

Akin et al confirmed the inverse correlation betwween Rb inactivation and p16 expression in NSCLC. The results in this study showed that there was not only loss of Rb and p16 proteins expression, but also a negative correlation between Rb and p16 expression was found ($P<0.05$) which is consistent with others[6,9]. These support the hypothesis that Rb and p16 genes adjust with each other by negative feedback in cell cycle regulation.

In conclusion, the present study points out that disruption of Rb/p16 pathway is a common event and plays important roles in NSCLC tumorigenesis. Further prospective studies with larger series are needed to confirm these results. Targeting checkpoint proteins in Rb/p16 pathway might represent a good therapeutic strategy for the development of new molecular treatments for lung cancer.

(本文图 1、2见封四)

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Rb/p16路径在非小细胞肺癌中的表达意义

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[摘要]**目的**: 探讨 Rb和 p16基因在非小细胞肺癌(NSCLC)中的表达及其与临床病理因素的关系。**方法**: 采用免疫组化 SP法检测 80例 NSCLC组织中 Rb和 p16的表达。**结果**: 在 80例 NSCLC组织中, 分别有 56例和 47例显示 Rb和 p16表达缺失。两者之间有一定相关($P<0.05$)。p16在鳞状细胞癌比腺癌中有较高的表达($P<0.05$), 而 Rb蛋白的表达异常与肿瘤分期有关($P<0.05$)。**结论**: Rb/p16路径的中断在肺癌的发生发展中是常见事件。

[关键词] 肺肿瘤; Rb; p16

[中国图书资料分类法分类号] R 734.2 [文献标识码] A

Rb/p16 pathing in non-small-cell lung cancers (正文见 256 页)

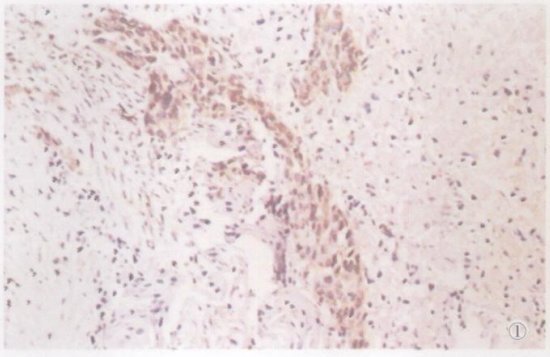

Figure 1 Rb protein is positive expression in NSCLC(×400)

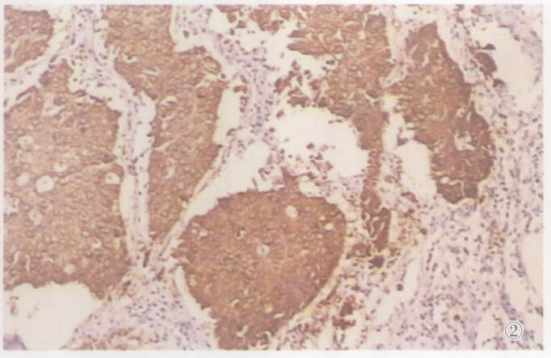

Figure 2 p16 protein is positive expression in NSCLC(×400)

近端型皮样肉瘤 4 例临床病理分析(正文见 309 页)

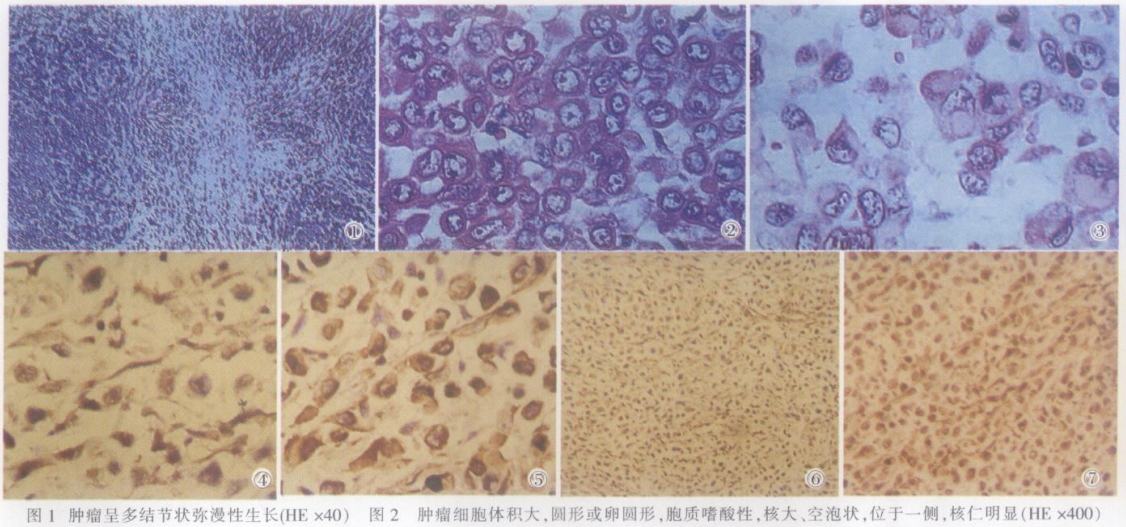

图 1 肿瘤呈多结节状弥漫性生长(HE ×40) 图 2 肿瘤细胞体积大,圆形或卵圆形,胞质嗜酸性,核大、空泡状,位于一侧,核仁明显(HE ×400) 图 3 横纹肌样细胞(HE ×400) 图 4 瘤细胞弥漫性表达 Vmentin(SP 法) 图 5 瘤细胞弥漫性表达 cytokeratin(SP 法) 图 6 瘤细胞表达 CD34(SP 法) 图 7 瘤细胞表达 EMA(SP 法)

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