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Expression and significance of cyclin D1, p27kip1 protein in bronchioloalveolar carcinoma

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Abstract: Purpose: To investigate the relationship between expression of cell cycle-related protein cyclin D1, p27kip1 and the pathogenesis of bronchioloalveolar carcinoma (BAC) and the value of prediction of prognosis. Methods: Cyclin D1 and p27kip1 protein were detected by immunohistochemical En Vision method in 43 BACs. Results: The positivity of cyclin D1 in BAC was 65.1% (28/43), which was significantly higher than that in normal pulmonary tissue (0/13), $P<0.01$. No statistically significant association was found between cyclin D1 expression data and sex, age, tobacco-use history, histologic subtype (mucinous vs nonmucinous), stromal fibrosis, lymph node metastasis, clinical stage or postoperative survival period ($P>0.05$), while cyclin D1 expression was found to be negatively correlated with tumor size ($P<0.05$). The positivity of p27kip1 in BACs was 51.2% (22/43), significantly lower than that in normal pulmonary tissue (12/13), $P<0.01$. p27kip1 expression level was not associated with sex, age, tobacco-use history, tumor size or histologic subtype ($P>0.05$), but was negatively correlated with stromal fibrosis, lymph node metastasis and clinical stage ($P<0.05$); and positively associated with postoperative survival period ($P<0.01$). The survival rate of p27kip1 positive group was significantly higher than that of p27kip1 negative group ($P<0.01$). No statistically significant correlation was found between cyclin D1 and p27kip1 expression. Conclusions: Increased cyclin D1 expression and decreased p27kip1 expression are related to the pathogenesis of BAC; decreased p27kip1 expression is associated with metastasis progression; immunodetection of p27kip1 is useful for assessment of prognosis.

Key words: Adenocarcinoma, Bronchioloalveolar, Lung neoplasms, Cyclin D1, p27kip1, Immunohistochemistryction
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INTRODUCTION

Bronchioloalveolar carcinoma (BAC) is a particular subtype of pulmonary adenocarcinoma derived from clara cell and type II pneumocyte. BAC cells grow along and within alveolar spaces while the alveolar framework of the lung is preserved. The incidence of BAC appears to be rising recently. The etiology and pathogenesis of this unique neoplastic disease are still unclear; many studies of oncogene and tumor suppressor gene expression include BAC with all adenocarcinoma or non-small cell lung cancer (NSCLC), rather than as a separate category.

Recent studies revealed that uncontrolled cell cycle is closely related to uncontrolled cell proliferation and carcinogenesis (Harris *et al*., 1995). Cyclin-dependent kinases (CDKs), together with cyclins and CDK inhibitors, govern cell cycle progression in eukaryotic cells. In the cell cycle, different phase is regulated by different factors, in them, G_1-phase regulators are most closely related to carcinogenesis. cyclin D1, one of the key cell cycle regulators, an important positive regulator in G_1/S transition, is a putative proto-oncogene overexpressed in a wide variety of human neoplasms. p27kip1 is an important CDK inhibitor, which binds to and inhibits preferentially G_1-phase kinases, thereby arresting the cell cycle at G_1-phase. Decreased p27kip1 expression has been shown to be associated with the development and progression of malignancy in many human malignant tumors, and provides independent prognostic information. To our knowledge, p27kip1 expression in BAC and cyclin D1 expression in BAC have not been reported in the literature, except for the 19 cases reported in McDonald and Pilgram (1999) and the 110 cases reported in Bombi *et al*.(2002). Here we evaluated the expression of the cell cycle related proteins cyclin D1, p27kip1 in 43 surgically resected BACs using immunohistochemical En Vision method, discuss the relationship between the expression of the genes and their clinicopathologic significances.

METHODS

Selection of cases

The archival files of the Department of Pathology in Sir Run Run Shaw Hospital were reviewed retrospectively, covering the period Jan., 1994−Nov., 2000. The formalin fixed, paraffin embedded, haematoxylin and eosin stained slides for all BAC cases were reviewed by the first author to verify the diagnosis made by the original pathologist, and to obtain histologic subtype (mucinous vs nonmucinous). Fourty-three surgically resected pure BACs were retrieved and used for IHC studies. According to the degree of septal fibrosis, these are further subclassified into non-sclerosing and sclerosing BAC. None of the patients had adjuvant chemotherapy or radiation therapy before operation. The patient charts and pathology reports were reviewed to obstain information on the social history, tumor diameter and location, lymph node status and clinical stage. Thirteen paracancerous normal pulmonary tissue were selected as control group.

Immunohistochemical staining

Four-micron thick unstained sections were cut from the paraffin blocks. The immunostaining used En Vision method. Antigen unmasking by heating in 10 mmol/L sodium citrate buffer (pH 6.0) in microwave oven (Galanz WD900BS) was used. For cyclin D1, 0.5% pancreatic proteinase digestion was conducted before microwave treatment. The following antibodies were applied to the section: cyclin D1 (1:50, purified mouse monoclonal antibody, DCS-6, NeoMarkers), p27kip1 (1:50, purified mouse monoclonal antibody, DCS-72.F6, NeoMarkers), which was then incubated at 25 ºC (cyclin D1, 60 minutes; p27kip1, 40 minutes). Positive and negative controls were designed and reviewed along with the test slides. For positive control of cyclin D1 immunostaining, poorly-differentiated colonic adenocarcinoma with known cyclin D1 overexpression was used, normal pulmonary tissue was used for positive control of p27 immunostaining. For negative control, the section was treated in the same way except that the primary antibody was substituted with phosphate-buffered saline.

Assessment of immunohistochemistry

Since the cell cycle proteins are produced and exert their regulatory effects in the nucleus, the measurement of importance in the review of IHC slides was the presence of positive nuclear staining. Computed quantitation was performed utilizing the HPIAS-1000 cell image analysis system wherein the total number of tumor cells was counted in 10 microscopic fields at high-power magnification, and then the positivity was expressed as the percentage of the positive cells. For each antibody, this was graded semiquantitatively on a four-tier scale: −, < 5% positive cells; +, 5% to 24% positive cells; ++, 25% to 49% positive cells; +++, ≥50% positive cells.

Statistical analysis

Chi-square test, Fisher's exact test and Log-rank test.

RESULTS

Clinicopathologic features

Among the 43 patients, 17 were men and 26 were women. Ages ranged from 30 to 79 years (mean±SD, 58.3±11.1 years). Ten patients were current or former smokers and 33 patients were nonsmokers; 40 patients with solitary nodule, 2 patients with multiple nodules and 1 with diffuse pneumonic-like infiltrate; 24 tumors were ≥3cm in maximal dimension, 19 were <3cm; 39 cases were of the nonmucinous type (Fig.1), 4 were mucinous (Fig.2); 18 were non-sclerosing BAC, 25 were sclerosing BAC. According to the international TNM system, 19 tumors were stage I, 12 stage II, 6 stage III, 6 stage IV. Thirty-three of the 43 patients had available follow-up information, with a 4 to 95 month follow-up period; among them, 19 patients survived ≥2 years, 14 patients <2 years.

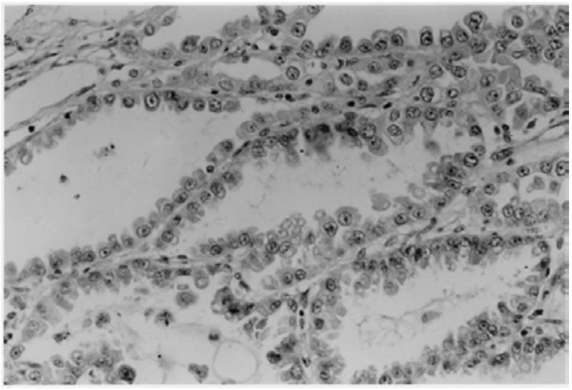

Fig.1 Nonmucinous bronchioloalveolar carcinoma. There is a proliferation of cuboidal cells with apical spouts growing along intact alveolar walls (HE×200)

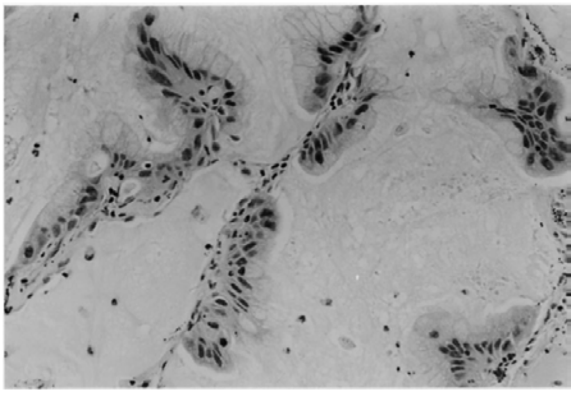

Fig.2 Mucinous bronchioloalveolar carcinoma. Well-differentiated mucin-containing columnar cells line intact alveolar wall, with accumulation of mucin within alveolar spaces (HE×200)

Immunohistochemical findings

Twenty-eight of 43 BACs (65.1%) showed nuclear cyclin D1 expression (Fig.3); while in the matched normal pulmonary tissue, no positive expression was found, although there were occasional normal bronchial epithelial cells whose nuclei stained positive (2 cases); the difference was very significant (Fisher's exact test, $P<0.01$). 22 of 43 BACs (51.2%) showed nuclear p27kip1 expression (Fig.4), while 12/13 normal lung tissue (92.3%) showed p27kip1 expression, the difference was very significant (Fisher's exact test, $P<0.01$); and the moderate to strong positivity in BAC (18.6%) was also obviously lower than that in the normal pulmonary tissue (84.6%) (Fisher's exact test, $P<0.01$) (Table 1).

Relationship between cyclin D1, p27kip1 expression and clinicopathologic parameters

No statistically significant association was found

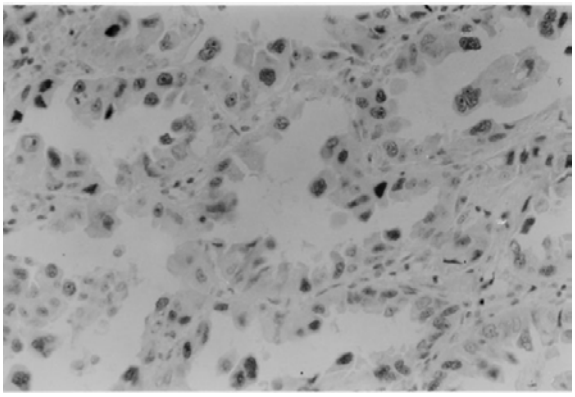

Fig.3 Strong (+++) immunohistochemical nuclear cyclin D1 positivity in Bronchioloalveolar carcinoma (En Vision×200)

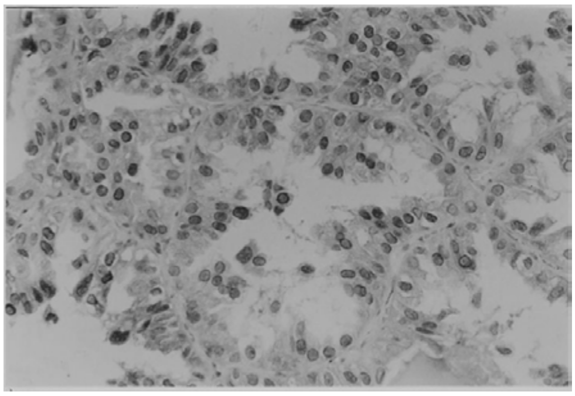

Fig.4 Strong (+++) immunohistochemical nuclear P27 positivity in bronchioloalveolar carcinoma (En Vision ×200)

Table 1 Expression of cyclin D1, p27kip1 in BAC and normal pulmonary tissue

	n	Cyclin D1				Positivity (%)	p27kip1				Positivity (%)
		−	+	++	+++		−	+	++	+++	
BAC	43	15	14	8	6	65.1	21	14	3	5	51.2
Normal lung tissue	13	13	0	0	0	0	1	1	5	6	92.3

between cyclin D1 expression data and sex, age, tobacco-use history, subtype of BAC (mucinous vs. nonmucinous), stromal fibrosis, lymph node metastasis, clinical stage or postoperative survival period; while cyclin D1 expression was negatively correlated with tumor size, the positivity in the <3 cm group was significantly higher than that in the ≥3 cm group (Fisher's exact test, P<0.05), and the moderate to strong positivity also showed the same tendency (Fisher's exact test, P<0.05). The expression of p27kip1 level was not associated with sex, age, tobacco-use history, tumor size or histologic subtype, but was negatively correlated with stromal fibrosis, lymph node metastasis (χ^2 test, P<0.05) and clinical stage (Fisher's exact test, P<0.05), and positively associated with postopera- tive survival period (Fisher's exact test, P<0.01) (Table 2).The survival curves using the Kaplan-Meier method showed that no significant difference was found between the cyclin D1 positive group and cyclin D1 negative group (Fig.5, Log-rank test, P> 0.05), while the survival rate of the p27kip1 positive group was significantly higher than that in p27kip1 negative group (Fig.6, Log-rank test, P<0.01).

Table 2 Relative analysis of cyclin D1, p27kip1 expression and clinicopathologic parameters

Clinicopathologic parameters	n	Cyclin D1				Positivity (%)	p27kip1				Positivity (%)
		−	+	++	+++		−	+	++	+++	
Sex											
Male	17	5	5	4	3	70.6	7	7	2	1	58.8
Female	26	10	9	4	3	61.5	14	7	1	4	46.2
Age(yrs)											
≥50	32	9	11	7	5	71.9	15	11	3	3	53.1
<50	11	6	3	1	1	45.5	6	3	0	2	45.5
Tobacco use											
Yes	10	2	2	3	3	80.0	5	3	2	0	50.0
No	33	13	12	5	3	60.6	16	11	1	5	51.5
Maximal tumor dimension											
≥3 cm	24	12	8	1	3	50.0$^{\Delta}$	11	6	2	5	54.2
<3 cm	19	3	6	7	3	84.2	10	8	1	0	47.4
Histologic subtype											
NM	39	14	12	7	6	64.1	19	12	3	5	51.3
M	4	1	2	1	0	75.0	2	2	0	0	50.0
Stromal fibrosis											
Non-sclerosing	18	7	6	2	3	61.1	5	7	2	4	72.2$^{\Delta}$
Sclerosing	25	8	8	6	3	68.0	16	7	1	1	36.0
LN metastasis											
Positiove	22	8	7	4	3	63.6	14	6	1	1	36.4$^{\Delta}$
Negative	21	7	7	4	3	66.7	7	8	2	4	66.7
Clinical stage											
I + II	31	9	10	6	6	70.9	12	11	3	5	61.3$^{\Delta}$
III + IV	12	6	4	2	0	50.0	9	3	0	0	25.0
Postop. survival period											
≥2yrs	19	6	5	5	3	68.4	6	6	3	4	68.4$^{\Delta\Delta}$
<2yrs	14	5	5	3	1	64.3	12	1	0	1	14.3

For the two BACs with multiple nodules, the aggregate tumor diameter was used (McDonald and Pilgram, 1999)

$^{\Delta}P$<0.05; $^{\Delta\Delta}P$<0.01

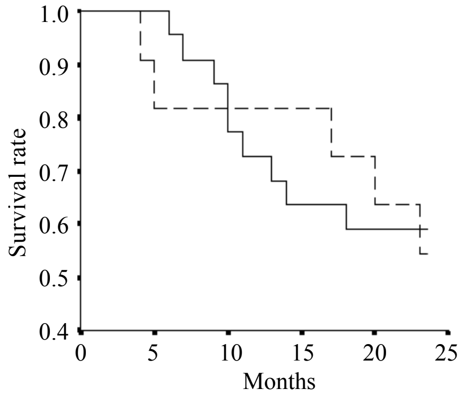

Fig.5 Survival for cyclin D1 positive versus cyclin D1 negative in BAC patients — Cyclin D1 +; - - - Cyclin D1 −

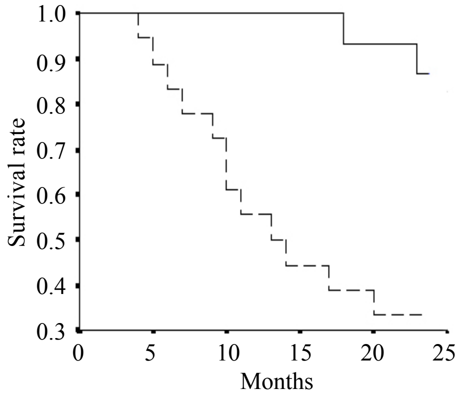

Fig.6 Survival for p27 positive versus p27 negative in BAC patients — p27 +; - - - p27 −

Association between cyclin D1 and p27kip1 expression

Of the 28 cyclin D1-positive cases, 15 also showed p27kip1 nuclear expression, the positivity was 53.6%; of the 15 cyclin D1-negative cases, 7 showed p27kip1 nuclear expression, the positivity was 46.7%. There was no statistically significant association between cyclin D1 and p27kip1 expression (χ^2=0.186, P>0.05).

DISCUSSION

Studies showed that cyclin D1 is usually expressed in NSCLC, while it is negative or slightly expressed in SCLC. McDonald and Pilgram (1999) reported in the study of 19 BACs that immunohistochemical nuclear cyclin D1 positivity was 32%; and that no statistically significant association was found between the expression data and subtype of BAC, tumor diameter or clinical stage. Bombi *et al.* (2002) reported that the positive expression rate of cyclin D1 in BAC was 70%, which was comparable to our result. Many studies showed that cyclin D1 nuclear expression in tumor cell population had considerable variability probably caused by cyclin D1 expression occurring in G_1 phase with a peak in late G_1 and degrading rapidly when transitting into S phase. Nguyen *et al.*(2002) reported that the expression rate of cyclin D1 in NSCLC was 51%; and that the expression data was not correlated with clinicopathologic parameters and prognosis. It was reported (Xie *et al.*, 1999; Li *et al.*, 1999; Fu *et al*, 2000) that cyclin D1 positivity in NSCLC or pulmonary adenocarcinoma was 54.3%−65.6%; our result was similar. As to the precancerous lesion of BAC, atypical adenomatous hyperplasia (AAH), the overexpression of cyclin D1 was frequently observed (Tominaga *et al.*, 2003). Kurasono *et al.* (1998) reported that the frequency of lesions with cyclin D1 overexpression was relatively high in AAH (62.5%), but was lower in adenocarcinoma (32.4%), which suggested that overexpression of cyclin D1 is an early event and plays an important part in the tumorigenesis in BAC. In our study, no expression waswas observed in the matched pulmonary tissue, while it was obviously overexpressed in BAC, the presence of immunohistochemical nuclear cyclin D1 expression in 65.1% cases suggested that overexpression of cyclin D1 is a common molecular abnormality and closely related to the development of BAC. No statistically significant association was found between cyclin D1 expression data and sex, age, tobacco-use history, subtype of BAC, stromal fibrosis, lymph node metastasis, clinical stage or postoperative survival period; no significant difference was found between the survival of the cyclin D1 positive group and the cyclin D1 negative group, which was consistent with reported observations. The cyclin D1 expression in our research was negatively correlated with tumor size, which is not in agreement with observations reported in literature. One interpretation for such finding is that overexpression of cyclin D1 is a common and important event in carcinogenesis in many tissues, including the lung. However, cyclin

D1 overexpression is not required for the maintenance of a malignant phenotype, so it is likely that cyclin D1 expression will gradually reduce or lose along with the growth of the tumor (Kurasono *et al.*, 1998). Another alternative explanation is that too much cyclin D1 may be toxic to the cell and leads to cell cycle arrest; there exists a certain upper threshold level for cyclin D1 protein above which it is no longer tolerable (Bartkova *et al.*, 1994). Whether this phenomenon exists in the ≤3 cm tumors in our study should be confirmed with further quantitative study.

p27kip1 is a non-specific cyclin-dependent kinase inhibitor, which binds to and inhibits preferentially G_1-phase cyclin-CDK complexes such as cyclin D-$CDK_{4/6}$ and cyclin E-CDK_2 thereby inhibiting G_1/S transition, and then suppresses cell proliferation. In addition, p27kip1 protein plays an important role in conduction of extracellular signals, and has a role in cell differentiation, so it is also regarded as a tumor suppressor gene. A large number of studies showed that p27kip1 gene mutations had been identified only rarely; its mRNA level keeps stable in the cell cycle, while the level of p27kip1 protein decreases obviously during development and progression in many tumors. This decrease occurs mainly at the post-translational level with protein degradation by the ubiquitin-proteasome pathway. Recent studies (Hommura *et al.*, 2000; Tsihlias *et al.*, 1998; Chiarle *et al.*, 2000) showed that low immunohistochemical expression of p27kip1 is associated with high tumor grade, lymph node metastasis and advanced clinical stage in a variety of human malignant tumors including breast, colon, lung, prostate, endometrium, gastric carcinomas and malignant lymphoma, which suggests that low p27kip1 is a powerful and independent prognostic marker of poor clinical outcome. Our study showed that p27kip1 expression rate in BAC was 51.2%, which was significantly lower than that in the normal pulmonary tissue (92.3%); and that the moderate to high positivity in BAC was also significantly decreased. This result suggested that decreased p27kip1 expression is associated with the development of BAC. Correlations with clinicopathologic parameters showed that the p27kip1 expression data was not associated with sex, age, tobacco-use history, tumor size and histologic subtype; but was negatively correlated with stromal fibrosis. p27kip1 positivity in lymph node positive group was significantly lower than that in lymph node negative group; the positivity in stage III+IV was significantly lower than that in stage I+II; the positivity in survival period in ≥2yrs group was significantly higher than that in <2yrs group; the survival rate of p27kip1 positive group was significantly higher than that in p27kip1 negative group. These data indicated that decreased p27kip1 expression is involved in metastasis and malignant progression, and that immunodetection of p27kip1 may be useful in the assessment of prognosis.

As to the relationship between cyclin D1 and p27kip1 expression, previous reports showed different results in different tumors. Nohara *et al.* (2001) found a positive association between p27kip1 and cyclin D1 expression, while Hui *et al.*(1999) reported that decreased p27kip1 was associated with cyclin D1 overexpression. In our research, there was no statistically significant association of cyclin D1 and p27kip1 expression with BAC. So the definite relationship between the two proteins should be further explored with more BAC cases.

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